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Cloning and Characterization of GL-7-ACA Acylase Gene from *Pseudomonas* sp. GK16

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The gene coding for glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase was cloned from *Pseudomonas* sp. GK16 and some of its characteristics were analyzed. The complete nucleotide sequence revealed that the putative open reading frame is 2160 bases long and encodes 720 amino acids. By SDS-PAGE three proteins, approximately corresponding to 70, 54 and 16 kDa of molecular weight, were detected in *E. coli* cells carrying pGAP18. The largest protein should be a precursor which is not processed yet, while the other two proteins must be derived from the precursor by the proteolytic processing.

7-Aminocephalosporanic acid (7-ACA) is a raw material used for the production of many semisynthetic cephalosporin drugs. Chemical deacylation of cephalosporin C, a fermentation product, is the primary method used to produce 7-ACA industrially (6, 16). To overcome the shortcomings of the chemical method and the environmental pollution caused by the byproducts, an enzymatic process consisting of two steps is hereby presented (12). In the first step, D-amino acid oxidase is used to deaminate oxidatively the cephalosporin C to yield the glutaryl-7-ACA (GL-7-ACA), and in the second step, the GL-7-ACA is deacylated to form the 7-ACA by hydrolysis of the GL-7-ACA acylase. It has been reported that the enzymatic conversion of GL-7-ACA to 7-ACA is highly desirable for the prevention of chemical contamination in the production of cephalosporin antibiotics (4).

Several groups had screened and isolated the strains producing GL-7-ACA acylase from soil niches (1, 15, 19). Among them, Matsuda and Komatsu reported the cloning of the gene encoding a GL-7-ACA acylase from a *Pseudomonas* strain and its biochemical characteristics. They showed that the GL-7-ACA acylase was composed of the two nonidentical subunits derived from the same precursor and that the enzyme was found in the periplasmic space. They, however, only published the partial N-terminus sequence of GL-7-ACA acylase. In this article, we report the molecular cloning of the gene encoding a GL-7-ACA acylase from *Pseudomonas* sp. GK16 and its entire nucleotide sequence and some of its characteristics.

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Key words: cloning, expression, nucleotide sequence, *Pseudomonas* sp. GK16, GL-7-ACA acylase, processing

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

The bacterial strains and plasmids used in this work are listed in Table 1. *Pseudomonas* sp. GK16 (obtained from ChongKunDang Pharmaceutical Co.) was grown in nutrient broth (Difco, Detroit, Mich.) at 32°C with aeration. L-broth was used as the basal medium for *E. coli* strain, which was grown at 37°C. Antibiotics were included to select for cells carrying plasmids; 50 μg/ml of tetracycline hydrochloride, 100 μg/ml of sodium ampicillin.

Enzymes, Chemicals and Radioisotope

Restriction endonuclease, calf intestinal alkaline phosphatase, exonuclease III, S1 nuclease, Klenow enzyme, T4 DNA polymerase and T4 DNA ligase were obtained from Promega and were used as recommended by the manufacturer. RNase, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-galactoside (IPTG) were obtained from Boerhinger-Mannheim. All antibiotics were purchased from Sigma Chemical Co. GL-7-ACA and *p*-dimethylaminobenzaldehyde were obtained from ChongKunDang Pharmaceutical Co. and Showa Chemicals Inc., respectively. Radioisotope was obtained from Amersham Corp..

Construction of *Pseudomonas* sp. GK16 Gene Library

Chromosomal DNA was isolated as described by Doi (5). The purified chromosomal DNA from *Pseudomonas* sp. GK16 was partially digested with *Pst*I, and DNA fragments ranging from 4 to 9 kb were ligated to *Pst*I-digested, dephosphorylated pBR322. The ligation mixture was used to transform *E. coli* HB101 by the $CaCl_2$ method (3).

Cloning of GL-7-ACA Acylase Gene

Table 1. Strains and plasmids.

