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Protein Engineering of Penicillinase as Affinity Ligands for Bioprocessing

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The active site and the substrate binding site of penicillinase (β-lactamase) from *Bacillus licheniformis* were altered in this study so that the enzyme retains the specific binding capability to the β-lactam antibiotics, but fails to hydrolyze them. When Lys47 in the enzyme molecule was replaced by Ala47, the mutant protein PenP(KA) lost not only its catalytic activity but also the substrate binding ability. In contrast, when Ser44 was replaced by Ala, the mutant protein PenP(SA) lost its catalytic activity but still kept the substrate binding ability. It was found that PenP(SA) exhibited the characteristic association and dissociation with penicillin G, but the dissociation constant was much larger than expected. Possible use of this mutant protein as an affinity ligand is also discussed.

The affinity adsorption for small molecular weight products such as antibiotics is often limited because of the lack of effective biospecific ligands. Conventional separation processes for these compounds mainly utilize a combination of non-specific methods such as chromatography, ion-exchange, and physical adsorption. Many of these compounds interact specifically with different enzymes in a number of biological systems. For instance, β-lactam antibiotics interact with microbial β-lactamases, which hydrolyze these antibiotics to biologically inactive molecules. Immobilized β-lactams have been used as affinity ligands to purify several types of β-lactamases (1-3). However, the reciprocal system using immobilized enzymes for purifying valuable β-lactams cannot be applied directly because these enzymes not only specifically bind to the β-lactams but also catalyze their hydrolyses.

Recent advances in protein engineering now permit the modification of any gene for designing novel proteins not found in nature using techniques such as site-directed mutagenesis (4). This had led to the possibility of designing enzymes by manipulating the active site so that it retains the capability of specific recognition and reversible binding to the substrate but fails to catalyze its degradation. Such a redesigned enzyme would be a powerful affinity ligand for its corresponding substrate. In this study we have demonstrated the feasibility of this concept using β-lactamase from *Bacillus licheniformis*.

MATERIALS AND METHODS

Bacterial strains and plasmids The bacterial strains and plasmids used are listed in Table 1. *penP*, *penI*, and *penJ* are the penicillinase (β-lactamase) gene, its repressor gene, and antirepressor gene from *B. licheniformis* ATCC 9945a, respectively.

Transformation Transformation of *Escherichia coli* with plsmid DNA was done as described previously (6). Transformants were selected on L agar (6) containing 20 μg of tetracycline (Tc) per ml. Transformation of competent *Bacillus subtilis* cells was also performed as previously described (5). *B. subtilis* transformants were selected on L agar containing 25 μg of Tc per ml.

DNA manipulations and analysis Preparation of plasmid DNA, cleavage and ligation of DNA, gel electrophoresis for DNA analysis and isolation were all performed as described previously (8). DNA was sequenced by the dideoxy method (7), with an M13 sequencing kit (Takara Shuzo Co., Kyoto). The sequencing was done on both strands. Site-directed mutagenesis was performed with the *in vitro* mutagenesis system (Amersham Japan, Tokyo).

Penicillinase production and enzyme assay *B. subtilis* carrying the *penP* gene was cultivated overnight in CH/S medium (8) at 37°C. A sample (50 ml) of the preculture was inoculated in the fresh medium (5 *l*) and cultivated in a 10 *l* fermentor (Type MD-500, Marubishi Bioeng Co., Tokyo) for 18 h. The supernatant of the culture broth obtained by centrifugation (8000 *g*, 10 min) was used as the enzyme source. Penicillinase was purified by phosphocellulose treatment, carboxymethylcellulose chromatography and Sephadex G-100 chromatography as described previously (9).

Penicillinase was assayed by the iodometric method as described previously (6). The method for detection of penicillinase-positive colonies on LP plates (L agar containing 0.75% (w/v) polyvinyl alcohol) has also been described previously (6).

