Production of 3-Aminonocardinic Acid from Nocardicin C by Microbial Enzymes

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Penicillin acylase has been used on an industrial scale to catalyze the hydrolytic removal of the side chain of penicillins to yield the β -lactam nucleus, 6-aminopenicillanic acid.¹⁾ This nucleus is the starting material of semi-synthetic β -lactam antibiotics, some of which are superior to the natural penicillins in their antibacterial activity and/or resistance to β -lactamases.

Nocardicin A is an antibiotic with a monocyclic β -lactam nucleus having an N-acyl side chain.²⁾ We have tried to isolate microorganisms which produce nocardicin A acylase to give the β -lactam nucleus, 3-aminonocardinic acid (3-ANA). About 100 strains of bacteria, 1000 strains of actinomycetes, 20 strains of yeasts and 100 strains of fungi were examined for their activity to deacylate nocardicin A, but none of them was found to catalyze the deacylation of nocardicin A to yield 3-ANA.

Then, the search for enzymes that catalyze the deacylation of nocardicin C was undertaken. Nocardicin C (Fig. 1) is a minor component of nocardicin complex produced by *Nocardia uniformis* subsp. *tsuyamanensis*,³⁾ and also can be prepared by chemical hydrogenation of nocardicin A.⁴⁾

Microorganisms were cultivated in the following media; 1% peptone, 1% meat extract, 0.5% yeast extract and 0.2% sodium chloride, pH 7.0 for bacteria and actinomycetes; 1% polypeptone, 0.5% yeast extract, 3% sucrose and 0.2% potassium biphosphate for yeasts and fungi. Cells of each culture were collected by centrifugation (3000 rpm, 10 min) and suspended in 0.02 M Tris-hydrochloric acid buffer pH 8.0. The reaction mixture for the assay of nocardicin C acylase consisted of 0.2 ml of the cell suspension and 0.2 ml of nocardicin C solution at a concentration of 50 mg/ml in 0.1 N sodium hydroxide. The mixture was kept standing at 37°C for 4 to 20 hr. After incubation, a portion of the reaction mixture was spotted on a cellulose sheet (Eastman Chromagram No. 6065) which was developed with solvent system of 50% propanol. The developed sheet was dried and sprayed with ninhydrin

FIG. 1. Structure of Nocardicin A and C, and 3-Aminonocardinic Acid (3-ANA).

or ferric chloride reagent. The R_f value of standard 3-ANA, prepared chemically,⁵⁾ was 0.65.

Strains tested for their nocardicin C acylase are as follows; *Pseudomonas schuylkilliensis* IAM 1126, *Xanthomonas campestris* IAM 1671, *Acetobacter acetosum* IAM 1803, *Gluconobacter capsulatus* IAM 1823, *Escherichia coli* IAM 1204, *Proteus vulgaris* IID OX 19 US, *Staphylococcus aureus* IAM 1101, *Aeromonas hydrophila* IAM 1018, *Protaminobacter alboflavus* IAM 1040, *Bacillus subtilis* IAM 1069, *Bacillus cereus* IAM 1072, *Bacillus megaterium* IAM 1166, *Azotobacter vinelandii* IAM 1078, *Agrobacterium tumefaciens* IAM 1037, *Brevibacterium ammoniagenes* IAM 1641, *Micrococcus luteus* IAM 1097, *Microbacterium flavum* IAM 1642, *Mycobacterium tuberculosis* IID H 37 RV, *Aerobacter aerogenes* IAM 1019, *Alcaligenes faecalis* IAM 1015, *Corynebacterium fascians* IAM 1079, *Kluyvera citrophila* ATCC 21285, *Arthrobacter simplex* IAM 1660, *Sarcina lutea* IAM 1099, *Serratia marcescens* IAM 1067, *Streptomyces fradiae* IFO 3123, *Streptomyces olivaceus* IFO 3200, *Nocardia asteroides* IFO 3384, *Micromonospora purpurea* IFO 12575, *Thermopolyspora polyspora* IFO 12134, *Amorphosporangium auranticolor* IFO 12245, *Actinoplanes armeniacus* IFO 12555, *Aspergillus oryzae* IAM 2630, *Penicillium chrysogenum* IAM 7106, *Rhizopus delmar* IAM 6038, *Mucor fragilis* IAM 6140, *Rhodotorula rubra* IFO 0002, *Endomycopsis capsularis* IFO 0672 and *Lipomyces starkeyi* IFO 0678.

It was found that all of the microorganisms examined produced the enzymes which decomposed nocardicin C to yield a substance corresponding to 3-ANA on thin layer chromatogram. An aliquot of each reaction mixture (40 μ l) was then spotted at the "start" of cellulose sheet as a band. After the chromatogram was

TABLE I. PURIFICATION OF NOCARDICIN C ACYLASE

Step	Total volume (ml)	Total protein ^a (mg)	Activity ^b (unit)	Specific activity (unit/mg)
I. Culture filtrate	9,500	29,920	120,600	4.0
II. (NH ₄) ₂ SO ₄ fractionation	54	1,780	103,400	58.1
III. DEAE-Sephadex chromatography	52	297	79,860	269
IV. Hydroxyapatite chromatography-1	30	65.0	38,710	595
V. Hydroxyapatite chromatography-2	99	6.20	10,700	1,726
VI. Sephadex G-100 chromatography	47	1.98	4,830	2,440

^a Measured by the method of Lowry *et al.*³⁾

^b One unit is tentatively defined as an amount of enzyme which produces 1 μ mole (485 μ g) of 3-ANA in 60 min under optimum condition.

developed as mentioned above, the separated materials were detected under a UV lamp (2536Å) and the material of the spot corresponding to that of 3-ANA was recovered by scraping the zone from the sheet into a test tube and extracting with 4 ml of 0.1N sodium hydroxide. The UV absorption spectrum of the extract solution showed a maximum at 245 nm and found to be identical with that of authentic 3-ANA.

Among the microorganisms tested, *Pseudomonas schuyllkilliensis* IAM 1126 showed the strongest deacylase activity. Nocardicin C acylase of this organism was purified about 600 fold by fractionation with ammonium sulfate (40~70%) and column chromatography on DEAE Sephadex, Hydroxyapatite and Sephadex G 100. Summary of purification of the enzyme is presented in Table I. The purified enzyme solution gave a single protein band when applied to polyacrylamide gel electrophoresis according to the method of Reisfeld *et al.*⁷⁾ Its molecular weight was determined to be 64,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Weber and Osborn.⁸⁾ The pH optimum for the deacylase activity is 8.0~9.0 and the optimum temperature is 50°C. The *K_m* value for the enzyme is 1.1×10^{-2} M and *V_{max}* is 0.211 mm/min. It was inhibited by the following divalent metal ions; Co, Ni, Zn, Cd, Pb, and Ag. No stimulatory effect of metal ions was observed. The enzyme was examined for its activity to synthesize nocardicin C from 3-ANA and its acyl side chain, but it was incapable of catalyzing the reverse, synthetic reaction.

3-ANA produced by the enzymatic deacylation of

nocardicin C was extracted and purified from the reaction mixture according to the procedure described previously,³⁾ to yield colorless crystal. The crystal decomposed at 203~205°C and was found to have empirical formula C₁₁H₁₂N₂O₄. The IR spectrum was found to be identical with that of an authentic sample of 3-ANA.

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