Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 5, 439-443 (1991)

Characters of Extracellular β-Lactamase Obtained from a Strain of *Streptomyces* sp.

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방선균의 일주가 생성하는 균체외 β-Lactamase의 특성

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Abstract — A strain of *Streptomyces* sp. isolated from soil was found to produce extra-cellular β-lactamase associated partially to the cell growth. The β-lactamase was purified from the culture supernatant through ammonium sulfate fractionation, ion-exchange chromatographies and gel filtration. The final purification fold and recovery yield were 57 and 6.2%, respectively. Molecular weight of the β-lactamase was estimated to be about 67,000 by SDS-polyacrylamide gel electrophoresis. The optimal reaction condition was at pH 7~8 and at 35~45℃. The K_m and V_{max} values of the enzyme for penicillin G were estimated to be 3 mM and 1.3×10^3 μM/min/mg protein, respectively. The purified β-lactamase was classified to the class A enzyme hydrolyzing only penicillin.

β-lactam antibiotics are hydrolyzed and converted to inactive forms by β-lactamase which was firstly identified before the discovery of the β-lactam antibiotics (1). However, various β-lactamases have been characterized after the wide uses of the β-lactam antibiotics and it was found that the β-lactamases were the major factor acquiring resistancy to the antibiotics (2, 3). β-lactamases are very diverse enzymes having distinct substrate specificity and biochemical property, hence, they can be classified into class A, B, and C (4-6). Class A enzymes are serine β-lactamases hydrolyzing only penicillins and class B enzymes are metallo β-lactamases requiring Zn^{2+} ions for their activities. Class C are cephalosporinases having very limited substrate specifity to only cephalosporines, which are produced mainly from Gram negative bacteria (7).

It has been reported that *Streptomyces* spp. produce β-lactam antibiotics as well as β-lactamases, however, the productions are very closely related to culture conditions (8-12). Furthermore, it is very interesting to note that inhibitors to the β-lactamases are also produced by *Streptomyces* spp. and the reaction mechansims of the compounds at molecular level were reported (13-16). However the regulatory mechanisms to biosynthesize those compounds in *Streptomyces* have not been well elucidated.

In this connection, strains of *Streptomyces* spp. producing β-lactamase and the inhibitor were selected for the elucidation of the biosynthetic regulatory mechanisms in *Streptomyces* spp. and as the first step the characters of β-lactamase purified from the selected strain were identified.

Key words: *Streptomyces*, β-lactamase, β-lactam antibiotics
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Materials and Methods

Microorganism and culture conditions

Streptomyces sp. KIS-13 isolated from soil was selected as a strain producing extra--cellular β-lactamase (16). Stock and seed cultures were carried

out with a rich medium formulated as follows: glucose 15 g, corn steep liquor 10 g, yeast extract 5 g, $CaCO_3$ 3 g, and distilled water 1 *l*. Medium used for the production of β-lactamase was formulated as follows: glycerol 25 g, peptone 10 g, yeast extract 2 g, $CaCO_3$ 3 g, and distilled water 1 *l* (9). The culture for the enzyme production was carried out using a fermentor (Korea Fermentor Co.) where agitation and aeration were controlled to 200 rpm and 1 vvm, respectively. Temperature was maintained at 30℃ and the initial pH was adjusted to 8.0.

Determination of β-lactamase activity

β-Lactamase activity was determined by the iodometric method (17). One unit of β-lactamase was defined as the amount of enzyme which hydrolyzes 1 μmol of penicillin G per min at 37℃. Protein was determined by the method of Bradford (18) where ovalbumin (Sigma Co.) was used as a standard protein.

Purification of enzyme

Protein in the cell free culture supernatant was fractionated with $(NH_4)_2SO_4$. The active fractions were dialyzed and loaded to DEAE-Sephadex A-50 ion-exchanger equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The active fractions were collected and loaded again to SP-Sephadex C-50 (ϕ1.3×7 cm) equilibrated with 50 mM citrate buffer (pH 5.5). The column was eluted with 100 m*l* of 50 mM citrate buffer and then followed with NaCl gradient (0-1 M) using 200 m*l* of the citrate buffer. The active fractions were chromatographed on the Sephadex G-75 which was previously swollen in 0.1 M Tris-HCl buffer (pH 7.0) and packed into a column (ϕ1.5×70 cm) equilibrated with the same buffer. The flow rate was controlled to 3 m*l*/h and fractions of 1.0 m*l* were collected.

