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Identification of the active site of *Citrobacter freundii* β-lactamase using dansyl-penicillin

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The active site sequence of a β-lactamase encoded by chromosomal gene(s) in *Citrobacter freundii* GN346 was determined using dansyl-penicillin as a fluorescent probe. The tryptic digest of the labelled enzyme gave a fluorescent peptide containing 22 amino acids. The sequence of this peptide was identical to the consensus sequence of class C β-lactamases, Gly-Ser-X-Ser-Lys. The residue labelled was the serine adjacent to the glycine. The active site sequence corresponded to positions 46–67 of the entire sequence of the *Citrobacter freundii* β-lactamase determined on the basis of the DNA sequence of the structural gene [(1986) Eur. J. Biochem. 156, 441–445]. The labelled serine corresponded to Ser-64.

Active site; Dansyl-penicillin; βLactamase; Amino acid sequence; (C. freundii)

1. INTRODUCTION

Most chromosomal β-lactamases in gram-negative bacteria are cephalosporinase-type enzymes [2–4]. The clinical importance of these cephalosporinases is increasing because cephalosporinase-producing bacteria are generally resistant to both cephalosporins and penicillins [5].

The *Citrobacter freundii* cephalosporinase is a typical cephalosporinase with respect to substrate specificity and physical properties [2]. We have studied the mechanism of cephalosporinase inactivation by novel mechanism-based inactivators [6–8] using the *C. freundii* GN346 enzyme as a model enzyme. In order to clarify the interaction between the inactivators and the enzyme at the molecular level, it is necessary to know the amino acid sequence around the active site of the enzyme. Recently, the entire base sequence of the *C. freundii* cephalosporinase gene was reported by Lindberg and Normark [1]. Although the active site was identified on the basis of the known active sites of cephalosporinases [9,10], the active site sequence should be directly determined after isolation of the active site peptide.

We tried to determine the active site sequence of the *C. freundii* cephalosporinase using dansyl-penicillin as a fluorescent probe for detection of the active site. Although dansyl-penicillin has previously been employed only for a penicillinase-type enzyme [11], our study revealed that the fluorescent method is also applicable to cephalosporinase-type enzymes, and that it is a convenient technique.

In this paper, we report the determination of the 22-amino acid sequence around the active site of the cephalosporainse produced by *C. freundii* GN346.

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2. MATERIALS AND METHODS

2.1. *Enzyme and chemicals*

β-Lactamase from *C. freundii* GN346 was purified by the procedure described in [6]. Dansyl-penicillin was synthesized from 6-aminopenicillanic acid and dansyl chloride according to Cartwright and Fink [11]. Tosylphenylalanylchloromethane (TPCK)-treated trypsin and thermolysin

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were purchased from Sigma (St. Louis, MO, USA).

2.2. *Fractionation of peptides*

HPLC of peptides digested with trypsin or thermolysin was carried out with a Spectra Physics model 8700 apparatus on a column (25 cm × 0.46 cm in diameter) filled with Bakerbond wide-pore butyl (C4) (5 μm) (J.T. Baker, Phillipsburg, NH, USA) unless otherwise stated. Elution was performed with a linear gradient of 5–50% acetonitrile containing 0.1% trifluoroacetic acid for 30 min at a flow rate of 1.5 ml/min, and 1 min fractions were collected.

2.3. *Polyacrylamide gel electrophoresis, amino acid composition analysis and sequencing of labelled peptides*

Electrophoresis at pH 11.7 of the peptides, which had been labelled with 8-isothiocyanate-1,3,6-trisulfonylpyrene (TSPITC), was carried out according to Tsugita et al. [12]. The amino acid composition was determined with a Hitachi model 835 amino acid analyzer after hydrolysis of the peptides in 6 N HCl in vacuo at 110°C for 24 h. Automatic amino acid sequencing was performed with an Applied Biosystems gas-phase sequencer [13].

3. RESULTS

3.1. *Preparation of the labelled enzyme*

Prior to labelling of the enzyme with dansyl-penicillin, the roles of dansyl-penicillin as a substrate and an inhibitor for the *C. freundii* cephalosporinase were investigated. The k_{cat} value was 5.2×10^{-2} s^{-1}, and the K_i value for a competitive inhibitor was determined to be 3.2 μM. Dansyl-penicillin was also found to act as a progressive but reversible inhibitor of the enzyme. The enzyme activity was completely lost when the molar ratio of the probe to the enzyme was 20. However, the inactivated enzyme gradually regained its activity after removal of the free probe from the reaction medium, the rate of the restoration being approx. 1.2×10^{-4} s^{-1}.

