HOKKAIDO UNIVERSITY

Title	Inhibition of β-Glucuronidase by Extracts of Chondria crassicaulis
Author(s)	SEKIKAWA, Chisato; KURIHARA, Hideyuki; GOTO, Keiichiro; TAKAHASHI, Koretaro
Citation	北海道大学水産科学研究彙報, 53(1), 27-30
Issue Date	2002-03
Doc URL	http://hdl.handle.net/2115/21961
Type	bulletin (article)
File Information	53(1)_P27-30.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Bull. Fish. Sci. Hokkaido Univ.
53(1), 27-30, 2002.

Inhibition of β-Glucuronidase by Extracts of *Chondria crassicaulis*

Chisato SEKIKAWA[1], Hideyuki KURIHARA[1], Keiichiro GOTO[1]
and Koretaro TAKAHASHI[1]

Abstract

Methanol extracts of the red alga *Chondria crassicaulis* inhibited *Escherichia coli* β-glucuronidase. Five inhibitory fractions obtained from the extracts showed inhibitory potency similar to that of D-glucaro-1,4-lactone, a known β-glucuronidase inhibitor. Inhibitory components of the fractions were not isolated, but the different inhibition mechanisms of the fractions indicate that the inhibitory components differ from each other.

Key words: β-glucuronidase, Inhibition, *Chondria crassicaulis*

Introduction

Beta-glucuronidase, widely distributed in animals, plants and bacteria, catalyzes hydrolysis of β-glucuronides (Levvy and Conchie, 1966). Beta-glucuronides are final metabolites of hydrophobic xenobiotics (Kato and Kamataki, 1996). Because β-glucuronides are excreted via urine and feces, they play important roles in excretion of xenobiotics. In animals, low β-glucuronidase activity is maintained through localization in lysosomes (Yasuoka, 1982). However, β-glucuronidase produced by intestinal bacteria hydrolyzes glucuronide to liberate xenobiotics. The xenobiotics liberated exhibit toxicity in the intestine and decrease excretion rate of xenobiotic via reabsorption. Inhibition of bacterial β-glucuronidase in the intestine will promote excretion of xenobiotics and thus decrease their toxicity.

Several β-glucuronidase inhibitors have been isolated from nature. The inhibitors are categorized as terpenoids and their glucuronides (Nawaz et al., 2000; Shim et al., 2000; Kim et al., 1996; Hayashi et al., 1992), flavonoids and their glucuronides (Kim et al., 1994; Narita et al., 1993), and pseudo-sugar containing nitrogen (Cenci di Bello et al., 1984). Crude drug containing β-glucuronidase-inhibitory activity decreased toxic side effects of the metabolite SN-38 of the anticancer agent CPT-11 (Narita et al., 1993). This finding supports the idea that β-glucuronidase inhibitor decreases toxicity of xenobiotics. In our search for β-glucuronidase inhibitors, we found that extracts of the red alga *Chondria crassicaulis* showed inhibitory activity. No secondary metabolites have been obtained from *C. crassicaulis* except for arsenic compounds (Edmonds et al., 1997). In the present study, we obtained five inhibitory fractions against β-glucuronidase from the extracts of *C. crassicaulis* and elucidated inhibitory mechanisms of those fractions.

Materials and Methods

General

High-performance liquid chromatography (HPLC) was carried out with a SHIMADZU LC-10AT$_{VP}$ chromatograph equipped with a SHIMADZU SPD-10A$_{VP}$ ultra violet (UV) detector and a SHIMADZU RID-10A refractive index (RI) detector. Liquid chromatography-mass spectroscopy (LC-MS) was performed with a Tosoh CCPS chromatograph-JEOL JMX-SX102A mass spectrometer.

Assay of β-glucuronidase activity

The reaction mixture (total volume of 2.5 ml) consisted of 10 mM phosphate buffer (pH 7.0), 0.4 mM *p*-nitrophenyl β-D-glucuronide (Nacalai Tesque), test material in 4% methanol, and 2.0 Fishman unit/ml *Escherichia coli* β-glucuronidase (Sigma Chemical). One Fishman unit liberates 1.0 μg of phenolphthalein from phenolphthalein glucuronide for 60 min at 37°C and pH 6.8. Methanol did not affect enzyme activity under this condition. The reaction was carried out at 37°C for 7 min. The reaction was terminated by adding 0.25 M Na_2CO_3 (1.5 ml), and then the absorbance of the resulting solution was measured at 400 nm.

