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

OH SUNG KWON, SANG HO PARK[†], BONG-SIK YUN, YU RYANG PYUN[††] and CHANG-JIN KIM*

Korea Research Institute of Bioscience and Biotechnology, P. O. Box 115, Yusong, Taejon 305-600, Korea
[†] Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[††] Department of Biotechnology, Yonsei University, Seoul 120-749, Korea

(Received for publication August 23, 2000)

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 control[1]. 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 hydrolysis[2], oligosaccharide structure, and in medicinal chemistry[3,4].

In the course of our screening program for glycosidase inhibitors of microbial origin, we have isolated *cyclo*(D-Pro-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%, $MgSO_4$ 0.05%, KH_2PO_4 0.1%, at pH 6.0). After incubation at 27°C for 7 days on a rotary shaker (150 rpm), 5 ml of the seed culture was inoculated into thirty 500 ml Erlenmeyer flasks containing 100 ml of the production medium (glucose 2.0%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4$ 0.05%, KH_2PO_4 0.1%, at pH 6.0). The fermentation was carried out at 27°C for 7 days on a rotary shaker (150 rpm).

The enzymatic activities of glycosidases were determined colorimetrically by monitoring the release of *p*-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 50 mM phosphate buffer, pH 7.0 specified in enzyme source (Sigma). Incubations were for 30 minutes at 37°C, and the reactions were terminated by the addition of 2 ml of 0.4 M glycine buffer, pH 10.4. The released *p*-nitrophenol in the reaction was measured at 410 nm. The concentration showing 50% inhibition (IC_{50}) was determined from a plot of percent inhibition *vs* the concentration.

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 concentrate was subjected to a column of silica gel and stepwisely eluted with $CHCl_3$-MeOH (100 : 1→10 : 1). The active fractions were combined and chromatographed on a Sephadex LH-20 column with MeOH. Finally the active fraction was purified by preparative HPLC (Senshu pak ODS, i.d. 4.6×250 mm, eluted with 55% aq. MeOH). The peak with a retention time of 25 minutes was collected and concentrated to give a white powdery material of **1** (4.0 mg).

The physico-chemical properties of **1** are as follows: white powder; UV λ_{max} nm in MeOH: 210 (end absorption) nm; IR (KBr): 3200, 2928, 1678, 1637, 1430 cm^{-1}; $[\alpha]_D$= −22.3° (*c* 0.2, MeOH); ^1H NMR in $CDCl_3$ (ppm, 300 MHz) 0.92 (3H, d, *J*=6.9 Hz, Val γ_1), 1.07 (3H, d, *J*=7.5 Hz, Val γ_2), 1.90 (1H, m, Pro γ), 2.05 (1H, m, Pro β), 2.05 (1H, m, Pro γ), 2.39 (1H, m, Pro β), 2.64 (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); ^{13}C NMR in $CDCl_3$ (ppm, 75 MHz) 15.9 (CH_3, Val γ_1), 19.2 (CH_3, Val γ_2), 22.2 (CH_2, Pro γ), 28.3 (CH, m, Val β), 28.4 (CH_2, Pro β), 44.9 (CH_2, 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^+), $C_{10}H_{16}N_2O_2$.

The structure of **1** was determined to be *cyclo*(D-Pro-L-Val) on the basis of 2D-NMR spectra including ^1H-^1H

* Corresponding: changjin@mail.kribb.re.kr

COSY, HMQC, and HMBC, as summarized in Fig. 1. 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 source[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 manner 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*(D-Pro-L-Val) was presumed be a specific inhibitor of β-glucosidase.

Fig. 1. Structure of **1** elucidated by ^1H-^1H COSY and HMBC experiments.

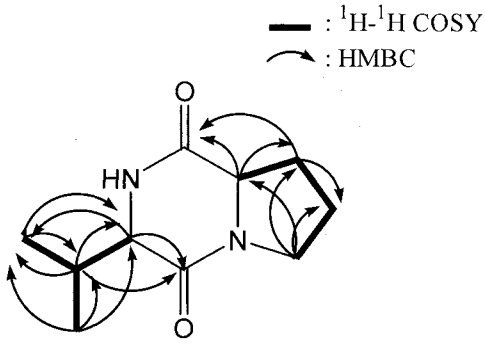

Fig. 2. Dose dependent inhibition of β-glucosidase by **1**.

●, Compound **1**; ○, castanospermine.

