Gene Cloning and Characterization of α-Amino Acid Ester Acyl Transferase in Empedobacter brevis ATCC14234 and Sphingobacterium siyangensis AJ2458

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The gene encoding α-amino acid ester acyl transferase (AET), the enzyme that catalyzes the peptide-forming reaction from amino acid methyl esters and amino acids, was cloned from Empedobacter brevis ATCC14234 and Sphingobacterium siyangensis AJ2458 and expressed in Escherichia coli. This is the first report on the aet gene. It encodes a polypeptide composed of 616 (ATCC14234) and 619 (AJ2458) amino acids residues. The V_{max} values of these recombinant enzymes during the catalysis of L-alanyl-L-glutamine formation from L-alanine methyl ester and L-glutamine were 1,010 U/mg (ATCC14234) and 1,154 U/mg (AJ2458). An amino acid sequence similarity search revealed 35% (ATCC14234) and 36% (AJ2458) identity with an α-amino acid ester hydrolase from Actobacter pasteurianus, which contains an active-site serine in the consensus serine enzyme motif, GxSYxG. In the deduced amino acid sequences of AET from both bacteria, the GxSYxG motif was conserved, suggesting that AET is a serine enzyme.

Key words: L-alanyl-L-glutamine; α-amino acid ester acyl transferase; Empedobacter brevis; Sphingobacterium siyangensis; α-glutamine

L-glutamine (Gln) is a conditionally essential amino acid due to its central function in nitrogen metabolism. Despite its nutritional importance, it is rarely used as a component of parenteral nutrition due to its low solubility and instability in solution. One approach to overcome its physicochemical limitations is to supply it as a dipeptide by conjugating it with other amino acids. Consequently, L-alanyl-L-glutamine (Ala-Gln) is one of the most suitable Gln-containing dipeptides.

Several methods of producing Ala-Gln have been reported, including Ala-Gln chemical synthesis via d-2-chloropropionyl-glutamine, as reported by Sano et al., but these methods require many complicated steps, due to the need to introduce protecting groups into the amino acids and to remove them from the resulting peptides. Recently, Tabata et al. reported a simple method of Ala-Gln synthesis through fermentative production from glucose and ammonia, but this system cannot recognize the fixation of the desired amino acid at a specified position. Hence this method has the potential disadvantage that undesired peptide byproducts may occur in accordance with the substrate specificity of the enzyme.

We have developed a novel enzymatic method to generate Ala-Gln that overcomes the disadvantages of conventional methods, using L-alanine methyl ester (Ala-OMe) as an acyl donor and Gln as a nucleophile. We found that α-amino acid ester acyl transferase (AET), purified from Empedobacter brevis ATCC14234 (EAET), generates large amounts of Ala-Gln from Ala-OMe and Gln in aqueous solution, with yields exceeding 80% relative to Ala-OMe. In addition, the enzyme has wide substrate specificity for both acyl donors and nucleophiles, and can catalyze peptide-forming reactions not only to produce various dipeptides from the corresponding amino acid esters and amino acids but also to produce various oligopeptides from the corresponding amino acid esters and peptides.

Here we report the gene cloning of AET from two strains, and the characterization of the resulting recombinant enzymes. First we cloned the EAET-encoding gene and expressed it in E. coli. Next, we hypothesized that forms of AET other than EAET are more highly expressed in E. coli and screened for AET homologs. Sphingobacterium siyangensis AJ2458 was selected as a representative strain, and the gene encoding AET from S. siyangensis AJ2458 (SAET) was cloned. The Ala-Gln producing activity in a culture broth of recombinant E. coli expressing SAET was higher than that of recombinant E. coli expressing EAET. These recombinant enzymes were then purified and characterized, and analysis of the deduced amino acid sequence indicated that AET is a serine enzyme.

