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A novel metagenome-derived gene cluster from termite hindgut: Encoding phosphotransferase system components and high glucose tolerant glucosidase

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ABSTRACT

Functional screening of a metagenomic library of termite hindgut identified an overlapping gene cluster encoding the phosphotransferase system (PTS) components, which consisted of a glucoside specific PTS enzyme II gene (*glu1923*) and a glycoside hydrolase gene (*glu1392*). Hydrolytic experiments revealed that the combined effect of Glu1923 and Glu1392 was responsible for the utilization of glucosidic substrates in recombinant *Escherichia coli* (*E. coli*) strains. Yeast two hybrid analysis suggested that there was an interaction between Glu1923 and Glu1392, and the domain EIIA of Glu1923 played an important role for the interaction. In addition, the hydrolase Glu1392 displayed hydrolysis ability toward cellobiose and maltose, and had a very high tolerance to glucose with a K_i value of 2.25 M. These properties make Glu1392 an interesting candidate in biotechnology applications after further study.

1. Introduction

In bacteria, glucoside sugar is most commonly taken up by the phosphoenolpyruvate (PEP)-sugar phosphotransferase system (PTS) [1], and the utilization of glucoside sugar can convert oligosaccharide into glucose and providing the carbon sources [2,3]. The PTS, which consists of three key components including enzyme I (EI), heat-stable phosphocarrier protein (HPr), and enzyme II (EII), plays an important role in carbohydates transport [4]. EI firstly autophosphorylates from PEP and transfers P to HPr protein, and then the phosphoryl group is delivered to the carbohydrates via EII. Finally, the phosphorylated carbohydrate was translocated into cytoplasm for specific PTS-dependent utilization [5]. To date, numbers of PTS genes/operons were identified by computational and functional analyses [6–8]. Further studies indicated that EI and HPr from different organisms were evolutionarily conserved [9]. However, the permease EII had substrate specificity, and only a few EIIs with unique structures or functions were identified until now. In addition, almost all of the PTS genes/operons reported so far were derived from cultured microorganisms, and little attention had been paid to discover novel PTS genes/operons from unculturable microorganisms, which account for over 99% of microorganisms in the environment. Therefore, some efforts should be made to discover novel PTS genes/operons. The metagenomic approach, featured by direct cloning of DNA from environment samples and thereby accessing the potential of unculturable organisms, was a powerful tool for the isolation of novel PTS genes/operons. Also, the study on novel PTS genes/operons from unculturable microorganisms will help us to better understand how bacteria absorb and utilize sugar substrates.

Previous studies had isolated and analyzed many bacteria β-glucoside operons that regulated the utilization of β-glucosides [10,11]. In enteric bacteria *Escherichia coli*, glucoside PTS operons contain all of the genetic elements for the uptake and utilization of glucosides, but the operons are cryptic and transcriptionally silent in nature [9]. Thus, the wild-type strains are normally unable to utilize the glucoside sugar as a carbon source. The modulation of β-glucoside (such as arbutin and salicin) PTS operon *gluGFB* have been extensively studied, and the result showed that an adaptive mutation by the insertion of an insertion sequence (IS) to the regulation region can activate these cryptic PTS genes [12]. Recently, another *E. coli* strain with β-glucoside sugar (cellobiose) utilization phenotype was also produced by mutations in cryptic operons *chb*

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and *asc*, or by replacing the cryptic promoters of these endogenous operons with a constitutive promoter [13].

Termites are very efficient decomposers of lignocellulosic plant materials and their high digestion capacity mainly results from the hindgut-associated obligate symbionts [14]. Therefore, these symbiotic microorganisms in the hindgut may contain rich enzymes and PTS genes/operons related with cellulose degradation. To discover novel PTS components from unculturable microorganisms of termite hindgut and give a deep insight into the regulatory mechanism of the bacterial PTS operons, we here identified a novel PTS gene cluster encoding two PTS components from metagenomic library of hindguts inhabiting microbes of lower termite, *Coptotermes formosanus*. Also, we investigated the interaction of two proteins encoded by two glucoside PTS components. Finally, the recombinant glucosidase Glu1392 with high glucose tolerance was characterized after being overexpressed in *E. coli* and purified.