Extraction and isolation

C. crassicaulis (5.94 kg air-dried weight), collected at the seashore of Hakodate in southern Hokkaido in 2000,

[1] *Laboratory of Biochemistry and Biotechnology, Graduate School of Fisheries Sciences, Hokkaido University* (北海道大学大学院水産科学研究科応用生物科学講座)

were cut into small pieces and extracted twice with methanol under ambient temperature for 10 days. With the guidance of inhibitory activity against β-glucuronidase activity, the combined extract (115.5 g) was separated into ethyl acetate-soluble fraction (2.57 g) with a series of solvent partitioning. The fraction was separated by reversed-phase (RP) column chromatography (Cosmosil 140 C_{18}-OPN, Nacalai Tesque, 6.0×44.0 cm, eluted with methanol : water=3 : 7-methanol : water=1 : 9), to give fraction I (611.0 mg), followed by Sephadex LH-20 column chromatography (Pharmacia, 1.8×50.0 cm, eluted with chloroform : methanol=1 : 1) to afford three active fractions, fraction II (550.9 mg), fraction III (9.0 mg) and fraction E (3.4 mg). Fraction III was divided into fraction D (1.9 mg) by RP HPLC [column, ULTRON VX-ODS (5 μm) 4.6×250 mm, Shinwa Chemical Industries, LTD.; mobile phase, methanol : water=65 : 35; detection, UV at 210 nm]. Fraction II was separated by silica gel column chromatography (Wakogel C-100, Wako Pure Chemicals, 1.8×50.0 cm, eluted with chloroform : acetone=19 : 1-chloroform : acetone=85 : 15), to give fraction IV (87.9 mg), followed by RP HPLC [column, ULTRON VX-ODS (5 μm) 20.0×250 mm, Shinwa Chemical Industries, LTD.; mobile phase, acetonitrile : 0.1% phosphoric acid : tetrahydrofuran=88 : 10 : 2; detection, RI] to obtain fraction V (32.7 mg). Fraction V was fractionated by silica gel HPLC [column, ULTRON VX-SIL (5 μm) 4.6×250 mm, Shinwa Chemical Industries, LTD.; mobile phase, chloroform : methanol=19 : 1; detection, RI], to give fraction VI (11.2 mg), followed by silica gel HPLC [column, ULTRON VX-SIL (5 μm) 4.6×250 mm, Shinwa Chemical Industries, LTD.; mobile phase, chloroform : 2-propanol=97.5 : 2.5; detection, RI] to afford fraction VII (8.3 mg). Fraction VII was subjected to silica gel preparative thin layer chromatography (TLC) (Kieselgel 60 F_{254}, E. Merck, developed with chloroform : methanol=85 : 15), to give two active fractions, fraction VIII (2.5 mg) and fraction C (2.0 mg). Fraction VIII was further separated by RP HPLC [column, ULTRON VX-ODS (5 μm) 4.6×250 mm, Shinwa Chemical Industries, LTD.; mobile phase, methanol : water=9 : 1; detection, UV at 210 nm], to obtain fractions A (0.4 mg) and B (0.8 mg).

LC-MS analysis

Fractions A, B and C were analyzed by LC-MS. The LC was carried out under the following conditions : column, Develosil ODS-5 (1.5×150 mm, Nomura Chemical); detection, UV at 210 nm; mobile phase, methanol : water=9 : 1; flow rate, 0.075 ml/min; temperature, ambient. The ionization method of the MS was electrospray ionization (ESI).

