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Functional Mapping of Amino Acid Residues Responsible for the Antibacterial Action of Apidaecin

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Functional mapping was carried out to address the amino acid residues responsible for the activity of the antibacterial peptide apidaecin from the honeybee by an in vivo assay system developed previously. The C-terminal region and many of the proline and arginine residues which are present at high frequency in apidaecin were found to play an important role in its antibacterial activity.

Various insects possess an intriguing innate immune response system that functions to eliminate invading bacteria. Apidaecin (AP), an antibacterial peptide from the honeybee (*Apis mellifera*), exists in three multiple isoforms (Ia, Ib, and II) that are each 18 amino acids long, proline rich, and basically charged (see Fig. 1). Production of AP in lymph fluid is inducible (2, 5). AP exhibits bacteriostatic effects on gram-negative bacteria such as *Escherichia coli* (2, 4, 5). Without any information on the structure-function relationship of AP, it would be time-consuming to carry out functional mapping to identify residues responsible for the activity of AP by analyzing all possible synthetic analogs of the 18-residue peptides.

We have already established an in vivo system for monitoring the antibacterial activities of the products of the randomly mutagenized gene for apidaecin Ib (AP1) to analyze the structure-function relationship of AP (8). This system is characteristic in the sense that *E. coli*, which is intrinsically sensitive to attack by AP1, is used as the recipient host. We qualitatively estimated the activity of each mutated AP1, the genes for which were inserted individually into secretory expression plasmids. The plasmid vector, pOSΔB-AP1, was constructed by fusing the chemically synthesized AP1 gene with that for a stable protection partner, *Streptomyces* subtilisin inhibitor (SSI) (6). As it were, it is a kind of suicide system that can be controlled by the concentration of the transcriptional inducer isopropyl-β-D-thiogalactopyranoside (IPTG). We demonstrated that, in principle, AP1 mutants with different activities can be screened on the basis of IPTG concentration-dependent growth inhibition of the host cell, as judged by colony size, on selective plates containing IPTG at various concentrations (primary screening).

Following several screening trials, we obtained four AP1 mutants with reduced levels of antimicrobial activity: one single-mutation-carrying AP1, P9L (abbreviation for its amino acid substitution, Pro-9 to Leu) and three double-mutation-carrying AP1s. P5L:R12Q, P11L:R17C, and P14S:R17C (Table 1). The antibacterial activity of purified P9L mutant AP1 for *E. coli* JM109 was found to be reduced to one-third of that of wild-type AP1 (8). However, we needed to clarify the effect on AP1 activity of the individual mutations of the doubly mutated AP1 molecules. In this study, to analyze the mutants other than P14S:R17C (not separable because of the absence of an adequate restriction enzyme site), the double point mutations in each mutant gene were separated by enzymatic digestion of the AP1 gene at the *Acc*I and *Not*I sites, as illustrated in Fig. 1A. The single-mutation-carrying genes obtained, P5L, P11L, and R12Q, were expressed in *E. coli* JM109 to examine the effect of expression of each mutant gene on the growth of the host cell (results are described below).

In a previous study (8), the sites of mutation were significantly concentrated in regions of the AP1 gene coding for Pro residues. Since we could not exclude the possibility that hydroxylamine, with its C→T converting activity, renders Pro codons hot spots for mutation, we adopted PCR, an alternative in vitro random mutation of the AP1 gene, in order to increase the probability of mutation. Since the frequency of misincorporation by *Taq* polymerase is high, PCR has been used to introduce random mutations into genes, and reaction conditions for enhancing the mutation frequency have been reported elsewhere (7). Figure 1B shows a scheme for achieving PCR-induced mutagenesis of the target region. An approximately 400-bp DNA fragment containing the SSI-AP1 gene fusion was isolated from plasmid pSI205ΔEAP1M (8) digested with *Sma*I and *Bam*HI and ligated into the same sites of pUC18 (9). The resultant plasmid, pU-AP1, was used for PCR mutagenesis with two universal sequencing primers (Takara Shuzo Co., Ltd.), M4 (5′-GTTTTCCCAGTCACGAC-3′ [near the *Bam*HI site]) and RV (5′-CAGGAAACAGCTATGAC [near the *Sma*I site]). The target region, including the AP1 gene, was amplified with the two primers under error-prone conditions: addition of 1% β-mercaptoethanol, 0.25 mM $MnCl_2$, and 10% dimethyl sulfoxide to the 100-μl reaction buffer containing 2.5 nM pU-AP1, 0.025 U of *Taq* polymerase (Boehringer Mannheim), 1 μM primers, a 0.2 mM concentration of each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was carried out using a program of 30 cycles of 93°C for 90 s, 43°C for 30 s, and 72°C for 3 min with a Program Temp Control System PC-700 (ASTEC Co.). After amplification, the product was digested with *Xho*I and *Bam*HI and ligated into expression plasmid pOSΔB-AP1 predigested with the same restriction enzymes. The resultant plasmid was introduced into *E. coli* JM109 cells which were then plated on Luria-Bertani plates containing 0.04 mM IPTG and 50 μg of ampicillin per ml.