SDS-polyacrylamide gel electrophoresis and characterization of the purified enzyme

SDS-PAGE was performed on 10% SDS slab gel (19). Phosphorylase b (97.4 K), bovine serum albumin (66.2 K), ovalbumin (42.7 K), carbonic anhydrase (31.0 K), and trypsin inhibitor (21.5 K) were used as molecular weight standards. The gel stain was achieved by silver staining method (20).

Results and Discussions

β-lactamase production and purification

Changes in cell concentration, pH, and β-lactamase activity during a batch culture of *Streptomyces* sp. KIS 13 are shown in Fig 1. β-lactamase production initiated coincidently with the cell growth start. However further production of β-lactamase was apparent although cell growth was apparently declined. It indicated that *Streptomyces* sp. KIS 13 produced extra-cellular β-lactamase as the mixed growth-associated type.

The culture broth was centrifuged at 6000 xg for 10 min and ammonium sulfate was added to the cell free supernatant. It was found that β-lactamase was well fractionated by the salt. The active fractions were dialyzed and loaded to DEAE-Sephadex A-50 ion-exchanger equilibrated with 50 mM citrate buffer (pH 7.0), where the most proteins were bound to the exchanger but β-lactamase was not. Therefore the effluent was collected and loaded again to SP-Sephadex C-50 equilibrated with 50 mM citrate buffer (pH 5.5). As shown in Fig 2, it was evident that β-lactamase was bound to the ex-