Protein assay Protein concentration was determined by the BCA method (protein assay kit, Pierce Co. Rockford, Illinois, U.S.A.). Bovine serum albumin was used as a standard.

Immunoprecipitation Anti-penicillinase serum was prepared by subcutaneously injecting a rabbit with 1.0 mg of purified penicillinase in complete Freund's adjuvant, boosting subcutaneously at two weeks with 1.0 mg of purified penicillinase in complete adjuvant, and bleeding beginning two weeks later. The sample was coagulated at room temperature for 30 min, and then kept at 4°C overnight. After centrifugation, the supernatant fraction was used as anti-penicillinase serum. Immunoprecipitation on

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TABLE 1. Bacterial strains and plasmids

Bacteria/Plasmids	Characteristics	Reference
Bacteria		
B. subtilis MI113	*trpC2 arg-15 thr-5 hsmM hsrM*	(5)
E. coli C600-1	*leu-6 thr-1 supE44 lacY1 tonA21 hsdR hsdM* Trp	(6)
E. coli JM109	*recA1 Δlacpro endA1 gyrA96 thi-1 hsdR17 supE44 relA1* F'[*traD36proAB lacI*^{q}*ZΔM15*]	(7)
Plasmid		
For *B. subtilis*		
pPTB50	Tc^r *penP*$^+$ *penI penJ*	(8)
pPTB50 SA	Tc^r *penP(SA)*, Ser44 was replaced by Ala	This work
pPTB50 ST	Tc^r *penP(ST)*, Ser44 was replaced by Thr	This work
pPTB50 KA	Tc^r *penP(KA)*, Lys47 was replaced by Ala	This work
For *E. coli*		
pTTE11	Tc^r *penP*$^-$ *penI penJ*	(6)
pTTE11 SA	Tc^r *penP(SA)*, Ser44 was replaced by Ala	This work
pTTE11 ST	Tc^r *penP(ST)*, Ser44 was replaced by Thr	This work
pTTE11 KA	Tc^r *penP(KA)*, Lys47 was replaced by Ala	This work

plate was performed by the gel-diffusion method (10).

Penicillin binding assay Characteristics of penicillin binding were examined by the gel filtration method (11) and the equilibrium dialysis method (12). Benzyl[^{14}C]-penicillin potassium (labeled PenG) (Amersham Japan, Tokyo) was used as rediolabeled substrate. A Sephadex G-25 column (7×250 mn) was used in the gel filtration method. The equilibrium dialysis method was performed under the following conditions using semipermeable membrane.

Compartment A: 2.6×10^{-5} M PenP(SA) and 3.7×10^{-5} M [^{14}C]-PenG in 0.1 M phosphate buffer (pH 7.0)

Compartment B: 0.1 M phosphate buffer (pH 7.0)

Chemicals Penicillin G was purchased from Meiji Seika Co., Tokyo. Chemically synthesized oligonucleotides were from Applied Biosystem's Japan Co., Osaka. The antibiotics, restriction endonucleases, ligase, and all of the reagents used here were purchased from the same companies and laboratories as in previous work (6, 8, 9), unless otherwise noted.

RESULTS AND DISCUSSION

Active site of *B. licheniformis* penicillinase Strominger *et al.* (13) compared the amino acid sequences of penicillin binding sites and their flanking regions for various β-lactamases, carboxy peptidase, and penicillin binding proteins (PBP) (Table 2). The amino acid sequence, Ser-X-X-Lys, was found to be the consensus sequence for the penicillin binding site. Since the active site of *Bacillus cereus* β-lactamase type I was known to be Ser91 of the immature enzyme (14), the amino acid sequence was aligned and compared with that of *B. licheniformis* penicillinase (data not shown). The sequence, Ser91-X-X-Lys94, and its flanking region of *B. cereus* enzyme are very similar to the sequence, Ser85-X-X-Lys88, and the flanking region of *B. licheniformis* immature penicillinase, respectively. Ser85 corresponds to Ser44 of exo-small penicillinase, because the immature enzyme is cleaved at Lys42 to give an extracellular enzyme (15). Accordingly, it was inferred that Ser44 of extracellular penicillinase of *B. licheniformis* might function as an active site.