In order to label the cephalosporinase with the fluorescent probe, the enzyme (5.4 mg, 120 nmol) was incubated with 12 μmol dansyl-penicillin in 0.37 ml of 50 mM sodium phosphate buffer (pH 7.0) at 30°C for 5 min. The reaction was stopped by adding 1 vol. of acetic acid. Gel filtration was then carried out on a Sephadex G-25 column (100 cm × 1.5 cm in diameter) in 15% acetic acid at 5°C to remove free dansyl-penicillin and the degradation product. The protein fractions were pooled and lyophilized.

To determine the extent of labelling, a part of the labelled enzyme was treated with 10% triethylamine at 37°C for 2 h and then fluorescence (excitation, 350 nm; emission, 550 nm) due to the liberated dansyl-penicilloic acid was measured. The molar ratio of the bound dansyl-penicillin to the enzyme was estimated to be 0.64.

3.2. *Isolation of the labelled peptide*

The labelled and lyophilized enzyme was dissolved in 2 ml of 30 mM ammonium bicarbonate buffer (pH 7.9) containing 2 M urea. Trypsin (50 μl; 1 mg/ml in 0.001 N HCl) was added and then the reaction mixture was incubated for 60 min at 37°C. Then a second portion of 50 μl of the trypsin solution was added and the incubation continued for a further 60 min. The tryptic digest was directly fractionated by HPLC, and one major peak (T1; eluted at 19 min) and one minor peak (T2; eluted at 10 min) were detected on the basis of the fluorescence (fig.1).

The T1 fractions (eluted between 18 and 20 min) were pooled and lyophilized (T1 peptides). One half of the T1 peptides was dissolved in 20% formic acid and then fractionated by a second HPLC under the conditions given above (fig.2A). From the three major peaks detected on the basis of absorbance at 210 nm, the first peak (eluted at 19 min) corresponded to the fluorescent peptide. However, the three peaks were too close for the respective components to be separated from each other.

The dansyl-penicilloyl moiety was removed from the T1 peptides by 10% triethylamine treatment. It was expected that the removal of the hydrophobic dansyl-penicilloyl moiety from the labelled peptide would result in shortening of the retention time on HPLC. As expected, a new peak appeared at 16 min with a concomitant decrease in the labelled peptide peak (the first of the three major peaks) (fig.2B). Thus, the peptide that eluted at 16 min was identified as the active site fragment.