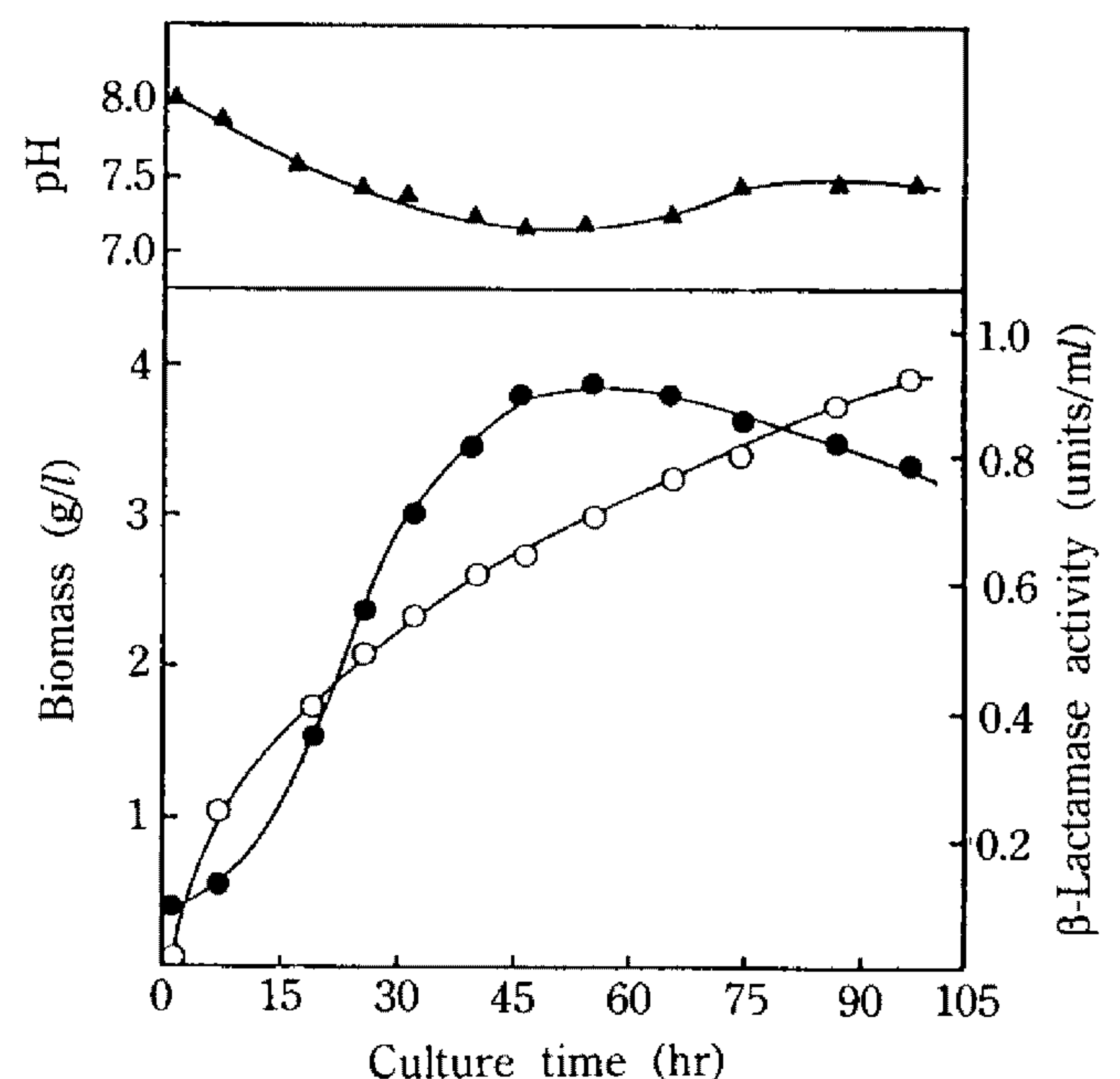

Fig. 1. Changes in biomass (●), β-lactamase activity (○) and pH (▲) in a batch culture of *Strpetomyces* sp. KIS 13.