Materials and Methods

Materials. The amino acid methyl esters used were hydrochloride salts obtained from Bachem (Bubendorf, Switzerland), and the amino acids were from Ajinomoto (Tokyo). A HiLoad 26/10 Q Sepharose column, a Resource PHE column, and a HiLoad 16/60 Superdex 200 pg column were purchased from GE Healthcare (Buckinghamshire, UK). A Bio-Scale CHT5-I column was from Bio-Rad Laboratories (Hercules, CA).

Microorganisms and culture conditions. E. brevis ATCC14234 and S. siyangensis AJ2458 were grown in Terrific Broth (TB) medium, containing glycerol 4 g/L, peptone 12 g/L, yeast extract 24 g/L, KH_{2}PO_{4} 2.3 g/L, and K_{2}HPO_{4} 12.5 g/L at 20°C. E. coli IM109, harboring expression plasmids, was grown in TB medium containing 100 mg/L of ampicillin sodium salt (amp) at 20°C.

Cloning of genes encoding α-amino acid ester acyl transferase. We cloned the gene encoding AET from E. brevis ATCC 14234 (eaet), as

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Abbreviations: AET, α-amino acid ester acyl transferase; amp, ampicillin sodium salt; ORF, open reading frame; TB, Terrific Broth
follows: Consensus primers (5′-TTTACGNCATHAYACRCC-3′
and 5′-TCGGGCATGTRTAGTNGACRTT-3′: H. A, T, or C; N: A,
C, G, T; R: A or G; Y: C or T) were designed based on the internal
amino acid sequence of Eaet. A 1.5-kb DNA fragment was amplified
from E. brevis ATCC 14234 genomic DNA, and a 4.5-kb DNA
fragment was hybridized to the 1.5-kb DNA fragment by Southern
hybridization. The 4.5-kb fragment was inserted into the HindIII site of
the pUC118 vector. Then pUC118 Eaet was obtained by colony
hybridization using the amplified DNA fragment as probe.

Expression plasmids for eaet were constructed as follows: Three
initiation codons were identified as candidates, with which we
designed corresponding primers, GTG2 5′-GGGAGGTCCTATAT-
AAAAATAATACATAAAAAATCT-3′ and 5′-GGGGGCTGCA-
GTACTTGTACGGTTTCGCCCGATAAA-3′, GTG1 5′-GGGAGGATT-
CCATATCCGACAAATTACGAAAAAATAGGAAA-3′ and 5′-GOG-
GGGCCGCTAGCTTGTACGGTTTCGCCCGATAAA-3′, and ATG
5′-GGGAGGATCCATATCCGACAAATTACGAAAAAATAGGAAA-3′
and 5′-GGGGGCTGCA-GTACTTGTACGGTTTCGCCCGATAAA-3′.
The eaet genes were amplified by PCR using the GTG2, GTG1, and
ATG primers and E. brevis ATCC14234 genomic DNA as template.
During PCR, the GTG initiation codon was intentionally converted
to ATG, because the former is used infrequently in
E. brevis
ATG primers and

The active fractions, collected from the non-adsorbed
buffer (pH 8.0). All procedures were carried out at 4°C;
the supernatant was ultracentrifuged at
195 W for 45 min. To remove the insoluble fraction, the supernatant was ultracentrifuged
at 100,000 × g for 20 min, and the resulting supernatant was used as
the cell-free extract.

The cell-free extract (47 mL) was applied to a Bio-Scale CHT5-1
column (1 × 6.4 cm) equilibrated with 100 mM potassium phosphate buffer
(pH 6.5). The column was washed with 100 mM potassium phosphate buffer containing 2 mM ammonium
sulfate (pH 6.5), and then the enzyme was eluted with a linear gradient of
2.0-0.5 M ammonium sulfate. The purified enzyme (0.6 mL) was
dialyzed against 20 mM potassium phosphate buffer (pH 6.5) and
stored at 4°C.

E. coli Saet was cultured under the same conditions as E. coli Gtg2,
harvested by centrifugation (8,000 × g, 10 min), and washed with 100 mM potassium phosphate buffer (pH 6.5).
All procedures were carried out at 4°C or on ice. Wet cells (5 g) were resuspended in 50 mL
of 100 mM potassium phosphate buffer (pH 6.5), and the cell
suspension was sonicated with an Insomator 201 at 195 W for 45 min.
To remove the insoluble fraction, the supernatant was ultracentrifuged
at 100,000 × g for 20 min, and the resulting supernatant was used as
the cell-free extract.