Results and Discussion

Separation and LC-MS analysis of the inhibitory fractions

We were unable to isolate each inhibitor because they became unstable during purification. All the fractions obtained showed several spots on TLC (RP-18 F_{254s}, E. Merck, developed with methanol : water=9 : 1 for fractions A and B, developed with methanol : water=65 :

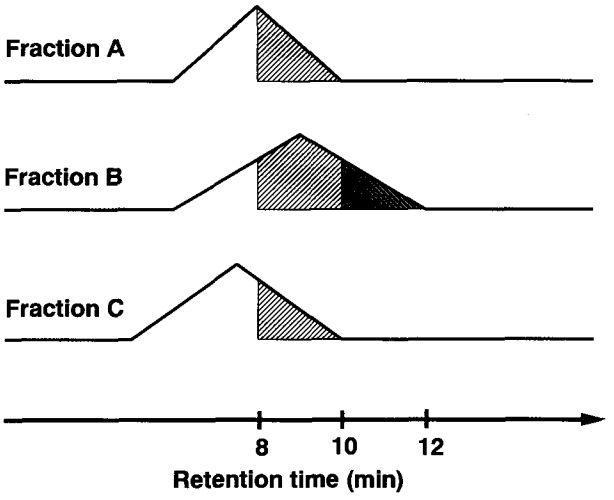

Fig. 1. Schematic total ion chromatograms of fractions A, B and C in LC-MS analysis.
Shaded parts indicate the peaks observed in mass chromatograms (MC) : ▨, observed in MC for m/z 294; ■, observed in MC for m/z 441.

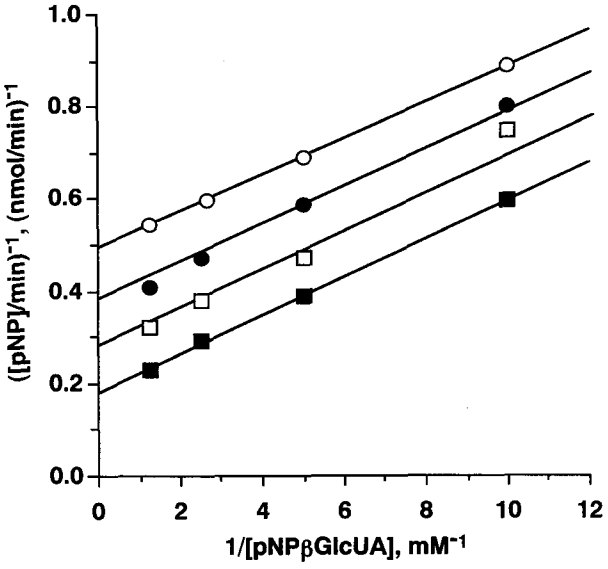

Fig. 2. Lineweaver-Burk plot of fraction B.
pNPβGlcUA, *p*-nitrophenyl β-D-glucuronide; pNP, *p*-nitrophenol.
■, [B] 0 μg/ml; □, [B] 5 μg/ml; ●, [B] 10 μg/ml; ○, [B] 20 μg/ml.

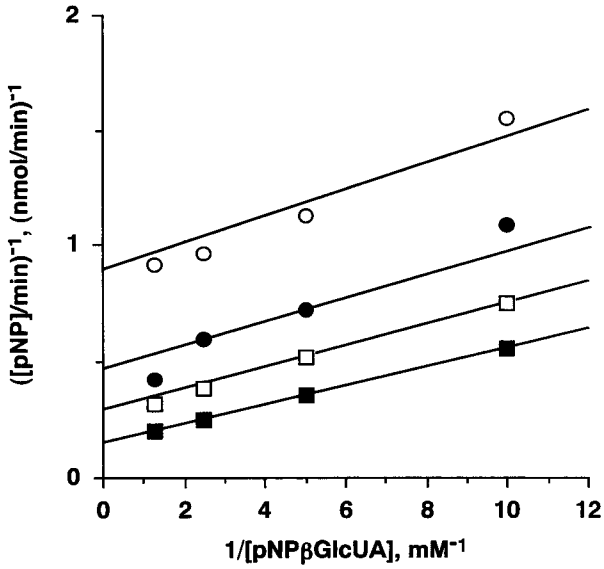

Fig. 3. Lineweaver-Burk plot of fraction C.
pNPβGlcUA, *p*-nitrophenyl β-D-glucuronide; pNP, *p*-nitrophenol.
■, [C] 0 μg/ml; □, [C] 5 μg/ml; ●, [C] 10 μg/ml; ○, [C] 20 μg/ml.

