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Extracellular Production of β-Lactamase by *Penicillium chrysogenum*

Sam Whan Lee, Kyu Chung Hur, Kil Soo Lee and W.S. Kim

Department of Biology, Yonsei University, Seoul 120, Korea

*Penicillium chrysogenum*에 의한 β-Lactamase 生成

李三煥·許奎晶·李吉洙·金銀壽

延世大學校 生物學科 微生物學教室

Abstract : β-Lactmase was isolated from the culture filtrate of the penicillin producing strain, *Penicillium chrysogenum* Q176. When the pH of the medium was adjusted to 5.0 at the start of culture, a rapid increase in pH accompanied by the synthesis of penicillin was observed in the first 2~4 days. When the pH of medium was brought to 6.0 or 7.0 the opposite was observed: high yield of the enzyme and little of the antibiotics in the medium. The optimum enzyme activity was at a temperature of 40°C and around pH 7.0. A partially purified enzyme was assayed on several different substrates including penicillins V and G, 6-aminopenicillanic acid, cephalosporin C. The V_{max} values calculated were 24.5, 20.4, 7.6, and 6.1 mmoles/hour, and the K_m values were 16.4, 12.6, 7.5, and 6.9 mM in the order given.

β-Lactamases have been isolated from many microorganisms including bacteria (Arcos *et al.*, 1968; Cornellis and Abraham, 1975; Davies and Abraham, 1974; Eriquez and D'amato, 1979; Sunaga *et al.*, 1976; Weinrich and Del Bene, 1976), Streptomyces (Ogawara *et al.*, 1978; Schwarz, 1979), Nocardia (Wallace *et al.*, 1978), and blue-green algae (Kushner and Breuil, 1977) but no report has yet been made on the production of β-lactamse by the penicillin producing strain *Penicillium chrysogenum.* This is to report a pH-dependent synthesis of β-lactamase in the culture filtrates of *P. chrysogenum* Q176.

Materials and Methods

P. chrysogenum Q176, an isolate of University of Wisconsin, was obtained from the Stock Culture Institute in Seoul and used for the source of antibiotics and the enzyme. *Staphylococcus aureus* ATCC 6538P was employed for the assay of antibiotics.

A 500ml Erlenmeyer flask containing 150ml sterile liquid media consisting 20.0g of lactose, 20.0g of corn steep solid, 0.5g of KH_2PO_4, 0.25g of $MgSO_4 \cdot 7H_2O$, 3.0g of $NaNO_3$, 0.04g of $ZnSO_4 \cdot 7H_2O$, 2.75g of $CaCO_3$ plus 2 drops of antifoam emulsion A(Sigma) in 1000ml H_2O, was shaken on a rotary incubator(150rpm) at 24°C for a period of 15 days. After the media were autoclaved(lactose and $CaCO_3$ were separately autoclaved) they were cooled down to room temperature and the pH's (5.0, 6.0 and 7.0) were adjusted using dilute sterile H_2SO_4 and NaOH before they were inoculated with 1.0ml inoculum taken from a 48 hour culture at pH 5.0. Every 24 hours after the start of the incubation of cultures of 3 initial pH's, the pH changes of the media were measured,and a 0.2ml aliquot was taken for analysis of the antibacterial activity using the cup method described by William and Moyer(1943). The diameters of the clear zone around the cups in the lawn of *S. aureus* were measured to compare the relative

antibacterial potency of the culture media of *P. chrysogenum*. Bioautography of antibiotics produced by *P. chrysogenum* along with standard antibiotics such as penicillin G and V was made with culture filtrates in order to check the identity of the antibacterial substances using the method of Wagman and Weinstein (1973).

After 5~7 days the cultures reached a full growth in the media and enzymes were isolated from the filtrates of 7~10 day cultures. After brief centrifugation(20, 000xg) followed by glass wool filtration, the mycelia and spores were removed and the filtrates were placed in the dialysis sacks(Sigma) and concentrated to about 20% volume in the Polyethylene glycol 6000(Hopkins & Williams Ltd., Eng.). The concentrates were cooled in the refrigerator overnight and centrifuged at 5, 000xg for 15 min. Enzymes were partially purified by Sephadex gel filtration and DEAE Sephadex column chromatography.

The activity of β-lactamase was analyzed by the hydroxylamine methods initially described by Boxer and Everett(1949) and Batchelor *et al.*(1961).