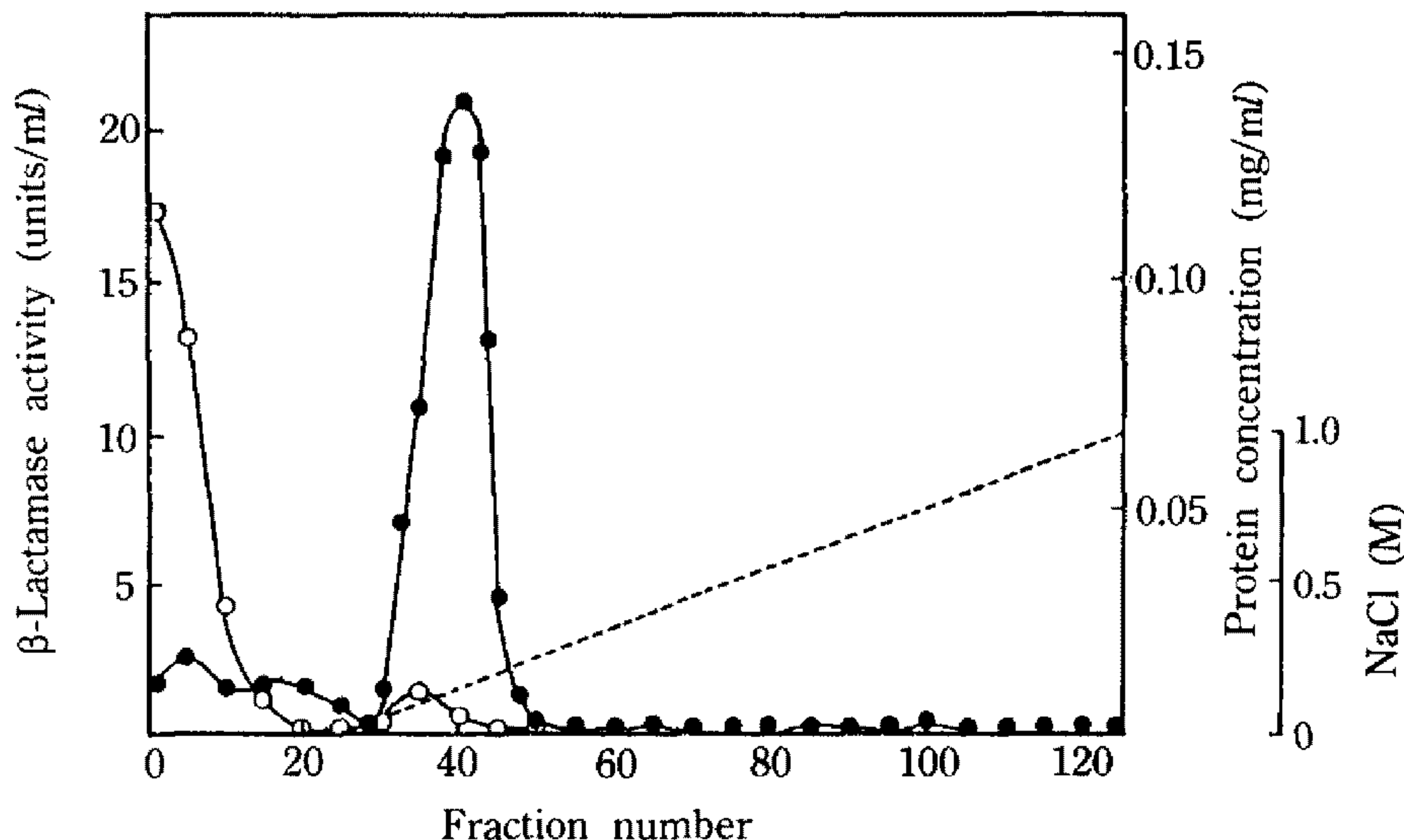

Fig. 2. Ion exchange chromatography of β-lactamase on SP-sephadex C-50.
The sample solution was applied to the colume (ϕ1.3×7 cm) equilibrated with 0.05 M Na-citrate buffer (pH 5.5). Then the column was washed with 50 ml of the same buffer and eluted with a 200 ml continuous linear gradient of NaCl (0-1 M) in the same buffer. Fractions of 2 ml were collected at a flow rate of 20 ml/h.
(●; β-lactamase activity, ○; protein, —; NaCl gradient)

