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Analysis of acetohydroxyacid synthase variants from branched-chain amino acids-producing strains and their effects on the synthesis of branched-chain amino acids in *Corynebacterium glutamicum*

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ABSTRACT

Acetohydroxy acid synthase (AHAS) controls carbon flux through the branch point and determines the relative rates of the synthesis of isoleucine, valine and leucine, respectively. However, it is strongly regulated by its end products. In this study, we characterized AHAS variants from five branched-chain amino acids-producing strains. Amino acid substitution occurred in both catalytic subunit and regulatory subunit. Interestingly, AHAS variants reduced sensitivity to feedback inhibition by branched-chain amino acids (BCAAs). Although AHAS with amino acid substitution in regulatory subunit showed higher resistance, amino acid substitution in catalytic subunit could also endow AHAS with resistance to feedback inhibition. In addition, AHAS variants from V2 and L5 displayed about 1.4-fold higher specific activity compared to other AHAS variants. On the other hand, AHAS variant from V1 exhibited the highest resistance to BCAAs, 87% of original activity left even in the presence of 10 mM BCAAs. Recombinant *Corynebacterium glutamicum* strains were further constructed to investigate the effects of expressing AHAS variants on the synthesis of BCAAs and alanine (main by-product) in *C. glutamicum*. BCAAs production was increased with the increase of resistance to feedback inhibition, although valine showed a significant increase. For instance, C. g-1BN could accumulate 9.51 g/l valine, 0.450 g/l leucine and 0.180 g/l isoleucine, and alanine was reduced to 0.477 g/l. These AHAS variants are important for further improving performance of BCAAs-producing strain.

Introduction

In *Corynebacterium glutamicum*, acetohydroxy acid synthase (AHAS, EC 2.2.1.6) catalyzes the first common reaction in the synthesis of BCAAs. It catalyzes decarboxylation of two molecules of pyruvate to form one molecule of 2-acetolactate (a precursor of valine and leucine), or decarboxylation of one molecule of pyruvate with one molecule of 2-ketobutyrate to form one molecule of acetohydroxy butyrate (a precursor of isoleucine) [1]. AHAS[1] is composed of large subunit and small subunit [2]. The large subunit (encoded by *ilvB*) is responsible for catalytic activity, while the small subunit (encoded by *ilvN*) mediates its regulation of feedback inhibition by the end products. In fact, AHAS is subjected to feedback inhibition by BCAAs in *C. glutamicum*. The concentrations of BCAAs required for 50% inhibition (IC_{50}) are 0.9 mM for valine, 3.1 mM for isoleucine and 6.0 mM for leucine [3]. Valine is the strongest inhibitor, and AHAS might be mainly regulated by valine. In *Escherichia coli*, AHAS I, AHAS II and AHAS III were found, differing in sequence, size and regulation of enzymatic activity [4]. These isoenzymes are also composed of two different polypeptides, large subunit (catalytic) and small subunit (regulatory). Interestingly, AHAS I and AHAS III are inhibited by valine, while AHAS II is insensitive to valine. Their preferences for ketobutyrate are also very different. Similar to *E. coli* AHAS II and AHAS III, *C. glutamicum* AHAS showed higher affinity to ketobutyrate than to pyruvate. In the presence of ketobutyrate, the synthesis of isoleucine is therefore preferred, while the synthesis of valine and leucine are reduced.

BCAAs (valine, isoleucine and leucine) are hydrophobic amino acids, due to their aliphatic chain with a branched alkyl group. BCAAs can be synthesized in plants, bacteria and fungi, but not by animals. Therefore, BCAAs are essential for vertebrates who must receive them from their diet [5]. Hence BCAAs are widely used for food ingredients, feed additives, nutraceuticals and phar-

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[1] *Abbreviations used:* AHAS, acetohydroxy acid synthase; BCAAs, branched-chain amino acids; LB, Luria–Bertani.

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maceuticals [6]. However, BCAAs production has not been developed as well as lysine and glutamic acid [7,8], due to multivalent regulation and longer synthesis pathway. Therefore, it is necessary to improve the performance of BCAAs-producing strains. Recently, increased understanding of the biosynthetic pathway for BCAAs has been gleaned from molecular breeding of *C. glutamicum*. This pathway can be divided into four reactions, which are catalyzed by AHAS, acetohydroxyacid isomeroreductase, dihydroxyacid dehydratase, transaminase, respectively [9]. AHAS is regulated by the end products in synthesis pathway [3]. Since acetohydroxy butyrate (a precursor of isoleucine) and 2-acetolactate (a precursor of valine and leucine) are formed in a parallel way by AHAS, it controls the flux through this branch point and determines the relative rates of the synthesis of isoleucine, valine and leucine, respectively. Therefore, AHAS is crucial for the synthesis of BCAAs and is an ideal target for genetic engineering.

AHAS has attracted a great attention since it is demonstrated to be a potential target for genetic engineering [1]. Since AHAS exhibits much higher affinity to ketobutyrate than to pyruvate, it plays a key role in determining relative the flux to different end products [10]. Several BCAAs-producing strains were developed by regulation of AHAS activity. For instance, co-expression of feedback resistant AHAS and threonine dehydratase could lead to high yield of isoleucine [11], while expressing feedback-resistant AHAS with acetohydroxyacid isomeroreductase or transaminase could significantly increase valine production [12–14]. On the other hand, deletion of the C-terminal domain of small subunit of AHAS could increase lysine production by reducing flux to the synthesis of BCAAs [15]. However, there was few report that AHAS is used to increase leucine production, might due to its longer synthesis pathway. From the aforementioned, AHAS is important for further improving the performance of BCAAs-producing strain.

