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Screening and identification of endophytes from the seeds of *Castonospermum austral* producing alpha-glucosidase inhibitors

Ren Peng*, Jianqiao Zhu, Yanchao Zhou, Menglu Chen, Jing Fu
College of Life Science, Jiangxi Normal University, Nanchang 330022, (CHINA)
E-mail : renpeng23@hotmail.com

ABSTRACT

Alpha-glucosidase inhibitors are potent inhibitors of HIV and HCV. Endophytes from the seeds of *Castonospermum austral* producing alpha-glucosidase inhibitors with high activity were screened. The strain which produced alpha-glucosidase inhibitors with the highest inhibition rate of 23.21% was obtained. The strain was identified as *Bacillus* cereus according to 16S ribosomal DNA sequence, morphological, physiological and biochemical features.

KEYWORDS

Alpha-glucosidase inhibitor;
Endophyte;
Castonospermum austral;
Screening;
Bacillus sp.

INTRODUCTION

Acquired immunodeficiency syndrome and hepatitis C have seriously threatened human health. Since the beginning of the epidemic, acquired immunodeficiency syndrome has accounted for about 35 million people direct and indirect deaths[1]. Furthermore, more than 350 000 people die every year from hepatitis C-related liver diseases[2]. Human immunodeficiency virus (HIV) and hepatitis C virus (HCV) are viruses which cause acquired immunodeficiency syndrome and hepatitis C, respectively. Alpha-glucosidase inhibitors such as castanospermine and celgosivir (Castanospermine-6-O-butyrate) are potent inhibitors of HIV and HCV[3,4]. Thus the discovery of novel alpha-glucosidase inhibitors is a focus of medicine development. Alpha-glucosidase inhibitors are obtained from microbial metabolites, plant extraction and chemical synthesis. Onose S *et al.* successfully isolated a *Bacillus* species producing alpha-glycosidase inhibitor[5]. Vichasilp C *et al.* optimized extraction of 1-deoxynojirimycin (a kind of alpha-glycosidase inhibitor) from mulberry leaves by using response surface methodology[6]. Cronin L *et al.* developed a new synthetic approach from a 5-C-methoxypyranosyl azide to castanospermine[7].

Endophytes constitute an important source of natural products, which exhibit all kinds of biological activities such as antibiotic, enzyme inhibition, antioxidant and anticancer effects[8-10]. However, there are few reports on the production of alpha-glycosidase inhibitor from endophytes. Nimal Christhudas I.V. S. *et al.* isolated an endophyte (*Streptomyces* sp. Loyola UGC) from *Datura stramonium* L, the methanolic extract of which showed remarkable inhibition of α-glucosidase (IC_{50} 730.21 ± 1.33 μg/ml)[11]. Artanti N *et al.* reported that endophytic fungi isolated from *T. sumatrana* exhibited alpha-glucosidase inhibitory activities[12]. Due to the potent alpha-glycosidase inhibitors in *Castonospermum*

austral, the present paper aimed to isolate endophytes from *Castonospermum austral* seed. The identification of endophytes was also described.

MATERIALS AND METHODS

Surface sterilization of the seeds of *Castonospermum austral*

Fresh seeds of *Castonospermum austral* were washed with tap water, and then were dried in sterile condition. Surface sterilization of *Castonospermum austral* seeds was carried out by immersing seeds in 99% (v/v) ethanol for 1 min, 3.125% NaOCl for 6min and 99% (v/v) ethanol for 30s again. The sterilized seeds were rinsed five times with saline water and blotted by blotting paper. 50μl aliquots of the final washing solution were plated on NA medium. A lack of colony confirmed the success of surface sterilization of *Castonospermum austral* seeds.

Isolation of endophyte from the seeds of *Castonospermum austral*

The sterilized seeds were cut into pieces and grinded in a sterilized mortar containing 10ml of saline water, and then rest for 15min. 50μl aliquots of supernant were transferred to Gause 1 cultural medium plate, followed by incubation at 28°C for 7~14d. The colonies were isolated, purified and deposited on PDA medium.

The first screening

The isolated endophytes were inoculated into fermentation medium (starch 1.0%, glucose 1.0%, peptone 0.8%, NaCl 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7.0~7.2) and incubated at 28 °C (120 rev/min) for 72 h. The culture was centrifuged and the resultant supernatant was mixed with amylase. The mixture was incubated at 37 °C for 20min. At the same time, Oxford cups were placed on the plates containing starch 1% and agar 2%. 200μl aliquots of the mixture solution were added into Oxford cups respectively and incubated at 37 °C for 12 h, and then the color was developed with KI-I_2. The amylase inhibitory activity was estimated based on the size of clear zone.

