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Production and Characterization of Extracellular β-Lactamase from *Streptomyces aureofaciens* SMF14

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A strain SMF14 producing an extracellular β-lactamase was isolated from soil and identified to be a strain of *Streptomyces aureofaciens*. β-Lactamase was purified from the cell free culture broth through batchwise hydroxyapatite adsorption, anion exchange chromatography on DEAE Sephadex A-50, gel filtration on Sephadex G-75, and adsorption chromatography on hydroxyapatite. The molecular mass was estimated to be about 43 kDa by SDS-PAGE. The β-lactamase had substrate specificity to penicillins and it was inhibited by clavulanic acid, being classified to the group 2a of penicillinase. The optimal reaction pH and temperature were pH 6.0~7.5 and 50°C. The K_m and V_{max} values of β-lactamase for penicillin G were calculated to be 1.72 mM and 5.4×10^5 $\mu M\cdot min^{-1}$, respectively.

β-Lactamases (β-lactam anhydrolases, EC 3.5.2.6) hydrolyze the cyclic amide bond in the β-lactam ring of a penicillin or a cephalosporin (1). β-Lactamases are found from a variety of different microorganisms, mostly from microorganisms having resistance to β-lactam antibiotics (22, 26). β-Lactamase activity has been detected in most of the *Streptomyces* spp. with a few exceptions (20), and other actinomycetes including *Nocardia* spp. (27). Because β-lactamases are very diverse enzymes with distinct substrate specificity and physicochemical properties, they can be classified into diverse groups (3-5). In some cases, the gene encoding of β-lactamase is located in the chromosome, in others it is mediated by extrachromosomal elements, either by plasmids or transposons (15). A significant number of β-lactamases were encoded by plasmids or transposons capable of widespread transfer to other genera of bacteria (7, 9). Genes related to antibiotic production in *Streptomyces* spp. are found to be physically linked with the resistance to the endogenous antibiotic (6, 24). It has been reported that *Streptomyces* spp. produce β-lactam antibiotics as well as β-lactamases. More interestingly, the inhibitors of β-lactamase are also produced by *Streptomyces* spp. (8). However, the regulatory reaction mechanisms used to biosynthesize those compounds at molecular level in *Streptomyces* have not been well elucidated.

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Therefore, strains of *Streptomyces* spp. which produce the β-lactamase were selected for the elucidation of the biosynthetic regulatory mechanisms at molecular level. And the strain was identified and the characteristics of the β-lactamase were invesigated.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The strain SMF14 isolated from soil was selected as the strain to be used for producing extracellular β-lactamase. Stock and seed cultures were carried out in a rich medium formulated with the following: glucose 15 g, corn steep liquor 10 g, yeast extract 5 g, $CaCO_3$ 3 g, and distilled water 1l. Medium used for the production of β-lactamase was formulated with the followings: glucose 10 g, glycerol 15 g, peptone 10 g, yeast extract 2 g, K_2HPO_4 0.5 g, and distilled water 1 *l*. The culture for the enzyme production was carried out by using a 5 *l* jar fermentor (Korea Fermentor Co.) and the agitation and the aeration were controlled at 200 rpm and 0.5 vvm, respectively. Temperature was maintained at 30°C and the initial pH was adjusted to 7.0.

Analytical Method

Cell mass was determined as dried cell weight (D.C.W) after drying at 80°C for 24 h. β-Lactamase activity was determined by the iodometric assay method (23). One unit of β-lactamase was defined as the amount of en-

zyme which hydrolyzes 1 μmol of penicillin G per min at 30℃. The concentration of protein was determined by the Bradford method (2) where ovalbumin (Sigma Chemical Co.) was used as the standard protein.

Identification of the Isolate SMF14

Cultural and morphological characteristics were observed with the media of the International *Streptomyces* Project (25). The solid cultures were carried out on agar plates, and the submerged cultures were studied in baffled flasks by using a rotary shaking incubator at 28℃. After an acid hydrolysis of cell wall, diaminopimelic acid and whole cell sugars were analyzed by doing thin layer chromatography (13). The taxonomic unit characters used for the numerical identification of *Streptomyces* were tested following Williams *et al.*, the identification scores of the isolate were determined using the Taxon program (28, 29).

