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**Communications to the Editor**

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**HISTARGIN, A NEW INHIBITOR OF  
CARBOXYPEPTIDASE B,  
PRODUCED BY ACTINOMYCETES**

Sir:

During the course of our screening studies for the isolation of carboxypeptidase B inhibitors, we were successful in obtaining an inhibitor, which we named histargin, from culture filtrates of *Streptomyces roseoviridis* MF118-A5.

In this paper, the fermentation and isolation procedures and some physico-chemical properties of histargin are reported.

Carboxypeptidase B inhibitory activity was measured using the following modification of the angiotensin I converting enzyme activity determination method<sup>1)</sup>. A mixed solution was prepared by adding 0.25 ml of 0.05 M Tris-hydrochloric acid buffer solution (pH 8.0) and 0.15 ml of water or 0.15 ml of test solution to 0.05 ml of 0.05 M hippuryl-L-lysine (Peptide Institute, Japan). The mixed solution was incubated for 3 minutes at 37°C, and 0.05 ml of a solution that contained about 0.3 µg/ml of purified carboxypeptidase B (Boehringer-Mannheim, West Germany) and 1 mg/ml of bovine serum albumin (Wako Pure Chemical Industries, Ltd., Japan) was added to the mixed solution. The resulting mixture was incubated for 30 minutes at 37°C, after which 0.03 ml of 1 N sodium hydroxide was added to terminate the reaction. Fifteen minutes later, 2 ml of 0.06 M phosphate buffer (pH 7.2) and 2 ml of 1% cyanuric chloride (2,4,6-trichloro-S-triazine), freshly dissolved in 2-methoxyethanol, were added to the mixed solution. After the mixture was allowed to stand for 15 minutes at room temperature, the absorbance at 382 nm was measured. Blanks were prepared corresponding to the above two sets of solutions (*i.e.* with and without test solution). This involved the same procedure except that the sodium hydroxide solution was added prior to the addition of the enzyme solution. The concentration of inhibitor required for 50% inhibition ( $IC_{50}$ ) was determined.

Histargin is produced by submerged culture of *S. roseoviridis* MF118-A5 in a stainless-steel

fermentor with the medium consisting of glycerol 2%, dextrin 2%, soy peptone 1%, yeast extract 0.3%,  $(NH_4)_2SO_4$  0.2% and  $CaCO_3$  0.2%, adjusted to pH 7.4 with 5 N NaOH before sterilization.

Six liters of seed culture prepared with a 30-liter jar fermentor containing 15 liters of the medium described above was used to inoculate a 200-liter fermentor containing 100 liters of the same medium. Fermentation was carried out for 42 hours at 28°C with agitation at 250 rpm and with air flow of 100 liters per minute.

One hundred liters of a culture filtrate was passed through a 9 liter column of Amberlite IRC-50(H<sup>+</sup>). The column was washed with 50% aqueous acetone and the active principle adsorbed to the resin was eluted with 0.5 N HCl in 50% aqueous acetone. The active eluate was concentrated to 6 liters and the concentrate was neutralized with 6 N NaOH. To this concentrate was added 180 g of activated carbon and the mixture was agitated for 30 minutes. The carbon was collected and washed with water. The inhibitor was eluted from the activated carbon with 50% aqueous acetone (pH 2 with 6 N HCl). The eluate was concentrated under reduced pressure to yield crude powder, 36.5 g. The crude material thus obtained was adsorbed on a column of Dowex 50WX4 (pyridinium form, 100~200 mesh, 500 ml). After washing the resin bed with 0.5 M pyridine - acetate buffer (pH 4.6), the inhibitor was eluted with 1 M pyridine - acetate buffer (pH 5.0), and the eluate was concentrated under reduced pressure. The crude material thus obtained (4.5 g) was dissolved in 30 ml of 10% aqueous ammonium acetate and the solution was divided into five equal portions. Each portion was applied to a Partisil-10-ODS-3/Magnum 20 preparative HPLC column (Whatman Ltd., U.S.A.) which had been equilibrated with 10% aqueous ammonium acetate. The column was developed with the same solution at a flow rate of 8 ml per minute. The active fractions were pooled and diluted ten fold with distilled water. The resulting solution was then adsorbed on a column of Dowex 50WX4(H<sup>+</sup>, 100~200 mesh, 500 ml). The resin bed was washed with water and the

Fig. 1-A. IR spectrum of histargin monohydrate (KBr).