By this PCR method, six single-mutation-carrying mutants and four double-mutation-carrying mutants were obtained with a 0.5% frequency (Table 1). For N3S:L18P, separation of the double point mutation was carried out to isolate N3S and L18P by the same strategy as described above. Mutations were found to overlap at positions 9 (Pro), 11 (Pro), 12 (Arg), and 14 (Pro) between the two distinct mutation methods of hydrox-

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TABLE 1. Summary of AP mutants

Method and mutant	Mutation point(s)	Category[a]	Reference
Wild type	None	1	8
Hydroxylamine			
P5L (separated from P5L:R12Q)	CCG→CTG	2	This study
P9L	CCG→CTG	2	8
P11L (separated from P11L:P17C)	CCG→TTG	2	This study
R12Q (separated from P5L:R12Q)	CGG→CAG	2	This study
P14S:R17C	CCG→TCG, CGC→TGC	NT	8
P11L:R17C	CCG→TTG, CGC→TGC	NT	8
P5L:R12Q	CCG→CTG, CGG→CAG	NT	8
PCR			
N3S (separated from N3S:L18P)	AAC→AGC	1	This study
P13Q	CCG→CAG	2	This study
P14T	CCG→ACG	3	This study
P14L	CCG→CTG	3	This study
P16T	CCG→ACG	2	This study
L18P	CTG→CCG	3	This study
L18Q	CTG→CAG	2	This study
I8V:R12Q	ATC→GTC, CGG→CAG	NT	This study
N3S:L18P	AAC→AGC, CTG→CCG	NT	This study
P11L:P14L	CCG→CTG, CCG→CTG	NT	This study
P9L:L18Q	CCG→CTG, CTG→CAG	NT	This study
Spontaneous: I8T	ATC→ACC	1	8

[a] Category numbers indicate the degree of growth inhibition by SSI-AP1 fusion proteins as shown in growth curves in Fig. 2. 1, strong inhibition; 2, mild inhibition; 3, slight and negligible inhibition; NT, not tested.

ylamine treatment and PCR. This suggested that the Pro and Arg residues present at high frequency in AP1 play an important role in its mode of action.

Figure 2 shows the growth curves of transformants harboring plasmids carrying the wild-type AP1 gene and 12 mutated AP1 genes, with pOS1 as a control, grown in liquid medium. Induction of the expression of mutant genes was achieved by the addition of IPTG (final concentration, 1 mM) to the medium

