Cloning and functional characterization of the β-amyrin synthase gene from *Bupleurum chinense*

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Abstract

*Bupleurum chinense* DC., a source of the traditional Chinese medicine *Bupleuri Radix*, is rich in triterpenoid saikosaponins with high pharmacological activities. The enzyme β-amyrin synthase (BAS), which converts the precursor 2,3-oxidosqualene to produce the triterpene skeleton, is crucial for the biosynthesis of triterpenoid saponins. In this study, we cloned the full-length sequence of the BAS gene from *B. chinense*, conducted a bioinformatics analysis, and expressed it in *Saccharomyces cerevisiae* to investigate its function. The cDNA of β-amyrin synthase (*BcBAS*, GenBank accession number: MN186093) cloned from aseptic seedlings of *B. chinense* was 2 307 bp with a 2 286 bp open reading frame coding for 761 amino acids. Phylogenetic analysis suggests that the BcBAS protein was closely related to the BAS proteins from *Panax ginseng* and *Betula platyphylla* as chromatography mass spectrometry analysis showed that the enzymatic product was indeed β-amyrin, the precursor of oleanane type triterpenes. Overall, our findings lay the foundation for in-depth analysis of the biosynthesis pathway of saikosaponins.

Additional key words: *Saccharomyces cerevisiae*, saikosaponins.

Introduction

*Bupleuri Radix*, a traditional Chinese herbal medicine, is widely used in China, Japan, South Korea, and countries with the same cultural influence. The most popular source of it, *B. chinense*, is rich in triterpenoid saikosaponins (Wang et al. 2018). Modern pharmacological research shows that saikosaponins isolated from *B. chinense* such as saikosaponin a (SSa), saikosaponin c (SSc), and saikosaponin d (SSd) have significant antiinflammatory (Chen et al. 2018), antidepressant (Sun et al. 2018), antitumor (Feng et al. 2017), hepatoprotective (Ashour and Wink 2011) and immunomodulatory (Yen et al. 2010) effects, with a broad application foreground and high development potential.

Saikosaponin is an oleanane type triterpene composed of isoprene units. Its biosynthesis pathway is consistent with the general rule of triterpenoid biosynthesis (Haralampidis et al. 2002), which is divided into three stages: 1) initial stage: synthesis of the precursors sopentenyl pyrophosphate and dimethylallyl diphosphate; 2) skeleton stage: squalene epoxidase (SE) catalyzes the production of 2,3-oxidosqualene promoting the formation of the oleanane type triterpene skeleton; 3) modification stage: variety of saikosaponins via modification by cytochrome P450s and UDP-glycosyltransferases (Lin et al. 2013). The enzyme β-amyrin synthase (BAS), which belongs to the oxidosqualene cyclase (OSC) superfamily, plays an important role in the biosynthesis of triterpenoid saponins. It converts the precursor 2,3-oxidosqualene to produce β-amyrin, the basic triterpene skeleton (Yan et al. 2015). The characterization of BAS is crucial for the functional analysis of downstream key enzyme genes cytochrome P450s (Jo et al. 2017) and UDP-glycosyltransferases (Xu et al. 2016).

In our research, we used the comparative transcriptome data of *B. chinense* to obtain the full-length sequence of the BAS gene, after which bioinformatics analysis was performed. The gene was subsequently introduced into *Saccharomyces cerevisiae* for heterologous expression, gas chromatography-mass spectrometry (GC-MS) was used to determine the product for functional verification. This research contributes to the analysis of saikosaponin biosynthesis.

Materials and methods

Plants: The aseptic seedlings of *Bupleurum chinense* DC. were collected in Fangshan District, Beijing, China

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Abbreviation: BAS - β-amyrin synthase; CAS - cycloartenol synthase; GC-MS - gas chromatography-mass spectrometry; LUS - lupeol synthase; OSC - oxidosqualene cyclase; SE - squalene epoxidase.

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Extraction of RNA and reverse transcription: Total RNA was extracted from the aseptic seedlings of *B. chinense* according to the instruction manual of the *Easpe Super* total RNA extraction kit (Promega, Madison, WI, USA), and reverse-transcribed using a SMARTer™ RACE 5′/3′ kit (TaKaRa, Tokyo, Japan).

Bioinformatic analysis: The full-length unigene annotated as BAS was selected from the *B. chinense* transcriptome and Primer Premier 5.0 software was used to design specific primers (Zhang and Gao 2004). The cdNA served as a template for PCR amplification using 2x Phusion Master Mix (NEB, Ipswich, MA, USA). The program encompassed denaturation at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 55 °C for 15 s and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The PCR product was subcloned into the cloning vector pEASY-Blunt (TransGen Biotech, Beijing, China), and then transformed into E. coli Trans1-T1 competent cells (TransGen Biotech). Positive clones were screened by colony PCR and confirmed by sequencing.

Functional characterization in *Saccharomyces cerevisiae*: The open reading frame of *BcBAS* was ligated into a double-digested ( BamHI and XhoI restriction enzymes) pYES2 expression vector (Invitrogen, Carlsbad, USA) using the Seamless cloning kit (Beyotime, Beijing, China). The recombinant plasmid was named pYES2-BcBAS and transformed into lanosterol synthase deficient *Saccharomyces cerevisiae* by Frozen-EZ Yeast Transformation II™ kit (Zymo Research, Los Angeles, CA, USA). The empty pYES2 plasmid was used as a control. Positive transformants were seeded into 20 cm² of SC-U dropout medium (URA minus medium 8 g dm⁻³ with 20 g dm⁻³ glucose) and cultivated at 30 °C for 48 h. Then, the cells were collected by centrifugation (3 000 g, 4 min), resuspended in 20 cm² of SC-U induction medium and grown at 30 °C for 48 h (Lu et al. 2018).

