Cyclo(d-Pro-L-Val), a Specific β-Glucosidase Inhibitor Produced by Aspergillus sp. F70609

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Glycosidases catalyze the hydrolysis of poly and oligosaccharides into monomers or cleave bonds between sugars and non-carbohydrate aglycons. Thus, a number of metabolic processes rely on these enzymes for their efficacy, selectivity and control1). There is now a great deal of interest in glycosidase inhibitors since these compounds have been shown to be important tools in studies on the mechanism of enzyme-catalyzed glycoside hydrolysis2), oligosaccharide structure, and in medicinal chemistry3'4).

In the course of our screening program for glycosidase inhibitors of microbial origin, we have isolated cyclo(p-?XO-L-Val) (1, Fig. 1), as a specific β-glucosidase inhibitor from the culture broth of Aspergillus sp. F70609. In this paper, we describe the fermentation, isolation, and β-glucosidase inhibitory activity of 1. In addition, we have synthesized the isomers of 1 and compared their activity against the enzyme.

Cultural characteristics of the strain F70609 were observed on potato-dextrose agar, malt extract agar and Czapek-Dox agar. Morphological characteristics of the spores and mycelia were observed with a scanning electron microscope (Philips Sem 515) and a light microscope (Nikon Labophot-2). From the observed results, the strain F70609 was identified as a member of Aspergillus sp.

A slant culture of the strain F70609 was used to inoculate two 500 ml Erlenmeyer flasks containing 100 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, polypeptone 0.5%, MgSO4 0.05%, KH2PO4 0.1%, at pH 6.0). After incubation at 27℃ for 7 days on a rotary shaker (150 rpm), the fermentation broth (3 liters) was centrifuged and the resulting mycelial cake was extracted with 70% aqueous acetone. After removal of acetone, the aqueous solution was extracted three times with ethyl acetate. The ethyl acetate-soluble portion was concentrated in vacuo.

The enzymatic activities of glycosidases were determined colorimetrically by monitoring the release of β-nitrophenol from the appropriate para-nitrophenyl (PNP)-glycoside substrate (Sigma). The reaction mixture contained 5 mM of PNP-glycoside and the enzyme in a final volume of 0.5 ml containing 50 mM acetate buffer, pH 5.0 or 30 mM phosphate buffer, pH 7.0 specified in enzyme source (Sigma). Incubations were for 30 minutes at 37℃, and the reactions were terminated by the addition of 2 ml of 0.4 M glycine buffer, pH 10.4. The released β-nitrophenol in the reaction was measured at 410 nm. The concentration showing 50% inhibition (IC50) was determined from a plot of percent inhibition vs the concentration.

The physico-chemical properties of 1 are as follows: white powder; UV λmax nm in MeOH: 210 (end absorption) nm; IR (KBr): 3200, 2928, 1678, 1637, 1430 cm⁻¹; [α]D = -22.3° (c 0.2, MeOH); 1H NMR in CDC13 (ppm, 300 MHz) 0.92 (3H, d, J=6.9 Hz, Val γ1), 1.07 (3H, d, J=7.5 Hz, Val γ2), 1.90 (1H, m, Pro γ), 2.05 (1H, m, Pro β), 2.05 (1H, m, Pro γ), 2.39 (1H, m, Pro β'), 2.49 (1H, m, Pro β'), 2.64 (1H, m, Val β'), 3.50~3.70 (2H, m, Pro δ), 3.94 (1H, br. s, Val α), 4.09 (1H, br. t, J=8.1 Hz, Pro α), 5.73 (1H, s, Val NH); 13C NMR in CDC13 (ppm, 75 MHz) 13.2 (CH3, Val γ1), 19.2 (CH3, Val γ2), 22.2 (CH2, Pro γ), 28.3 (CH3, m, Val β), 28.4 (CH2, Pro β), 44.9 (CH2, Pro δ), 58.7 (CH, Pro α), 60.3 (CH, Val α), 164.8 (CO, Val), 169.9 (CO, Pro); EI-MS: m/z 196 (M⁺), C10H16N2O2.

