Chemistry of Natural Compounds, Vol. 47, No. 2, May, 2011 [*Russian original No. 2, March-April, 2011*]

DEGLYCOSYLATION OF ISOFLAVONOID GLYCOSIDES FROM *Maackia amurensis* CELL CULTURE BY β-D-GLUCOSIDASE FROM *Littorina sitkana* HEPATOPANCREASE

M. I. Kusaikin,* A. M. Zakharenko, S. P. Ermakova, M. V. Veselova, E. V. Grigoruk, S. A. Fedoreev, and T. N. Zvyagintseva

UDC 577.151.45

Maakia amurensis *(strain A-18) cell culture synthesizes a significant quantity of isoflavonoids, a large part of which consists of isoflavone glucosides and malonylglucosides.* β-*D-Hydrolase enzyme complexes from the marine mollusk* Littorina sitkana *and the marine mycelial fungus* P. canescens *were used to obtain isoflavones from their conjugated forms. The specificity of* β-*D-glucanases from* L. sitkana *for various glycosides was studied. The deglycosylation efficiency depended on the aglycon structure. The deglycosylated fraction of isoflavonoids obtained from* M. amurensis *cell culture exhibited antitumor activity.*

Keywords: β-glucosidase, isoflavonoids, *Maackia amurensis*, *Littorina sitkana*, antitumor activity.

Isoflavonoids are an important class of secondary metabolites, the principal structure of which is the 3-phenylchromone moiety. Isoflavonoids are characterized by carbohydrate substituents on C-7 and C-4′. Substituents in other positions are encountered much more rarely [1, 2].

Isoflavonoids occur in plants, for example, in soy, mainly as glucosides and their conjugates whereas isoflavonoid algycons are used in most pharmacological investigations. This is explained by the more pronounced biological activity of the aglycons than the glycosides for treating estrogen-dependent diseases [2, 3].

The aglycons can be produced by both acidic and enzymatic hydrolysis of the isoflavonoid glycosides. Acid hydrolysis or sequential alkaline–acidic hydrolysis is used for total analysis of isoflavonoids in plant sources and biological additives [1]. Enzymatic hydrolysis has indisputable advantages over acidic hydrolysis. All enzymatic reactions occur at physiological temperatures and pH values. Under such conditions modification of the aglycons is practically excluded and the yield of the main product approaches 100%.

Multi-year studies by researchers at PIBOC, FEB, RAS, on the pharmacological activities of the polyphenol complex from *Maackia amurensis* wood resulted in the pharmacopoeial drug Maksar, which is registered as a hepatoprotective agent. Cell cultures of *M. amurensis* were grown at the BSI, FEB, RAS, in order to obtain an industrial source of polyphenols. A cell culture of *M. amurensis* (strain A-18) that was obtained from seed sprouts synthesized a significant amount of isoflavonoids, the composition of which differed from polyphenols isolated earlier from *M. amurensis* plant. Investigations showed that a large part of the polyphenols in strain A-18 was present as isoflavonoid glucosides and malonylglucosides [4].

Both pure glycosides (Table 1) and a fraction of isoflavonoid glycosides obtained from *M. amurensis* cell culture were used as potential substrates in order to find enzymes capable of catalyzing the hydrolysis of isoflavonoid glycosides. The fraction contained four glucosides and three malonylglucosides of isoflavonoids.

β-D-Hydrolase enzyme complexes from the marine mollusk *Littorina sitkana* and the marine mycelial fungus *P. canescens*, which contain β-D-glycosidases and 1,3-β-D-glucanases, certain properties and the optimal activity conditions of which were studied before [5], were used to obtain the isoflavonoid aglycons.

Use of the pure compounds as substrates showed that the enzyme complexes from *L. sitkana* and *P. canescens* were capable of cleaving the *O*-β-D-glycoside bond in glucosides of various structures. The glucoside hydrolysis efficiency was practically the same for both complexes and depended slightly on the structures of the aglycon and carbohydrate (Table 1).

Pacific Institute of Bioorganic Chemistry, Far-East Branch, Russian Academy of Sciences, 690022, Vladivostok, fax: (4232) 31 40 50, e-mail: mik@piboc.dvo.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 182–185, March–April, 2011. Original article submitted October 26, 2010.