**Results**

The cdNA of *BcBAS* was 2 307 bp, with a 2 286 bp (11-2296 bp) open reading frame encoding 761 amino acids. The molecular mass of BcBAS protein was 87.527 kDa and the theoretical pl was 6.05. The total number of negatively charged residues (Asp + Glu) was 89 and positively charged residues (Arg + Lys) 76. According to calculations, the instability index (II) was 44.35, the aliphatic index was 76.69, and grand average of hydropathicity (GRAVY) value of the protein was -0.328.

The amino acid sequence of *BcBAS* showed high similarity with the reported BAS protein sequences from other plants found in BLAST search. It had 83.38, 83, 81.6, and 80.5 % identity to the enzymes from *Betula platyphylla* (Q8W3Z1.1), *Bruguiera gymnorrhiza* (A8CDT2.1), *Panax ginseng* (Q082140.1), and *Glycyrrhiza glabra* (Q9MB42.1), respectively. Multiple sequence alignment showed that *BcBAS* protein contained conserved regions typical of the OSC gene superfamily, such as the MWCYCR motif, DCTAE motif, and QW (QXXXGXXW or QXGXXXW) motifs (Fig. 1).

The phylogenetic tree was constructed based on known OSC protein sequences from other plants downloaded from the National Center for Biotechnology Information. These OSC protein sequences were grouped into three main branches: BAS, lupeol synthase (LUS), and cycloartenol synthase (CAS). *BcBAS* clustered with other BAS proteins, showing a close relationship, while being far from the LUS and CAS proteins (Fig. 2).

The SOPMA analysis showed that the secondary structure of *BcBAS* protein encompassed 341 residues in α-helices (44.81 %), 100 residues in extended strands (13.14 %), 55 in β-turns (7.23 %), and 265 in random coils (34.82 %). The BcBAS protein had no transmembrane regions according to TMHMM analysis. The human
Fig. 1. The multiple sequence alignment of β-amyrin synthase proteins from different plant species. Four QXXXGXW motifs and one QXXGXXXW motif are shown in red boxes, one MWCYCR motif is shown in a green box, and one DCTAE motif is shown in a blue box.
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The CAS protein (1w6j.1.A), which shares 44.16% sequence similarity with BcBAS, was used as template. The amino acid residues 69-756 were utilized in the modeling process (Fig. 3).

The BcBAS coding sequence was ligated into the pYES2 yeast expression vector, namely pYES2-BcBAS, and the positive colony was verified (Fig. 4). The plasmid pYES2-BcBAS was transformed into Saccharomyces cerevisiae and the empty pYES2 plasmid was used as a control. The enzymatic product of the heterologously expressed BcBAS protein was detected by GC-MS. A single new compound was found in the extract of cells expressing BcBAS. It was confirmed as β-amyrin by comparing the retention time and ion fragment information with the authentic standard (Figs. 1 and 2 Suppl.). There were three major characteristic ion fragments of β-amyrin at m/z 189.0, 203.0, and 218.0 (Fig. 2 Suppl.).

Discussion

Triterpenoid saponins are a diverse class of plant secondary metabolites with significant pharmacological activities, including anti-tumor, anti-viral, and anti-inflammatory effects. Tetracyclic and pentacyclic triterpenes, whose main representatives are dammarane and oleanane, respectively, are two major categories of triterpenoid saponins. The manufacture of triterpenoid saponins mainly relies on extraction from plants, synthesis through chemical modification of natural raw materials, and biosynthesis (Augustin et al. 2011). However, the traditional extraction processes have low yields and waste large amounts of...
BcBAS. Therefore, the
co-expression of the
key enzyme in its synthesis pathway. Kushiro
et al. (1998) successfully cloned the full-length cDNA sequence of
BAS from ginseng hairy roots in 1998, and observed the
production of β-amyrin in mutant yeast lacking lanosterol
synthase for the first time. Liu et al. (2014) studied the
effect of the co-expression of the BAS and SQS genes on
the metabolic pathway of glycyrrhizic acid, discovering
that the co-expression contributed to the accumulation of
the metabolite β-amyrin. Sun et al. (2018) cloned the BAS
gene from Hedera helix for the first time and verified the
positive correlation between HhBAS expression detected by
RT-qPCR and the content of triterpenoid saponin.

In this study, we successfully cloned the BAS gene
from aseptic seedlings of Bupleurum chinense. The
bioinformatics analysis confirmed that the gene was a
member of the oxidosqualene cyclase family with more
than 80 % sequence similarity with other known BAS
proteins, containing the conserved DCTAE motif associated
with substrate binding, as well as QW motifs that stabilize
carbocations in the cyclization reaction (Sikkema et al.
1994). Phylogenetic analysis revealed that BcBAS was
closely related to B. gymnorhiza and B. platyphylla BAS.
We assumed that BcBAS could convert 2,3-oxidosqualene
to produce β-amyrin in B. chinense. Therefore, the
heterologous yeast expression of this gene was performed
to confirm our idea. The enzymatic product was detected
and confirmed to be β-amyrin by GC-MS, completing the
functional verification of the BAS gene. What's more,
the 3D modeling structure of BcBAS protein could be used
in molecular docking to predict enzyme active sites, which
laid a foundation for the modification of enzyme activity
by site-directed mutation technique in the future. In a
nutshell, the characterization of BcBAS gene could give a
new potential genetic engineering approach to improve the
accumulation of saikosaponins by gene over-expression or
other methods in engineered yeast and plant systems. This
study could greatly promote the analysis and regulation of
saikosaponin biosynthesis pathway.

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