The structure of 1 was determined to be cyclo(d-Pro-L-Val) on the basis of 2D-NMR spectra including 1H-1H
The configurations of component amino acids were established by chiral TLC analysis of the acid hydrolysate (121°C, 24 hours) of 1.

Cyclo(L-Pro-L-Val) has been isolated from various fungi and other natural sources\(^5,6\) and its isomer, cyclo(L-Pro-D-Val) from the Caribbean sponge Calyx cf. podatypa\(^7\). However, this is the first report on cyclo(D-Pro-L-Val) as an enzyme inhibitor from a fungus.

When various glycosidases were tested, compound 1 could inhibit β-glucosidase activity, but did not inhibit significantly other glycosidases such as yeast α-glucosidase, Aspergillus amyloglucosidase, Aspergillus α-galactosidase, Escherichia coli β-galactosidase, and jack bean α-mannosidase (Table 1). As shown in Fig. 2, compound 1 inhibited β-glucosidase activity with an IC\(_{50}\) value of 75 μg/ml, in a dose-dependent fashion. This activity is comparable with that of castanospermine which was used as the control. In order to elucidate the mechanism of β-glucosidase inhibition of 1, steady-state kinetics were obtained and the data are shown in Fig. 3. A reciprocal plot analysis revealed that 1 behaved as a non-competitive inhibitor with respect to the substrate PNP-β-glucopyranose. The Ki value of 1 was found to be 7.1 × 10\(^{-5}\) mol/liter.

To elucidate structure-activity relationships of 1 for β-glucosidase inhibition, two isomers of 1 were synthesized by the method of Nitecki et al.\(^8\), which is known to proceed without racemization. The optical rotation \([\alpha]_D\) of cyclo(L-Pro-L-Val) and cyclo(L-Pro-D-Val) was found to be −31.3° (c 0.2, MeOH) and −12° (c 0.4, MeOH), respectively. Table 2 shows the different activities of three isomers of cyclo(Pro-Val). Cyclo(L-Pro-L-Val) inhibited almond β-glucosidase activity slightly compared to 1, but cyclo(L-Pro-D-Val) was not active (Table 2). Thus, cyclo(P-Pro-L-Val) was presumed to be a specific inhibitor of β-glucosidase.

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**Table 1. Inhibitory effect of compound 1 on various glycosidase activities.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sources</th>
<th>% of inhibition (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloglucosidase</td>
<td><em>Aspergillus niger</em></td>
<td>0 7</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>Jack Bean</td>
<td>0 6</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td><em>Aspergillus niger</em></td>
<td>8 11</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>Brewers yeast</td>
<td>6 10</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td><em>Escherichia coli</em></td>
<td>0 5</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>Almond</td>
<td>36 79</td>
</tr>
</tbody>
</table>

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![Fig. 1. Structure of 1 elucidated by \(^1\)H-\(^1\)H COSY and HMBC experiments.](image)

![Fig. 2. Dose dependent inhibition of β-glucosidase by 1.](image)
Fig. 3. Lineweaver-Burk plot of almond \( \beta \)-glucosidase inhibition by 1.

- \( \bullet \), 0 \( \mu \)M (no inhibitor);
- \( \bigcirc \), 50 \( \mu \)M;
- \( \triangle \), 100 \( \mu \)M;
- \( \nabla \), 150 \( \mu \)M.

Table 2. \( \beta \)-Glucosidase inhibition activities of cyclo(Pro-Val) isomers.

<table>
<thead>
<tr>
<th>Cyclo dipeptides</th>
<th>% of inhibition (100 ( \mu )g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Pro-L-Val</td>
<td>15</td>
</tr>
<tr>
<td>D-Pro-L-Val</td>
<td>79</td>
</tr>
<tr>
<td>L-Pro-D-Val</td>
<td>0</td>
</tr>
</tbody>
</table>

References