Sir:

We have previously reported that various inhibitors of endopeptidases and exopeptidases have been found in culture filtrates of microbes\(^1-^3\). Recently, we have demonstrated that exopeptidases, alkaline phosphatase and esterase are located not only in cell but also on the cellular membrane of various kinds of mammalian cells\(^4-^6\) and that inhibitors of these enzymes modified immune responses\(^7,^8\). We thought that exopeptidase inhibitors should be useful in the analysis of biological functions.

In the present study, we searched for inhibitors of carboxypeptidase A (EC 3.4.17.1) and discovered (S)-\(\alpha\)-benzylmalic acid from the culture filtrate of Streptomyces hygroscopicus (resemble MG368-CF16). Although the inhibitor we obtained is a known substance\(^9\), we report its isolation and characterization of this agent because of the importance of its inhibitory actions.

The method described by HAYAKARI, et al.\(^{10}\) for measuring the inhibitory activity of carboxypeptidase A in culture filtrates was modified as follows: A reaction mixture consisting of 0.25 ml of 0.05 M Tris-HCl buffer containing 0.9 M NaCl (pH 7.5), 0.05 ml of 10 mM hippuryl-L-phenylalanine (Sigma Chem. Ltd., Saint Louis, U.S.A.), and 0.15 ml of distilled water with or without a test material was incubated at 37°C for 3 minutes. 0.05 ml of carboxypeptidase A (Type I from bovine pancreas, Sigma Chem. Ltd.) was added and mixed well. Exactly 30 minutes later, 0.03 ml of 1 N NaOH was added and the solution was removed from the incubator. After standing for 15 minutes at room temperature, 2 ml of 0.06 M phosphate buffer (pH 7.2) and 2 ml of 1% cyanuric chloride dissolved in ethylene-glycol monomethyl ester were added. After standing for 5 minutes at room temperature, absorbance at 382 nm was read. The amount of the enzyme was adjusted to give an absorbance of about 0.4. 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 culture medium used for the production of the inhibitor was as follows: glycerol 1.5%, soluble starch 1.5%, soybean meal 0.5%, fish meal 1.5%, CaCO\(_3\) (precipitate) 0.2% (adjusted to pH 7.4 with 6 N NaOH before sterilization). Maximum production was usually attained on the 5~7th day from the shaken culture (27°C) of the strain number MG368-CF16 in the Institute of Microbial Chemistry.

Chart 1. Purification of carboxypeptidase A inhibitor.

<table>
<thead>
<tr>
<th>Culture filtrate of MG368-CF16</th>
<th>Amberlite XAD-4</th>
<th>DEAE-Sephadex A-25</th>
<th>Amberlite XAD-4</th>
<th>Silica gel (CHCl(_3)-MeOH)</th>
<th>HPLC (Partisil 10)</th>
<th>(S)-(\alpha)-Benzylmalic acid</th>
</tr>
</thead>
</table>

Extraction and purification processes are outlined in Chart 1. A culture filtrate (6 days, 10 liters) was adjusted to pH 2.0 with 2 N HCl and passed through a column of Amberlite XAD-4 (1 liter, 8 x 20 cm) previously equilibrated with 0.01 N HCl. The column was washed with 1 liter of distilled water and was eluted with 4 liters of 0.5 N aqueous ammonia. The active eluate was concentrated under reduced pressure to give a brownish powder (7.1 g, IC\(_{50}\) 35 \(\mu\)g/ml). It was placed on a column of DEAE-Sephadex A-25 (250 ml, 3 x 35 cm) which was equilibrated with 0.05 M sodium phosphate buffer at pH 5.8, and the column was developed with a linear gradient of NaCl (0.05 ~ 1.0 M) in the same buffer. The active fractions were combined and adjusted to pH 2.0 with 2 N HCl and passed through a column of Amberlite XAD-4 (100 ml, 1.6 x 50 cm) previously equilibrated with 0.01 N HCl. The column was washed with 200 ml of water and the product was eluted with 1 liter of 0.5 N aqueous ammonia. The active eluate was concentrated under reduced pressure to give a light brownish powder (1.1 g, IC\(_{50}\) 0.79 \(\mu\)g/ml). It was dissoldd in 1.2 ml of distilled water and chromatographed on a Whatmann HPLC Prepacked Column (Partisil 10, ODS-2,
Table 1. Inhibitory activity of α-benzylmalic acid to exo- and endopeptidases.

<table>
<thead>
<tr>
<th>Exopeptidase</th>
<th>IC₅₀ (µg/ml)</th>
<th>Endopeptidase</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxypeptidase A</td>
<td>0.48</td>
<td>Trypsin</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>10</td>
<td>Pepsin</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Converting enzyme*</td>
<td>&gt;200</td>
<td>Papain</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Aminopeptidase A</td>
<td>&gt;100</td>
<td>Esterase</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Aminopeptidase B</td>
<td>&gt;100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Angiotensin I converting enzyme (EC 3.4.15.1).

M₉/₂₅) with a linear gradient ranging from 0 to 80% methanol using a Waters gradient generator (ALC/GPC 200 series). The active fractions were concentrated under reduced pressure to give white powder (27 mg, IC₅₀ 0.48 µg/ml).

'HNMR of this powder dissolved in D₂O: 7.83 ppm (s, 5H), 3.67 and 3.24 ppm (AB quartet, 2H, J=17 Hz), and 3.57 ppm (d, 2H). 'C NMR spectrum (D₂O) showed eleven carbons: 178.2 ppm, 174.9 ppm, 135.8 ppm, 131.4 ppm, 131.2 ppm, 129.4 ppm, 129.2 ppm, 128.1 ppm, 76.9 ppm, 45.5 ppm, 43.8 ppm. Mass spectrum (Fig. 1): m/z 225 (M+1, 4%), 206 (18%), 162 (28%), 161 (9%), 119 (13%), 116 (24%), 115 (30%), 92 (61%), 91 (100%), 87 (10%), 65 (30%), 43 (30%). Dehydration accounted for the peak at m/z 206 by methylation with diazomethane, but the expected peak at m/z 252 (M⁺) could not be detected. Fragment formulae of these peaks were obtained from high resolution mass spectrum. NMR spectra were obtained with a 400 MHz Jeol GX400 spectrometer. Low resolution mass spectra were determined with a Hitachi RMU-6M mass spectrometer. High resolution mass spectra were obtained with a Hitachi M80-H. [α]₂⁵° +13.7° (c 1.0, H₂O), mp 153~155°C. According to these results, we have established that the inhibitor of carboxypeptidase A produced by the strain MG368-CF16 is (S)-α-benzylmalic acid (C₁₁H₁₂O₅).

As shown in Table 1, this compound is a specific inhibitor for carboxypeptidases. Carboxypeptidase B was inhibited by α-benzylmalic acid but the IC₅₀ was 20-fold higher than that for carboxypeptidase A. Kinetic studies showed that α-benzylmalic acid inhibited carboxypeptidase A in a competitive manner with an apparent Kᵢ value of 6.7×10⁻⁸ M.

Oral administration of 0.005~5.0 µg/mouse of α-benzylmalic acid augmented delayed-type hypersensitivity (DTH) to sheep red blood cells in the footpad test using CDF₁ mice older than 10 weeks. α-Benzylmalic acid had no antimicrobial activity. It has low acute toxicity. No deaths occurred after an intraperitoneal injection of 500 mg/kg in mice.
References