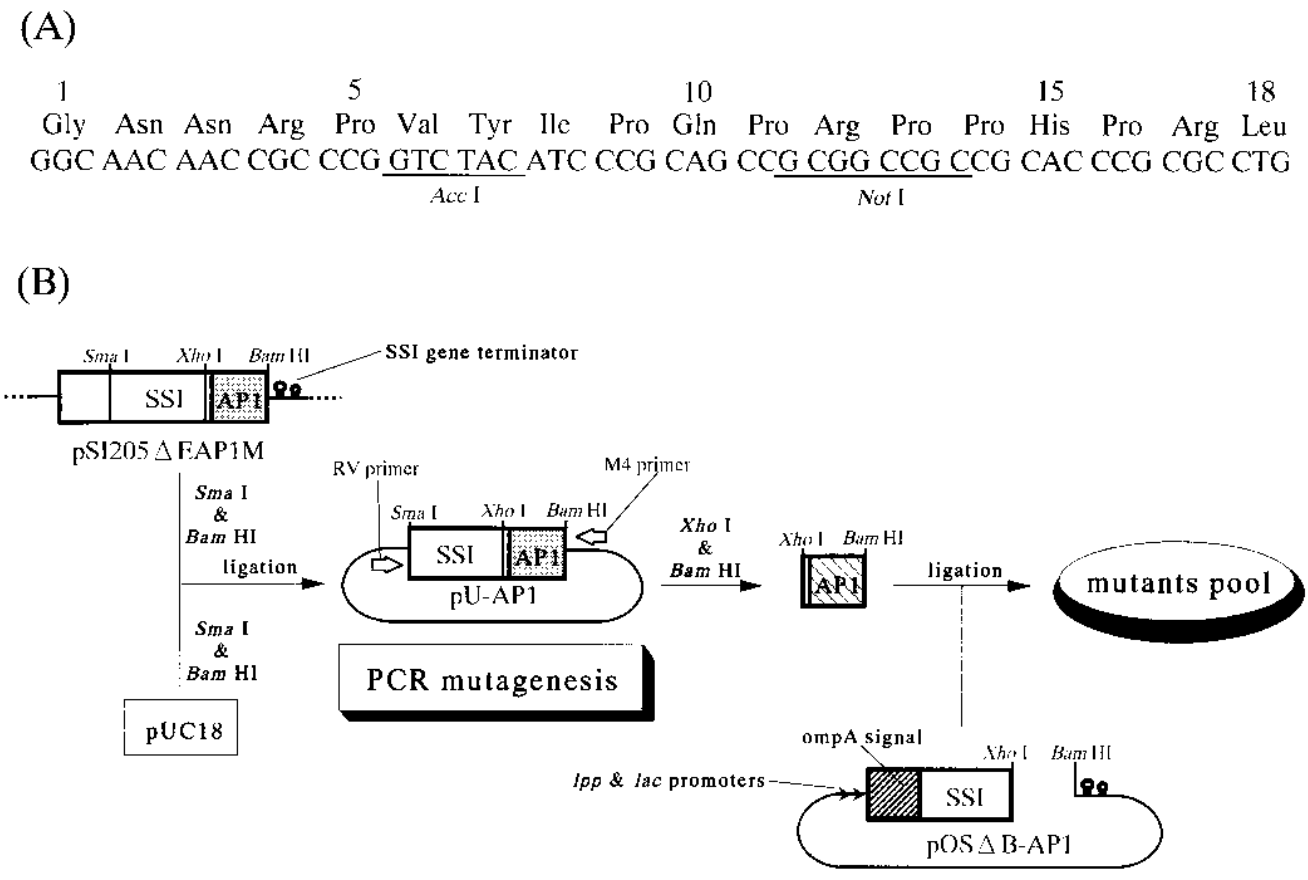

FIG. 1. (A) Primary structure of chemically synthesized AP gene; (B) scheme for PCR-induced mutagenesis. Please see the text for details.