The cell-free extract (44 mL) was applied to a Resource PHE column
(0.64 × 3 cm) equilibrated with 100 mM potassium phosphate buffer
containing 2 mM ammonium sulfate (pH 6.5). The column was washed with 100 mM potassium phosphate buffer containing 2 mM ammonium
sulfate (pH 6.5), and then the enzyme was eluted with a linear gradient of
2.0-0.5 M ammonium sulfate. The purified enzyme (0.6 mL) was
dialyzed against 20 mM potassium phosphate buffer (pH 6.5) and
stored at 4°C.

Protein analysis. Protein concentrations were determined by the Bradford method with bovine serum albumin as standard. Sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10–20% polyacrylamide gel (Davis Pure Chemicals,
Tokyo) with Precision Protein Marker (Bio-Rad Laboratories,
Hercules, CA) as marker proteins. The native relative molecular mass
was determined with a HiLoad 16/60 Superdex 200 pg column using
Gel Filtration LMW and Gel Filtration HMW calibration kits (GE
Healthcare, Buckinghamshire, UK).

Enzyme assay. To measure Ala-Gln producing activity, we used a mixture of 100 mM borate buffer (pH 9.0), 100 mM Ala-OMe, and
200 mM Gln at a final volume of 0.1 mL. Because Ala-OMe hydro-
chloride was used as substrate, the substrate solution was adjusted to
pH 9.0 with 6 N NaOH before it was added to the enzyme. The enzyme
reaction was carried out at 25°C for 10 min. To stop the reaction, 1 mL
of 1% w/v H2PO4 solution was added. One unit of Ala-Gln producing
activity was defined as the amount of enzyme that produces 1 µmol
of 100 mM potassium phosphate buffer containing 4 mM ammonium sulfate (pH 6.5).

The optimal reaction pH values for the reactions were determined at 25°C using various buffers at
100 mM: sodium acetate (pH 4.0, 4.5, 5.0, and 5.5), MES (pH 5.5, 6.0,
and 6.5), potassium phosphate (pH 6.5, 7.0, and 7.5), Tris–HCl (pH 7.5 and 8.0), and borate (pH 8.0, 8.5, 9.0, 9.5, and 10.0). The
optimal reaction temperature was selected from among

Kinetic assay. The kinetic parameters with regard to the Ala-Gln
forming reaction were determined by measuring the Ala-Gln produced
by varying the concentration of Ala-OMe (4.6, 9.1, 18.2, 36.4, and 72.7 mM) against several fixed concentrations of Gln (4.6, 9.1, 18.2, 36.4, and 72.7 mM) at 25°C in 100 mM borate buffer (pH 9.0). The $K_m$ values toward Ala-OMe and Gln and the $V_{max}$ values were determined from secondary plots of Lineweaver-Burk plots.

High-performance liquid chromatography analysis. The amounts of dipeptides produced were measured by high-performance liquid chromatography under the following conditions: column, Inertsil ODS-2 (4.6 × 250 mm; GL Science, Tokyo); mobile phase, $\text{H}_3\text{PO}_4$ solution containing a 10:1.5–5 mixture of 5 mM sodium 1-octanesulfonate (pH 2.1) and methanol; flow rate, 1.0 mL/min; temperature 40°C; detection, 210 nm.

Results

Cloning and expression of the gene encoding α-amino acid ester acyl transferase from *E. brevis* ATCC14234

To analyze the N-terminal amino acid sequence of EAET, EAET was purified as described previously, but we could not determine whether the N-terminal was protected. Next, we analyzed fragments of EAET after cleavage with lysyl endopeptidase and determined the internal amino acid sequences of EAET to be LFTAIYQPK and ETNVTYTMPD. The gene was obtained as described above in ‘Materials and Methods.’ The nucleotide sequence contained a 1,851-bp ORF, encoding a polypeptide of 616 amino acids (Fig. 1). The estimated molecular mass was approximately 71 kDa.