Strains or plasmids	Relevant genotype or characteristics	Source or reference
Escherichia coli		
HB101	*hsd-20 rec13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	(2)
DH5α	ϕ80d*lacZ* Δ*M15* [*endA1 recA1 hsdR17* (r^-m^-) *supE44 thi-1* λ^- gyrA relA1 F^- Δ (*lacZYA-argF*) U169]	(3)
Pseudomonas sp.		
GK16	GL-7-ACA acylase producer	(10)
Plasmids		
pBR322	Ap^r, Tc^r	Laboratory stock
pUC18/19	Ap^r	Laboratory stock
pPR217	pUC19-217 bp N-terminal fragment of GL-7-ACA acylase gene, Ap^r	This study
pGA1	pBR322-7.45 kb fragment containing GL-7-ACA acylase gene, Tc^r, Ap^s	This study
pGAP18	pUC18-2.8 kb fragment containing GL-7-ACA acylase gene, Ap^r	This study

The 217 bp probe was prepared by PCR with forward (5'-ATCTACGGCGTCGACGCGCCC-3') and reverse primers (5'-CGCCGCGAAGGCGTCGAGGTT-3') using *Pseudomonas* sp. GK16 chromosomal DNA as a template. We used the commercial DIG DNA labeling and detection kits supplied by Boehringer-Mannheim to label the 217 bp probe. It was labeled by random-primed incorporation (7) of digoxigenin-labeled dUTP. Colony hybridization was performed as described by Maniatis *et al.* (13). Detection of specific hybridization was done by immunoassaying with anti-digoxigenin-alkaline phosphatase conjugate and staining with 5-bromo-3-indolyl phosphate (8).

Determination of GL-7-ACA Acylase Activity

The positive clone was grown on a selective plate (L-broth containing 50 μg/ml of tetracycline or 100 μg/ml of ampicillin) for 16 h. Then the plate was exposed to chloroform vapor for 15 min, scraped with a toothpick, and suspended in 100 μl of GL-7-ACA (1 mg of GL-7-ACA per ml of 0.1 M phosphate buffer, pH 7.0). The mixture was incubated at 37°C for 60 min, and the reaction was terminated by addition of 120 μl of acetic acid-0.25 M NaOH (2:1, v/v), followed by addition of 40 μl of *p*-dimethylaminobenzaldehyde (0.5 % in methanol). *p*-Dimethylaminobenzaldehyde makes a yellow condensation product with 7-ACA (14).

Nucleotide Sequence Determination and Analysis

Digestion of the recombinant plasmid with restriction enzymes and serial deletion with exonuclease III were employed for subcloning in both orientations into pUC19 and for deletion, respectively. Nucleotide sequence was determined by using Sanger's dideoxy chain termination method (17) with [^{35}S-α]dATP and DNA sequencing kit (Sequenase™ version 2.0 kit from United States Biochemical Co.). Sequencing reaction mixtures were electrophoresed on a 6% polyacrylamide gel containing 8 M urea. Sequence data were analyzed by DNASIS program (Hitachi software engineering Co. LTD.). The nucleotide and amino acid sequences were scanned with the databases available at the National Center for Biotechnology Information (NCBI) of the National Institute of Health (NIH) through Experimental GENINFO (R) BLAST Network Service (Blaster).

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli with 10% or 14% polyacrylamide containing 0.1% SDS (11). The proteins were stained with Coomassie brilliant blue R-250.

RESULTS

Cloning of a GL-7-ACA Acylase Gene from *Pseudomonas* sp. GK16

As the GL-7-ACA acylase activity was detected with a *Pseudomonas* sp. GK16 (data not shown), we synthesized PCR primers on the basis of partial nucleotide sequence reported by Matsuda and Komatsu (14) and carried out the PCR with the chromosomal DNA of *Pseudomonas* sp. GK16. As the expected size of PCR product (217 bp, Fig. 1) was generated, a plasmid gene library of *E. coli* was screened with the PCR product as a probe. As a result, one positive clone was obtained (data not shown) and confirmed by analyzing the restriction map and comparing the nucleotide sequence of a part of the recombinant plasmid, named pGA1, with the partial nucleotide sequence of GL-7-ACA acylase gene mentioned above. As shown in Fig. 2, pGA1 contained a 7.45 kb insert, but its restriction map showed differences from that reported by Matsuda and Komatsu (14) at the restriction sites for *Sal*I, *Mlu*I and *Xho*I. There were five restriction sites for *Sal*I, and two sites for *Mlu*I and *Xho*I, respectively, in the cloned segment, while there were three sites for *Sal*I, one site for *Mlu*I, and three sites for *Xho*I in the previously reported gene (14).

