PRODUCTION OF ACTINONIN, AN INHIBITOR OF AMINOPEPTIDASE M, BY ACTINOMYCETES

Sir:

As we have previously reported, inhibitors of various endopeptidases and exopeptidases have been found in culture filtrates of microorganisms[1~3], and all of them were new compounds as shown by determination of their structures. But as reported in this paper, searching for inhibitors of aminopeptidase M (EC 3.4.11.2), we isolated the known actinonin[4] and found that this antibiotic inhibits aminopeptidases. We report the screening method and isolation and identification of actinonin because of the importance of its inhibitory activities.

The screening method is as follows: A mixture consisting of 0.25 ml of 2 mM L-leucine β-naphthylamide (purchased from Protein Research Foundation, Osaka, Japan), 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.0), 0.2 ml of a mixture of water; the test solution was incubated at 37°C for 3 minutes; aminopeptidase M (0.05 ml, 4 U/mg from hog kidney, Boehringer Mannheim GmbH, West Germany) was added and mixed well; exactly 30 minutes later, 1.0 ml of a solution of the stabilized diazonium salt Garnet GBC in 1 M acetic acid buffer at pH 4.2, containing 10% Tween 20, was added; after standing for 15 minutes at room temperature, the absorbancy at 525 nm was read; the amount of the enzyme was adjusted to give an absorbance of around 0.35, that is, about 25 nmol of β-naphthylamine was released; the reaction was also carried out in the absence of the enzyme solution to obtain the blank value; the concentration of the inhibitor required for 50% inhibition (IC_{50}) was calculated.

The inhibitor was produced by shaken culture of MG848-hF6 in a medium containing glucose 1.0%, corn starch 2.0%, corn steep liquor 2.0%, corn gluten meal 2.0%, NH_4Cl 0.25%, NaCl 0.3%, $CaCO_3$ 0.6% (adjusted to pH 6.2 with 5 N NaOH before sterilization). Maximum production was usually attained on the 8~10th day at 27°C. The crude filtrate (11 liters) was passed through a column of Amberlite XAD-4 (1 liter, 8×20 cm). After washing the resin with 2 liters of distilled H_2O, the inhibitor was eluted with 4 liters of 80% aqueous MeOH. The active eluate was concentrated under reduced pressure to give a brownish powder (10.7 g, IC_{50}=10.5 μg/ml). The powder was chromatographed on a column of silanized silica gel (500 ml, 4.6×30 cm) equilibrated with 1% citrate and 2% potassium acetate buffer at pH 4.9. A linear gradient of acetonitrile from 0~50% in the same buffer was used as the developer. The active eluate was concentrated under reduced pressure and the concentrate was passed through a column of Amberlite XAD-4 (100 ml, 3×14 cm). The column was washed with 500 ml of distilled H_2O and eluted with 400 ml of 80% aqueous MeOH. The effluent was concentrated under reduced pressure to give a light brownish powder (0.96 g, IC_{50}=1.1 μg/ml). It was placed on a column of silica gel (200 ml, 2.4×44 cm) and was eluted with the mixture of $CHCl_3$ and MeOH (95: 5). The active eluate was concentrated under reduced pressure to give a slightly yellow powder (213 mg, IC_{50}=0.6 μg/ml). The inhibitor was purified by repeated crystallization from MeOH and benzene to yield 146 mg, IC_{50} against aminopeptidase M = 0.4 μg/ml (white needles, mp 148~149°C).

The purified material was soluble in H_2O, MeOH and EtOH, but insoluble in $CHCl_3$ and benzene. It gave positive color reaction with ferric chloride and Rydon-Smith reagent, but negative with ninhydrin. On thin-layer chromatograms on Silica gel 60 (E. Merck, West Germany), it gave a single spot at Rf 0.66 (1-BuOH - AcOH - H_2O, 4: 1: 1) the Rm value (Ala 1.0) on high-voltage paper electrophoresis (3,500 V, 10 minutes, HCOOH - AcOH - H_2O, 1: 3: 36, pH 1.8) was 0.49. It showed only end absorption in the UV spectrum; $[\alpha]_D^{20}$ −50° (*c* 2.0, EtOH); *pKa* 9.30 (H_2O); IR ν_{max}^{KBr} cm^{-1} 3375, 3310, 3080, 2970, 2940, 2880, 1655, 1620, 1580, 1560, 1480, 1455; ^1H NMR (400 MHz, CD_3OD, external TMS; δ = 0) 0.63 (3H), 0.72 (6H), 1.01 (12H), 1.22 (2H), 1.68 (4H), 1.79 (1H), 2.01 (2H), 2.55 (1H), 3.33 (2H), 3.47 (2H), 3.87 (1H), 4.12 (1H); ^{13}C NMR 14.3, 18.9, 19.6, 23.5, 25.1, 27.8, 28.0, 31.8, 32.8, 32.8, 33.5, 36.5, 43.7, 58.2,

Fig. 1. Structure of actinonin.

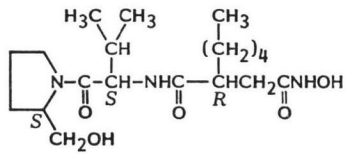

Table 1. Inhibitory activity of actinonin to aminopeptidases.

Enzyme	IC_{50} (μg/ml)
Aminopeptidase M	0.4
Leucine aminopeptidase	1.0
Aminopeptidase A	>100
Aminopeptidase B	>100
Methionine aminopeptidase	>100
Formylmethionine aminopeptidase	>100

60.7, 63.5, 170.7, 173.0, 177.1; SIMS (*m/z*) 386 (M+1). The elemental analysis was as follows; Found: C 59.5, H 8.8, N 10.5. Calcd for $C_{19}H_{35}N_3O_5$: C 59.2, H 8.9, N 10.9. The acid hydrolysis with 6 N of hydrochloric acid at 110°C for 15 hours gave the following four hydrolysis products; an ether extractable acid, an amino acid and two basic substances. ^{1}H NMR of the ether extractable acid was as follows: (400 MHz, $CDCl_3$, external TMS; $\delta = 0$) 0.89 (3H), 1.31 (6H), 1.64 (2H), 2.65 (2H), 2.84 (1H); SIMS (*m/z*) 189 (M+1); $[\alpha]_D$ +24° (*c* 1.0, EtOH); mp 82.5~83.5°C. This was identified as D-pentylsuccinic acid. The amino acid was identified as (+)-L-valine. Two basic substances were identified as hydroxylamine and L-prolinol by comparison with authentic samples. Thus, the inhibitor of aminopeptidase M produced by the strain MG848-hF6 was identified as actinonin (Fig. 1).

The inhibitory activities of actinonin against aminopeptidases were examined by the method previously described and shown in Table 1. It is a strong inhibitor for aminopeptidase M and leucine aminopeptidase. Inhibition of actinonin against aminopeptidase M is competitive with the substrate. The *Ki* and *Km* values are 1.7×10^{-7} M and 8.0×10^{-5} M, respectively. In tests described previously[5], intraperitoneal administration of 0.01~1 mg/mouse of actinonin augmented delayed-type hypersensitivity (DTH) and it also increased antibody formation. It has low acute toxicity. No deaths occurred after an intraperitoneal injection of 250 mg/kg in mice.

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(Received July 19, 1985)

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