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Purification of branched-chain amino acid aminotransferase from *Helicobacter pylori* NCTC 11637

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Summary. Branched-chain amino acid aminotransferase was purified by several column chromatographies from *Helicobacter pylori* NCTC 11637, and the N-terminal amino acid sequence was analyzed. The enzyme gene was sequenced based on a putative branched-chain amino acid aminotransferase gene, *ilvE* of *H. pylori* 26695, and the whole amino acid sequence was deduced from the nucleotide sequence. The enzyme existed in a homodimer with a calculated subunit molecular weight (MW) of 37,539 and an isoelectric point (pI) of 6.47. The enzyme showed high affinity to 2-oxoglutarate ($K_m = 0.085$ mM) and L-isoleucine ($K_m = 0.34$ mM), and V_{max} was 27.3 μmol/min/mg. The best substrate was found to be L-isoleucine followed by L-leucine and L-valine. No activity was shown toward the D-enantiomers of these amino acids. The optimal pH and temperature were pH 8.0 and 37 °C, respectively.

Keywords: Branched-chain amino acid aminotransferase – Purification – *Helicobacter pylori* – *ilvE* gene

Introduction

Helicobacter pylori is a microaerophilic gram-negative bacterium that lives in the mucous layer of the human stomach and duodenum (Warren and Marshall, 1983). The organism has been implicated in the pathogenesis of chronic gastritis, gastric and duodenal ulcers, and stomach cancer (Warren and Marshall, 1983; Marshall and Warren, 1984; Parsonnet et al., 1991; Wotherspoon et al., 1993), and it appears that more than half of the world's population is infected with the pathogen (Kelly, 1998). Branched-chain amino acid aminotransferase [EC 2.6.1.42] (BCAT) is an enzyme that converts isoleucine, leucine and valine into their corresponding 2-oxo acids, with 2-oxoglutarate as the amino group acceptor. The catabolism of branched-chain amino acids plays a major role in the formation of aroma and flavor compounds in fermented products. *Lactococcus lactis* (Yvon et al., 2000) is widely used as a starter microorganism in the dairy industry, and *Staphylococcus carnosus* (Madsen et al., 2002) has been used in meat products such as dried sausages. Apart from dairy strains, which are auxotrophic for branched-chain amino acids, the original role of BCAT was probably the biosynthesis of these amino acids since expression of the *ilvE* gene is repressed by isoleucine (Yvon et al., 2000). In the present study, we isolated BCAT from *H. pylori* cells, characterized its enzymic properties, and cloned the gene to read the amino acid sequence. Although the rate of incorporation of branched-chain amino acids into *H. pylori* cells is low (Stark et al., 1997), suggesting that these amino acids are not used for energy production in the bacteria, the findings of the present study will contribute to the knowledge of aminotransferase involved in amino acid metabolism in *H. pylori.* To the best of our knowledge, this is the first report on the purification of BCAT from organisms in the order *Helicobacter.*

Materials and methods

Culture

H. pylori NCTC 11637, the type strain, was cultured on Brucella agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) containing Campylobacter Selective Supplement (Oxoid, Hampshire, UK) and 5% horse serum (Sigma-Aldrich, St. Louis, MO, USA) under 10% CO_2 at 37 °C for 48 h. Cultured cells were harvested by centrifugation at 8,000 *g* for

20 min and suspended in 50 mM Na-phosphate buffer containing 0.9% NaCl (pH 7.0). The cells were washed once with the same buffer before being stored at −80 °C until use.