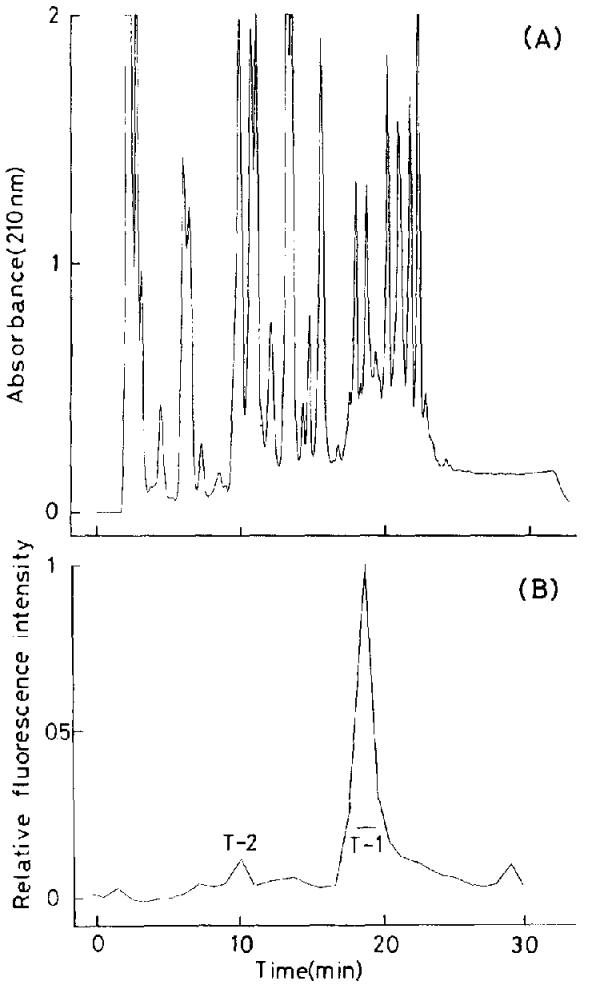

Fig.1. Fractionation of tryptic peptides by HPLC. The tryptic digest of the labelled enzyme (500 μl) was applied to a reverse-phase wide-pore butyl (C4) column, and eluted as described in section 2. (A) UV detection with an ISCO V^4 UV detector at 210 nm. (B) Fluorescence detection with a Hitachi MPF4 fluorescence spectrophotometer (excitation 350 nm; emission, 550 nm) after dilution of each fraction with 2 ml of acetonitrile.

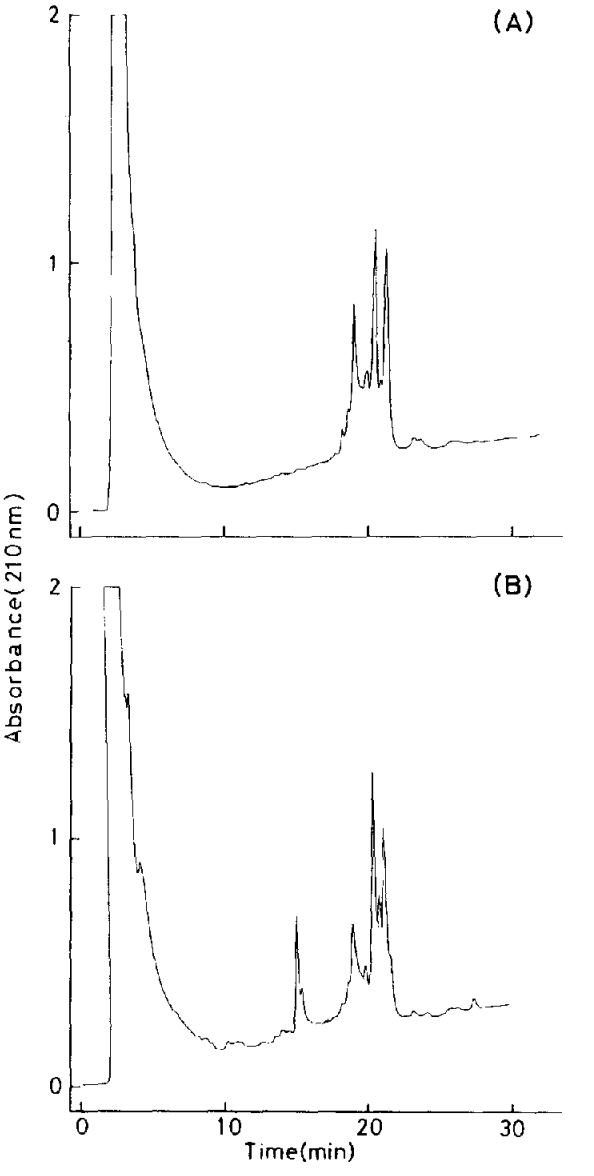

Fig.2. HPLC of T1 peptides. T1 peptides (A) or triethylamine-treated T1 peptides (B) were subjected to a second HPLC, as described in the text, under the same conditions as in fig.1.

Polyacrylamide gel electrophoresis of the active site fragment labelled with TSPITC gave a single band. The molecular mass was determined to be about 2600 Da, which corresponded to a peptide composed of 22 amino acids. The amino acid composition of this peptide was analyzed and the results are shown in table 1.

The amino acid sequence of the active site fragment was determined, with an automatic sequencer, to be as shown in fig.3. The amino acid composition calculated from the sequence was in good agreement with the results obtained with the amino acid analyzer except for a small difference (table 1).

