

Communications to the Editor

DIPROTINS A AND B,
INHIBITORS OF DIPEPTIDYL
AMINOPEPTIDASE IV,
PRODUCED BY BACTERIA

Sir:

In the course of screening studies for inhibitors of dipeptidyl aminopeptidase IV (EC 3.4.14.5), new peptides which we named diprotins A and B were isolated from culture filtrates of *Bacillus cereus* BMF673-RF1. In this paper, the isolation, physico-chemical properties and structure determination of diprotins are reported. Dipeptidyl aminopeptidase IV (DAP-IV) was prepared from rat kidney homogenate by ammonium sulfate precipitation (30~80%)¹⁾. In the assay for DAP-IV and its inhibition, a reaction mixture (1 ml) containing 50 mM Tris-maleate-NaOH (pH 7.2), 0.4 mM Gly-Pro- β NA (Bachem.), DAP-IV, with or without a test material was incubated at 37°C for 30 minutes¹⁾. The reaction was stopped by adding 1 ml of 1 M acetate buffer (pH 4.2) containing 0.1% (w/v) Fast Garnet GBC salt (Sigma) and 10% (w/v) Tween 20. The amount of naphthylamine liberated was spectrophotometrically measured²⁾ and the concentration of inhibitor required for 50% inhibition (IC_{50}) was determined.

Diprotins were produced by reciprocal shaking culture of the strain BMF673-RF1 for 2 days at 27°C in a medium containing 1% glucose, 1% glycerol, 1% potato starch, 0.5% Polypepton, 0.5% meat extract, 0.5% NaCl, 0.32% CaCO₃ and 0.05% silicon oil KM-70 (Shin-Etsu Chemical Co. Ltd., Japan), adjusted to pH 7.4 with 5 N NaOH before sterilization.

Inhibitors in 56 liters of culture filtrate were adsorbed on activated charcoal and eluted with 90% aqueous methanol adjusted to pH 2 with 6 N HCl. The eluate was concentrated under reduced pressure to yield a crude powder. Inhibitors in the crude powder were adsorbed on a column of Dowex 50WX8 (free acid form, 20~50 mesh) and the adsorbed material was eluted with 1.5 N aqueous ammonia. The active material was adsorbed on a Dowex 50WX4 (100~200 mesh) column equilibrated with 0.2 M pyridine - acetate buffer (pH 3.2), washed with the same buffer and

then eluted with 0.5 M pyridine - acetate buffer (pH 4.5). The active fractions were collected and concentrated under reduced pressure. The concentrate thus obtained was chromatographed on a Sephadex G-25 column with distilled water, and on a Silica gel 60 (E. Merck) column with ethanol - 28% (w/v) aqueous ammonia (50:1), and on a Dowex 50WX4 (pyridinium form, 100~200 mesh) column with 0.2 M pyridine - acetate buffer (pH 3.2), successively. In this last step, the activities appeared in two separate fractions. The earlier fraction contained diprotin B and the later, diprotin A. Each of them was concentrated and chromatographed on a Silica gel 60 column with chloroform - methanol - acetic acid - water (60:8:2:1) and on a Dowex 50WX4 (pyridinium form, 100~200 mesh) column with 0.2 M pyridine - acetate (pH 3.2). Each active fraction was desalted with Dowex 50WX4 (free acid form, 100~200 mesh) and 1 N aqueous ammonia, and then concentrated under reduced pressure and lyophilized, yielding 22.7 mg of diprotin A and 64.8 mg of diprotin B. Diprotins A and B were obtained as colorless powder. Diprotins A and B are soluble in water, methanol, ethanol but insoluble in ethyl acetate, chloroform and ether. They give weekly positive ninhydrin reaction and positive RYDON-SMITH reaction. On thin-layer chromatograms on Silica gel 60 (E. Merck), diprotins A and B give a single spot at Rf 0.42 and 0.38 (1-BuOH - AcOH - H₂O, 4:1:1) and Rf 0.66 and 0.63 (EtOAc - pyridine - AcOH - H₂O, 5:5:1:3), respectively.

