ISOFLAVONE RHAMUNOSIDES, INHIBITORS OF β-GALACTOSIDASE PRODUCED BY ACTINOMYCETES

Sir:

As reported previously, we have isolated many kinds of inhibitors against protease and glycosidase in culture filtrates of actinomycetes[1~4]. Chemical structures of inhibitors produced by microorganisms are quite interesting and could be hardly imagined from the structures of the enzyme substrate. We screened microbial culture filtrates for activity to inhibit β-galactosidase and found pyridindolol[4], a specific inhibitor against the enzyme under acidic condition. β-Galactosidase (EC 3.2.1.18) is a glycosidase which release β-galactose from the terminal carbohydrate chains of glycoprotein or glycolipid under acidic and neutral conditions. We have continued further screening for inhibitors of β-galactosidase and recently isolated inhibitors, isoflavonoids, which were active under both acidic and neutral conditions. In this paper, the isolation and the characterization of isoflavonoids are described. Although inhibitory activity against β-galactosidase was found in culture filtrates from various species of actinomycetes, one strain (MD865-C3) was chosen and used as the source for further production and isolation. Strain MD865-C3 was isolated from a soil sample collected in Sengakuji Temple, Tokyo and found to be closely related to *Streptomyces xanthophaeus*.

Activity to inhibit β-galactosidase was determined according to the following procedures; to 0.05 ml of 0.05 M *p*-nitrophenyl-β-D-galactopyranoside (B.D.H. Chemical Ltd., England) in 0.05 M phosphate buffer (pH 7.0) was added 0.44 ml of the sample solution in the same buffer. After 3 minutes at 37°C, 0.01 ml of the enzyme solution (10 μg of β-galactosidase from bovine liver, obtained from Sigma Chemical Co., U.S.A.) was added and the reaction mixture incubated for 15 minutes at 37°C. Incubation was stopped by adding 2 ml of 0.4 M glycine-sodium hydroxide buffer (pH 10.5) and the optical density of liberated *p*-nitrophenol was measured at 400 nm. The reaction was also carried out without addition of enzyme solution and the result was taken as control blank. The concentration of the inhibitor required for 50 % inhibition (IC_{50}) was calculated as described in previous paper[5].

The inhibitors were produced by shaking culture or tank fermentation of the strain MD865-C3. The medium contained lactose 2.0 %, soybean meal 1.5 %, NaCl 0.3 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, K_2HPO_4 0.1 %, $CuSO_4 \cdot 5H_2O$ 0.0007 %, $FeSO_4 \cdot 7H_2O$ 0.0001 %, $MnCl_2 \cdot 4H_2O$ 0.0008 %, $ZnSO_4 \cdot 7H_2O$ 0.0002 % and pH was adjusted to 7.2 with 2 N NaOH. The maximum production of inhibitors was attained 2~3 days after inoculation in shaking culture or 1~2 days in tank fermentation and maintained for 2~3 days thereafter.

Isoflavonoids in a culture filtrate (28 liters) were passed through a column of Amberlite XAD-2 (10×28 cm). The adsorbed inhibitors were eluted with methanol, and the active eluate was evaporated under reduced pressure (powder I, 5.72 g, IC_{50}=6 μg/ml). Powder I thus obtained was subjected to Sephadex LH-20 chromatography (4×120 cm), using methanol as the solvent. The active fraction was evaporated under reduced pressure. As shown in Fig. 1, four active peaks were obtained. Active fractions from each peak were pooled and dried (peak II; 226.7 mg, IC_{50}=10 μg/ml: peak III; 653.3 mg, IC_{50}=3.2 μg/ml: peak IV; 289.7 mg, IC_{50}=2.0 μg/ml). Peak III was subjected to silica gel column chromatography (22×1.7 cm), using chloroform-methanol (10:1) as the solvent. Two active fractions were obtained and dried (inhibitor III-2 299.4 mg; inhibitor III-3, 107.9 mg). Both active fractions were dissolved in a small amount of methanol and crystallized by adding chloroform drop-

Fig. 1. Elution pattern of inhibitors on Sephadex LH-20 column chromatography

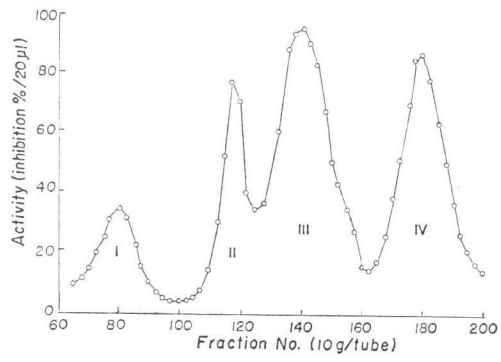

Fig. 2. Infrared absorption spectrum of β-galactosidase inhibitor (KBr)