Reaction mechanism of penicillinase The mechanism of reaction of *B. cereus* penicillinase was inferred as follows: The hydroxyl group of the active site Ser is used to form acyl-enzyme intermediate. The intermediate is hydrolyzed, giving penicilloic acid as the product (16).

Herzberg and Moult (17) determined the tertiary structure of *Staphylococcus aureus* β-lactamase. The enzyme structure was compared with that of the serine protease from *Streptomyces griseus* (18). It was found that the steric conformation of Glu166, Lys73 and Ser70 (active site) of the β-lactamase was nearly identical to that of Asp102, His57 and Ser195 (active site) of the serine protease, all of which are essential for catalytic activity (Fig. 1) (17, 18). His57 of the serine protease activates Ser195 by providing an electron. Likewise, Lys73 of the β-lactamase might function to activate Ser70. Therefore, it was inferred that Lys47 of *B. licheniformis* penicillinase could function to activate the putative active site Ser44.

TABLE 2. Consensus sequence of penicillin binding regions

Enzyme	Microorganism	Active site amino acid sequence
		★
Penicillinase (β-lactamase)	*B. licheniformis*	-Phe-Ala-Phe-Ala-Ser-Thr-Ile-Lys-Ala-
	S. aureus	-Phe-Ala-Tyr-Ala-Ser-Thr-Ser-Lys-Ala-
	B. cereus	-Phe-Ala-Phe-Ala-Ser-Thr-Tyr-Lys-Ala-
	E. coli	-Phe-Pro-Met-Met-Ser-Thr-Phe-Lys-Val-
CPase	*E. coli* (PBP-5)	-Arg-Asp-Pro-Ala-Ser-Leu-Thr-Lys-Met-
	B. subtilis	-Leu-Pro-Ile-Ala-Ser-Met-Thr-Lys-Met-
	B. stearothermophilus	-Leu-Gly-Ile-Ala-Ser-Met-Thr-Lys-Met-

★, Active center.

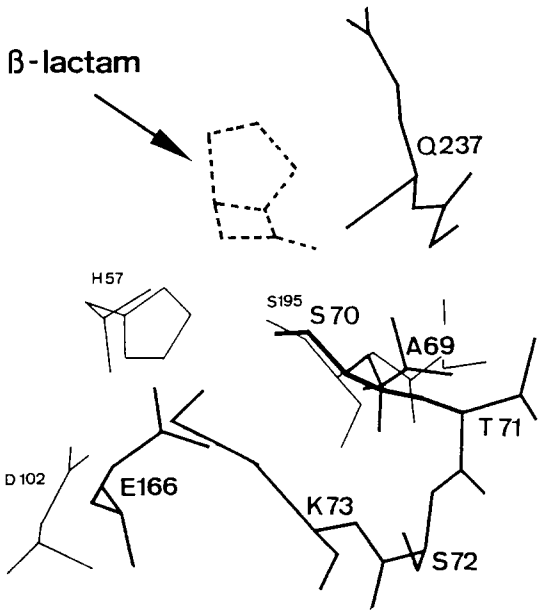

FIG. 1. Comparison of three dimensional structures in the active center regions of *Staphy. aureus* β-lactamase (——) (17) and serine protease of *S. griseus* (——) (18).

Creation of mutant proteins of penicillinase To eliminate the catalytic activity of *B. licheniformis* penicillinase, the following amino acid replacements were attempted.

TABLE 3. Synthetic oligonucleotides used

	Oligonucleotide
Ser44 → Ala44	5'GCTTTTGCGGCGACGAT3' (★)
Ser44 → Thr44	5'GCTTTTGCGACGACGAT3' (★)
Lys47 → Ala47	5'TCGACGATTGCGGCTTAAAC3' (★★)

★, Base substitution.