Physico-chemical properties of diprotins were as follows: Diprotin A; mp 178~180°C (dec), $[\alpha]_D^{25} -70.7^\circ$ (*c* 1, 1 N HCl). Anal Calcd for C₁₇H₃₁N₃O₄: C 59.80, H 9.15, N 12.32, O 18.74, found: C 59.30, H 9.10, N 12.53, O 18.80. The molecular formula was determined to be C₁₇H₃₁N₃O₄ by the elemental analysis and secondary ion mass spectrometry, *m/z* 342 (M+1). Diprotin B; mp 158~160°C (dec), $[\alpha]_D^{25} -94.1^\circ$ (*c* 1, 1 N HCl). Anal Calcd for C₁₆H₂₉N₃O₄: C 58.69, H 8.92, N 12.83, O 19.54, found: C 58.20, H 8.88, N 12.52, O 20.03. The molecular formula was determined to be C₁₆H₂₉N₃O₄ by the elemental analysis and secondary ion mass spectrometry, *m/z* 328 (M+1).

Amino acid analyses showed that diprotin A is composed of one residue of proline and two residues of isoleucine, and diprotin B is composed of one residue each of valine, proline and leucine. All amino acids were found to be L-form by the measurement of their specific rotation. From the results of EDMAN degradation and anhydrous hydrazine degradation and secondary ion mass spectra of diprotins A and B, the structures of diprotins A and B were elucidated to be L-isoleucyl-L-prolyl-L-isoleucine and L-valyl-L-prolyl-L-leucine, respectively.

The IR and secondary ion mass spectra of diprotins A and B are shown in Figs. 1 and 2, respectively.

Table 1. Inhibitory activity of diprotins against exopeptidases.

Exopeptidase	IC ₅₀ (μg/ml)	
	Diprotin A	Diprotin B
Dipeptidyl aminopeptidase IV	1.1	5.5
Aminopeptidase A	>100	>100
Aminopeptidase B	>100	43.7
Leucine aminopeptidase	>100	>100
Carboxypeptidase A	>100	>100
Carboxypeptidase B	>100	>100
Dipeptidyl carboxypeptidase	92	>100

Fig. 1-A. IR spectrum of diprotin A (KBr).

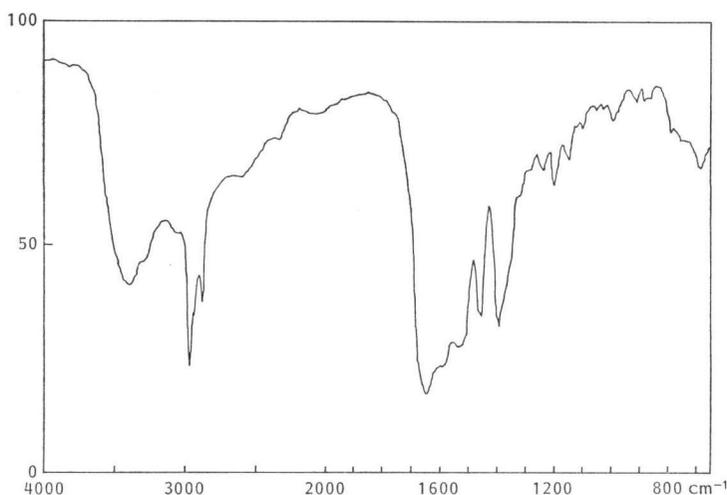


Fig. 1-B. IR spectrum of diprotin B (KBr).

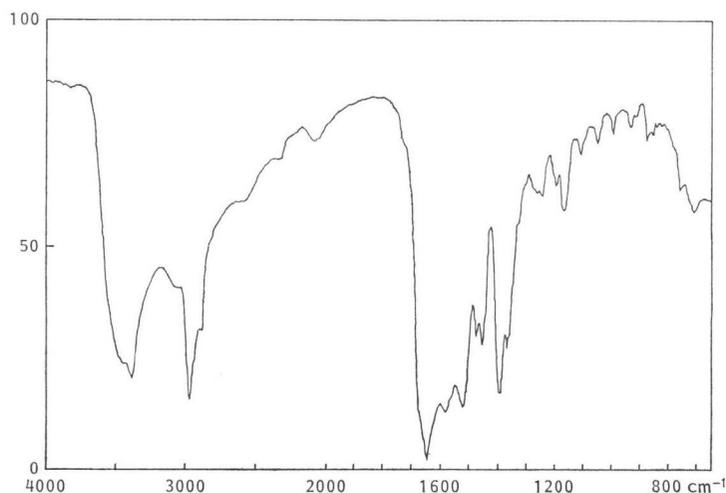


Fig. 2-A. Secondary ion mass spectrum of diprotin A.