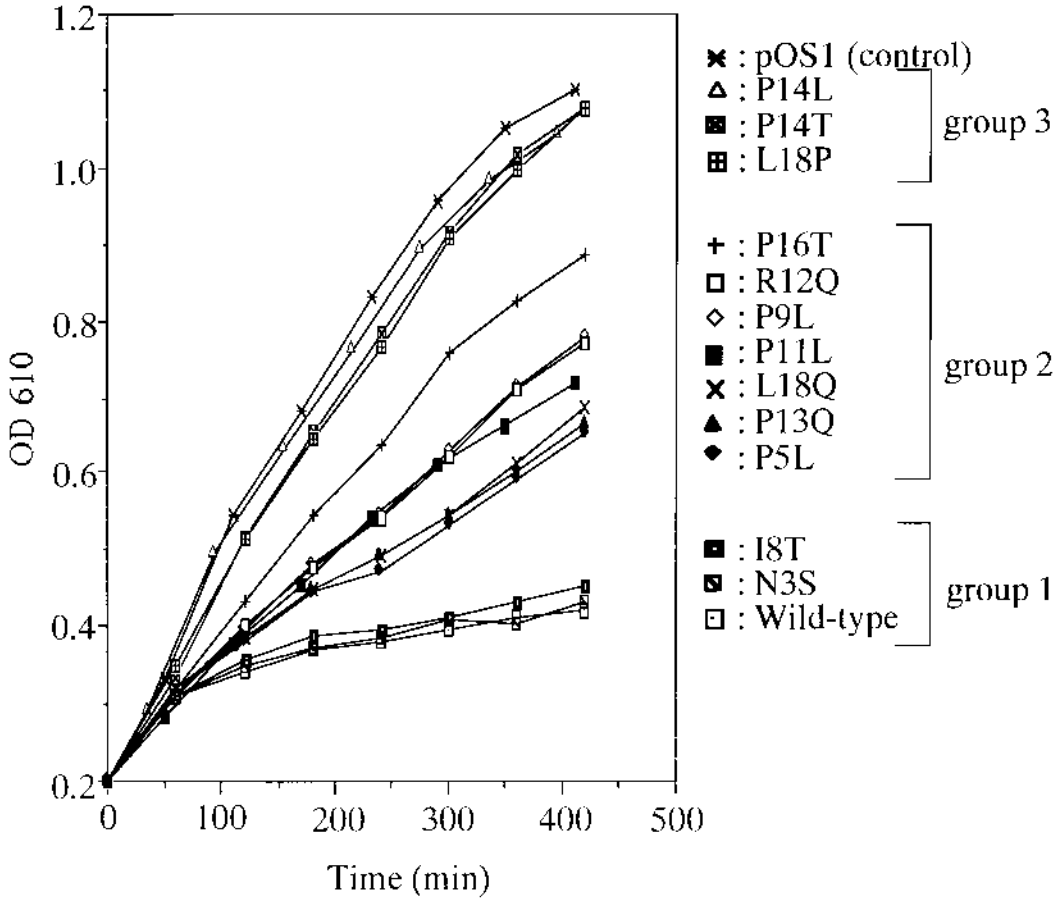

FIG. 2. Growth curves of transformants producing mutant APs fused with SSI. Growth curves are constructed for transformants harboring 12 plasmids carrying SSI-mutated AP1 fusion genes, the SSI–wild-type AP1 fusion gene, and the SSI gene alone. OD 610, optical density at 610 nm.

when the optical density for cell growth at 610 nm reached 0.2. Growth inhibition patterns observed were categorized into three groups in good agreement with those observed in the primary plate assay (data not shown). Construction of growth curves can be considered to be a secondary screening method for more precise estimation of antibacterial activities of various AP1 mutants screened by the primary plate assay.

No significant variation in the amounts of SSI-AP1 fusions was observed among the mutants tested except P5L, judging from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3), in which extraction efficiency of SSI-AP1 fusion protein from the periplasmic space can be normalized by the density of total protein bands. Markedly reduced production of SSI-AP1 in P5L was supposed to be caused by the proteolytic degradation during the purification process of the fusion protein. Bands probably corresponding to slightly degraded SSI-AP1 species were also detected in the lower zone of the SSI-AP1-specific band in the wild type and mutants N3S, I8T, P11L, P16T, L18P, and L18Q. This suggests that the growth inhibition of host cells was caused by SSI-AP1 itself and/or by degraded SSI-AP1 species. The amount of each AP1 mutant protein released into the medium was nearly proportional to that accumulated in the periplasmic space under the experimental conditions employed. Furthermore, in mutants P14L, P14T, L18P, and L18Q, protein band patterns distinctly different from those of other mutants were observed in the upper zone of the SSI-AP1-specific bands. Since we