(i) To block the formation of the acyl-enzyme intermediate, the hydroxyl group of Ser44 was eliminated, *i.e.* Ser44 was replaced by Ala44.
(ii) To inhibit the formation of the acyl-enzyme intermediate, a methyl group was added to Ser44 as the steric interference, *i.e.* Ser44 was replaced by Thr44.
(iii) To eliminate the catalytic activity, Lys47 was replaced by Ala47.

These amino acid substitutions were performed by site-directed mutagenesis as shown in Fig. 2. *B. licheniformis* penicillinase gene, *penP*, was cloned in pPTB50. The *Pst*I-*Cla*I fragment of *penP* was cloned in *Pst*I-*Acc*I sites of phage M13. Single-stranded DNA was isolated from the recombinant phage M13*penP*, and was annealed by the synthetic oligonucleotide carrying mutation (Table 3). After *in vitro* mutagenesis, the DNA was used for the transfection of *E. coli* TG1. The mutation was confirmed by DNA sequencing. The *Pst*I-*Bgl*II fragment of pTTE11 was substituted with the *Pst*I-*Bgl*II fragment containing mutation. The mutant plasmids (amino acid substitutions, Ser44 → Ala44, Ser44 → Thr44, Lys47 → Ala47) were

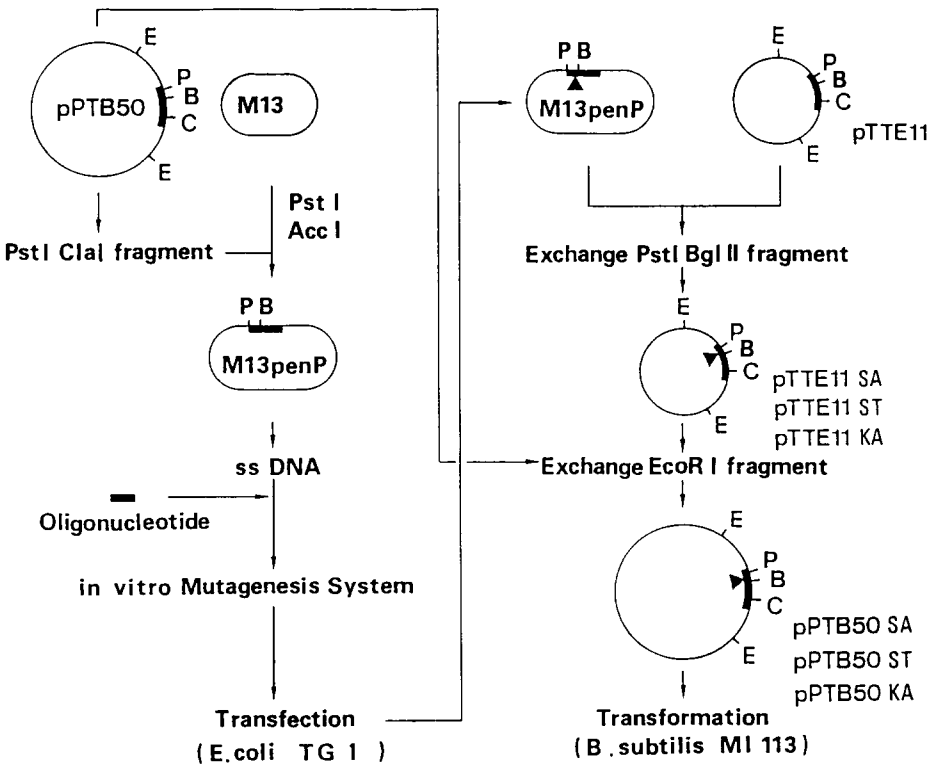

FIG. 2. Strategy for the construction of mutant enzyme genes by site-directed mutagenesis. For details, see text.