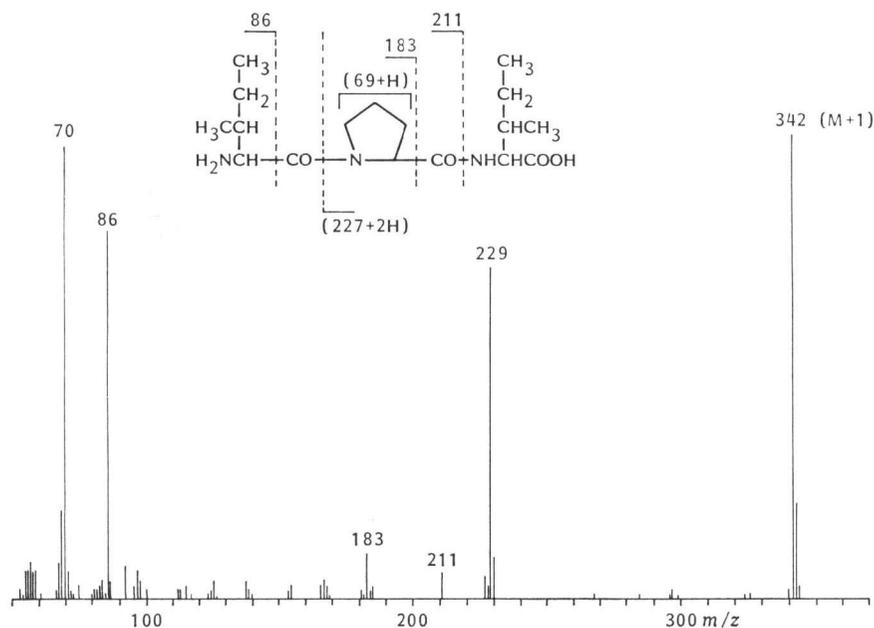
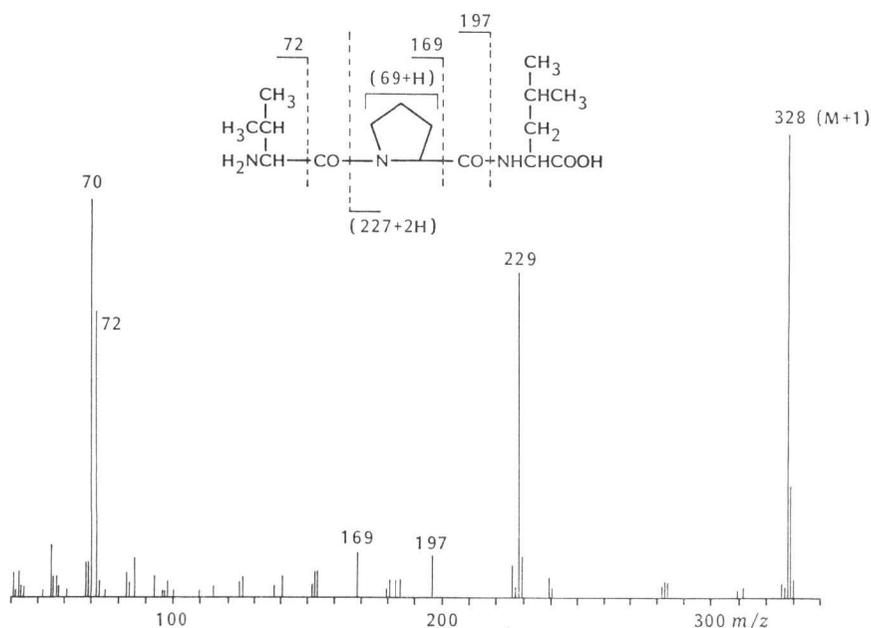


Fig. 2-B. Secondary ion mass spectrum of diprotin B.



The purified diprotins A and B showed IC_{50} values of $1.1 \mu\text{g/ml}$ and $5.5 \mu\text{g/ml}$ against DAP-IV, respectively. Inhibition by diprotins is competitive with the substrate. The K_i value of diprotin A is $2.2 \times 10^{-6} \text{ M}$ and that of diprotin B is $7.6 \times 10^{-6} \text{ M}$. Activities of diprotins A and

B in inhibiting peptidases are summarized in Table 1. Intraperitoneal administration of $0.1 \sim 4 \mu\text{g/mouse}$ of diprotin A augmented delayed type hypersensitivity to sheep red blood cells in footpad test⁴⁾ using male JCL:ICR mice 6 weeks old. Diprotins A and B at $100 \mu\text{g/ml}$ had no

antimicrobial activity. They have low toxicity; no deaths after intravenous injection of 250 mg/kg to mice.

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