Enzyme purification from H. pylori

Harvested cells of *H. pylori* NCTC 11637 (wet weight, 120 g) were suspended in 240 ml of 50 mM Na-phosphate buffer (pH 7.0) containing 1 mM EDTA, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, and 50 μM pyridoxal 5′-phosphate (PLP) (EGPP), and were disrupted by passing them twice through a French press (Ohtake Works, Tokyo, Japan) at 135 megapascals. All of the purification procedures described hereafter were carried out at 4 °C. After the removal of unbroken cells by centrifugation at 8,000 *g* for 20 min, the supernatant solution was centrifuged at 140,000 *g* for 60 min. Subsequently, the protein in the supernatant was fractionated by ammonium sulfate with 45–80% saturation. The resultant precipitate was dissolved in 20 mM Tris-HCl buffer (pH 8.5) containing EGPP (buffer A), and dialyzed against buffer A before being applied to a DEAE Toyopearl column (25 i.d. × 420 mm; Tosoh, Tokyo, Japan) equilibrated with buffer A. The enzyme was eluted with a linear gradient of 0 to 300 mM NaCl in buffer A after washing the column with the same buffer. The active fractions were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing EGPP (buffer B) and 40% ammonium sulfate for 16 h. The dialysate was loaded onto a Phenyl Sepharose column (25 i.d. × 420 mm; Amersham, Piscataway, NJ, USA) previously equilibrated with the dialysis buffer. The enzyme fraction was eluted by applying buffer B containing a linear gradient of 40 to 0% ammonium sulfate. The active fractions eluted with 15 to 8% ammonium sulfate were combined and dialyzed against 20 mM Tris-HCl buffer (pH 8.5) containing EGPP (buffer C). The dialysate was then applied to a DEAE Sepharose column (16 i.d. × 100 mm; Amersham) equilibrated with buffer C, and was eluted with a linear gradient of 0 to 200 mM NaCl in buffer C. The active fraction was further purified with a ceramic hydroxyapatite column (12 i.d. × 150 mm; BioRad, Hercules, CA, USA) equilibrated with 10 mM K-phosphate buffer (pH 7.0) containing 50 μM PLP after being dialyzed against the same buffer. Elution was performed with a linear gradient of 0 to 400 mM K-phosphate. The fraction eluted with 140 to 200 mM K-phosphate was followed by gel filtration with a Shodex KW-2003 column (20 i.d. × 300 mm; Showa Denko, Tokyo, Japan), which had been equilibrated beforehand with 50 mM Na-phosphate buffer (pH 7.2) containing 50 μM PLP and 200 mM NaCl. Finally, an active preparation was obtained after hydrophobic chromatography with a Butyl Toyopearl column (8 i.d. × 75 mm; Tosoh) performed in the same manner as the above chromatography with the Phenyl Sepharose column. The preparation was used in electrophoresis and enzymatic characterization, after being dialyzed against buffer C and concentrated by ultrafiltration with a Vivaspin concentrator (Viva Science, Hanover, Germany).

Enzyme assay

The assay mixture contained 10 μg of enzyme sample, 10 mM L-isoleucine (or 10 mM other L-amino acids), 10 mM 2-oxoglutarate, 10 μM PLP, and 50 mM Tris-HCl buffer (pH 8.0) in a final volume of 100 μl. After incubation at 37 °C for 20 min, trichloroacetic acid was added to make a 5% solution to stop the reaction. The supernatant solution yielded by centrifugation at 8000 *g* for 10 min was analyzed for L-glutamate by high performance liquid chromatography (HPLC) (Nagata et al., 1992) as follows. The 200 μl supernatant was applied to a Dowex 50W-X8 (6 i.d. × 10 mm) column (Dow Chemical, Midland, MI., USA) to purify glutamate, and was eluted with 2 M NH_4OH. The eluate was dried in a centrifugal evaporator, and the resultant residue was dissolved in a small amount of water and modified with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA; Sigma-Aldrich). The FDAA glutamate was separated on a thin layer chromatography plate (Merck, Darmstadt, Germany) by developing with phenol-water (3:1, v/v), recovered from the plate, and analyzed by HPLC using a reversed-phase column (Nova-Pak C18, 3.9 i.d. × 150 mm; Waters, Milford, MA, USA), and a JASCO (Tokyo, Japan) or Hitachi (Tokyo, Japan) gradient HPLC system. Elution from the column was carried out with a linear gradient from 10 to 40% acetonitrile in 50 mM triethylamine phosphate buffer (pH 3.5) for 40 min at a flow rate of 1.0 ml/min at 25 °C. The eluate was monitored at 340 nm by a UV detector L-7400 (Hitachi). The amounts of L-glutamate were obtained based on the peak area given by a D-2500 Chromato-Integrator (Hitachi) and the standard curve.

Protein concentrations were determined by the method described by Lowry et al. (1951) using bovine serum albumin as a standard.

Electrophoresis and N-terminal protein sequencing

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 12.5% polyacrylamide gel, following the method described by Laemmli (1970). A low-molecular weight calibration kit for SDS-PAGE (Amersham) was used as molecular marker proteins. The proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride membrane (Sequi-Blot™; Bio-Rad) by a semi-dry system, and were stained with Coomassie brilliant blue. The bands were cut out and the N-terminal amino acid sequence of the enzyme was analyzed with an automated Edman degradation protein sequencer (PPSQ-21; Shimadzu, Kyoto, Japan).