The second screening

The candidates with high amylase inhibitory activity were selected for assaying alpha-glucosidase inhibitory activity. Alpha-glucosidase inhibitory assay was conducted according to Kim *et al*. (2004) with a minor modification[13]. Sample (0.2 mL), 0.1 mL of enzyme solution and 1.0 mL of 50 mM phosphate buffer at pH 6.8 were added to a test tube, and then incubated for 10 min at 37°C. The reaction was initiated by addition of 0.1ml of 116 mM pNPG (p-Nitrophenyl α-D-glucopyranoside), followed by 10 min incubation at 37°C. The reaction was stopped by addition of 2.0 mL of 100 mM Na_2CO_3. The absorbance of p-nitrophenol released from PNPG at 405nm was measured with a spectrophotometer. Phosphate buffer in place of enzyme and sample was used as the experimental blank. Due to the effects of pigment in sample, the background absorption was also measured by displacing sample with phosphate buffer. Alpha-glucosidase inhibitory rate = [absorption in the presence of alpha-glucosidase-(absorption in the presence of alpha-glucosidase and sample- background absorption)]/ absorption in the presence of alpha-glucosidase*100%

Identification of the isolated endophyte and phylogenetic tree analysis

The endophyte was identified based on the method described in "Bergey's Manual of Determinative Bacteriology"[14] and analysis of 16S rDNA sequence. The genomic DNA was extracted using TIANamp Bacteria DNA Kit according to the manual of manufacturer.

16S rRNA gene of the endophyte was amplified using the universal forward primer (5' -AGAGTTTGATCCTGGCTCAG-3') and reverse one (5' -ACGGTTACCTTGT TACGACTT-3')[15]. The amplification was done by initial denaturation at 95 °C for 3 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s and final extension at 72 °C for 5 min. The PCR product was checked by agarose gel electrophoresis and recovered from the gel by using the quick gel extraction kit. The purified DNA fragment was sequenced using an automated sequencer.

RESULTS AND DISCUSSION

Screening of endophyte from *Castonospermum austral* seed

Generally, Saccharide hydrolase inhibitors have several cooperative inhibition effects such as alpha -glucosidase and alpha-amylase inhibition[16]. In the first

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screening procedure, if alpha-amylase completely degraded starch into monosaccharide, the color don't developed with KI-I_2, thus a clear zone was formed near Oxford cups. A smaller clear zone indicated higher amylase inhibitory activity. The candidates with high amylase inhibitory activity were selected for assaying alpha -glucosidase inhibitory activity in the second screening procedure. The size of a clear zone was easily measured and the price of starch is far lower than that of pNPG, thus the combination of the first screening and the second screening decreased labor and lowered cost in the experiments. TABLE 1 showed the screening result of endophytes from *Castonospermum austral* seeds. Nine strains were selected based on the size of clear zone formed near Oxford cups. Among these strains, strain No.10 had the smallest clear zone and its alpha-glucosidase inhibitory rate was 13.87%. However, strain No.12 showed the highest alpha-glucosidase inhibitory rate (23.21%) with the clear zone of 10 mm. The date suggested that amylase inhibitory activity alpha-glucosidase inhibitory activity was loosely related.

TABLE 1 : Screening results of endophyte from *Castonospermum austral* seed

Strain	Control	No.1	No.2	No.6	No.7	No.8	No.9	No.10	No.11	No.12
Size of a clear zone (mm)	17	11	12	10	11	10	11	6	12	10
Alpha-glucosidase inhibitory rate (%)	0	13.70	3.76	0.62	1.95	0.98	5.30	13.87	14.25	23.21

IDENTIFICATION OF THE ISOLATED STRAIN NO.12

Strain No.12 had the highest alpha-glucosidase inhibitory, thus the strain was used for identification. Strain No.12 is Gram positive, motility positive and facultative aerobic bacteria. The strain is rod-shaped and capsule deficient. It was able to produce spore. It can produce acids in broth containing glucose when cultured in anaerobic condition, but can not produce acids in the presence of arabinose, mannitol and xylose. It was not aerogenesis when degrading carbohydrate. It also could secret catalase. The strain colony was large, surface irregularity and flat.

To further confirm the identity of the isolate, PCR amplification and sequencing of the 16S rRNA gene were done. 16S ribosomal RNA gene of the strain was submitted to GenBank under the accession number of KF668650. On the basis of comparison of 16S ribosomal DNA sequences of strain No.12 and other *Bacillus* species available in GenBank, strain No.12 had homology (99%) with *B.cereus* Y1 (the accession number of KC247316.1 in GenBank). According to the Bergey's manual of systematic bacteriology and 16S ribosomal RNA sequence of the strain, it was tentatively named as *Bacillus cereus* SD6.

Until now, Some microorganisms including *Streptoverticillium verticillus*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Streptomyces nigrifaciens* and *Streptomyces sp.* were found to produce alpha-glucosidase inhibitors[17-20]. Furthermore, the metabolites form endocytes such as *Streptomyces* sp. Loyola UGC, *Aspergillus terreus* and *Aspergillus aculeatus* were potent alpha-glucosidase inhibitors[11,21,22]. To our knowledge, *Bacillus cereus* from *Castonospermum austral* seeds was reported to produce alpha-glucosidase inhibitor for the first time. The optimization of alpha-glucosidase inhibitor production from the strain and the purification and characterization of alpha-glucosidase inhibitor are under investigation.

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