PRODUCTION OF ACETYL-L-LEUCYL-L-ARGININAL, INHIBITOR OF DIPEPTIDYL AMINOPEPTIDASE III BY BACTERIA

Sir:

As part of our continuous effort toward the discovery of novel enzyme inhibitors from microbial cultures[1~3], we have searched for inhibitors against dipeptidyl aminopeptidases. We wish to report the discovery of acetyl-L-leucyl-L-argininal as a specific inhibitor against dipeptidyl aminopeptidase III (DAP-III, EC 3.4.14.4) from the culture filtrate of a bacterium (BMG520-yF2). Although this inhibitor is identical to a known analogue of leupeptin[4,5], we also wish to report its isolation and characterization because of the important inhibitory actions.

DAP-III was partially purified from rat pancreas by ammonium sulfate fractionation according to the method of ELLIS and NUENKE[6]. The method of testing the enzyme inhibitory activity was as follows: a mixture consisting of 0.1 ml of 2 mM L-arginyl-L-arginine β-naphthylamide (purchased from Bachem.), 0.5 ml of 110 mM Tris-HCl buffer (pH 9.0 containing with 5.6 mM 2-mercaptoethanol), 0.3 ml of a mixture of water and the test solution and 0.1 ml of DAP-III enzyme solution (34 μg protein in 10 mM phosphate buffer, pH 7.0) was incubated at 37°C for 30 minutes. Then 10 μl of 40 mM $NaIO_4$ was added and incubated at 37°C for 5 minutes. Finally 1.0 ml of 3 mM fast garnet solution was added and the absorbance at 525 nm was determined after 15 minutes at room temperature. The concentration of the inhibitor required for 50% inhibition (IC_{50}) was determined.

The inhibitor was produced by shaken culture of BMG520-yF2 in a medium containing glycerol 2%, dextrin 2%, soy peptone 1%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2%, pH 7.4 with 5 N NaOH before sterilization. The maximum production was attained after 14~20 hours of culture at 27°C. The inhibitor was adsorbed onto activated carbon and eluted with 80% acetone solution. The active eluate was evaporated under reduced pressure to a brown powder (IC_{50} 18 μg/ml; 61% yield). The crude powder was dissolved in water and applied onto a column of Amberlite XAD-7. After washing the column with water and 80% methanol, the inhibitor was eluted with 0.05 N HCl in 80% methanol. The active eluate was adjusted to pH 4.0 with Dowex WGR and concentrated under reduced pressure. The concentrate was rechromatographed by the same column using a linear gradient of 80% methanol to 0.05 N HCl in 80% methanol. The active fraction gave a pale brown powder (IC_{50} 1.2 μg/ml; 16% yield). Further purification was achieved by column chromatography over Sephadex LH-20 developed with 80% methanol, giving a white powder (IC_{50} 0.04 μg/ml; 13% yield, mp 129~132°C).

The inhibitor is soluble in water, methanol, ethanol and butanol and is insoluble in acetone, ethyl acetate and chloroform. It gives positive color reactions with red tetrazolium, RYDON-SMITH's and SAKAGUCHI's reagents, but negative with ninhydrin. A thin-layer chromatogram of the inhibitor and leupeptin is shown in Fig. 1. The pure inhibitor was separated into two spots on TLC as did leupeptin. In the case of leupeptin this observation was rationalized by assuming an equilibrium mixture of the aldehyde, hydrate, and cyclic alkanolamine forms in aqueous solution[7]. The present inhibitor showed no absorption maximum between 210~400 nm and $[\alpha]_D^{16}$ −45.2° (*c* 0.5, H_2O). Amino acid analysis of the acid hydrolysate of the oxidized compound prepared

Fig. 1. Thin-layer chromatogram on silica gel. Solvent: BuOH - BuOAc - AcOH - H_2O, 4: 2: 1: 1. Reagent: RYDON-SMITH or SAKAGUCHI.

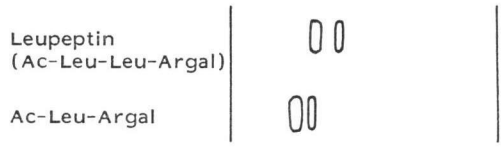

Table 1. Inhibitory effect against DAP-III.

Enzyme	IC_{50} (μg/ml)				
	Ac-L-Leu-L-Argal	Leupeptin	Antipain	Chymostatin	Elastatinal
DAP-III	0.04	0.026	0.045	11	>100

by a permanganate-oxidation showed leucine and arginine in a molar ratio of 1: 1. Approximately 10% of the arginine was D-arginine. This may be an artifact of the isolation procedure. The optical configurations of both amino acids were determined using HPLC on the diastereomeric peptides formed after derivatization with *t*-butoxycarbonyl-L-phenylalanine *N*-hydroxysuccinimide, by the modified method of MITCHELL *et al.*[8]. Molecular weights of the inhibitor and oxidized compound were shown by the secondary ion mass spectrometry as MH^+ m/z at 314 and 330, respectively.

From these results, the structure of the inhibitor was deduced to be acetylleucyl-argininal ($C_{14}H_{27}N_5O_3$, MW 313).

In order to confirm the proposed structure of the inhibitor, acetyl-L-leucyl-L-argininal [Ac-L-Leu-L-Argal] was synthesized from L-leucyl-L-argininal dibutylacetal [H-L-Leu-L-Argal$(OBu)_2$] which was obtained from leupeptin by the procedure of SAINO *et al.*[9] by acetylation of H-L-Leu-L-Argal$(OBu)_2$ with acetylimidazole followed by the silica gel chromatography. The purified Ac-L-Leu-L-Argal$(OBu)_2$ was then treated with 1 N HCl to afford Ac-L-Leu-L-Argal as white powder (mp 130~133°C dec). The $[\alpha]_D^{16}$ of the synthesized Ac-L-Leu-L-Argal was $-51.4°$ (c 1, H_2O). The inhibitor was found to be identical to synthetic Ac-L-Leu-L-Argal by TLC, HPLC[9] (column; Develosil 50DS 6 mm×200 mm, solvent=1% H_3PO_4 - CH_3CN, 85: 15, v/v detected at 205 nm) and IR.

The inhibitory activities of Ac-L-Leu-L-Argal, leupeptin, antipain[10], chymostatin[11] and elastatinal[12] against DAP-III are summarized in Table 1. DAP-III is known as a thiol enzyme inhibited by *p*-chloromercurisulfonate and *N*-ethylmaleimide[6]. Leupeptin and antipain (inhibitors to serine-thiol enzyme) were also strong inhibitors of DAP-III.

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TAKAAKI NISHIKIORI
FUMIKA KAWAHARA
HIROSHI NAGANAWA
YASUHIKO MURAOKA
TAKAAKI AOYAGI
HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23, Kamiosaki, Shinagawa-ku,
Tokyo 141, Japan

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