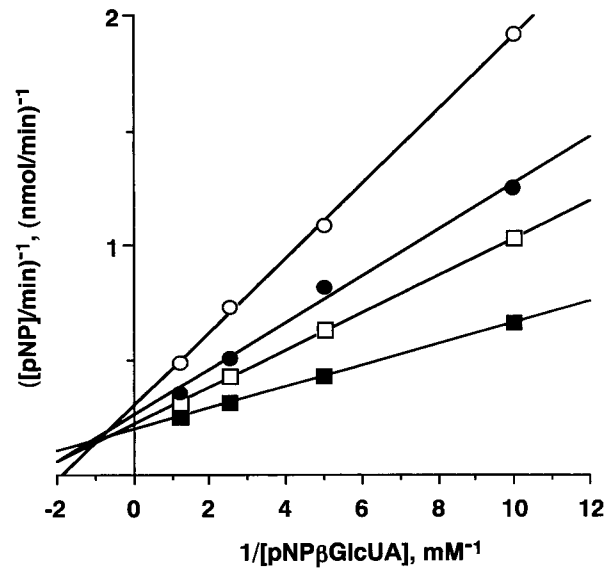

Fig. 4. Lineweaver-Burk plot of fraction D.
pNPβGlcUA, p-nitrophenyl β-D-glucuronide; pNP, *p*-nitrophenol.
■, [D] 0 μg/ml; □, [D] 6 μg/ml; ●, [D] 12 μg/ml; ○, [D] 24 μg/ml.

35 for fraction D, Kieselgel 60 F_{254}, E. Merck, developed with chloroform : methanol = 85 : 15 for fractions C and E). We attempted to identify components in fractions A, B and C as mixtures by LC-MS analysis. Fractions A, B and C contained a same component (component-294), showing the same retention time and the same ion peak at *m/z* 294 in LC-MS analysis (Fig. 1). Fraction B contained another component which showed longer retention time than the component-294 and an ion peak

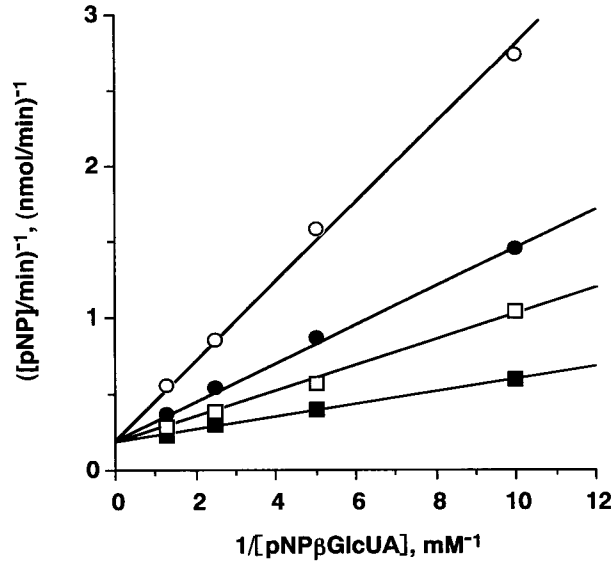

Fig. 5. Lineweaver-Burk plot of fraction E.
pNPβGlcUA, *p*-nitrophenyl β-D-glucuronide; pNP, *p*-nitrophenol.
■, [E] 0 μg/ml; □, [E] 6 μg/ml; ●, [E] 12 μg/ml; ○, [E] 24 μg/ml.

Table 1. Inhibitory activity of the fractions obtained from *C. crassicaulis* against *E. coli* β-glucuronidase

Fraction	IC_{50}[a](μg/ml)	Inhibitory activity[b](%)
A		40.8
B	18.0	
C	7.26	
D	18.9	
E	13.8	
D-Glucaro-1,4-lactone[c]	10.5	53.6

[a] Concentration of substrate was 0.4 mM.
[b] Concentration of the test compounds was 12.5 μg/ml.
[c] A known inhibitor.

at *m/z* 441.