Expression of GL-7-ACA Acylase and Determina-

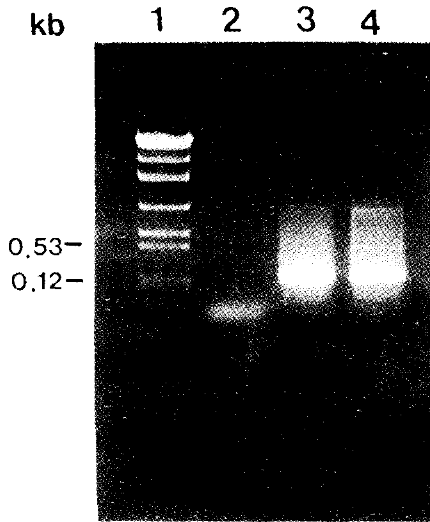

Fig. 1. Agarose gel electrophoresis of PCR product.
Lane 1, DNA size marker (λ DNA cut with *Dra*I); lane 2, oligonucleotide primers; lane 3 and 4 , PCR product.

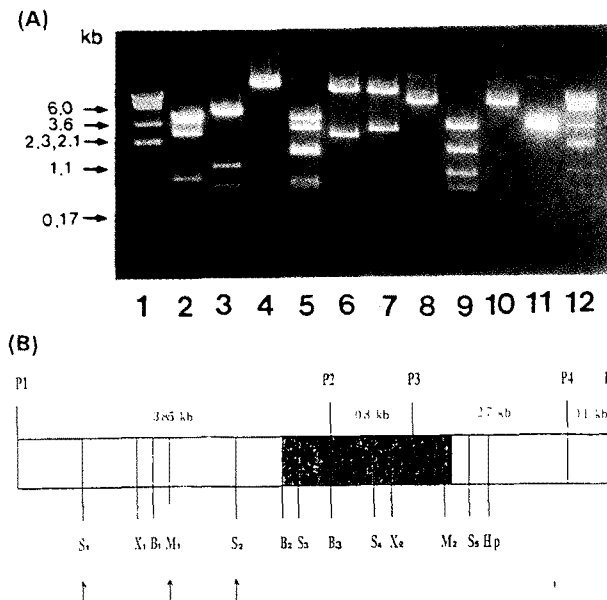

Fig. 2. Agarose gel electrophoretic analysis of restriction fragments of pGA1 and restriction map of its insert.
(A) Lane 1 and 12, DNA size marker (λ DNA cut with *Dra*I); lane 2, pGA1 cut with *Pst*I; lane 3, pGA1 cut with *Bam*HI; lane 4, pGA1 cut with *Hpa*I; lane 5, pGA1 cut with *Sal*I; lane 6, pGA1 cut with *Xho*I; lane 7, pGA1 cut with *Mlu*I; lane 8, pUC19 containing a 3.85 kb *Pst*I fragment cut with *Xho*I; lane 9, pUC19 containing a 3.85 kb *Pst*I fragment cut with *Sal*I; lane 10, pUC19 containing a 3.85 kb *Pst*I fragment cut with *Mlu*I; lane 11, pUC19 containing a 0.8 kb *Pst*I fragment cut with *Xho*I. (B) Thick and thin arrows indicate more or less endonuclease sites existing in the insert of pGA1, respectively, than those in the GL-7-ACA acylase gene-bearing DNA fragment cloned by Matsuda and Komatsu (14). The black box indicates the structural region of GL-7-ACA acylase gene. Abbreviations: P, *Pst*I; B, *Bam*HI; S, *Sal*I; X, *Xho*I; M, *Mlu*I; Hp, *Hpa*I.