Purification of β-Lactamase and Determination of Molecular Mass

The culture broth was harvested and the cell-free culture broth was collected by centrifugation at 6,000 g for 20 min. The hydroxyapatite was added into the culture supernatant and then mixed thoroughly for 4~5 h. The protein binding hydroxyapatite was collected by centrifugation. Elution of β-lactamase from hydroxyapatite-protein complex was carried out with a 0.3 M phosphate buffer (pH 7.0). The eluates were concentrated and dialyzed by doing an ultrafiltration through a Diaflo membrane (Amicon Co.). The concentrated solution was applied to a column of DEAE Sephadex A50 equillibrated with 20 mM Tris HCl buffer (pH 8.3) and eluted with the same buffer at a flow rate 24.0 $ml \cdot h^{-1}$ followed by a linear gradient of 0~0.5 M NaCl. The flow rate was maintained at 24.0 $ml \cdot h^{-1}$. The active fraction was c. ncentrated and dialyzed against the same buffer. The sample solution was applied to a column of Sephadex G-75 equillibrated with 20 mM Tris HCl buffer (pH 8.3) and eluted with the same buffer at a flow rate of 15.0 $ml \cdot h^{-1}$. The active fractions were collected and concentrated by ultrafiltration. The solution was exchanged with 1 mM potassium phosphate buffer (pH 6.8) by repeated dilution and ultrafiltration. The enzyme solution was applied to a column of hydroxyapatite equillibrated with 1 mM potassium phosphate buffer (pH 6.8) and eluted with the same buffer followed by a linear gradient of 0~0.3 M phosphate. The flow rate was maintained at 5.0 $ml \cdot h^{-1}$.

The apparent molecular mass of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis according to the method proposed by Laemmli (11). A 12% polyacrylamide gel was run and stained with coomassie brilliant blue R-250. As standard marker, proteins such as ovalbumin (97.4kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa) purchased from Sigma Chemical Co., were used.

RESULTS AND DISCUSSION

Selection and Identification of Strain Producing β-Lactamase

Over 200 strains of *Streptomyces* spp. were tested to select the strains which produce β-lactamase. The β-lactamase activity on the agar plate was detected by Ogawara method (21). The incubated plate was overlaid with soft agar containing 0.1% phenol red (pH 9.0) and 10% penicillin G. After 10 min, the color around the colony changed from red to yellow, showing β-lactamase activity (Fig. 1). As a result, an isolate SMF14 was selected as the strain to use for producing extracellular β-lactamase.

The isolate SMF14 produced typical streptomycete mycelia and spores on solid media. Colonies which developed on the media were tough and leathery. The isolate SMF14 developed long rectiflexible spores in chains, and the spore surface ornament was spiny (Fig. 2).

The diaminopimelic acid (DAP) in the cell wall was identified to be a LL-DAP isomer, and the diagnostic sugars in the whole cell hydrolysate were not detected. From the morphological and chemotaxonomical characterization, it was concluded that the isolate SMF14 belongs to genus *Streptomyces* (Table 1).

In order to identify the isolate to species level, taxono-

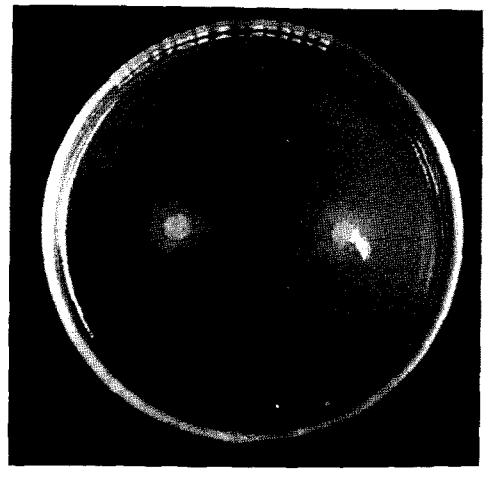

Fig. 1. β-Lactamase activity of the isolate SMF 14 cultured on Bennet agar medium at 30℃ for 4 days.
The incubated plate were overlaid with soft agar containing 0.1% phenol red (pH 9.0) and 10% penicillin G. After 10 min, β-lactamase acitivity was shown by formation of yellow color around the colonies.

Fig. 2. Spore chains of isolate SMF 14.
The sample cultured on inorganic-salt starch agar medium (ISP 4) at 30℃ for 14 days was observed using Scanning electron micrograph (Stereoscan 260 SEM) after gold coating.

mical unit characters of the isolate SMF14 were numerically analysed by using the Taxon program (12). The willcox probability of the isolate SMF14 to cluster14 was over 0.999999 and much higher than those of next nearest cluster (Table 2). This indicated that the isolate SMF14 belongs to cluster14. In recent reports, the member species in cluster14 were reclassified as *Streptomyces aureofaciens*, according to the numerical analyses (14). Therefore we concluded that the isolate SMF14 was a strain of *Streptomyces aureofaciens*.

