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Gene cloning, functional expression and characterisation of a novel glycogen branching enzyme from *Rhizomucor miehei* and its application in wheat breadmaking

Shupeng Wu [a], Yu Liu [b], Qiaojuan Yan [b], Zhengqiang Jiang [a,*]

[a] Department of Biotechnology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Bioresource Utilization Laboratory, College of Engineering, China Agricultural University, Beijing 100083, China

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ABSTRACT

A gene (*RmGBE*) encoding a glycogen branching enzyme from *Rhizomucor miehei* was cloned into the pET28a (+) vector and expressed in *Escherichia coli*, and biochemically analysed. *RmGBE* had an open reading frame of 2097 bp encoding 698 amino acid residues. The purified enzyme was a monomer of 78.1 kDa. RmGBE was optimally active at 25 °C and pH 7.5. It displayed excellent cold adaptation over a low temperature range of 10–30 °C, retaining over 85% of its relative activity. RmGBE showed the highest specificity to amylose, about ten times higher than to amylopectin. Addition of RmGBE to wheat bread resulted in a 26% increase in specific volume and a 38% decrease in crumb firmness in comparison with the control. Besides, the retrogradation of bread was significantly retarded along with the enzyme reaction. These properties make RmGBE highly useful in the food and starch industries.

1. Introduction

Glycogen is a highly branched polysaccharide composed of α-1,4-linked glucans with branches via α-1,6-glycosidic linkages which is used as a storage carbohydrate by many microbes. There are three different enzymes responsible for biochemical conversion of glucose-1-phosphate to glycogen: (i) ADP or UDP-glucose pyrophosphorylases (EC 2.7.7.27 or EC 2.7.7.9) for the synthesis of ADP (or UDP)-glucose, (ii) synthases (EC 2.4.1.21) which can create new α-1,4-glycosidic linkages between the glucosyl unit from ADP-glucose and the preexisting α-1,4-glucan, and (iii) branching enzymes (EC 2.4.1.18, α-1,4-glucan: α-1,4 glucan 6-α-glucanotransfereases) which catalyses the formation of α-1,6-glycosidic linkages (Prieiss, 1984).

Branching enzymes are carbohydrate-active enzymes belonging to the α-amylase family. This family comprises hydrolases, transglycosylases and isomerases such as α-amylases, isoamylases, α-glucosidase, pullulanase, cyclodextrin glycosyltransferases and branching enzymes (Svensson, 1994). Branching enzymes possess both α-amylolysis and glucosyl-transferactivities, which catalyse the formation of branch points in glycogen or amylopectin by cleavage of α-1,4-glycosidic bonds and subsequent transfer of the cleaved oligosaccharide to α-1,6 positions (Fox, Kawaguchi, Greenberg & Preiss, 1976; van der Maarel, van der Veen, Uitdehaag, Leemhuis & Dijkhuizen, 2002). Glycogen branching enzymes sometimes are also called starch branching enzymes, which catalyse the formation of α-1,6-branch point into amylose and amylopectin (Eun-Joo, Soo-In, & Hyun-Ah, 2008). There are several differences between glycogen branching enzymes and starch branching enzymes: the degree of branching in glycogen (8–9%) is higher than that of starch amylopectin (3.5%), but the branches of glycogen tend to be shorter (8–10 glucose units) than the amylopectin (12–20 glucose units) (Murakami, Kanai, Takata, Kuriki, & Imanaka, 2006). These structure differences between glycogen and amylopectin are thought to be primarily due to different actions and specificities of glycogen branching enzymes and starch branching enzymes. Intermolecular reactions of glycogen branching enzyme have been demonstrated using a potato branching enzyme (Borovsky, Smith, Whelan, French & Kikumoto, 1979). Intramolecular reactions have been analysed using a *Bacillus* branching enzyme (Takata, Takaha, & Okada, 1996a; Takata, Takaha, Okada, Hizukuri, Takagi & Imanaka, 1996b). These intramolecular

Abbreviations: DP, degree of polymerisation; GH, glycoside hydrolase; HPAEC, high performance anionic-exchange chromatography; RACE, Rapid Amplification of cDNA Ends; MES, 2-(N-morpholino) ethane sulphonic acid; RmGBE, α-1,4-glycogen branching enzyme from *Rhizomucor miehei*.