Table 1

Amino acid composition of the active site peptides of *Citrobacter freundii* cephalosporinase

Amino acid	Number of residues per molecule		
	Trypsin fragment		Trypsin-thermolysin fragment
	From amino acid analysis	From sequencer	From amino acid analysis
Asp	2.4	3	–
Thr	1.5	2	–
Ser	2.4	2	1.4
Glu	3.0	3	0.5
Pro	0.9	1	–
Gly	1.8	1	0.9
Ala	1.9	2	–
Val	2.1	2	0.4
Ile	1.4	1	–
Leu	2.0	2	1.0
Phe	1.3	1	–
Lys	1.0	1	0.6
His	0.7	1	–

3.3. *Determination of the labelled residue*

The T1 peptides (approx. 10 nmol) were digested with thermolysin (0.2 mg) in 2 M urea at 37°C for 2 h. The digest was fractionated by HPLC. One major peak (TH; eluted at 16.8 min) was detected on the basis of the fluorescence (not shown). The dansyl-penicilloyl moiety was removed from the TH peptides by 10% triethylamine treatment. The HPLC pattern of the treated peptides was compared with that of the untreated TH peptides. In this experiment, HPLC was performed under the conditions described in section 2 except that a Zorbax C8 column (25 cm × 0.46 cm in diameter) was used. Several new peaks appeared after the triethylamine treatment (not shown). The amino acid composition of the smallest component (thermolytic peptide) among those in these new peaks is shown in table 1. On the basis of the sequence of the tryptic peptide, the thermolytic peptide was confirmed to coincide with the Leu-Gly-Ser fragment (fig.3). The residue labelled with dansyl-penicillin was the serine between glycine and valine.

4. DISCUSSION

The sequences around the active sites of the class C β-lactamases of *Pseudomonas aeruginosa, Escherichia coli* K12 (amp C) and *Enterobacter cloacae* P99 have been established [9,10]. The corresponding sequence determined in our work is very similar to that of the amp C β-lactamase; 19 out of 22 residues are identical. Similarly, 18 out of 22 residues and 12 out of 14 residues are the same as in the sequences of the *E. cloacae* P99 and *P. aeruginosa* β-lactamases, respectively.

The entire sequence of the structural gene of the *C. freundii* OS60 β-lactamase was recently determined by Lindberg and Normark [1]. The active site sequence directly determined from the peptide by us is consistent with the sequence at positions 46–67, determined from the DNA sequence. The labelled serine corresponding to Ser-64 was confirmed to be the active site serine.

Dansyl-penicillin had previously only been used as a probe for a penicillinase-type enzyme [11]. The present work revealed that dansyl-penicillin exhibits far smaller values for both k_{cat} (5.2×10^{-2} s^{-1}) and K_i (3.2 μM) toward the cephalosporinase than the values for k_{cat} (2×10^3 s^{-1}) and K_m (200 μM) toward *Bacillus cereus* β-lactamase I [11]. i.e.,

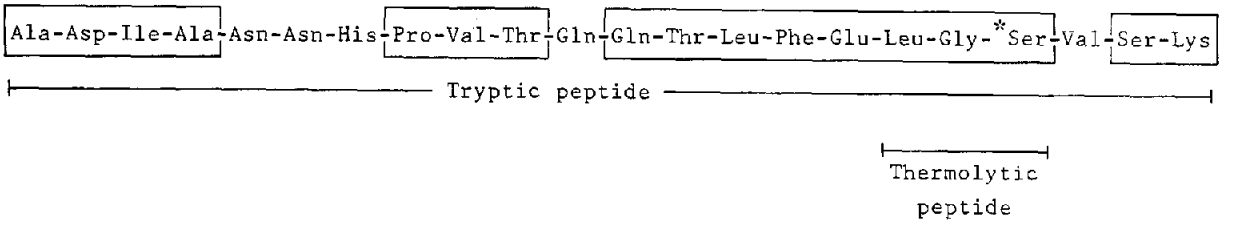

Fig.3. The active site sequence of the *C. freundii* β-lactamase. The common sequences with the *E. coli* amp c and *E. cloacae* P99 β-lactamases are boxed. An asterisk indicates the labelled residue.

it forms a far more stable complex with the former enzyme than with the latter. Dansyl-penicillin may be useful for the identification of the active sites of other cephalosporinase-type enzymes.

Dansyl-penicillin can be converted into a mechanism-based inactivator for cephalosporinases (unpublished). Dansyl-penicillin sulfone is assumed to be a better probe than dansyl-penicillin. Fisher et al. [14] suggested that a second binding site for a sulfone-type inactivator, i.e., other than the serine residue, may play an important role in the stable formation of the inhibitor-enzyme complex. Dansyl-penicillin may be useful for the investigation of this additional residue.

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