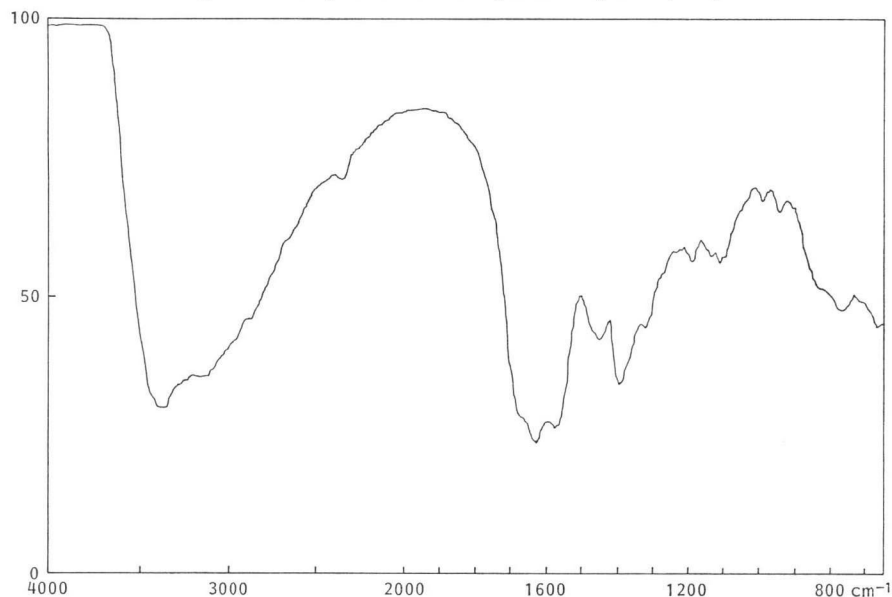
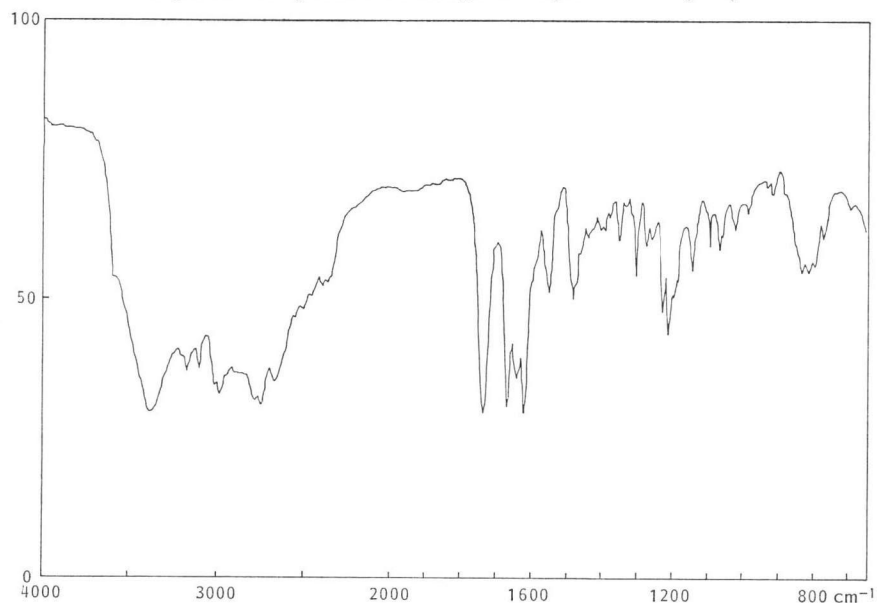


Fig. 1-B. IR spectrum of histargin tetrahydrochloride (KBr).



inhibitor was eluted with 1.5 N aqueous ammonia. The active eluate was concentrated under reduced pressure and lyophilized, yielding 1.92 g of pure histargin. Histargin monohydrate was obtained as a colorless powder.