Inhibitory activity and inhibition mechanisms of the fractions against E. coli β-glucuronidase

Five fractions obtained from *C. crassicaulis* showed comparable inhibitory potency against *E. coli* β-glucuronidase to that of D-glucaro-1,4-lactone, which is a typical β-glucuronidase inhibitor (Table 1). However, Lineweaver-Burk plot analysis showed that the individual fraction had different inhibition mechanisms. Fractions B and C showed uncompetitive inhibition (Figs. 2 and 3). Fraction D showed mixed inhibition (Fig. 4). Fraction E showed competitive inhibition (Fig. 5). There was an insufficient amount of fraction A for testing. LC/MS analysis showed that both fractions B

and C contained component-294, and both fractions showed uncompetitive inhibition. These results suggested that component-294 will show uncompetitive inhibition. The other fractions showed different inhibition mechanisms. Therefore, this indicates that the components in the other fractions were different from component-294.

In this study, inhibitory activity against *E. coli* β-glucuronidase was found in extracts of *C. crassicaulis*. The alga *C. crassicaulis* is edible in some regions of Japan, and may be used as functional food that reduces toxicity of xenobiotics. It is not clear why the inhibitors showed different inhibition mechanisms. Isolation and identification of the individual components will be carried out.

Acknowledgment

We are grateful to Mr. Kenji Watanabe of the GC-MS and NMR Laboratory, Graduate School of Agriculture, Hokkaido University, for LC-MS measurement.

References

Cenci di Bello, I., Dorling, P., Fellows, L. and Winchester, B. (1984) Specific inhibition of human β-D-glucuronidase and α-L-iduronidase by a trihydroxy pipecolic acid of plant origin. *FEBS Lett.*, **176**, 61-64.

Edmonds, J.S., Shibata, Y., Yang, F. and Morita, M. (1997) Isolation and synthesis of 1-deoxy-1-dimethylarsinoylribitol-5-sulfate, a natural constituent of *Chondria crassicaulis* and other red algae. *Tetrahedron Lett.*, **38**, 5819-5820.

Hayashi, T., Kawasaki, M., Okamura, K., Tamada, Y. and Morita, N. (1992) Scoparic acid A, a β-glucuronidase inhibitor from *Scoparia dulcis*. *J. Nat. Prod.*, **55**, 1748-1755.

Kato, R. and Kamataki, T. (1996) *Drug Metabolism* (in Japanese). Tokyo Kagaku Dojin, Tokyo.

Kim, D.-H., Shim, S.-B., Kim, N.-J. and Jang, I.-S. (1999) β-glucuronidase inhibitory activity and hepatoprotective effect of *Ganoderma lucidum*. *Biol. Pharm. Bull.*, **22**, 162-164.

Kim, D.-H., Jin, Y.-H., Park, J.-B. and Kobashi, K. (1994) Silymarin and its components are inhibitors of β-glucuronidase. *Biol. Pharm. Bull.*, **17**, 443-445.

Levvy, G.A. and Conchie, J. (1966) β-glucuronidase and the hydrolysis of glucuronides. p. 301-364, Dutton, G.J. (ed) *Glucuronic acid*. Academic Press, New York.

Narita, M., Nagai, E., Hagiwara, H., Aburada, M., Yokoi, T. and Kamataki, T. (1993) Inhibition of β-glucuronidase by natural glucuronides of *Kampo* medicines using glucuronide of SN-38 (7-ethyl-10-hydroxycamptothecin) as a substrate. *Xenobiotica*, **23**, 5-10.

Nawaz, H.R., Malik, A., Khan, P.M., Shujaat, S. and Rahman, A.-u. (2000) A novel β-glucuronidase inhibiting triterpenoid from *Paeonia emodi*. *Chem. Pharm. Bull.*, **48**, 1771-1773.

Shim, S.-B., Kim, N.-J. and Kim, D.-H. (2000) β-glucuronidase inhibitory activity and hepatoprotective effect of 18β-glycyrrhetinic acid from the rhizomes of *Glycyrrhiza uralensis*. *Planta Medica*, **66**, 40-43.

Yasuoka, S. (1982) β-glucuronidase (in Japanese). *Rinsho Kensa Mook*, (**11**), 183-191.