tion of Enzyme Activity in *E. coli*

The ability of pGA1 to express GL-7-ACA acylase

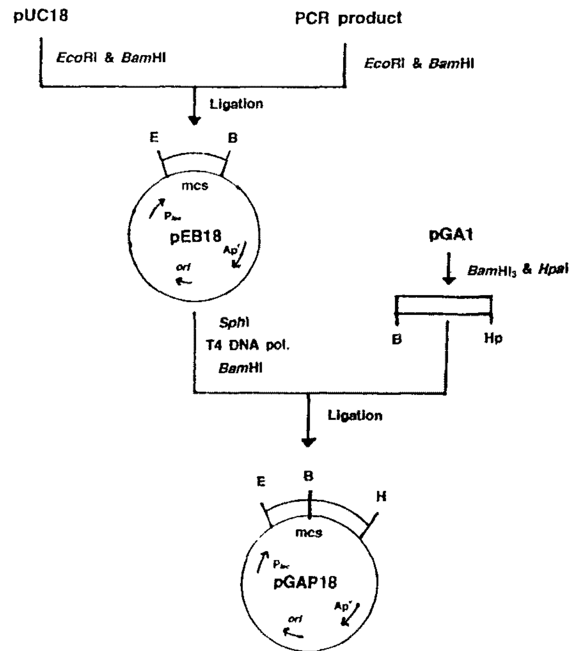

Fig. 3. Schematic pathway for the construction of pGAP18.
Abbreviations: E, *Eco*RI; B, *Bam*HI; Hp, *Hpa*I; H, *Hin*dIII.

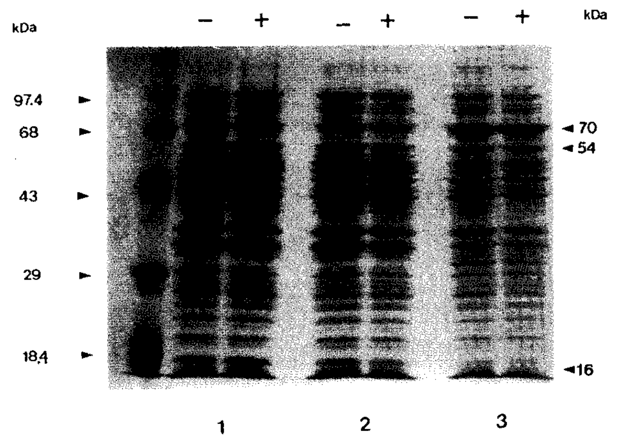

Fig. 4. SDS-polyacrylamide gel electrophoresis protein profiles.
The production of GL-7-ACA acylase in *E. coli* DH5α harboring pGAP18 was analyzed by SDS-10% polyacrylamide gel electrophoresis with its cell extract. After *E. coli* DH5α cells harboring pGAP18 were cultured to an OD_{550} of 0.5 at 37°C, the culture was further continued for 3 h without IPTG or in the presence of IPTG at a final concentration of 1 mM. Cell lysate was prepared from 1 ml of each culture and was subjected to SDS-PAGE. The precursor of GL-7-ACA acylase with a MW of 70 kDa and its small (α) and large (β) subunits with MWs of 16 and 54 kDa, respectively, are shown. Number 1, *E. coli* DH5α; number 2, *E. coli* DH5α harboring pUC18; number 3, *E. coli* DH5α harboring pGAP18. +, IPTG induction; -, no induction.

was tested by enzyme activity assay. Cell-free extract from the clone harboring pGA1 showed the enzyme activity of GL-7-ACA acylase (data not shown). To reduce the size of a fragment containing GL-7-ACA acylase gene, we constructed pGAP18, a derivative of pUC18 (Fig. 3). A 0.6 kb *Bam*HI_2-*Bam*HI_3 region of