Purification of β-Lactamase

The batch culture data for the production of β-lactamase are shown in Fig. 3. When the β-lactamase activity reached maximum, the culture broth was harvested by centrifugation and the proteins were absorbed to hydroxyapatite. Elution of β-lactamase from hydroxyapatite-protein complex was carried out with a 0.3 M phosphate buffer and 61% of elution was recovered. More purifications were carried out by doing anion exchange chromatography, gel filtration, and adsorption chromatography.

Table 1. Comparison of diagnostic characteristics between *Streptomyces*, *Streptoverticillium*, and isolate SMF 14.[a]

Characteristics	*Streptomyces*	*Streptoverticillium*	SMF 14
Colony size	Discrete	Discrete	Discrete
Substrate mycelium	+	+	+
Spore	+	−	+
Sporangia	−	−	−
Motile spore	−	−	−
Aerial mycelium	+	+	+
Chains of arthrospores	+	+	+
Arthrospore in verticils	−	+	−
Spore surface smooth	+	+	−
Spore surface hairy, spiny, or warty	+	−	+
Motile spores	−	−	−
Sugar in cell hydrolysates			
Arabinose,galactose, xylose	−	−	−
DAP isomer in cell wall			
LL-DAP	+	+	+
meso-DAP	−	−	−

[a]Symbol: +, 90% or more of strains are positive; −, 10% or less of strains are positive

Table 2. Identification of *Streptomyces* sp. SMF14 to the clusters of *Streptomyces*

Clusteter (centrotype)	Tax distance	95% Taxon radius	% Prob of strain further away	Willcox Probability
14 (*S. aureofaciens*)	0.3560	0.2869	0.0016	>0.999999
2 (*S. aburaviensis*)	0.4977	0.3972	0.0009	0.000000
39 (*S. longisporoflavus*)	0.5273	0.3570	0.0000	0.000000
36 (*S. thermovulgaris*)	0.5300	0.3351	0.0000	0.000000
38 (*S. prasinosporus*)	0.5617	0.3972	0.0000	0.000000

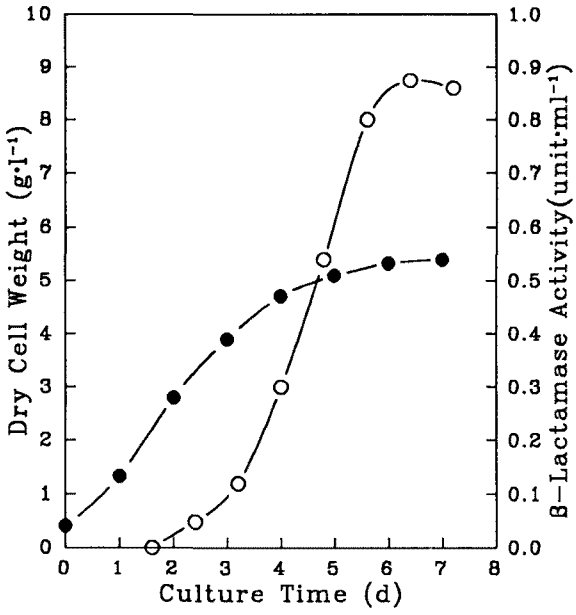

Fig. 3. Growth and β-lactamase production in a batch culture of *Streptomyces aureofaciens* SMF 14 using production medium at 30℃.

Aeration and agitation were controlled to give 0.5 vvm and 200 rpm, respectively.
(●): dry cell weight
(○): β-lactamase activity

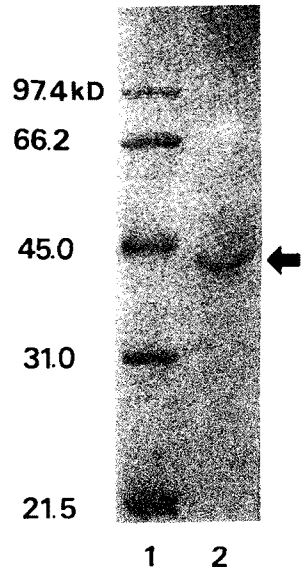

Fig. 4. SDS-polyacrylamide gel electrophoresis of β-lactamase produced from *Streptomyces aureofaciens* SMF 14.