To confirm that the DNA fragment we cloned was indeed *eaet*, expression plasmids for *eaet* were constructed. Because some initiation codons were observed (Fig. 1), *E. coli* JM109 was transformed with the various expression plasmids and grown at 20°C in TB medium containing 100 mg/L of amp for 40 h. After culture, the Ala-Gln producing activities of *E. coli* Gtg2, *E. coli* Gtg1, and *E. coli* Atg culture broths were examined. Only that of *E. coli* Gtg2 demonstrated similar activity (0.4 U/mL of culture broth), approximately 11 times higher than that of the wild-type strain. Based on these results, we concluded that the DNA fragment we cloned was indeed *eaet*, expression plasmids for *eaet* were constructed.
fragment contained a 1.8-kb ORF corresponding to eaet and that GTG2 was the initiation codon, as shown in Fig. 1.

**Screening of AET from Cytophaga, Flavobacterium, and Sphingobacterium**

We hypothesized that forms of AET other than EAET are more highly expressed in E. coli. Hence we screened strains that demonstrated Ala-Gln producing activity. Because E. brevis belongs to the phylum CFB, which includes the genera Cytophaga, Flavobacterium, and Sphingobacterium,9 we examined 57 such strains for their ability to produce Ala-Gln from Ala-OMe and Gln, as described previously.6) S. siyangensis AJ2458 produced amounts of Ala-Gln equal to E. brevis ATCC14234, prompting us to select it as a representative producer of Ala-Gln.

**Cloning and expression of the gene encoding AET from S. siyangensis AJ2458**

The gene encoding S. siyangensis AJ2458 α-amino acid ester acyl transferase (SAET) was cloned as described in “Materials and Methods.” We noted a 1,860-bp ORF in the nucleotide sequence, encoding a polypeptide of 619 amino acid residues whose estimated molecular mass was 71 kDa, the same as EAET.

To confirm that the DNA fragment was saet, a saet expression plasmid was constructed, and E. coli harboring this plasmid (E. coli Saet) was grown at 20 °C in TB medium containing 100 mg/L of amp for 40 h. E. coli Saet demonstrated Ala-Gln producing activity, leading us to conclude that the DNA fragment was saet. The Ala-Gln producing activities of E. coli Saet and E. coli Gtg2 were 39.4 and 0.4 U/mL of culture broth respectively, and the activity of E. coli Saet was 98.5 times higher than that of E. coli Gtg2.

**Purification and characterization of recombinant enzymes**

To determine whether the difference in Ala-Gln producing activity of culture broth as between E. coli Gtg2 and E. coli Saet depended on the specific activity of the enzyme, recombinant enzymes were purified (Table 1). The enzymes were purified in 3-column chromatography runs, and the purified enzymes appeared to be homogeneous, as determined by SDS-
Discussion

In this study, we isolated two novel genes encoding AET from *E. brevis* ATCC14234 and *S. siyangensis* AJ2458. The Ala-Gln producing activity of an *E. coli* Saet culture broth was 98.5 times higher than that of an *E. coli* Gtg2, although the specific activities of EAET and SAET were nearly equal. In addition, the codon usages of *saet* and *eaet* in *E. coli* were very similar (data not shown). Based on these findings, the difference in the Ala-Gln producing activity varies between *E. coli* Saet and *E. coli* Gtg2. While the reason for these differences in the Ala-Gln producing activity of culture broth as between *E. coli* Saet and *E. coli* Gtg2 is not clear at present, we believe that a difference in the manners of processing of EAET and SAET in *E. coli* causes the disparity.

Through database searches using BLAST (blastp 2.1.2), EAET and SAET were found to be homologous with several proteins (Table 2). Though the homology was 35%, the most homologous protein was the a-amino acid ester acyl transferase (AEH) from *Acetobacter pasteurianus*. AEH catalyzes the transfer of the acyl group from an a-amino acid ester to amine nucleophiles, such as 7-aminocepham and 6-penam compounds (synthesis), or to water (hydrolysis). AEH has not been reported to catalyze peptide-forming reactions with amino acids as a nucleophile.