The mycelia of *P. chrysogenum* were harvested, washed, and assayed for the enzyme following the method of Erickson and Bennet(1965). Silica Gel thinlayer chromatograph(TLC) of penicillin and the enzyme products were run by methods of Nara *et al.*, (1971). Two different solvent systems were used; Isoamylacetate: methyl alcohol: formic acid: water =65 : 20 : 5 : 10, v/v and Propanol: pyridine: acetic acid: water=15 : 10 : 3 : 12, v/v.

The TLC plates of 0.25mm Silica(Sigma) were activated by heat(100°C) for 30 min. and after 90 min. run, the chromatograph plates were dried at room tempeature, exposed to 30% ammonium gas for 20 min. and sprayed with 2% solution of soluble starch in 35% NaCl followed by a spray of 0.01N iodine solution. Spots were shown white in the blue-violet background.

Penicillin G and V, 6-aminopenicillanic acid (6-APA), β-lactamase(Penicillinase Type I), cephalosporin C were purchased from Sigma and penicilloic acid was donated by the Korea Advanced Institute of Science in Seoul.

Results

Antibacterial Activity

Cultivating *P. chrysogenum* in three sets of media each of which was adjusted to pH 5.0, 6.0 and 7.0, the antibacterial activities and pH changes of the media were traced every 24 hours for a period of 15 days (Fig. 1). After 5 days the pH 5.0 culture filtrates gave maximum antibacterial acitvity while those of other pH series indicated no bacteriocidal activity on *S. aureus*. In the pH 5.0 culture, as the antibacterial activity increased, the pH of the media rapidly increased to 7.0~7.5 and leveled off after 5 days. The culture started at pH 6.0 showed only small pH changes for a week but slowly the pH increased to 7.8 at the end of cultivation. With the culture media of pH 7.0, the pH fluctuated between 6.5 and 8.0 but the pH of the media remained essentially in the neutral area. The mycelial growth in all the cultures appeared same. Bioautography of concentrated filtrates along with penicillin G and V indicated that the antibacterial substance was found to be exclusively the penicillin G type.

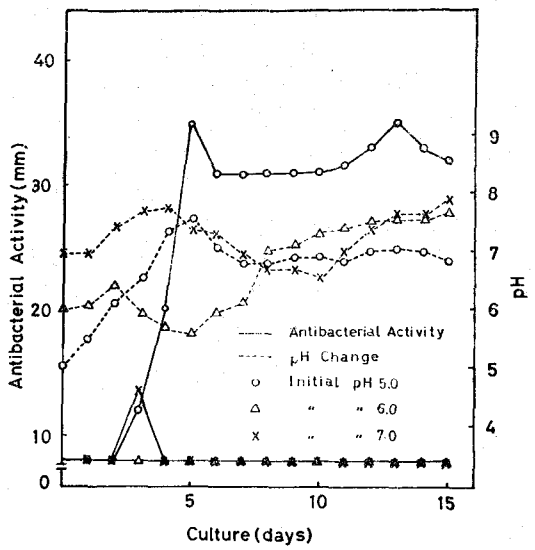

Fig. 1. Production of penicillin from the culture filtrates of *P. chrysogenum* and the change of pH of media during 15 day culture.

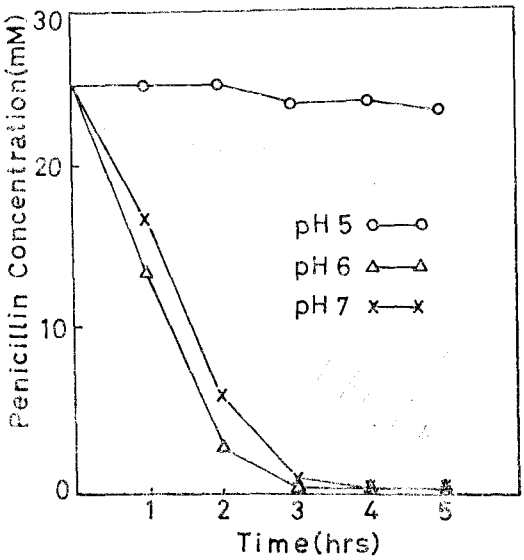

Fig. 2. Activities of extracellular enzyme on penicillin V. The enzymes were prepared from 7 day old culture filtrates of different initial pH's indicated.