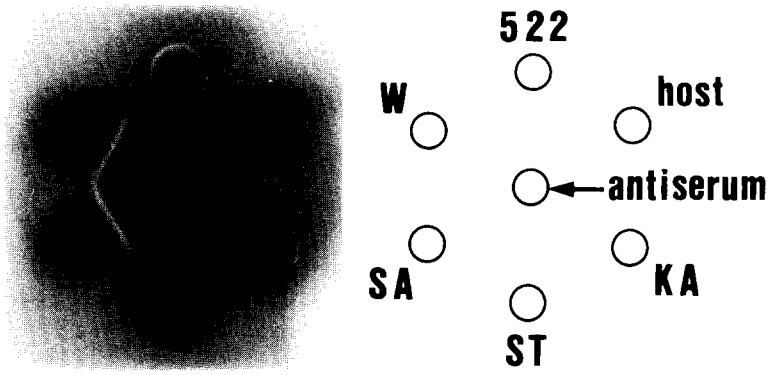

FIG. 3. Immunoprecipitation by gel-diffusion method. Culture supernatants of *B. subtilis* without plasmid and the strain carrying pTB522 (vector), pPTB50, pPTB50SA, pPTB50ST, or pPTB50KA are indicated by host, 522, W, SA, ST, or KA, respectively.

designated pTTE11SA, pTTE11ST, and pTTE11KA, respectively. The *Eco*RI fragment of pPTB50 was replaced by the *Eco*RI fragments of these plasmids, pTTE11SA, pTTE11ST, and pTTE11KA. The mutant plasmids thus obtained in *B. subtilis* were designated pPTB50SA, pPTB50ST, and pPTB50KA, respectively.

B. subtilis MI113 carrying wild-type *penP* or its mutants was cultivated in L broth at 37°C for 12 h, and the penicillinase activity was assayed. Although wild-type *penP* carrier produced a large amount of penicillinase (2250 units/ml), no enzyme activity was observed for three mutant plasmid carriers. To examine whether or not the mutant *penP* protein could be secreted from host cells, immuno-precipitation was performed (Fig. 3). Anti-penicillinase serum was in the center well, and the culture supernatants of plasmid carriers were placed in the surrounding wells. No precipitation was observed for *B. subtilis* (host) and the pTB522 carrier, while others exhibited fused precipitation bands. It is clear from Fig. 3 that the mutant proteins PenP(SA), PenP(ST), and PenP(KA) from pPTB50SA, pPTB50ST, and pPTB50KA, respectively, were secreted in the culture media. These results showed that the mutant proteins were secreted but had lost the catalytic activity entirely.

Purification of mutant proteins of penicillinase Purification of mutant proteins was performed by the method used for the wild-type enzyme. Since PenP(ST) was found to be difficult to be purified in a large amount, subsequent work was focused on PenP(SA) and PenP(KA) only. These two mutant proteins were purified and subjected to SDS-polyacylamide gel electrophoresis (Fig. 4). Both proteins showed single bands, with the same migration. PenP(SA) and PenP(KA) exhibited fused precipitation bands with purified penicillinase (wild type) (Fig. 4).

Penicillin binding assay for PenP(SA) and PenP(KA) For the penicillin binding assay, a Sephadex G-25 column