0009-3130/11/4702-0197

TABLE 1. Hydrolysis of Isoflavonoids by Enzyme Complexes and Preparations. Accumulation (% of Theoretically Possible) of Isoflavonoid Aglycons from Their Glucosides and Malonylglucosides

Compound	Enzyme complex, %		*L. sitkana*, %	
	L. sitkana	*P. canescens*	enzyme preparation G-I	enzyme preparation G-II
1	100	100	100	100
2	100	100	100	100
3	99.63	99.47	92.38	76.78
4	8.02	100	44.00	27.40
5	90.66	100	100	100
6	38.88	52.68	100	10.55
7	100	100	100	100

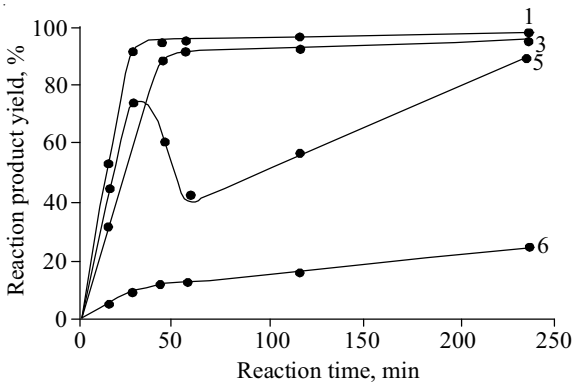

Fig. 1. Effect of β-D-glucosidase G-II from *L. sitkana* on isoflavonoid glycosides. Accumulation (% of theoretically possible) of daidzein from daidzin (**1**), genistein from genistin (**3**), formononetin from ononin (**5**) and from 6″-*O*-malonylononin (**6**).

For example, the degree of hydrolysis of genistin (**3**) was less than that of daidzin (**1**). Modification of the carbohydrate by introducing a malonyl group into the glucose C-6 position decreased noticeably the degree of enzymatic hydrolysis of modified genistin and ononin. The hydrolysis efficiency of these substrates was reduced significantly if the enzyme complex from *L. sitkana* and pure *O*-glycoside hydrolases G-I and G-II that were isolated from it were used (Table 1).

Enzyme preparations obtained from the marine mollusk *L. sitkana*, *endo*-laminarinase G-I (EC 3.2.1.39) and β-D-glucosidase G-II (EC 3.2.1.21), which can participate in cleavage of the glycoside bond, were selected for further experiments. The selection was based on the knowledge of their catalytic and molecular properties in addition to the ability to obtain these enzymes in a homogeneous state [5]. Both enzymes are capable of catalyzing cleavage of isoflavonoid glycosides to form the aglycons (Table 1). The efficiencies of both enzymes for hydrolysis of the glycosides as functions of the aglycon structure were practically identical. The enzymes also catalyzed cleavage of the glycoside bond with C-6 hydroxyl substitution in glucose by malonate (**4**, **6**, and **7**). However, the enzymes were much less active toward these substrates than toward the unsubstituted compounds (**3** and **5**, Table 1).

A comparison of the activity of the enzyme complexes obtained from the marine sources on the total isoflavonoid glycoside fraction showed that both selected sources contained enzymes that catalyzed efficiently cleavage of the isoflavonoid glycosides to form the aglycons (Table 1).

β-D-Glucosidase G-II, which is most sensitive to structural features of isoflavonoid glycosides, was selected for a detailed study of the influence of structural features of the isoflavonoid glycosides on the rate of the enzyme reaction (Table 1). Pure preparations of daidzin (**1**), genistin (**3**), ononin (**5**), and 6″-*O*-malonylononin (**6**) were used as substrates. The first three compounds differed in the aglycon structure; the last, the structure of the carbohydrate, which had a malonic acid substituent on the glucose C-6 atom.

Figure 1 shows that the rates of hydrolysis of the isoflavonoid glucosides by G-II enzyme were different. The hydrolysis rate was fastest for **1**; slower by 1.7 times, for **3**. The initial hydrolysis rate for **5** was less than that of **1** by 1.25 times; for **6**, almost 10 times.

Thus, the aglycon structure had a substantial influence on the hydrolysis rate of the isoflavonoid glycosides by β-D-glucosidase G-II. An electron-donating substituent in the 5-position of the aglycon reduced the deglycosylation rate by almost two times. Replacement of the hydroxyl by methoxyl in the 4″-position had an insignificant influence of the reduction of the hydrolysis rate in the initial stages. However, the hydrolysis curve for ononin (**5**) had an unusual shape (Fig. 1). The hydrolysis probably occurred in several stages. The first stage had the fastest reaction rate. Then, the accumulation of formononetin (**11**) stopped. The amount of it in the incubation medium even decreased slightly. Then, it began to increase again.

The following scenarios explaining such enzyme activity can be envisioned. The decreased hydrolysis rate of ononin (**5**) is probably due to inhibition of enzyme G-II by the hydrolysis reaction product formononetin (**11**). The decrease in the concentration of **11** during the reaction may be due to an acceleration of the reverse reaction rate, the synthesis of the starting glycoside, in a certain stage.