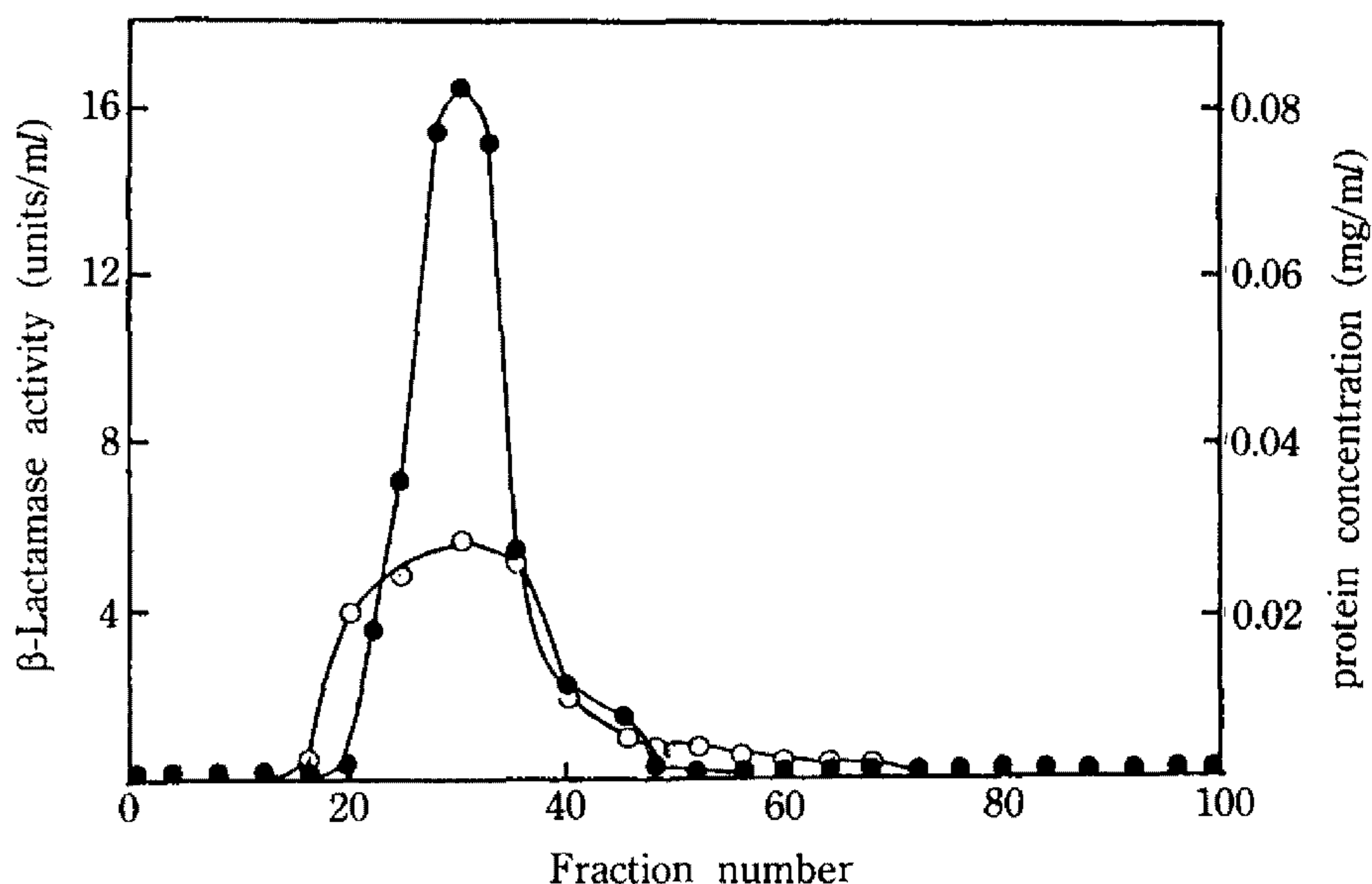

Fig. 3. Gel permeation chromatography of β-lactamase on sephadex G-75.
The sample solution was applied to the column (ϕ1.5×70 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.0) and then eluted with the same buffer. The flow rate was 3 ml/h and fractions of 1.0 ml were collected.
(●; β-lactamase activity, ○; proteins)

changer and also that β-lactamase was eluted to form single peak at 0.1-0.3 M NaCl concentration. The active fraction was chromatographed on the Sephadex G-75 (Fig 3). As results, β-lactamase was concentrated 57.30 fold with 6.2% recovery yield (Table 1).

Characterization of β-lactamase

The purified β-lactamase was stained as a single band and the molecular weight was estimated to be 67.000 by SDS-PAGE (Fig. 4). And it was worth to note that the molecular weight of the β-lactamase produced by *Streptomyces* sp. KIS 13 was larger than other β-lactamases produced by other strains of *Streptomyces* spp. (7, 21).

Substrate specificity of the β-lactamase was eva-

Table 1. Purification of β-lactamase from *Streptomyces* sp. KIS 13.

Purification step	Volume (ml)	Total activity (unit)	Total Protein (mg)	Specific activity (U/mg protein)	Purification fold	Recovery (%)
Culture supernatant	1510	2985	415.00	7.2	1.0	100.0
$(NH_4)_2SO_4$ fractionation	14	2588	117.00	22.2	3.1	86.7
DEAE-sephadex A-50 chromatography	60	749	9.25	81.0	11.3	25.1
SP-Sephadex C-50 chromatograghy	31	316	0.84	379.0	52.6	10.6
Sephadex G-75 chromatography	20	186	0.45	412.0	57.3	6.2

*. 1 unit; the amount of β-lactamase that hydrolyze 1 μM Penicillin G within 1 min.