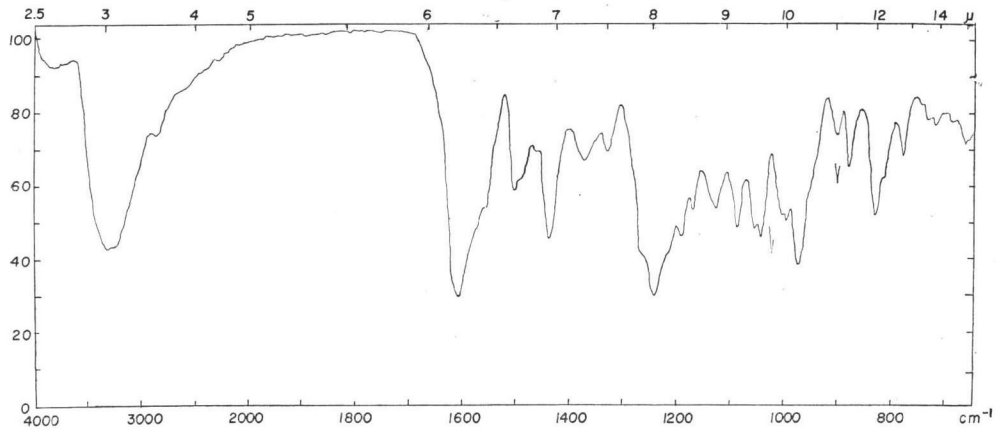

Table 1. Inhibitory activities of β-galactosidase inhibitors on various glycosidase

	ID_{50} (μg/ml)				
	Sialidase			β-Galactosidase	
				Bovine liver	
	Cl. perfringens	*Streptomyces*	A/Aichi/2/68	pH 4.2	pH 7.0
Inhibitor III-2	>250	>250	>250	14	3.6
Pyridindolol	>250	>250	>250	2.0	>250
Siastatin B	3	10	>250	>250	>250

The inhibitory activities of pyridindolol and siastatin B were determined by methods described previously[8-10].

wise. White needle crystals of inhibitors were obtained, which showed 50% inhibition of β-galactosidase (pH 7.0) at 3.6 μg/ml inhibitor III-2 and 1.2 μg/ml inhibitor III-3.

Properties of the inhibitor III-2 were as follows: m.p. 146°C; $[\alpha]_D^{20}$ −164° (*c* 1.0, methanol); maxima at 231 nm ($E_{1cm}^{1\%}$ 545), 262 nm ($E_{1cm}^{1\%}$ 765) in methanol (pH 7.0) and it showed maxima at 247 nm ($E_{1cm}^{1\%}$ 460), 282 nm ($E_{1cm}^{1\%}$ 780) in methanol (pH 10). The IR spectrum is shown in Fig. 2. The elemental analysis was as follows: Calcd. for $C_{21}H_{20}O_8$ (MW 400): C 62.91, H 5.04, O 31.97. Found: C 62.04, H 5.02, O 31.85. It was soluble in dimethylsulfoxide, methanol and ethanol, slightly soluble in ethyl acetate, acetone and water, insoluble in chloroform, benzene and ethyl ether. It gave positive anisaldehyde-sulfuric acid and 2,4-dinitrophenyl hydrazine reactions and negative ninhydrin, EHRLICH, RYDONSMITH and SAKAGUCHI reactions. On thin-layer chromatography using silica gel G (E. Merck), the inhibitor III-2 gave a single spot at Rf 0.46 with chloroform-methanol (5:1) and 0.68 with chloroform-methanol-water (65:25:4). It did not move in formic acid-acetic acid-water (25:75:900) under 3,500 V electrophoresis for 15 minutes. Inhibitor III-2 was hydrolyzed with 0.5 N HCl-dry methanol at 80°C for 3 hours. Degradation products indicated the presence of one mole of rhamnose and daizein. The structure of the inhibitor III-2 was determined as 7-O-rhamnopyranoside-4′,7-dihydroxyisoflavone. Details of the chemical studies on inhibitor and also general properties of another inhibitors will be reported in the next paper.

The inhibitory activity of inhibitor III-2 against β-galactosidase and other glycosidases are studied in comparison with pyridindolol[4] and siastatin B[3]. The methods employed for testing these activities have been described in previous papers[3,4,6-8]. The results in Table 1 show that the inhibitor III-2 is a specific inhibi-

tor against β-galactosidase. LINEWAVER-BURK plot of the kinetic study showed that the inhibitor III-2 inhibited the reaction in a competitive manner with the substrate. *Ki* is 7.1×10^{-6} M with *p*-nitrophenyl-β-D-galactopyranoside. It does not inhibit various kinds of sialidase. Inhibitors at 100 μg/ml showed no antibacterial and no antifungal activities. They have low toxicity and the intravenous injection of 250 mg/kg to mice caused no deaths.

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