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A glycosyltransferase gene responsible for pullulan biosynthesis in *Aureobasidium melanogenum* P16

Xi Chen, Qin-Qing Wang, Nan-Nan Liu, Guang-Lei Liu, Zhe Chi, Zhen-Ming Chi *

College of Marine Life Sciences, Ocean University of China, Yushan Road, No. 5, Qingdao, China

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ABSTRACT

In this study, one of the glucosyltransferase genes for pullulan production was cloned from *Aureobasidum melanogenum* P16 and charaterized. It was found that the *UGT1* gene had 4774 bp with four introns (47, 52, 54 and 46 bp). The N-terminal part of the protein displayed a conserved sequence controlling both sugar donor and accepter for substrate specificity whereas its C-terminal part carried a DXD motif that coordinated donor sugar binding. After complete removal of the gene *UGT1*, the mutant 1152-3 still produced 27.7 ± 3.1 g/L of pullulan and 4.6 U/g of the specific glucosyltransferase activity while its wild type strain P16 yielded 63.38 ± 2.0 g/L of pullulan and 5.7 U/g of the specific glucosyltransferase activity. However, after overexpression of the gene *UGT1*, the transformant G63 could produce 78.0 ± 3.01 g/L of pullulan and 19.0 U/g of the specific glucosyltransferase activity. It is interesting to note that the molecular weight of the produced pullulan by the wild type strain was 4.6×10^5 while that of the produced pullulan by the transformant G63 was 6.2×10^5. During the 10-Litter fermentation, the pullulan titer produced by the transformant G63 reached 80.2 ± 2.0 g/L within 132 h.

1. Introduction

Pullulan is an extracellular, regularly repeating, linear and biological macromolecule primarily consisting of maltotriose repeating units interconnected by α-1,4 glucosidic bond whereas consecutive maltotriose units are connected to each other by α-1,6 glycosidic linkages. The pullulan producers were viewed as different strains of *Aureobasidium pullulans* [1,2]. However, recently the high-pullulan producers have been found to be different strains of *Aureobasdium melanogenum* [3,4]. Because of its mechanical flexibility, oxygen impermeability and easy derivatibility, pullulan is highly valued for its applications in food, pharmaceutical, cosmetic and nanomaterial industries [5]. Recently, it has been found that pullulan that can form an oxygen-impermeable solid after drying can be used in the next-generation point-of-care testing for storage of labile biomolecules as stable water-soluble pellets for a long time [6]. The drug capsules made of pullulan are welcome by the vegetarian and muslims. Although this basic structure of pullulan was resolved in 1960 [7] and the commercial production of pullulan began in 1976 by the Hayashibara Company in Japan, its biosynthesis pathway, relevant enzymes and genetic mechanisms behind pullulan production still remain completely unclear to date [1]. In 2008, Duan et al. [8] proposed a pullulan biosynthesis pathway in *A. pullulans* Y68, a high pullulan producing yeast and found that α-phosphoglucose mutase; UDPG-pyrophosphorylase, glucosyltransferase were involved in pullulan biosynthesis. Prajapati et al. [1] thought that the proposed pathway would be very helpful for metabolism. In 2011 and 2012, a pullulan synthetase was found to be implicated in pullulan biosynthesis, but its exact function in pullulan biosynthesis is still unknown [9,10]. Although the exact pathway responsible for pullulan biosynthesis in different strains of *Aureobasidium* spp. is still a matter of debate, there is a convincing agreement that α-phosphoglucose mutase, UDPG-pyrophosphorylase, different glucosyltransferases and pullulan synthetase are involved pullulan biosynthesis [11]. The first glucosyltransferase catalyzes transfer of a glucosyl from UDP-glucose to an acceptor Lph (the lipid linked to cellular membrane) to form Lph-glucose. The second glucosyltransferase catalyzes transfer of a glucosyl from UDP-glucose to the Lph-glucose to yield Lph-glucose-α-1,6-glucose. Then, the third glucosyltransferase catalyzes transfer of a glucosyl from UDP-glucose to Lph-glucose-α-1,6-glucose for make Lph-glucose-α-1,6-glucose-α-1,4-glucose. Finally, the two fragments (glucose-α-1,6-glucose-α-1,4-glucose) are linked to each other by α-1,4 glycosidic linkage, resulting in

* Corresponding author.
E-mail addresses: zhenming@sdu.edu.cn, chi@ouc.edu.cn (Z.-M. Chi).

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formation of glucose-α-1,6-glucose-α-1,4-glucose-α-1,4-glucose-α-1,6-glucose-α-1,4-glucose under the catalysis of the pullulan synthetase. In this proposed pathway, it is strongly suggested that several glucosyltransferases take part in the pullulan biosynthesis. However, so far, no glucosyltransferase gene relevant to the pullulan biosynthesis in *Aureobasidium* spp. has been cloned and characterized. We think if all the glucosyltransferases and their function in pullulan biosynthesis can be elucidated, it will be possible to know the whole pullulan synthetic pathway and be of importance to enhance pullulan production by genetic engineering of the producers. Therefore, in this study, one of the glucosyltransferase genes responsible for pullulan biosynthesis was cloned from *A. melanogenum* P16, another high-pullulan producing yeast isolated from a mangrove ecosystem, characterized, deleted and over-expressed. This is the first time to show that the glucosyltransferase gene responsible for pullulan biosynthesis is elucidated.