With the advent of recombinant DNA techniques, genetic engineering strains of *C. glutamicum* can be constructed to enhance carbon flux to BCAAs by over-expressing AHAS. However, detailed amino acid substitution in AHAS and their influences of holoenzyme activity were seldom reported. In this study, AHAS from five BCAAs-producing strains were purified and analyzed. Recombinant *C. glutamicum* strains were further constructed to evaluate the effects on the synthesis of BCAAs by over-expressing AHAS variants.

Materials and methods

Strains and media

The bacterial strains and plasmids used in this study are listed in Table S1 in the Supplemental material.

V1, V2 and V3 are valine-producing strains. I4 is an isoleucine-producing strain, while L5 is used to produce leucine. *E. coli* DH5α was used as the host cell for propagating plasmids and *E. coli* BL21 (DE3) was used as the host cell for protein expression. The *E. coli* strain was cultivated in Luria–Bertani (LB) medium [16] at 37 °C and 200 rpm. The *C. glutamicum* strain was grown in LBG medium (LB containing 5 g/l glucose) at 30 °C. When appropriate, kanamycin (30 μg/ml for *C. glutamicum*, 50 μg/ml for *E. coli*) and 0.5 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) were added to the medium.

LBG medium (LB containing 5 g/l glucose) was used as seed culture medium. The fermentation medium was composed of 90 g/l glucose, 35 g/l $(NH_4)_2SO_4$, 20 g/l corn steep liquor, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 10 g/l $CaCO_3$, 50 μg/l D-biotin, and 100 μg/l thiamine-HCl. D-biotin and thiamine-HCl were filter sterilized and aseptically added to the medium. The medium component of $CaCO_3$ was sterilized alone by dry heat sterilization for 90 min at 160 °C before added to the medium. Both media were adjusted to pH 7.5 with KOH.

Constructions of plasmids and strains

Routine methods of molecular cloning (PCR, DNA restriction and ligation) were carried out according to published method [17]. EcoR I and Hind III sites were introduced into *ilvBN*-F (5′-GGAATTCGAAAGGAGATATACCGTGAATGTGGCAGCTTC-3′) and *ilvBN*-R (5′-CCCAAGCTTTTAGATCTTGGCCGGAGC-3′), respectively. Genes of *ilvBN* were amplified with primers of *ilvBN*-F containing an SD sequence and *ilvBN*-R by PCR, using genomic DNA of BCAAs-producing strains as template. After digestion with EcoR I and Hind III, the final PCR product was ligated into vector pDXW-8 (similarly digested), resulting in recombinant plasmids (Table S1 in the Supplemental material). All plasmids were sequenced for identification and then transferred to *C. glutamicum* ATCC 14067 by electrotransformation method [18].

Site-directed mutagenesis

Site-directed mutagenesis was carried out essentially by the published method [19]. With primers Mu41-N-R, (containing a mutation, 5′-GCCGAGTGTTTCGGTCTTTGCAACCACGAGGGA-3′), and *ilvBN*-F, the upstream fragment (2062 bp) was amplified by PCR, using genomic DNA of *C. glutamicum* ATCC 14067 as template. With primers of Mu41-F (containing a mutation, 5′-TCCCTCGTGGTTGCAAAGACCGAAACACTCGGC-3′) and *ilvBN*-R, the downstream fragment (416 bp) was amplified by PCR, using genomics of *C. glutamicum* ATCC 14067 as template. The final fragment (2446 bp, S41V in small subunit) was amplified by overlap extension PCR, using primers of *ilvBN*-F and *ilvBN*-R. Similarly, G156V in small subunit was produced with primers of *ilvBN*-F, Mu156-N-R (containing a mutation, 5′-CGCGGTTGAGTGCAATCTGTACGGATTGGAT-3′), Mu156-N-F (containing a mutation, 5′-ATCCAATCCGTACAGATTGCACTCAACCGCG-3′) and *ilvBN*-R. Finally, the two overlapping fragments cleaved with EcoR I and Hind III were ligated into vector pDXW-8, respectively. Plasmids of pDXW-8-*ilvBN* (S41V) and pDXW-8-*ilvBN* (G156V) were sequenced for identification and transferred to *C. glutamicum* ATCC 14067 by electrotransformation method [18].

Protein purification

Protein purification was performed according to the published method with slight modification [11]. Genes of *ilvBN* were amplified by primers of *ilvBN*-F1 (containing EcoR I site, 5′-GGAATTCGTGAATGTGGCAGCTTC-3′) and *ilvBN*-R, using genomic DNA of BCAAs-producing strains as template. The PCR product was cloned into vector pET28a. Subsequently, pET28a harboring *ilvBN* was transformed to *E. coli* BL21 (DE3), resulting in recombinant strains (Table S1 in the Supplemental material). The recombinants *E. coli* BL21 (DE3) with different *ilvBN* were cultured in LB medium and induced by the addition of 0.5 mM IPTG to medium at OD_{600} = 0.6. Cells were collected by centrifugation and disrupted by sonication in buffer A (containing 0.5 M NaCl, 10 mM imidazole, 20 mM HEPES, pH 7.8). AHAS protein was purified by a nickel-chelating Ni–NTA affinity column under buffer B (containing 0.5 mM NaCl, 0.5 mM imidazole, 20 mM HEPES, pH 7.8) after they were desalted. Protein concentrations were determined following the published method [20].

Enzyme assays

AHAS activity was determined according to the published method with slight modification [3]. Enzyme assay was performed