* Corresponding author. Address: PO Box 294, China Agricultural University, No. 17 Qinghua Donglu, Haidian District, Beijing 100083, China. Tel.: +86 1062737689; fax: +86 1082388508.
E-mail address: zhqjiang@cau.edu.cn (Z. Jiang).

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branching reactions produced mainly cyclic glucans from amylose and amylopectin.

Glycogen branching enzymes are widely distributed in microorganisms, plants and animals. Various glycogen branching enzymes from *Cyanobacterium synechococcus* (Kiel, Elgersma, Beldman, Vossen, & Venema, 1989), hen oviduct (Brockhausen et al., 1989), *Bacillus stearothermophilus* (Takata, Takaha, Kuriki, Okada, Takagi & Imanaka., 1994), wheat (Nair, Båga, Scoles, Kartha & Chibbar, 1997), *Neisseria denitrificans* (Buttcher, Quanz & Willmitzer, 1999), *Anaerobranca gottschalkii* (Thiemann et al., 2006), *Thermococcus kodakaraensis* (Murakami et al., 2006), *Mycobacterium tuberculosis* (Garg, Alam, Kishan, & Agrawal, 2007), *Oryza sativa* L. (Vu et al., 2008), *Streptococcus mutans* (Eun-Joo et al., 2008) and *Rhodothermus obamensis* (Roussel et al., 2013) have been identified and biochemically characterised, but only one fungal glycogen branching enzyme from *Neurospora crassa* (Kawabata, Toeda, Takahashi, Shibamoto, Kitao & Kobayashi, 2002) has been purified and characterised. So far, there are no reports on genes encoding glycogen branching enzymes in fungi. Except for a few thermostable enzymes from archaebacteria (Murakami et al., 2006), most of the glycogen branching enzymes display maximal activity at neutral pH and within the mesophilic temperature range of 35–55 °C (Kiel et al., 1989; Lee et al. 2010; Thiemann et al., 2006).

On the basis of their unique α-1,6 transglycosylation activity, glycogen branching enzymes may find new applications in starch-related industries. There is approximately 18–33% amylose in starch (Buleon, Colonna, Planchot & Ball, 1998). Due to the low solubility of amylose, the maximum concentration of starch in starch-degrading procedures is limited. Moreover, amylose tends to retrograde, resulting in lower product quality and more staling of food products. Glycogen branching enzymes specifically catalyse the α-1,4-glycosidic linkages of amylose, changing its structure and decreasing its percentage in starch. Several potential applications of glycogen branching enzymes have therefore been investigated. Starch modified by the glycogen branching enzyme has been applied in the coating step of paper manufacture (Nichols, 2000). Kawabata et al. (2002) demonstrated that the glycogen branching enzyme from *N. crassa* can change the structure of starch, thereby increasing the solubility and stability of starch solutions. Glycogen branching enzyme from *Streptococcus mutans* may act on rice starch and retard its retrogradation (Eun-Joo et al., 2008). Similarly, several glycogen branching enzymes from bacteria and plants have been found to improve the quality of food products such as cookies, cakes and breads (Okada, Kitahata, Yoshikawa, Sugimoto, & Sugimoto 1984; Spendler & Joergensen, 1997). However, few applications of fungal glycogen branching enzyme are available, and the detailed mechanisms require further investigation.

Rhizomucor miehei is a thermophilic fungus which has been reported to secrete multiple types of hydrolytic enzymes, such as β-1,3-1,4-glucanase (Tang, Yang, Yan, Cui & Jiang, 2012). Here we report on the molecular cloning and heterologous expression of a glycogen branching enzyme gene (*RmGBE*) from *R. miehei*. The recombinant glycogen branching enzyme was purified and characterised, and it was found to improve bread quality and increase its shelf life due to a staling-retardation effect. This is the first report of a fungal glycogen branching enzyme that efficiently improves bread quality with an antistaling effect.