Lane 1: molecular weight standard 97.4 kD: phosphorylase b, 66.2 kD: bovine serum albumin, 45.0 kD: ovalbumin, 31.0 kD: carbonic anhydrase, 21.5 kD: trypsin inhibitor
Lane 2: purified β-lactamase

Table 3. Purification of β-lactamase from *Streptomyces aureofaciens* SMF14

purification step	Total Activity (unit)	Total protein (mg)	Specific Activity (unit/mg)	Recovery Yield (%)	Fold
Culture broth	1752.2	341.0	5.13	100	1
Batchwise hydroxylapatite	1068.0	186.4	5.73	61.0	1.12
Anionexchange chromatography (DEAE-A 50)	400.0	14.5	27.59	22.8	5.38
Gel filtraton (Sephadex G-75)	151.9	6.37	23.85	8.7	4.65
Adsorption chromatography (hydroxylapatite)	24.3	1.12	21.70	1.4	4.23

As shown in Table 3, the enzyme was purified 4.23 fold compared with the culture filtrate, with an overall yield of 1.4%. The purity of the enzyme was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which gave a single protein band as the results (Fig. 4). The molecular mass of the enzyme was determined to be 43 kDa.

Characteristics of β-Lactamase

The enzyme exhibited high activity at the range of pH 6.0~7.5 but showed relatively wide range of pH stability between pH 5~9. The activation energy of β-lactamase between 30℃ and 50℃ was 0.66 Kcal·mol^{-1} (Fig. 5). The enzyme kept over 90% of its activity after 1 h treatment at below 50℃ in the absence of substrate. The substrate specificity of the enzyme was determined with various β-lactam antibiotics (6 mM final concentration). As shown in Table 4, the enzyme was able to hydrolyze penicillins but not cephalosporins. Therefore, the β-lactamase was considered to be a penicillinase. The enzyme was significantly inhibited by I_2 and clavulanic acid, the potent β-lactamase inhibitor, but not by EDTA (Table 5). The K_m and V_{max} values of β-lactamase for penicillin G were 1.72 mM and 5.4×10^5 μM·min^{-1}, respectively (Fig. 6).

There have been several classification schemes developed for β-lactamases. The most comprehensive of

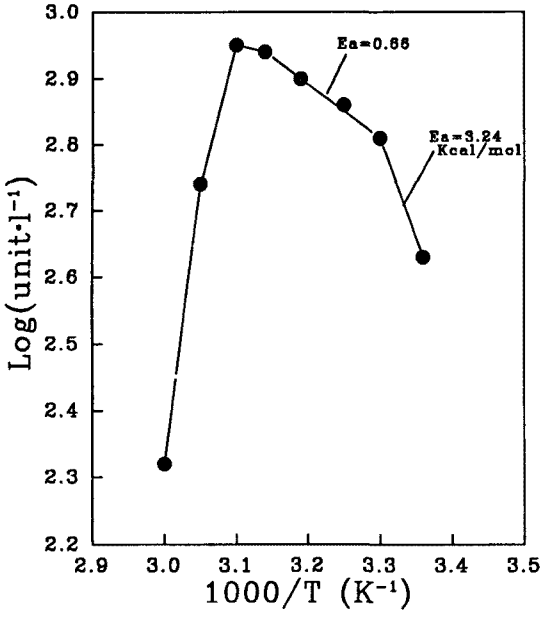

Fig. 5. Arrhenius plot of the β-lactamase activity.

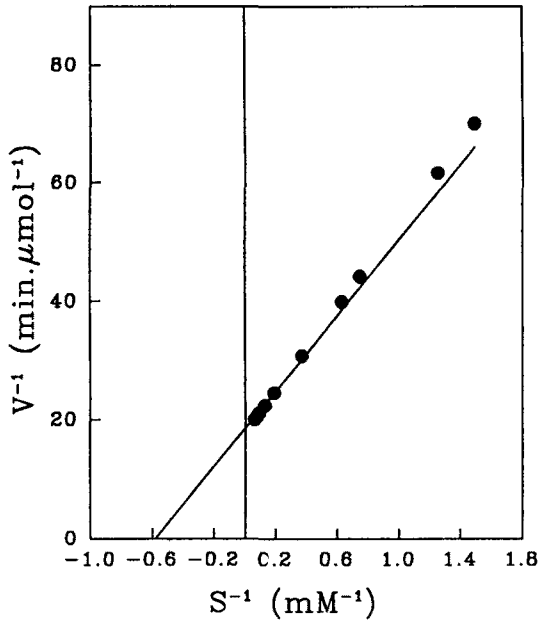

Fig. 6. Lineweaver-Burk plot for the hydrolysis of penicillin G by β-lactamase.