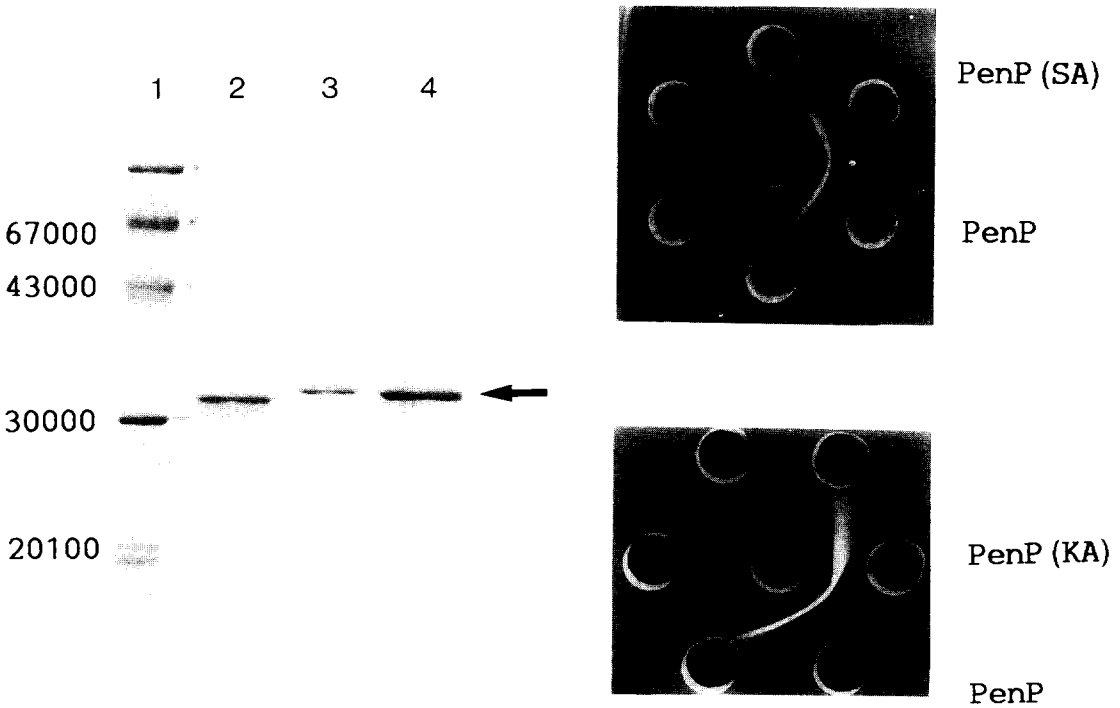

FIG. 4. SDS-polyacrylamide gel electrophoresis and immunoprecipitation of penicillinase and its mutant proteins, PenP(SA) and PenP(KA). Lane 1, molecular weight standard; lane 2, wild-type penicillinase (PenP); lane 3, PenP(SA); lane 4, PenP(KA).

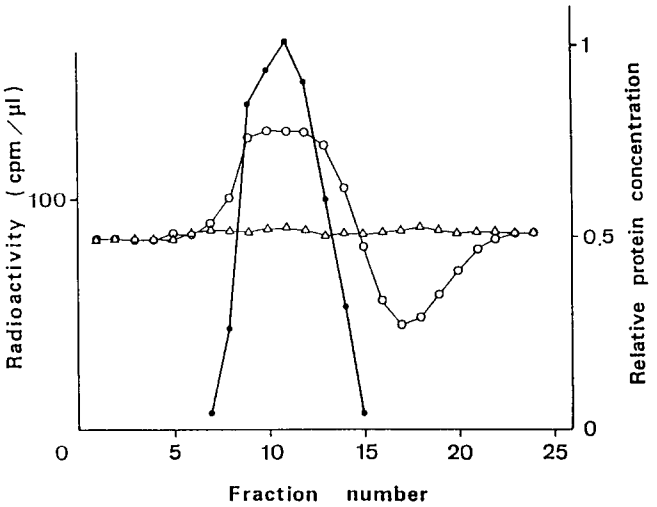

FIG. 5. Penicillin binding assay for PenP(SA), using gel filtration column chromatography. ○, PenP(SA) (2.2 n mole)+[^{14}C]-PenG; △, BSA (200 μg)+[^{14}C]-PenG. Radioactivity of [^{14}C]-PenG was measured. ●, PenP(SA).