2. Materials and methods

2.1. Strains, vectors and chemicals

E. coli DH5α was used as the cloning host, and BL21 (DE3) was served as the expression host. The vector pET-32a (+) was used for protein expression. *Saccharomyces cerevisiae* AH109 strains, pGBKT7 and pGADT7 vectors were used for yeast two hybrid assay. All restriction endonucleases and ligases were purchased from TakaRa (Dalian, China). All other chemical and reagents were from Sangon (Shanghai, China) unless indicated otherwise.

2.2. Construction of metagenome library and screening of glucosidase

Wood-feeding *C. formosanus* termites were collected from Dinghu Mountain, Guangdong province of China (22°10′26″N, 112°31′15″E). Approximately 500 worker termites were dissected and the aqueous content of the hindgut were collected as previously reported [15]. A total genomic DNA from the hindgut was isolated using E.Z.N.A.™ Bacterial DNA isolation kit (Omega). Then, the genomic DNA was partially digested with *Eco*RI and the DNA fragments of 2–8 kb were purified by Omega Gel Extraction Kit (Omega) and inserted into pUC118 vector (TaKaRa), which had been previously digested with *Eco*RI and dephosphorylated with calf intestine alkaline phosphatase. Next, the recombinant plasmid was transformed into *E. coli* DH5α and the transformants were cultured on lysogeny broth (LB) agar plate containing 0.75 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), 0.1% esculin hydrate, 0.25% of ferricammonium citrate and 100 μg/ml ampicillin at 37 °C overnight. Those colonies forming clear black halos were selected as positive colonies that have glucosidase activity [16] and DNA inserts in plasmids were sequenced on ABI 377 DNA sequencer.

2.3. Sequence and structure analysis of the overlapping gene cluster

The open reading frames (ORFs) of DNA sequences were identified using ORF-finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted ORFs were submitted to the NCBI website (http://blast.ncbi.nlm.nih.gov) for similarity analysis and homology search. Structure-based sequence alignment was performed by using CLUSTAL W (www.phylogeny.fr) [17]. Protein sequence analyses were performed at the Expasy proteomics server (http://www.expasy.org/) [18].

2.4. Expression of the overlapping gene cluster and individual genes and purification of recombinant proteins

The individual gene *glu1923*, *glu1392*, and the overlapping gene cluster *glu3311* were both amplified via PCR and the reaction conditions were: 98 °C, 5 min; 98 °C, 10 s; 55 °C, 10 s; 72 °C, 15 s, repeated for 30 cycles; 72 °C, 5 min. The PCR products were digested using *Kpn*I/*Eco*RI (for Glu1923), *Bam*HI/*Hin*dIII (for Glu1392), and *Kpn*I/*Hin*dIII (for Glu3311), and then ligated to expression vector pET-32a (+). The recombinant vectors were transformed into *E. coli* BL21 (DE3), and the cells were plated on LB agar containing 100 μg/ml ampicillin. The transformants were grown in a 100-ml flask containing 10 ml LB medium (a final concentration of 100 μg/ml ampicillin) at 37 °C until an OD_{600} reached to 1.0. Next, induction was initiated by adding IPTG to a final concentration of 1.0 mM and the cultures were incubated at 30 °C for 8 h (for *glu1392* and *glu3311* expression) or at 16 °C for 12 h (for *glu1923* expression) with shaking at 200 rpm. To purify expression products of *glu1392* and *glu3311*, cells were collected by centrifugation (8000 × g for 10 min at 4 °C). Finally, purification of the recombinant proteins was performed using a His·Bind Purification Kit (Novagen) according to the product manual. To purify recombinant Glu1923, the induced cells were resuspended in PBS buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO4 10 mM, KH_2PO4 1.76 mM, pH 7.4) with 0.1% *n*-dodecyl-β-D-maltopyranoside (DDM) for protein solubilization. Then, the cells were harvested by centrifugation (8000 × *g* for 10 min at 4 °C) and sonicated with EDTA-lysozyme treatment. Ultimately, purification of the recombinant proteins was performed using a His·Bind Purification Kit (Novagen) according to the product manual.

2.5. SDS-PAGE and western blotting analyses of target proteins

Molecular mass of the denatured proteins was determined by SDS-PAGE. Proteins were visualized following Coomassie brilliant blue G-250 staining. The products of the intact overlapping gene *glu*3111 were also analyzed by Native-PAGE. In addition, western blot analysis of target proteins was performed according to the standard protocol. Briefly, the recombinant proteins were separated on a 12% SDS-PAGE gel, which was semi-dry transferred at 15 V for 30 min to 0.45 mm PVDF membrane (Bio-Rad, USA), immunoblotted with anti-His Tag mouse Monoclonal antibody (dilution to 1:5000) (BOSTER, China). Next, the IgG goat anti-mouse antibody conjugated with HRP (Horseradish peroxidase) was used as a secondary antibody (dilution to 1:5000), with a DAB (Diaminobenzidine) Kit used for visualization of the protein band.