Histargin is soluble in water but insoluble in methanol, ethanol, ethyl acetate, chloroform, ether and dimethyl sulfoxide. Histargin gives positive PAULI, SAKAGUCHI and RYDON-SMITH

reactions. On thin-layer chromatograms on Silica gel 60 (E. Merck), histargin gives a single spot at  $R_f$  0.55 (EtOH - 25%  $\text{NH}_4\text{OH}$ , 1: 1) and  $R_f$  0.32 (1-propanol-pyridine-acetic acid-water, 15: 10: 3: 12), respectively. Physico-chemical properties of histargin were as follows: Histargin monohydrate; mp  $161 \sim 165^\circ\text{C}$  (dec);  $[\alpha]_D^{25} +36.3^\circ$  ( $c$  1, constant boiling HCl); Anal calcd for  $\text{C}_{14}\text{H}_{25}\text{N}_7\text{O}_4 \cdot \text{H}_2\text{O}$ : C 45.03, H 7.29, N 26.26,

O 21.42, Found: C 45.53, H 7.53, N 26.28, O 20.80. The molecular formula was determined to be  $C_{14}H_{25}N_7O_4$  by the elemental analysis and secondary ion mass spectrometry,  $m/z$  356 ( $M+1$ ). Potentiometric titration gave  $pK_a'$  values of <3.5, <3.5, 4.4, 6.8, 9.0 and >11. By addition of two mol equivalents of 2-nitroindan-1,3-dione (NID) as an aqueous solution to an aqueous solution of histargin, pale yellow crystals of histargin di-NID salt monohydrate were obtained. The chemical properties of this salt are as follows: mp 192~194°C (dec);  $[\alpha]_D^{20} + 16.8^\circ$  ( $c$  1, constant boiling HCl, after removal of the precipitated nitroindandione with glass filter). *Anal* calcd for  $C_{14}H_{25}N_7O_4 \cdot 2(C_8H_5NO_4) \cdot H_2O$ : C 50.86, H 4.93, N 16.68, O 27.52, Found: C 50.87, H 5.22, N 16.76, O 27.60.

By addition of excess hydrochloric acid solution to histargin and concentration of the resulting solution under reduced pressure, colorless crystals of histargin hydrochloride were obtained. The chemical properties of this salt are as follows: mp 166~173°C (dec);  $[\alpha]_D^{20} + 25.7^\circ$  ( $c$  1, constant boiling HCl). *Anal* calcd for  $C_{14}H_{25}N_7O_4 \cdot 4HCl$ : C 33.54, H 5.83, N 19.56, O 12.76, Cl 28.29. Found: C 33.59, H 5.76, N 19.23, O 13.53, Cl 28.12.

The IR spectra of histargin monohydrate and histargin tetrahydrochloride are shown in Fig. 1.

Determination of the chemical structure of histargin as *N*-[(*S*)-1-carboxy-4-guanidinobutyl]-*N'*-[(*S*)-1-carboxy-2-(imidazol-4-yl)ethyl]-ethylenediamine will be described in the following paper<sup>5)</sup>.

Histargin monohydrate showed an  $IC_{50}$  value of 17  $\mu\text{g/ml}$  against carboxypeptidase B. Inhibition of histargin is competitive with the

Table 1. Inhibitory activities of histargin against exopeptidases.

Exopeptidase	$IC_{50}$ ( $\mu\text{g/ml}$ )
Carboxypeptidase A	>100
Carboxypeptidase B	17
Aminopeptidase A	>100
Aminopeptidase B	>100

The enzyme inhibition assays above other than carboxypeptidase B were undertaken by the methods we described previously<sup>2~4)</sup>.

substrate. The  $K_i$  value of histargin was estimated to be  $3.2 \times 10^{-5}$  M. Activities of histargin in inhibiting exopeptidases are shown in Table 1.

As readily inferred from the structure, an asymmetrical ethylenediamine derivative, histargin has a metal chelating activity. The carboxypeptidase B inhibitory activity of histargin (37.3  $\mu\text{g/assay}$ ,  $2 \times 10^{-4}$  M) is completely abolished by addition of metal salts such as  $ZnCl_2$ ,  $CuCl_2$  and  $CoCl_2$  and partially abolished by  $FeCl_2$  but not by  $MgCl_2$ ,  $CaCl_2$  nor  $FeCl_3$  (all at  $2 \times 10^{-4}$  M).

Histargin at 100  $\mu\text{g/ml}$  had no antimicrobial activity. It has low acute toxicity. No deaths occurred after intravenous injection of 1 g/kg in mice.

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