Molecular weight estimation

A Shodex KW-2003 column (Showa Denko) was calibrated using a molecular weight marker kit (MW-Marker (HPLC); Oriental Yeast, Tokyo, Japan) to estimate the molecular weight of the native enzyme.

Cloning of the BCAT gene from H. pylori

The genomic DNA of *H. pylori* NCTC 11637 was isolated using a GenTLE yeast kit (Takara Shuzo, Kyoto, Japan). The oligonucleotide primers were designed for polymerase chain reaction (PCR): 5′-TGCGTA GAGTTTATGCGTTTCA-3′ as the forward primer and 5′-GCTTTGA GACTTCGCCCTTATT-3′ as the reverse primer based on the upstream and downstream regions of the *H. pylori* 26695 BCAT gene (DDBJ accession No. AE000603). The amplification of nucleotides between the two primers was performed by PCR using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) and the genome DNA of *H. pylori* NCTC 11637 as the template. The PCR program consisted of 30 cycles of a combination of denaturation at 94 °C for 20 sec, annealing at 55 °C for 20 sec, and extension at 65 °C for 70 sec. The amplified product was recovered from a 1% agarose gel and purified using a Suprec PCR kit (Takara Shuzo). The sequence of the purified product was analyzed by a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a Big Dye™ Terminator version 3.1 (Applied Biosystems).

Results and discussion

The N-terminal sequence of the purified *H. pylori* NCTC 11637 protein recovered from the SDS-PAGE gel (Fig. 1) was ADLENLDWKNLGFSYIKTD. This sequence coincided with the N-terminal amino acid sequence deduced from the nucleotide sequence of the *ilvE* gene of *H. pylori* NCTC 11637 except that the first amino acid, methionine residue, is deleted in the purified BCAT protein. The sequence of *H. pylori* NCTC 11637 *ilvE* gene was submitted to DDBJ, GenBank™ and BBI data banks (acces-

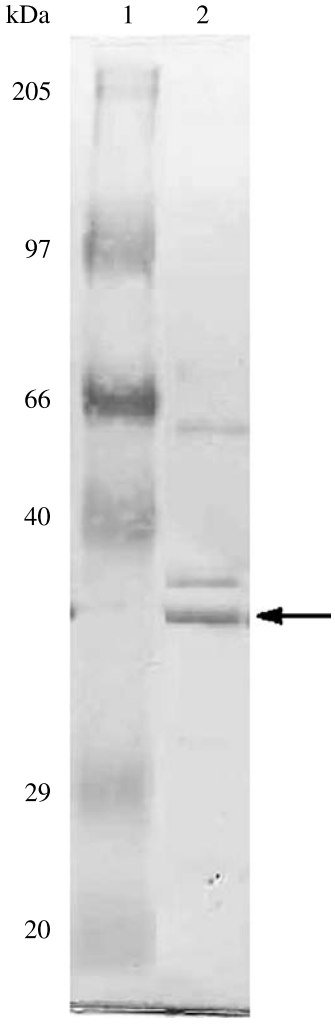

Fig. 1. SDS-PAGE (12.5% polyacrylamide) with Coomassie brilliant blue staining. *1* Marker proteins; *2* BCAT from *H. pylori* NCTC 11637 (arrow). The marker proteins are myosin (205,000), phosphorylase b (97,000), albumin (66,000), alcohol dehydrogenase (40,000), carbonic anhydrase (29,000), and trypsin inhibitor (20,000)

sion No. AB274528). The deduced protein is composed of 340 amino acid residues with a calculated molecular weight (MW) of 37,539, a similar value to 36,000 shown by the SDS-PAGE analysis (Fig. 1). The calculated isoelectric point (pI) of the deduced protein is 6.47. Since the MW of the purified BCAT obtained by gel filtration analysis was 68,500, the enzyme was found to exist in a homodimer, like BCAT isolated from *L. lactis* (Yvon et al., 2000), and unlike *Escherichia coli* BCAT, which exists in a hexamer (Inoue et al., 1988).