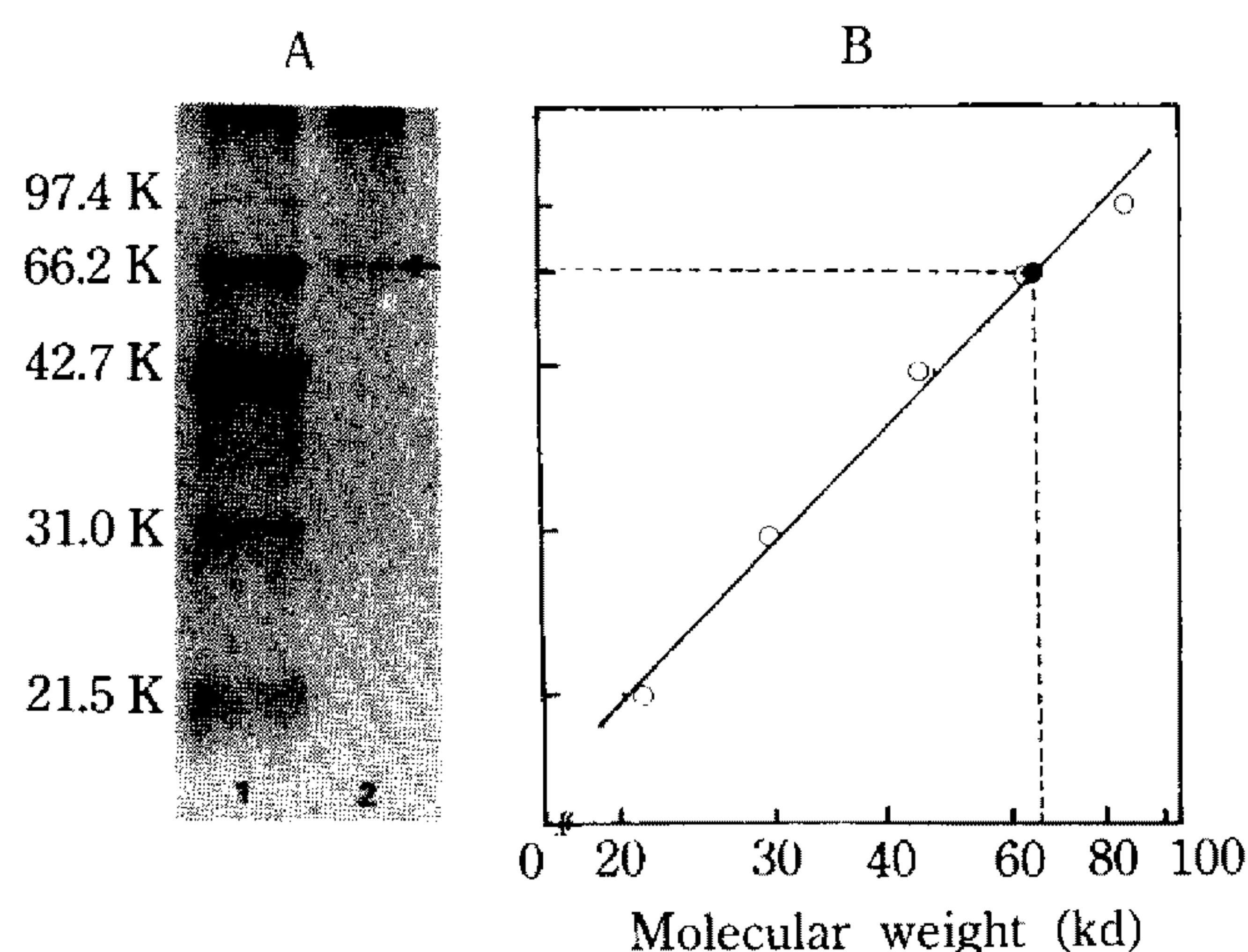

Fig. 4. (A) SDS-polyacrylamide gel electrophoresis of the purified β-lactamase. (B) Estimation of molecular weight of the β-lactamase.

(A) lane 1; molecular weight standards (97.4 K; phosphorylase b, 66.2 K; bovine serum albumin, 42.7 K; ovalbumin, 31.0 K; carbonic anhydrase 21.5 K; trypsin inhibitor), lane 2; purified β-lactamase

(B) (●; purified β-lactamase, ○; molecular weight standards)

Table 2. Activities of β-lactamase obtained from *Streptomyces* sp. KIS 13 on various β-lactam antibiotics.

Substrate	Specific activity (U/mg protein)	Relative activity (%)
Penicillin G	412.0	100
Ampicillin	223.0	54
Carbenicillin	117.0	28
Cloxacillin	8.3	2
Cephalothin	71.9	17

Table 3. Effects of EDTA and various metal ions on the activity of β-lactamase isolated from *Streptomyces* sp. KIS 13.

EDTA and metal ion	Concentration (μM)	Inhibition* (%)
Control		100
Ca^{2+}	100	65
Co^{2+}	100	80
Cu^{2+}	100	80
Mg^{2-}	100	100
Zn^{2+}	100	100
EDTA	3,000	100

*Relative activity was defined as the comparisons of the activities between without metal ions and with ions

luated with various β-lactam antibiotics. As compared in Table 2, the enzyme showed high specificity to penicillins. And it had relatively low specificity to cephalosporin. The β-lactamase was strongly inhibited by iodine and partially inhibited by Ca^{2+}, Co^{2+}, and Cu^{2+}. However, it was insensitive to EDTA (Table 3) which indicated that the β-lactamase isolated from *Streptomyces* sp. KIS 13 was not metallo enzyme (class B) but penicillinase (class A) which hydrolyzes only penicillin. Maximal activity of β-lactamase on penicillin G as a substrate occurred at pH 7.0~8.0 and at 35~45℃ (data not shown). The effect of penicillin G concentration on the enzyme activity was also tested and the K_m