We checked both these hypotheses experimentally and found **11** did not inhibit (up to a concentration of 1.25 mg/mL) the hydrolysis of a synthetic substrate, *p*-nitrophenyl-β-D-glucopyranoside, by G-II. It was also established that β-D-glucosidase G-II was not capable of synthesizing ononin (**5**) from *p*-nitrophenyl-β-D-glucopyranoside, which was used as a glucose and formononetin (**11**) donor. Therefore, the question about the reasons for the unusual behavior of β-D-glucosidase G-II in the hydrolysis of **5** remains open.

We showed earlier that Amur Maackia Dry Extract prepared from *M. amurensis* exhibits antitumor activity. It seemed interesting to study the antitumor activity of the isoflavonoid fraction obtained from *M. amurensis* cell culture and the aglycons obtained from its enzymatic hydrolysis. Glycoside, isoflavonoid, and aglycon fractions obtained from it by the action of β-D-glucosidase G-II did not exhibit cytotoxicity at concentrations up to 28 μg/mL. Also, both fractions did not affect the growth of cells at concentrations up to 28 μg/mL over 72 h.

The ability of the glycosides and the aglycons obtained from them to inhibit the growth of human breast cancer cells MDA-MB-231 was determined using the soft agar method that is based on the fact that cancer cells that are not fixed in the agar layer allow cell colonies to grow whereas compounds with potential antitumor activity inhibit the growth of cell colonies. The experiment was carried out using MDA-MB-231 cells as before [6] with certain modifications. All compounds were dissolved in DMSO. DMSO was used as a control for treating cells. Cells (2.4×10^4 cells/mL) were treated with the test compounds at a concentration of 28 μg/mL per milliliter of BME (Basal Medium Eagle) agar (0.33%) over BME agar (0.5%, 3.5 mL) containing extracts of the compounds at a concentration of 28 μg/mL. Cells were cultivated at 37°C and 5% CO_2 for 21 d. Cell colonies were estimated using an inverted microscope (Motic AE20, China) and Motic Images Plus software. Two independent experiments with three samples at each concentration were used for each compound.

Aglycon fractions at a concentration of 28 μg/mL inhibited growth of MDA-MB-231 human breast cancer cell colonies by 60% compared with the control whereas the glycoside fraction at this concentration did not inhibit the growth of their colonies. These results indicated that the aglycon fraction had antitumor activity against MDA-MB-231 human breast cancer cells.

EXPERIMENTAL

Analytical Methods. Reducing sugars were determined by the Nelson method [7]; protein content in preparations, by the Lowry method [8].

Substrates. *p*-Nitrophenyl-β-D-glucopyranoside was purchased from Sigma (USA). Pure isoflavonoid glucosides were prepared by the literature method [4].

Preparation of Isoflavonoid Glucoside Fraction. We used strain A-18 of *M. amurensis* cell culture. Dried cell culture was extracted three times with $CHCl_3$:EtOH (3:1) over 2 h. The combined extracts were lyophilized and separated preparatively over a column of Toyopearl 50F (3 × 40 cm) with elution by a linear gradient of H_2O:EtOH (containing 0.04% HCOOH). The fraction eluted by 20% aqueous EtOH contained only isoflavonoid glucosides. The composition of the fraction was determined using HPLC.

Enzyme complexes of β-D-hydrolases from *L. sitkana* and *P. canescens* in addition to β-D-glucosidases from *L. sitkana* were obtained by the literature method [5].

Determination of Enzyme Activity. The β-glucosidase activity was determined from the amount of *p*-nitrophenol released by the action of the enzyme on *p*-nitrophenyl-β-D-glucopyranoside [5]. The activity unit was taken as the amount of enzyme that catalyzed the formation of 1 nmol of product (*p*-nitrophenol) during 1 h under the determination conditions.

Products from Enzymatic Hydrolysis of Isoflavonoid Glucosides. A weighed portion (4 mg) of isoflavonoid glycosides (4 mg) was dissolved in sodium succinate buffer (0.025 M, pH 5.4), treated with β-D-glucosidase (2×10^{-2} U), and

incubated for 18 h at 37°C. The reaction was stopped by adding CH_3CN. Then the mixture was lyophilized and analyzed by HPLC.

Analytical HPLC was performed on an Agilent Technologies chromatograph (Series 1100) equipped with a QuatPump G1311A high-pressure pump system, G1322A Degasser, G1328B injector, and G1314A VWD detector. The analysis results were processed using ChemStation® software ver. 09 (Germany). We used the literature method [4] and dihydroquercetin (DHQ) as an internal standard.