Table 4. Substrate specificity of β-lactamase

Substrate	Concentration (mM)	Relative hydrolysis rate*
Benzylpenicillin	6	100
Penicillin V	6	124.2
Ampicillin	6	118.2
Cloxacillin	6	0
Carbenzcillin	6	0
Cephalosphorin C	6	0
Cephalothin	6	0
Cefotaxime	6	0
Cephaloridine	6	0

*The relative hydrolysis was calulated assuming that the hydrolysis rate of benzylpenicillin taken as 100.

these, a recent one proposed by Bush (3-5), is the one which was used in this work. The major subdivisions of the Bush scheme are based on preferred substrate group, susceptibility to inhibition by clavulanic acid, and the requirement for a metal ion for activity. Unlike other schemes, it incorporates both plasmid and chromosomally specified enzymes, as well as those produced by Gram-positive bacteria. Following to Bush scheme, as β-lactamase from *Streptomyces aureofaciens* SMF14 preferentially hydrolyzed penicillin and was susceptible to inhibition by clavulanic acid, it was classified to group 2a (PEN-Y type) enzyme.

Streptomyces spp. have been of interest as an antibio-

Table 5. Effects of various compounds on β-lactamase activity

Compounds	Concentration (mM)	Remanimg enzyme activity (%)
None		100.0
Clavulanic acid	0.67	14.0
Cloxacillin	0.61	81.4
Boric acid	0.54	37.2
EDTA	5.0	100.0
	10.0	100.0
CDTA	1.0	100.0
	5.0	100.0
Na-citrate	5.0	100.0
	10.0	100.0
I_2	0.4	0.0
	0.04	7.0
Urea	1.0	100.0
DTNB(Dithio-bis-nitrobenzoic acid)	1.0	87.2
pCMB(p-chloromercuribenzoate)	0.5	31.8
	1.0	23.9
Iodoacetic acid	1.0	100.0
$CuCl_2$	0.5	17.0
$ZnCl_2$	0.5	100.0
$CoCl_2$	0.5	100.0
$CaCl_2$	0.5	100.0
$FeCl_2$	0.5	95.7
$MgCl_2$	0.5	100.0
$MnCl_2$	0.5	100.0

Table 6. Properties of β-lactamases produced by *Streptomyces* spp.

Organism	Relative hydrolysis rate[a]					Inhibited by[b]		MW (kDa)	Opimum condition		Reference
	PEN	AMP	CLO	CAR	CEP	CA	EDTA		pH	Temp (℃)	
S. cacaoi	100	38	38	66	3	S	R	34	6-7	40-45	Ogawara (18)
S. albus G	100	120	2	10	1	S	R	30	6-8	ND	Johnson (10)
S. cellulosae	100	37	7	4	1	ND	R	27	7	ND	Ogawara (19)
S. sp. E750-3	100	28	6	13	2	ND	R	20	7-8	40	Ogawara (17)
S. sp. KIS-13	100	54	2	28	17	ND	R	67	7-8	35-45	Moon (16)
S. aureofaciens SMF 14	100	120	0	0	0	S	R	40	6-7.5	45-50	This work

[a]The relative hydrolysis was calulated assuming that the hydrolysis rate of benzylpenicillin taken as 100: PEN; Benzylpenicillin, AMP; Ampicillin, CLO; Cloxacillin, CAR; Carbenicillin CEP; Cepharoridine [b]CA; Clavulanic acid, EDTA; Ethylenediamine tetraacetic acid, S; Sensitive, R; Resistant

tic-producing bacteria and a possible source of resistance enzymes in pathogenic bacteria. In general, the enzymes are released extracellularly, produced constitutively and they show a good deal of species specificity among the groups. As the results shown in Table 6. the β-lactamase from *Streptomyces aureofaciens* SMF14 has properties similar to those of other *Streptomyces* spp.. Therefore, a purified β-lactamase was concluded to be a typical *Streptomyces* β-lactamase.

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