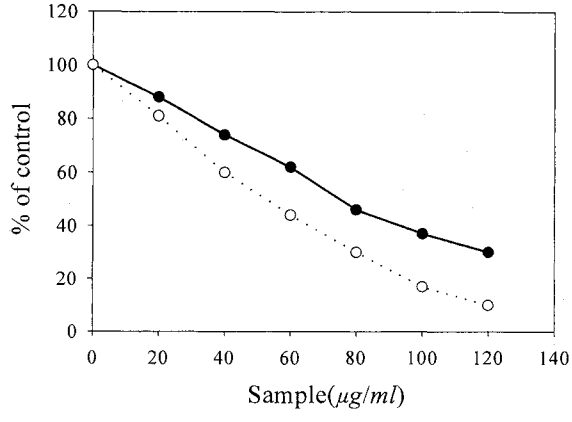

Table 1. Inhibitory effect of compound **1** on various glycosidase activities.

Enzyme	Sources	% of inhibition (μg/ml)	
		50	100
Amyloglucosidase	*Aspergillus niger*	0	7
α-Mannosidase	Jack Bean	0	6
α–Galactosidase	*Aspergillus niger*	8	11
α-Glucosidase	Brewers yeast	6	10
β-Galctosidase	*Escherichia coli*	0	5
β–Glucosidase	Almond	36	79

Fig. 3. Lineweaver-Burk plot of almond β-glucosidase inhibition by **1**.

●, 0 μM (no inhibitor); ○, 50 μM; ▲, 100 μM; ▽, 150 μM.

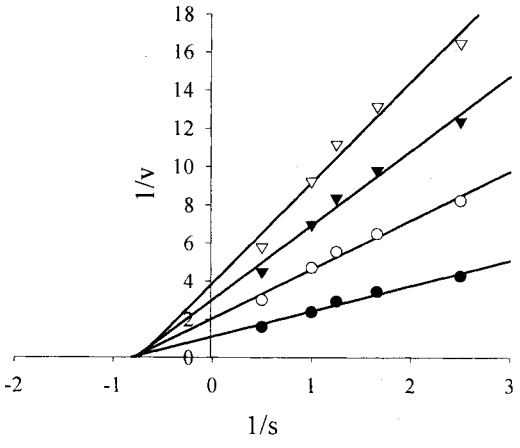

Table 2. β-Glucosidase inhibition activities of *cyclo*(Pro-Val) isomers.

Cyclo dipeptides	% of inhibition (100 μg/ml)
L-Pro-L-Val	15
D-Pro-L-Val	79
L-Pro-D-Val	0

References

1) FUHRMANN, U.; E. BAUSE & H. PLOEGH: Inhibitiors of oligosaccharide processing. Biochim. Biophys. Acta 825: 95~110, 1985
2) MULLER, O. T. & A. ROSENBERG: β-Glucoside hydrolase activity of normal and glucosylceramidotic cultured human skin fibroblasts. J. Biol. Chem. 252: 825~829, 1977
3) GRUTER, R. A.; J. J. NEEFJES, M. TERSMETTE, R. E. Y. GOEDE, T. ABRAHAM, H. G. HUISMAN, F. MIEDEMA & H. L. PLOEGH: Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature 330: 74~77, 1987
4) HUMPHRIES, M.; K. MATSUMOTO, S. L. WHITE & K. OLDEN: Inhibition of experimental metastasis by castanospermine in mice: Blockage of two distinct stages of tumor colonization by oligosaccharide processing inhibitors. Cancer Res. 46: 5215~5222, 1986
5) MUNEKATA, M. & G. TAMURA: Selective inhibition of SV40-transformed cell growth by diketopiperazines. Agric. Biol. Chem. 45: 2613~2618, 1981
6) STIERLE, A. C.; J. H. CARDELLINA & F. L. SINGLETON: A marine *Micrococcus* produces metabolites ascribed to the sponge *Tedania ignis*. Experientia 44: 1021, 1988
7) ADAMCZESKI, M.; A. R. REED & P. CREWS: New and known diketopiperazines from the *Caribban sponge*, Calyx cf. podatypa. J. Nat. Prod. 58: 201~208, 1995
8) NITECKI, D. E.; B. HALPERN & J. W. WESTLEY: A simple route to sterically pure diketopiperazines. J. Org. Chem. 33: 864~866, 1968