Inhibition of β-D-Glucosidase G-II by Formononetin. Enzyme solution (20 μL, 10^{-3} U) in sodium succinate buffer (0.025 M, pH 5.2) was treated with inhibitor solution (10 μL) containing from 0.5 to 200 μg of compound in sodium succinate buffer (0.025 M, pH 5.2). The mixture was held at 37°C for 10 min, treated with *p*-nitrophenyl-β-D-glucopyranoside (200 μL, 1 mg/mL) in sodium succinate buffer (pH 5.2), and incubated at 37°C for 30 min. The residual activity was determined.

Enzymatic Synthesis of Glucosides by β-D-Glucosidase G-II. The appropriate aglycon (1 mg) and *p*-nitrophenyl-β-D-glucopyranoside (5 mg) were dissolved in enzyme solution (1 mL, 5×10^{-5} U) and incubated at 37°C for 20, 40, and 120 min and 24 h. Production of the glycosides was monitored by HPLC.

Cell Cultivation. HCT-116 human colon cancer cells (ATCC No. CCL-247) and MDA-MB-231 human breast cancer cells (ATCC No. HTB-26) were cultivated in McCoy 5a and DMEM (Dulbecco's modified Eagle's medium), respectively, and 10% phosphate buffer (FBS) with added penicillin (100 U/L) and streptomycin (100 μg/L) in an MCO-18AIC incubator (Sanyo, Japan) at 37°C with 5% CO_2 content.

Determination of Isoflavonoid Cytotoxicity. HCT-116 and MDA-MB-231 cells (1.5×10^5/mL) were inoculated into 96-well plates and cultivated in 10% McCoy 5a and DMEM (200 μL), respectively, in a CO_2 incubator at 37°C for 24 h. Then, cells were treated with isoflavonoids at concentrations of 3, 5, 7, 14, and 28 μg/mL in fresh medium and incubated for 24 h. Each well was treated after incubation with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS reagent) (15 μL) and incubated for 4 h (37°C, 5% CO_2). The optical density was measured on a Bio-Tek Instruments spectrophotometer (USA) at wavelength 490/630 nm ($A_{490/630}$).

Neoplastic Cell Transformation (Soft Agar Method). The effect of the isoflavonoids on the formation and growth of colonies of MDA-MB-231 human breast cancer cells was determined by the soft agar method [6]. MDA-MB-231 cells (2.4×10^4/mL) were treated with isoflavonoids (28 μg/mL) in agar (1 mL, 0.33%) containing BME (1.8%), containing 10% over agar (3.5 mL, 0.5%) with BME (1.8%), and containing FBS (10%) and isoflavonoid (28 μg/mL). Controls were treated with the corresponding amount of DMSO. Cells were cultivated at 37°C and 5% CO_2 for 21 d. Human colon cancer cell colonies were estimated using a Motic AE20 inverted microscope (China) and Motic Images Plus software.

Statistical data treatment was carried out using the Student t-criterion with 95% probability limits (SigmaPlot 2000, version 6, SPSS Inc., USA).

ACKNOWLEDGMENT

The work was supported by RFBR grant 09-04-00761, RAS Presidium Program Molecular and Cellular Biology, and Presidential Grant MK 1472.2009.4.

REFERENCES

1. P. Delmonte and J. I. Rader, *J. AOAC Int.*, **4**, 89, 1 (2006).
2. A. J. Day, M. S. DuPont, S. Ridley, M. Rhodes, M. J. C. Rhodes, M. R. A. Morgan, and G. Williamson, *FEBS Lett.*, **436**, 71 (1998).
3. A. J. Day, F. J. Canada, J. C. Diaz, P. A. Kroon, R. Mclauchlan, C. B. Faulds, G. W. Plumb, R. A. Morgan, and G. Williamson, *FEBS Lett.*, **468**, 166 (2000).
4. S. A. Fedoreyev, V. P. Bulgakov, O. V. Grishchenko, M. V. Veselova, O. E. Krivoschekova, N. I. Kulesh, V. A. Denisenko, G. K. Tchernoded, and Yu. N. Zhuravlev, *J. Agric. Food Chem.*, **56**, 16, 7023 (2008).
5. M. S. Pesentseva, M. I. Kusaykin, S. D. Anastyuk, V. V. Sova, and T. N. Zvyagintseva, *Carbohydr. Res.*, **343**, 2393 (2008).
6. N. H. Colbrun, E. J. Wendel, and G. Abruzzo, *Proc. Natl. Acad. Sci. USA*, **78**, 11, 6912 (1981).
7. T. E. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 1, 265 (1951).