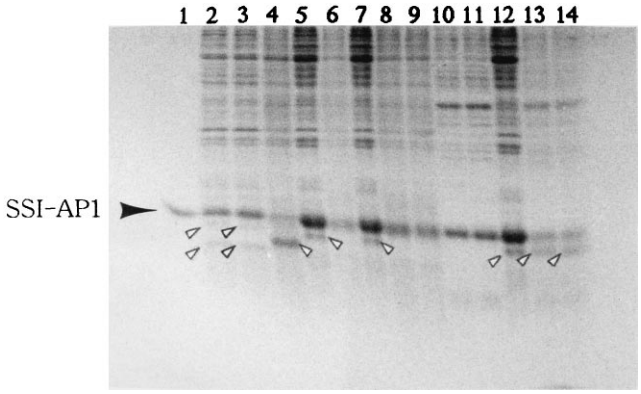

FIG. 3. SDS-PAGE analysis of wild-type and mutant recombinant SSI-AP1s. Each transformant was cultivated in 5 ml of Luria-Bertani medium containing 50 μg of ampicillin per ml at 30°C. A final concentration of 1 mM IPTG was added when the optical density at 610 nm reached 0.2. After measurement of cell numbers of transformants cultivated for 5 h, the periplasmic fraction was extracted from the cell pellet by a method reported previously (8). Extracted protein samples were precipitated by an 8% final concentration of trichloroacetic acid. Precipitates were resuspended in an adequate volume of TE buffer (10 mM Tris-Cl, pH 8.0) by adjustment to the same cell number (9.0×10^7 cells per 1 μl of TE buffer) among transformants, and 4 μl of each sample solution was subjected to SDS-PAGE. Lanes: 1, purified wild-type AP1 fusion protein (2 μg); 2, wild-type AP1; 3, N3S; 4, P5L; 5, I8T; 6, P9L; 7, P11L; 8, R12Q; 9, P13Q; 10, P14L; 11, P14T; 12, P16T; 13, L18P; 14, L18Q. Closed and open arrowheads indicate the SSI-AP1 fusion proteins and their detectable degraded species, respectively.

confirmed no contamination of other bacteria, it seems that this resulted from the different effects of the mutant AP1s on the physiological characteristics of the cells. Thus, we examined the correlation between growth and in vitro activity by using the previously characterized wild-type AP1 and P9L mutants as references (8). In addition, two representative mutants, P13Q and P14L, selected from groups 2 and 3, respectively, were purified from transformants by essentially the same method as those described previously (8). With an automated protein sequencer (8), predicted amino acid substitutions were confirmed for two recombinant mutant AP1s purified by reverse-phase high-performance liquid chromatography. From the results of the in vitro assay, the AP1 activity of mutant P13Q was reduced by one-third compared with that of wild-type AP1 (corresponding to a 4-μg/ml concentration of P13Q molecule) and no antibacterial activity was detected in the presence of even 20 μg of P14L per ml. Therefore, it was concluded that the antibacterial activity of each mutant can be estimated conveniently on the basis of the growth curves.

Functional mapping of AP1 by in vivo and in vitro assays resulted in the following findings. (i) Since the mutations of all group 3 mutants with the lowest AP1 activity are localized near the region of the AP1 gene encoding the C terminus, the C-terminal region of AP1 may play an important role in its antibacterial activity. (ii) Many of the Pro residues present at high frequency in AP1, the 12th Arg, and the C-terminal Leu residue contribute to the antibacterial activity. (iii) The degree of AP1 activity change depends on the amino acids substituted, as shown in the cases of L18P (group 3) and L18Q (group 2). (iv) The findings described above are in good agreement with the results of structure-function relationship analysis of naturally occurring AP-type peptides reported by Casteels et al. (3).

The final goal of the present study was to clarify the mode of action of AP1. We have evidence that the OmpA signal sequence-mediated direct production of AP1 without its fusion partner, SSI, effects almost the same growth inhibition pattern in each mutant AP1 as the SSI-AP1 fusion protein, although it is difficult to determine where and how inhibition occurs (this will be published elsewhere). This suggests that both cases have the same mode of action. It was reported that hymenoptaecin, a larger (93-amino-acid), cationic, and Pro-rich polypeptide found in AP1-producing honeybees, displays a lethal effect on *E. coli* through sequential permeabilization of the outer and inner membranes (1), while AP1 does not permeabilize the membrane and functions through a non-pore-forming mechanism (3). Taken together with our results, we speculate that, if any, an unknown target molecule(s) against AP1 attack is present on the bacterial cell surface and that the antibacterial action of SSI-AP1, once SSI-AP1 is excreted into the medium, leads to the growth inhibition of host cells by affecting some target molecule(s). Characterization of the distribution pattern of proteins in AP1-resistant mutant strains would enable us to identify the target molecule(s) of the antibacterial action of AP1. In addition, the isolation of mutants with antibacterial activities higher than that of wild-type AP1 using newly devised positive selection methods is in progress.

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