2.6. Hydrolysis activity of the recombinant proteins toward different substrates

The recombinant *E. coli* strains expressing *glu1923*, *glu1392*, and the overlapping gene cluster (*glu3311*) respectively were plated on LB agar plate containing 1.0 mM IPTG, 0.1% esculin hydrate, 0.25% of ferric ammonium citrate and 100 μg/ml ampicillin to test their hydrolysis activity according to the existence of black halo around corresponding colonies or not. Simultaneously, the recombinant expression vectors carrying different antibiotic resistance including pET-32a-glu1392 (with Ampicillin resistance) and pET-28a-glu1923 (with kanamycin resistance), were co-transformed to *E. coli* BL21 (DE3). Then, the co-transformants were also streaked on esculin plate for testing hydrolysis activity in vivo. In addition, abovementioned four recombinant strains were cultured on M9 minimal medium containing cellobiose, maltose, or trehalose for hydrolysis activity assay. M9 minimal medium was prepared according to the protocol described by Charusanti et al. [19].

2.7. Interaction assay in vivo between Glu1392 and Glu1923 by yeast two hybrid method

To test the interactions between Glu1392 and Glu1923, the bait vector pGBKT7 containing *glu1392* and the prey vector pGADT7 inserted *glu1923*, were transferred into auxotrophic yeast strain AH109 by LiAc method according to the instruction manual of Clontech (Yeast Protocols Handbook, Protocol No. PT3024-1). Then, the positive transformants were selected from a nutritionally selective plate deficient in tryptophan and leucine (SD/-Leu-Trp) and streaked on SD/-Trp/-Leu/-His/-Ade plates to further detect the protein interaction. The plates were incubated for 2–3 days at 30 °C for β-galactosidase activity assay via colony lift method [20]. In addition, to find out which domain of Glu1923 involved in the interaction with Glu1392, three deletion mutants containing different domains of Glu1923 were constructed by PCR and then ligated to the prey vector pGADT7, respectively. The first deletion mutant have the PTS-IIA domain deleted, this deletion mutant consisted of the first 464 residues at the N-terminal region of Glu*1923* . The second deletion mutant contained the PTS-IIC domain and was composed of 125–464 residues at the central region of Glu1923. The third deletion mutant included the PTS-IIC&IIA domain of Glu1923, which contained 125–641 residues at the C-terminal region of Glu1923. The prey vectors containing three deletion mutants were respectively transformed to auxotrophic yeast strain AH109 that harboring bait vectors pGBKT7-*glu1392* for the interaction assay.

2.8. Enzymatic activity and substrate specificity assay

The enzymatic activity of Glu1392, Glu1923, and the mixture of Glu1392 and Glu1923 toward aryl-glucoside substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma) were measured by following *p*-nitrophenol (*p*NP) release at 405 nm in a 96-well microtiter format based method [21]. Each enzyme solution contained 150 ng of purified enzyme, and the mixed enzyme solution was composed of purified Glu1392 (150 ng) and Glu1923 (150 ng). The 5 μl (30 ug/ml) of enzyme solution was mixed with 0.1 ml of 5 mM *p*NPG solution in 0.1 M citrate phosphate buffer at pH 8.0. After incubation at 50 °C for 20 min, the reaction was subsequently terminated by adding 200 μl of 1.0 M Na_2CO_3. The *p*NP released from the mixture was measured in a microplate reader (Thermo Scientific Inc., U.S.A). One unit of enzyme activity toward *p*NPG was defined as the amount of enzyme needed to produce 1 μmol of *p*NP per minute under the assay condition. Each experiment was repeated for three times with duplicate replicates, and Duncan's test ($p < 0.05$, ANOVA) was used for significant difference analysis. Substrate specificity of the enzymes toward natural substrates including oligosaccharide cellobiose, maltose and trehalose was determined by incubating the purified enzyme with respective substrates (5 mM) in 0.1 M citrate phosphate buffer at 50 °C for 30 min and measuring the released glucose by the standard procedures [22].