The second most homologous protein was *Bacillus laterosporus* 7β-(4-carboxybutanamido) cephalosporanic acid ester hydrolase (AEH) from *Acetobacter pasteurianus*. AEH catalyzes the transfer of the acyl group from an a-amino acid ester to amine nucleophiles, such as 7-aminocepham and 6-penam compounds (synthesis), or to water (hydrolysis). AEH has not been reported to catalyze peptide-forming reactions with amino acids as a nucleophile.
acids, which hydrolyzes glutaryl 7-ACA to 7-amino cephalosporanic acid, a 
starting material for semisynthetic cephalosporin antibiotics. According to the Structural Classification of 
Proteins (SCOP) classification, GA belongs to the N-terminal nucleophile amidohydrolase superfamily. In 
addition, it belongs to the penicillin acylase catalytic domain family, which includes penicillin G acylase 
(PGA). GA and PGA catalyze not only the hydrolysis of amide bonds but also, like AET, the synthesis of amide 
bonds. PGAs synthesize ampicillin from phenylglycine methyl ester and 6-amino penicillanic acid 
(6-APA) in a suitable solvent. Conversely, 6-APA is a competitive inhibitor; enzymes that are bound to 6-APA 
do not hydrolyze ampicillin. 

Ala-Gln was not hydrolyzed in the presence of Gln during Ala-Gln production by AET. Hence the reaction 
mechanism of AET has been suggested to resemble those of GA and PGA. However, AET can synthesize 
Ala-Gln at high yields in aqueous solution, unlike GA and PGA. A comparative analysis of the reaction 
mechanisms of AET, GA, and PGA might lead to further consideration of these enzymes.

The third most homologous protein was cocaine esterase from Rhodococcus sp. strain MB1. This enzyme 
hydrolyzes the ester bond in cocaine to benzoate and ecgonine methyl ester. Cocaine esterase and AET both 
contain an active-site serine in the consensus serine enzyme motif (GxSYxG, where x is a nonconserved 
amino acid). In GA, the GxSYxG motif is not completely conserved, and the last glycine is replaced 
by alanine. EAET and SAET contain the GxSYxG motif. Asp and His residues, which constitute the catalytic 
residues of serine enzymes, are also conserved (Fig. 2). In addition, AET activity was inhibited by 
4-nitrophenyl 4-guanidinobenzoate (p-NPGB), a serine enzyme inhibitor (data not shown). Based on these 
results, we propose that AET is a serine enzyme.

The amino acid adjacent to the catalytic His differs between EAET and SAET (EAET, F330; SAET, Y328). Nakagawa et al. reported that a mutation at the residue next to the catalytic His caused a selective increase in 
the amidase activity of lipase, and the effect of this mutation on the function of catalytic His was essential to 
the increased activity. We propose, then, that the difference in substrate specificity between EAET and SAET is caused by the difference in the amino acid adjacent to the catalytic His. Site-directed mutagenesis 
of EAET F330 and SAET Y328 may help to elucidate the function of the residue next to the catalytic His in 
AET.

This is the first report on the aet gene. Directed evolution, such as site-directed mutagenesis, might 
increase the Ala-Gln producing activity of the enzyme to improve the production yield of Ala-Gln.

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References


Table 2. Proteins Homologous to EAET and SAET

<table>
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<th>Protein IDa</th>
<th>Organism</th>
<th>Function</th>
<th>Homology (%)</th>
<th>Motifb</th>
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<td>GSSYG</td>
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<td>Brevibacillus laterosporus</td>
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<td>Rhodococcus sp.</td>
<td>Cocaine esterase16</td>
<td>26</td>
<td>GVSYLG</td>
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</tbody>
</table>

'aAccession number in the protein database of the National Center for Biotechnology Information.
bBold, consensus sequence of serine enzyme.