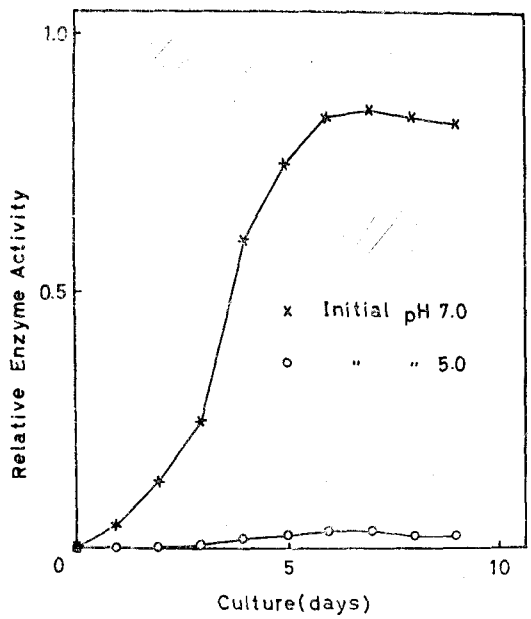

Fig. 3. Production of extracellular enzyme β-lactamase in the culture filtrates of *P. chrysogenum* which had been cultured in media of two different pH's, 5.0 and 7.0

Enzyme Activities on Penicillin

Enzymes were prepared from 7 day old culture filtrates of *P. chrysogenum* that had been grown on the media of different pH's. Every 60 min after incubation of enzyme and penicillin V mixture at 40°C, the enzyme activity was measured by assaying the residual penicillin in the reaction mixture to determine the level of destruction of the β-lactam by the cultures (Fig. 2). After 3 hours enzymes prepared from pH's 6.0 and 7.0 culture filtrates completely destroyed the penicillin, wherease the enzyme prepared from the filtrates of pH 5.0 media gave no activity. Fig. 3 shows the results of enzyme activity on penicillin G with the culture filtrates of the 5.0 and 7.0. During 9 day culture period the filtrates of pH 5.0 media gave little enzyme activity while those of pH 7.0 showed a sharp increase in enzyme activity reaching maximum after 6~7 days.

Identification of the Penicillin Destroying Enzyme

The penicillin destroying enzyme was identified as β-lactamase from analyses of thinlayer chromatography(Fig. 4). It indicated that the enzyme is not the penicillin acylase which is commonly found in bacteria and fungi (Erickson *et al.*, 1965; Hamilton-

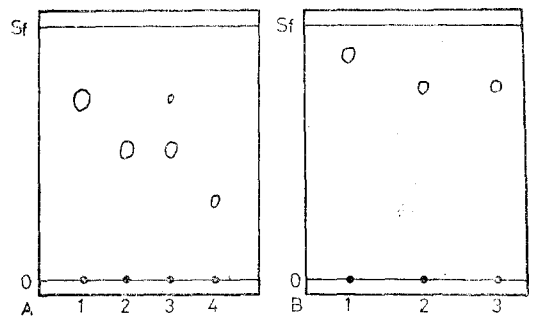

Fig. 4. Silica gel TLC of penicillin G and the reaction products of β-lactamase isolated from the culture filtrates. Fig. 4A solvent systems; isoamylacetate : methyl alcohol : formic acid : water=65 : 20 : 5 : 10, v/v; 0, spot origin; sf, solvent front; 1, penicillin G (Rf=0.70); 2, penicilloic acid (0.49); 3, enzyme substrate mixture (0.70 & 0.49); 4, 6-aminopenicillanic acid or 6-APA (0.30); Fig. 4B solvent system; pyridine : acetic acid : water=15 : 10 : 3 : 12, v/v; 1, penicillin G (0.85); 2, penicilloic acid (0.74); enzyme substrate mixture (0.74). TLC plates were treated and spots were developed by the methods described in the text.

Miller, 1966). Since the enzyme substrate mixture invariably produced penicilloic acid and no 6APA in two different solvent systems, we conclude that the pH-dependent extracellular enzyme detected in the culture media (pH's 6.0 and 7.0) of *P. chrysogenum* is β-lactamase.

Partial Purification and Determination of V_{max} and K_m of Enzyme.

The β-lactamese was partially purified by ammonium sulfate precipitation, Sephadex gel filtration and DEAE sephadex column chromatography. The optimum enzyme activity was at a temperature of 40°C and around pH 7.0 which was in general agreement with data reported on bacterial enzyme(Citri *et al.*, 1966; Jack *et al.*, 1970; Richmond *et al.*, 1973; Sunaga *et al.*, 1976). The V_{max} and K_m values were determined using several substances including penicillin V, G, 6-APA, and cephalosprin C and the data are summarized in Table 1.