The level of DNA homology between the *ilvE* genes of *H. pylori* NCTC 11637 and *H. pylori* 26695 is 93%. The sequence homology of the protein with those of three *H. pylori* strains, 26695, HPAG1 and J99 was 97%, 96% and 95%, respectively, and with the other *Helicobacter* species, *H. acynonichis* and *H. hepaticus*, 90% and 26%, respectively. The amino acid sequence of the BCAT of *H. pylori* NCTC 11637 exhibited 61% and 59% homology with those of *Haemophylus influenzae* and *L. lactis*, respectively, while identity with other bacteria such as *Bacillus subtilis* (38%), *Mycobacterium tuberculosis* (37%) and *Escherichia coli* (31%) was low, as shown in parentheses. The BCATs of *H. influenzae*, *L. lactis*, *B. subtilis* and *M. tuberculosis* belong to aminotransferase family III, and fall into subfamily IIIa together with *H. pylori* (Yvon et al., 2000; Berger et al., 2003). Surprisingly, only 26% homology was found between the BCATs of *H. pylori* and *H. hepaticus*. The sequence identity between the BCATs of *H. hepaticus* and *E. coli* (41%) is higher than the identity between *H. hepaticus* and *H. pylori* (26%). Furthermore, the amino acid sequence of *B. subtilis* D-amino acid aminotransferase, a member of subfamily IIIb, showed a higher similarity to that of *H. hepaticus* BCAT (25%) than that of *H. pylori* BCAT

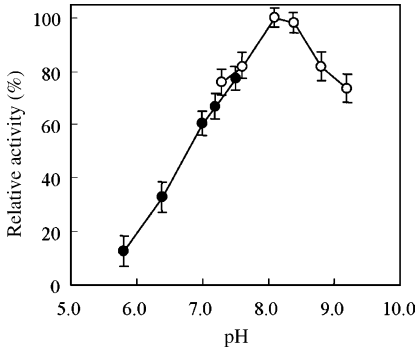

Fig. 2. pH dependency of BCAT activity. BCAT was purified from *H. pylori* NCTC 11637. The buffers used were 50 mM MOPS-NaOH (●) between pH 5.7 and 7.5, and 50 mM Tris-HCl (○) between pH 7.2 and 9.1, in order to obtain a good buffer action for each pH range. Each point represents the mean ± S.D. of triplicate assays

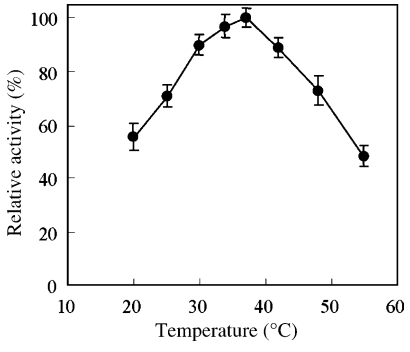

Fig. 3. Temperature dependency of BCAT activity. BCAT was purified from *H. pylori* NCTC 11637. The buffer used was 50 mM Tris-HCl (pH 8.0). Each point represents the mean ± S.D. of triplicate assays

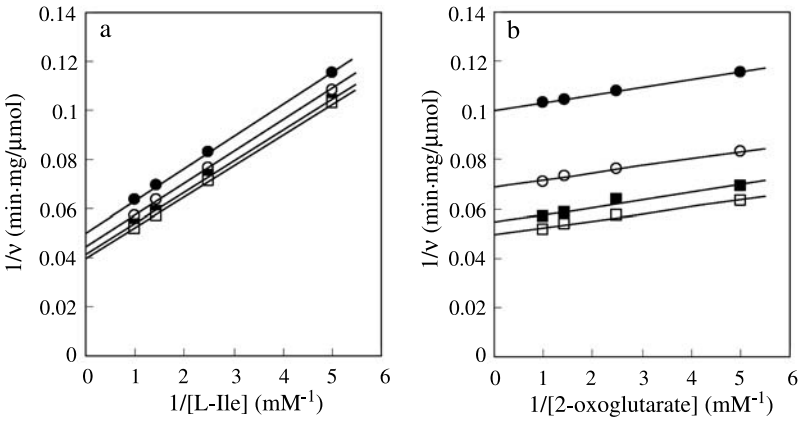

Fig. 4. Effect of different concentrations of 2-oxoglutarate (**a**) and L-isoleucine (**b**) on the initial velocities in the transamination reaction of BCAT from *H. pylori* NCTC 11637. Double reciprocal plots of velocity versus substrate concentration are shown. ●, 0.2 mM; ○, 0.4 mM; ■, 0.7 mM; □, 1.0 mM 2-oxoglutarate (**a**) or L-isoleucine (**b**). Each point represents the mean ± S.D. of triplicate assays, although vertical bars for S.D. values are not expressed for a clear appearance of the figure. **a** and **b** were drawn based on the same experimental data