was equilibrated with 0.1 M phosphate buffer (pH 7.0) containing [^{14}C]-PenG (0.85×10^{-6} M). A mixture (100 μl) of PenP(SA) (2.2 nmol) and [^{14}C]-PenG (0.85×10^{-6} M) in 0.1 M phosphate buffer (pH 7.0) was applied to the above-mentioned column, and eluted by the phosphate buffer plus [^{14}C]-PenG. The elution pattern of [^{14}C]-PenG is shown in Fig. 5. When protein PenP(SA) was eluted, high radioactivity was detected. Following this peak, a decrease in radioactivity was observed. In contrast, when PenP(SA) was replaced by bovine serum albumin (BSA, 200 μg), no such increase and decrease of [^{14}C]-PenG were observed. These results show that PenG interacted specifically with PenP(SA). When PenP(KA) was used instead of PenP(SA) in a similar column, no such association between PenP(KA) and PenG was observed. This fact indicates that Lys47 in the penicillinase is responsible for the binding of PenG.

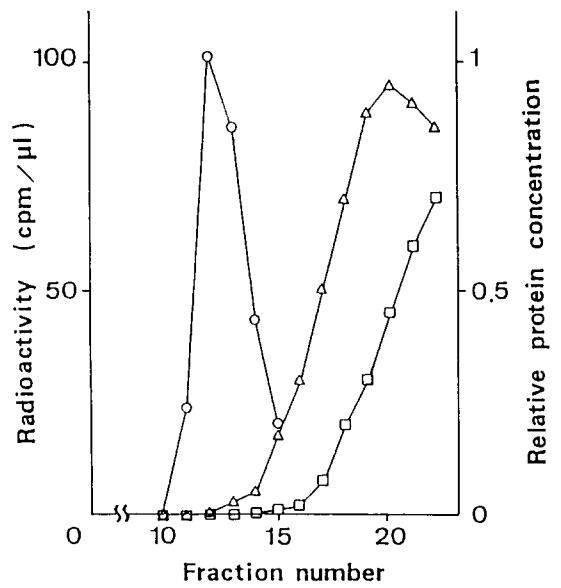

FIG. 6. Penicillin binding assay for PenP(SA). □, [^{14}C]-PenG; △, [^{14}C]-PenG+PenP(SA). Radioactivity of [^{14}C]-PenG was measured. ○, PenP(SA). For details, see text.

To examine the characteristics of the association and dissociation between PenP(SA) and PenG, different types of column chromatography were carried out. A Sephadex G-25 column was equilibrated with 0.1 M phosphate buffer (pH 7.0), and a mixture (100 μl, prewarmed at 30°C for 10 min) of PenP(SA) (0.5 nmol) and [^{14}C]-PenG (4 nmol) in 0.1 M phosphate buffer (pH 7.0) was applied to the column. The elution pattern is shown in Fig. 6. The peak fractions of PenP(SA) and PenG were no. 12 and no. 20, respectively. In other words, the protein passed through the column first, and then PenG was eluted. When only [^{14}C]-PenG was applied to the same column, the antibiotic was eluted more slowly than in the former case. These data indicate that PenG-PenP(SA) complex was dissociated during the process of column chromatography.

To assess the dissociation constant for the PenG-PenP(SA) system, the equilibrium dialysis method was performed. The dissociation constant was determined to be 2.0×10^{-3} M. If the mutant protein is used as an affinity ligand for PenG, the dissociation constant should be, in general, less than 10^{-5} M. In fact, the dissociation constant for the IgG-domain B (of protein A) complex is reported to be 2.2×10^{-8} M (19).

Despite the weak binding of PenP(SA) with PenG, it may have more proper binding constants with respect to other β-lactam antibiotics. This mutant protein may be useful in affinity chromatographic separation and bioscreening for new, potent β-lactam antibiotics from nature. We are currently investigating the possibilities in our laboratory.

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