A glucosyltransferase is an enzyme that catalyzes the sequential transfer of glucose from a specific activated donor (usually a nucleotide sugar or lipid-linked phosphor-sugar, such as UDP-glucose) to a specific acceptor molecule to form oligosaccharides, polysaccharides and other biological macromolecules. It has been demonstrated that the glucosyltransferases are a highly diverse group of enzymes with little homology even among the enzymes that share the same substrate specificity [12,13]. Now, all the glycosyltransferases according to their sequence homology have 86 families; however, out of the 86 families, only 27 families have members with known structure [13]. This means that it is very difficult to identify the glucosyltransferases responsible for biosynthesis of different kinds of oligo and polysaccharides with different chemical structures.

2. Materials and methods

2.1. Microbial strains and media

Aureobasdium melanogenum P16, a high pullulan producing yeast, was isolated from a mangrove ecosystem in Province of Hainan, China [3,4]. The yeast strain P16 was grown in a seed culture medium at 28 °C and 180 rpm for 48 h [3]. The pullulan production medium consisted of 120.0 g/L sucrose, 3.0 g/L yeast extract, 5.0 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L NaCl, and 0.6 g/L $(NH_4)_2SO_4$ [3]. The *Escherichia coli* DH5α was grown in a Luria-Bertani broth (LB) and used for amplification of the recombinant plasmids. The *E. coli* transformants were grown in the LB medium with 100 μg/mL of ampicillin. The yeast transformants were grown in a YPD medium containing 100 μg/mL of hygromycin B [3].

2.2. Gene and plasmids

A glucosyltransferase gene (*UGT1*) (accession number: KX421262) was cloned from the genomic DNA of *A. melanogenum* P16 as described below. A plasmid pMD 19-T simple vector for cloning of PCR products was purchased from TaKaRa (Japan). A plasmid pF14a-UGT (5664 kb) and a plasmid pPAX13-NS-UGT (10.313 kb) for knock-out and overexpression of the *UGT1* gene were constructed as described below.

2.3. Cloning of the glucosyltransferase gene (UGT1) responsible for pullulan biosynthesis from A. melanogenum P16

The yeast genomic DNAs for amplification of the glucosyltransferase genes in *A. melanogenum* P16 were isolated as described by Ma et al. [3]. It has been well documented that a specific activated donor for the glucosyltransferase is usually UDP-glucose. Therefore, the genes that have been annotated as UDP-glucose: glycoprotein glucosyltransferase-like proteins (accession numbers: KEQ61572, KEQ64507, KEQ59287 and KEQ57977) were searched from the sequenced and annotated DNAs of *Aureobasdium melanogenum* CBS 110374 (accession number: KEQ67433.1). To obtain the full-length of the glucosyltransferase genes from the genomic DNAs of the yeast strain P16, the glucosyltransferase genes were PCR amplified using the degenerate primers Clone-1152-F and Clone-1152-R, Clone-1731-F and Clone-1731-R, Clone-3915-F and Clone-3915-R, Clone-4337-F and Clone-4337-R designed according to the sequenced genomic DNAs of *A. melanogenum* CBS 110374 (accession number: KEQ67433.1) and the genomic DNAs of *A. melanogenum* strain P16 as the template (Table 1). The reaction system and the conditions for the PCR amplification were described by Chi et al. [14]. The PCR products were cloned into the pMD19-T simple vector and sequenced by BIOSUNE CO. Ltd (Beijing, China). After the sequence of the PCR products was analyzed with a NCBI ORF finder program and aligned with the known sequences of the genes encoding the glucosyltransferases from different strains of the fungi by using a NCBI BLASTn, the full length (4774 bp) of one of the glucosyltransferase genes (accession number: KX421262) from *A. melanogenum* P16 was obtained and was named *UGT1*.

2.4. Bioinformatic analysis of the cloned glucosyltransferase genes

The BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI) were used for the nucleotide sequence analysis of the cloned glucosyltransferase genes, deduction of the amino acid sequence from the cloned glucosyltransferase genes and database searches. Multiple sequence alignments of the amplified DNA and the amino acid sequence of the deduced proteins were carried out using the programs of a DNAMAN 6.0 (http://www.lynnon.com) and a Clustal X 1.8 [15]. A phylogenetic tree of the glucosyltransferases from different yeasts, filamentous fungi and bacteria was constructed by using a MEGA 4.0.

2.5. Construction of the knock-out vector pF14a-UGT for disruption of the cloned UGT1 gene

A DNA fragment [5′-arm-Poly(A)-hygromycin B phosphotransferase (*HPT*) gene–TEF promoter-arm-3′) for disruption of the *UGT1* genes was constructed based on the procedures described by Chi et al. [14] (Fig. 1). For example, the 5′-arms of the *UGT1* gene (accession number: KX421262) cloned above were PCR amplified from the genomic DNA of *A. melanogenum* P16 using the primers 1152-f1 and1152-r1 (Table 1). The 3′-arms of the *UGT1* gene were PCR amplified from the genomic DNA of *A. melanogenum* P16 using the primers 1152-f2 and 1152-r2 (Table 1). The 5′-arm and 3′-arm obtained were digested with *Sal*I and *Pst*I, *Bam*HI and *Eco*RI, respectively. The digested 5′-arm and 3′-arm were ligated into a plasmid pFL4a (digested with the same enzymes) to form pFL4a-UGT (Fig. 1). Finally, the 5′-arm-polyA-HPT-TEF-3′-arm fragments were obtained by digestion of the plasmid pFL4a-UGT with the enzymes *Eco*RI and *Pst*I (Fig. 1).

2.6. Disruption of the UGT1 genes in A. melanogenum P16

Preparation and transformation of the competent yeast cells of *A. melanogenum* P16 were performed using the procedures described by Chi et al. [14]. The transformation of the competent yeast cells of *A. melanogenum* P16 was accomplished by incubating 0.2 mL of the spheroplast suspension at 22 °C with at least 1.0 μg of the 5′-arm-polyA-HPT-TEF-3′-arm fragments obtained