Table I. The values of V_{max} and K_m obtained from several substrates of β-lactamase isolated from the culture filtrates of *P. chrysogenum*

Substrate	V_{max} (mmol/hr)	K_m (mM)
Penicillin V	24.5	12.6
Penicillin G	20.4	10.4
6-APA	7.6	7.5
Cephalosporin C	6.1	6.9

Detection of Penicilloic Acid from the Culture Filtrates of *P. chrysogenum*.

Culture filtrates of *P. chrysogenum* derived from the culture media of pH 5.0 and 7.0 were analyzed on the Silica gel TLC. Fig. 5 shows the results with the 7 day culture filtrates of the fungus cultured in pH 7.0 medium. The filtrates were applied on the TLC plate either directly or concentrated isoamylacetate extracts of the penicilloic acid. Results of 3 TLC analysesgave the same pattern; only the penicilloic acid was detected from the filtrates. Similar experiments with the filtrates of pH 5.0 culture media of the fungus showed no penicilloic acid spot on the TLC.

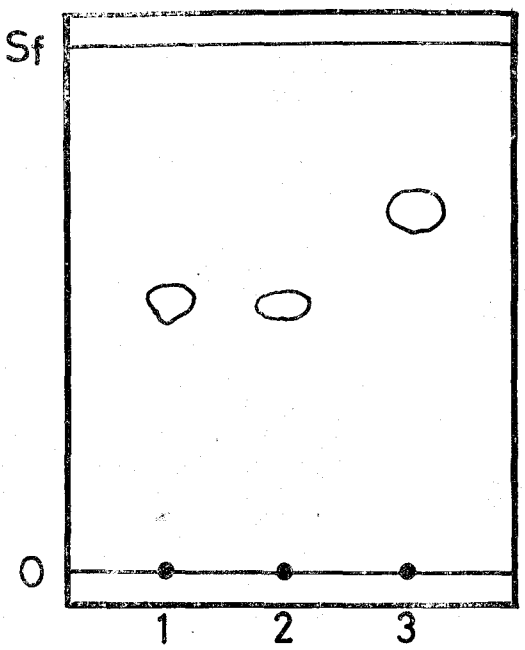

Fig. 5. Silica gel TLC of culture filtrates of *P. chrysogenum* demonstrating the presence of penicilloic acid. Solvent system, isoamylacetate : methyl alcohol : formic acid : water=65 : 20 : 5 : 10, v/v; O, spot origin: Sf, solvent front; 1, pH 7.0 culture filtrate; 2, penicilloic acid control; 3, penicillin G. The Rf values are 0.49, 0.49 and 0.70 respectively. No penicilloic acid was detected from the pH 5.0 culture filtrates.

Detection of β-Lactamase from Mycelia of *P. chrysogenum*.

Attempts to isolate β-lactamase from 7 day old mycelia from pH 5.0 media were failed; small amount of enzyme could be detected from mycelia grown in the media of pH 6.0 and 7.0.

Discussion

The dependence of the synthesis of penicillin and β-lactamase on the pH of culture media of *P. chrysogenum* is quite significant. The present data of experiment indicate that the starting pH of the culture medium effects the extracellular production of penicillin and β-lactamase in the culture of *P. chrysogenum;* at pH 5.0 only the penicillin and little β-

lactamase and at pH's 6.0 and 7.0 only the enzyme and no penicillin were produced. Production of penicillin and the enzyme from the mycelia was negligible in all cultures. Kushher and Breuil(1977) reported that the blue-green algae cells produced extracellular β-lactamaese and the pH rose from neutral to 10 during the cell growth. In the present experiment the rise of pH was indeed significant when penicillin was produced but the pH change was not so drastic when the enzyme was produced.

The penicillin producing strain *P. chrysogenum* Q176 was obtained from the Microbiology Laboratory of Yonsei University Medical School where the strain was kept as a lyophilized spore form. Maximum care was taken to avoid contamination of organisms especially those produce β-lactamase. Subcultures were started from the stock culture mycelia and the experiments were repeated five times. Except occasional minor variations in the amounts of penicillin and the enzyme, produced, the basic nature of pH dependency on the production of these chemicals was consistent. Physical data obtained from the enzyme were in general agreement with data published on β-lactamases from other sources (Citri *et al.*, 1966; Jack *et al.*, 1970; Richmond *et al.*, 1973). It hydrolyzes penicillin V, G, 6APA, and cephalosporin C favorably in the order given.

Penicilloic acid was detected only from the culture filtrates of the fungus grown in the media of neutral pH's and not from the media of pH 5.0, suggesting that penicillin may still be produced by the fungus grown in the media of neutral pH's as well as in the pH 5.0 media, but it is destroyed by the presence of the extracellular enzyme.

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