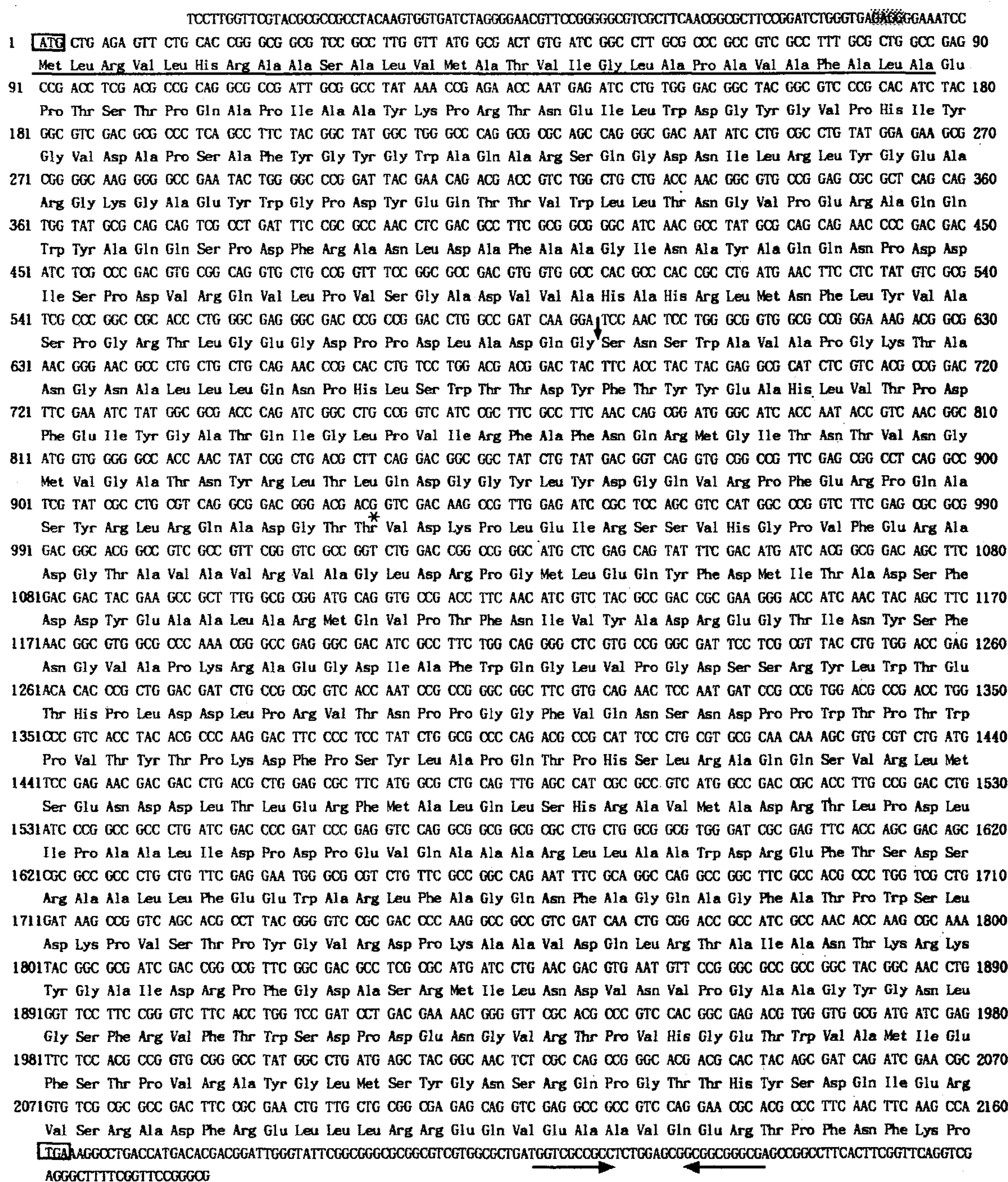

Fig. 5. Complete nucleotide and deduced amino acid sequences of GL-7-ACA acylase gene from *Pseudomonas* sp. GK16.
The DNA sequence is shown in the 5' to 3' orientation with respect to the transcription of GL-7-ACA acylase gene. The numbering of nucleotides and amino acids starts with the beginning of the coding region and the first Met, respectively. The amino acid sequence of signal peptide is underlined. The putative translation initiation and termination codons are indicated by boxes. Shaded box, putative ribosome-binding site; vertical arrow, putative processing site of the precursor; horizontal arrows, one inverted repeat sequence possibly related to transcription termination; asterisk, the end point of nucleotide sequence (933 nucleotides) published by Matsuda and Komatsu (14).

Fig. 6. Comparison of the amino acid sequence of GL-7-ACA acylase from *Pseudomonas* sp. GK16 with those of other closely related proteins.