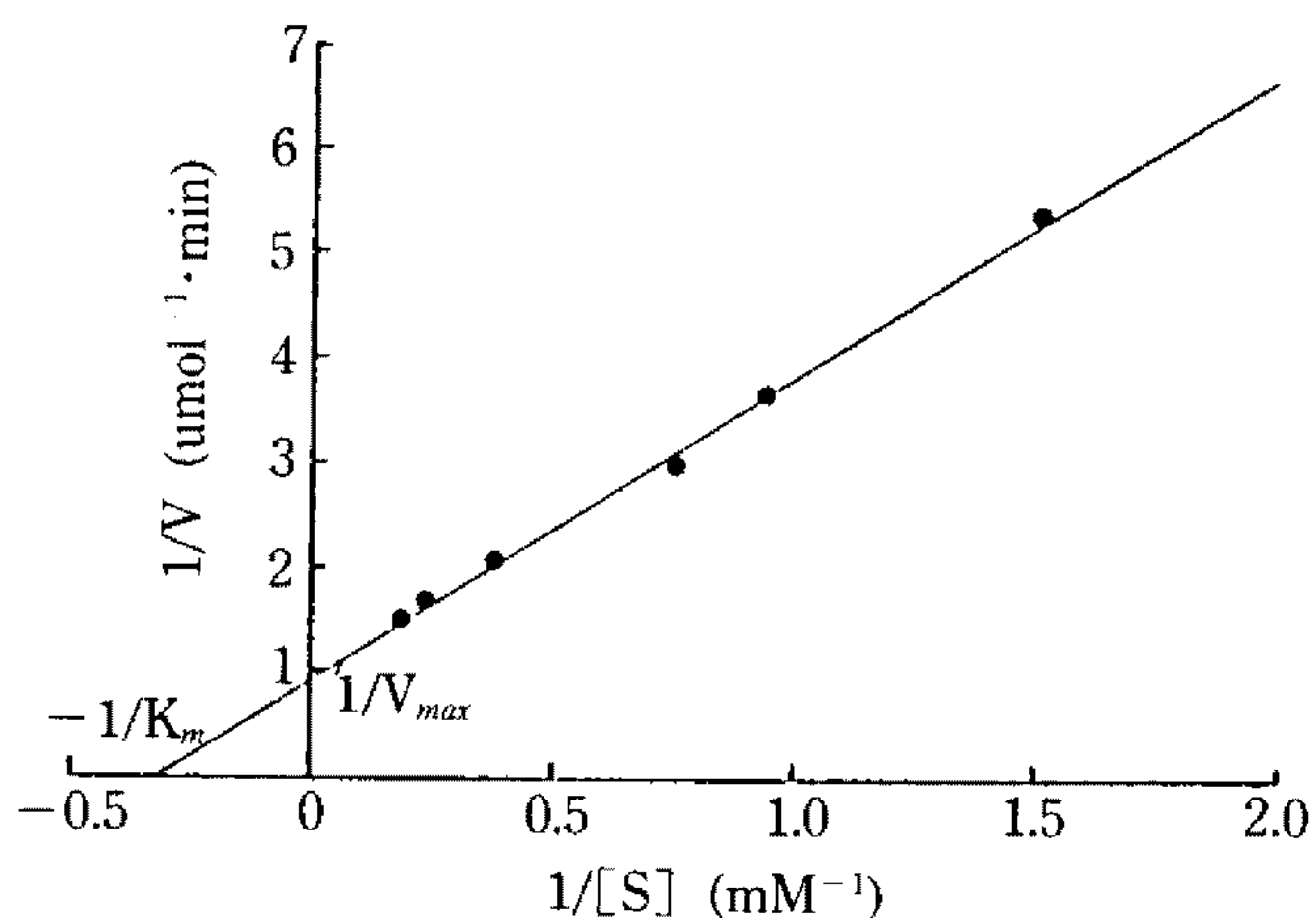

Fig. 5. Lineweaver-Burk plot for the hydrolysis of penicillin G by β-lactamase.
The estimated values of K_m and V_{max} were 3 mM and 1.3×10^3 μM/min/mg protein, respectively.

and V_m values were calculated to be 3 mM and 1.3×10^3 μM/min/mg protein, respectively (Fig 5).

It was thought that the β-lactamase obtained in this studies could be very useful as an enzyme in the development of β-lactamase inhibitor

Acknowledgement

The current study was supported by the Ministry of Education as the Genetic Engineering Program and Korea Foundation of Science and Engineering. The authors are appreciating for the supports.

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(Received July 16, 1991)