(15%). Therefore, *H. hepaticus* BCAT appears to belong to subfamily IIIb together with *E. coli*. All the BCAT enzymes possess pyridoxal 5′-phosphate as a coenzyme, and the coenzyme binding site, lysine residue, is conserved among all these proteins.

The optimal pH (Fig. 2) and temperature (Fig. 3) of the purified enzyme were pH 8.0 and 37 °C, respectively. The enzyme catalyzes a transamination reaction from a branched-chain amino acid to 2-oxoglutarate, producing L-glutamate. When 2-oxoglutarate or L-isoleucine concentration was varied from 0.2 to 1.0 mM at fixed concentrations of L-isoleucine or 2-oxoglutarate between 1 and 5 mM, a parallel pattern of linear double reciprocal plots was obtained as shown in Fig. 4a or b, respectively. These results fitted well to the Michaelis–Menten equation for a bireactant mechanism. Based on these results, Lineweaver-Burk plots were deduced (Cornish-Bowden, 1975) at infinite concentrations of substrates, and kinetic constants were calculated as follows: the K_m values for L-isoleucine and 2-oxoglutarate were 0.34 mM and 0.085 mM, respectively, and the V_{max} value was 27.3 μmol/min/mg (Fig. 5).

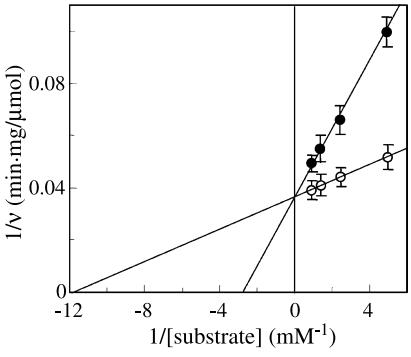

Fig. 5. Secondary Lineweaver-Burk plots of the transamination reaction of BCAT from *H. pylori* NCTC 11637 at infinite concentrations of 2-oxoglutarate (●) or L-isoleucine (○). The lines are deduced from the results shown in Fig. 4a and b. Each point represents the mean ± S.D. of triplicate assays

Table 1. Substrate specificity of branched-chain amino acid aminotransferase

Amino acids	Relative activity (%)
L-isoleucine	100 ± 1.8
L-leucine	70.1 ± 2.5
L-veline	47.9 ± 4.1
L-methionine	2.9 ± 0.9
L-aspartate	1.7 ± 0.8
L-phenylalanine	0.9 ± 0.4
Glycine	0.4 ± 0.2

Assayed with 10 mM amino acids and 10 mM 2-oxoglutarate. Each value represents the mean ± S.D. of triplicate assays

L-isomers of isoleucine, leucine and valine were found to be the most effective substrates (Table 1). L-methionine, L-aspartate, L-phenylalanine, and glycine were found to have much lesser ability to transaminate 2-oxoglutarate. This substrate specificity of BCAT is consistent with results obtained for other organisms such as *L. lactis* (Yvon et al., 2000), *Lactobacillus paracasei* (Thage et al., 2004), *S. carnosus* (Madsen et al., 2002), *B. subtilis* (Berger et al., 2003), and *M. tuberculosis* (Venos et al., 2004). The D-isomers of isoleucine, leucine and valine, and both enantiomers of alanine, proline and serine, as well as L-asparagine, L-histidine and L-lysine were inactive.

Polyamine synthesis and the associated methionine regeneration pathway are known to be potential drug targets in a variety of microorganisms. The BCAT enzyme may be involved in methionine production in *H. pylori*, since isoleucine, leucine, valine, glutamate and phenylalanine are preference amino donors for BCAT-catalyzed synthesis of methionine from ketomethiobutyrate in *B. subtilis*, *B. cereus*, and *B. anthracis* (Berger

et al., 2003; Venos et al., 2004), although the BCAT-catalyzed reactions in the reverse direction, i.e., syntheses of these amino acids were not examined in the present study.

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