2. Materials and methods

2.1. Strains, vectors and reagents

Escherichia coli strains DH5α and BL21 were used for propagation of the plasmids and as hosts for expression of the glycogen branching enzyme gene, respectively. PrimeSTAR HS DNA polymerase and restriction endonucleases were purchased from TaKaRa (Tokyo, Japan). T4 DNA ligase was from New England Biolabs (Ipswich, MA, USA). Vector pET-28a (+) was obtained from Novagen (Madison, WI, USA). Ni Sepharose iminodiacetic acid (IDA) resin matrix and Sephacryl S-200 resins were from GE Life Sciences (Pittsburgh, PA, USA). The substrates, amylose (type III from potato, A0512), amylopectin (from potato, A8515), soluble starch (S9765) and glycogen (from Oyster, type II, G8751) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Corn starch and potato starch were purchased from Beijing Aoboxing Bio-Tech Co., Ltd. Wheat starch was obtained from Taizhou good food condiment Co., Ltd. All other chemicals used were of analytical grade unless otherwise stated.

2.2. Microorganism and culture conditions

R. miehei CAU432 was deposited in the China General Microbiological Culture Collection Center (http://www.cgmcc.net/) under CGMCC no. 4967. For isolation of genomic DNA, the strain was grown at 50 °C for 2 days in medium (g L^{-1}): oat flour, 30; soybean peptone, 10; $MgSO_4{\cdot}7H_2O$, 0.3; KH_2PO_4, 5; $CaCl_2$, 0.3. For RNA isolation, the strain was cultured as above, and fungal mycelium was collected by centrifugation (5000×*g*, 10 min).

2.3. Cloning of a fungal glycogen branching enzyme gene and sequence analysis

DNA manipulations were performed according to the recombinant DNA techniques described by Sambrook and Russell (2001). Genomic DNA of *R. miehei* CAU432 was isolated using a standard method. For isolation of RNA as a template for RT-PCR, cells were grown and collected as described in Section 2.2. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and mRNAs were purified using the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany).

To clone a glycogen branching enzyme gene (*RmGBE*) from *R. miehei*, degenerate primers GBEDF (5′-GACGGCTTCCGAT TCgayggngtnac-3′, Y = C/T,N = A/T/C/G,R = A/G) and GBEDR (5′-TC GCCGACCAGGgcytgrtcrtg-3′) were designed on the basis of the conserved sequences (FDGFRFDGVTSMLY and ESHDQALVGDKT) of putative fungal glycoside hydrolase (GH) family 13 glycogen branching enzymes. The PCR conditions were as follows: a hot start at 94 °C for 5 min, five cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by 20 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was purified, ligated to pMD18-T vector, and sequenced.

The full-length cDNA sequence of the glycogen branching enzyme was obtained by 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) using a BD SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). To amplify the 5′ end of the cDNA, RACE product was amplified with the primer GBE5′GSP (5′-TGA TCATGCGATTCACAGTACG-3′) and adapter primer UPM and then subjected to nested PCR using the nested gene-specific primer GBE5′NGSP (5′-GCGATTCACAGTACGCGATAGCTT-3′) and adapter primer NUP. The PCR condition for RACE was 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C, and finally, 10 min at 72 °C. For the 3′ RACE, the primary PCR was performed using the primers GBE3′GSP (5′-GCTTTACAAGC ATCATGGTATTGGC-3′) and UPM, followed by a nested PCR using nested gene-specific primer GBE3′NGSP (5′-GGTATTGGCT ATGGCTTTTCAGG-3′) and NUP. The PCR product was purified, cloned and sequenced. The 5′ and 3′ flanking sequences obtained by 5′and 3′ RACE were assembled with that of the consensus region to form a full-length cDNA sequence containing the open reading frame (ORF) of the glycogen branching enzyme. The cDNA