The amino acid sequences are designated by the standard one-letter symbols. Identical amino acids are shaded. The putative processing sites of each precursor are indicated by a vertical arrow. (A), the amino acid sequence of the GL-7-ACA acylase of *Pseudomonas* sp. GK16; (B), the amino acid sequence of the cephalosporin C acylase of *Pseudomonas* sp. SE83 (15); (C), the amino acid sequence of the penicillin G acylase of *E. coli* ATCC 11105 (18).

pGA1 was amplified as a *Eco*RI-*Bam*HI_3 fragment by PCR. As primers for PCR, the following oligonucleotides were used; forward primer, 5'-AAAGAATTCTTGGTTCG-TACGCGCCGCCT-3' and reverse primer, 5'-GTTGGA-TCCTTGATCGGCCA-3'. The amplified fragment was subcloned into pUC18 and subsequently digested with *Sph*I. After treatment of T4 DNA polymerase to make the blunt end and then digestion with *Bam*HI, this plasmid was ligated with a *Bam*HI_3-*Hpa*I fragment of pGA1 and transformed into *E. coli* DH5α. Cell-free extract from the transformant harboring pGAP18 gave a higher enzyme activity than that from the strain harboring pGA1 (data not shown). We are not sure whether the GL-7-ACA acylase gene is transcribed by its own promoter or by *lacZ* gene promoter in pUC18.

SDS-PAGE was carried out to elucidate the protein profiles produced from pGAP18. As shown in lane 3 of Fig. 4, three proteins, approximately 70, 54 and 16 kDa of molecular weight, were detected in *E. coli* cells carrying pGAP18.

Nucleotide Sequence of the Cloned GL-7-ACA Acylase Gene and Amino Acid Sequence Comparison with Other Proteins

The complete nucleotide sequence and open reading frame of GL-7-ACA acylase gene and flanking regions are shown in Fig. 5. As expected from the preliminary experiment, the sequence of amino acid residue 1 to 311 exactly corresponded to that reported by Matsuda and Komatsu (14). The putative translation initiation and termination codons are found at 1 and 2161 base, respectively. From this open reading frame we conclude that the gene encodes 720 amino acids. The amino acid sequence of signal peptide is underlined and the vertical arrow between Gly-198 and Ser-199 indicates the putative processing site of GL-7-ACA acylase.

The amino acid sequence deduced from the ORF was compared with those of other closely related proteins, cephalosporin C acylase of *Pseudomonas* sp. SE83 (15) and penicillin G acylase of *E. coli* (18). The amino acid sequences of these enzymes lack an overall homology, but a computer search with the PC GENE program revealed that high similarities reside in a region (Fig. 6). Some identical and conserved amino acids are found around the region where the processing sites of three enzymes exist. We suggest that this region may be the domain required for the processing of the precursor rather than substrate binding or catalysis.

DISCUSSION

It is thought that GL-7-ACA acylase gene may be the same gene as that previously reported (14), since the nucleotide sequence encoding the N-terminus of the enzyme exactly corresponds to the partial sequence published by Matsuda and Komatsu. However, we are not sure whether there is any difference in the region which has not been reported, since the restriction map of flanking sequences revealed differences.

Matsuda and Komatsu reported that the active enzyme of GL-7-ACA acylase was found in the periplasmic space and that it is composed of two molecules of each of the two nonidentical subunits, when the cloned gene was expressed in *E. coli*. They also reported that the two nonidentical subunits were generated by proteolytic cleavage of the GL-7-ACA acylase between Gly-198 and Ser-199 (indicated by a vertical arrow in Fig. 5). On our SDS-PAGE three protein bands, 70, 54 and 16 kDa, were identified from the recombinant clone. Considering the entire coding sequence, we think the largest protein of 70 kDa is the precursor which is not proteolytically processed yet and the other two proteins are the products resulting from proteolytic processing.

In the comparison of GL-7-ACA acylase to other closely related proteins, all three enzymes contain the signal peptide sequence at their N-termini, and they are commonly processed to form the functional enzyme in the periplasmic space. The high sequence homologies at their processing and flanking regions strongly suggest that they might be processed through a common mechanism. What kind of a common mechanism works in two genera that are not closely related to each other in the evolutionary hierarchy? We suggest that the processing may be the enzyme-intrinsic ability, that is, autocatalytic processing, although it remains to be investigated further.

Now we are trying to overexpress the active GL-7-ACA acylase through *E. coli* and *B. subtilis* expression system for industrial use and also elucidate exactly what kind of a processing mechanism is working in both *Escherichia and Pseudomonas* genera.

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