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Aryl Sulfatase of Unusual Specificity from the Liver of Marine Mollusk *Littorina kurila*

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Abstract—An aryl sulfatase of unusual specificity has been isolated from the liver of marine mollusk *Littorina kurila*. It hydrolyzes *p*-nitrophenyl sulfate, does not affect the natural fucoidan, and catalyzes splitting off the sulfate group in position C4 of xylose residues within the carbohydrate chains of holostane triterpene glycosides from sea cucumbers. The properties of the enzyme were studied at pH 5.4. The protein is homogeneous according to electrophoresis and has M 45 ± 1 kDa. The semiinactivation time of the enzyme at 60°C is 20 min, and its K_m value for the hydrolysis of *p*-nitrophenyl sulfate is 8.7 ± 1 mM. It was shown that natural sulfated polyhydroxysteroids inhibit activity of the sulfatase; their I_{50} values depend on their structures and are within the range from 10^{-3} to 10^{-5} M.

Key words: aryl sulfatase, specificity, inhibition; marine mollusk Littorina kurila; sulfated polyhydroxysteroids, triterpene glycosides

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INTRODUCTION

Sulfatases (EC 3.1.6) form a large class of enzymes that catalyze the hydrolytic splitting off of sulfate from various substrates. They are found in both prokaryotes [1, 2] and eukaryotes: invertebrates [3–5] and mammals [6–8]. Bacterial sulfatases predominantly split off sulfate from synthetic substrates, such as 4-nitrophenyl sulfate and 4-nitrocatechol sulfate [9, 10]. Mammalian sulfatases are involved in transformations of mucopolysaccharides, sulfolipids, and sulfosteroids [6–8]. In invertebrates, arylsulfatases, steroidsulfatases, and glycosulfatases of different specificity have been identified, which affect either sugar sulfates or sulfated polysaccharides [3–5].

Comparative studies of structures of the sulfatases showed that they are evolutionarily conservative enzymes [11]. The active site of sulfatases contains an unusual C^{α}-formylglycine residue, which is formed during the posttranslational modification from cysteine in eukaryotic enzymes and from serine in prokaryotic enzymes [11, 12]. A structural similarity between the known aryl sulfatases and phosphatases was reported. It was hypothesized that these enzyme groups are evolutionarily related [13, 14].

Some authors believe that the existing nomenclature of sulfatases does not correspond to their specificity [15]. For example, none of shellfish and mammalian steroid sulfatases could be obtained thus far in the state free from an aryl sulfatase activity, whereas the homogeneous aryl sulfatases exhibited traces of the steroid sulfatase activity [16]. Many naturally occurring sulfated sugars appear to be substrates for aryl sulfatases but not for glycosulfatases, etc.

At present, the attention of researchers is focused on sulfatases that can simplify the structure of sulfated polysaccharides and thereby facilitate the structural analysis of these complex molecules. Reports on polysaccharide sulfatases and, in particular, fucoidan sulfatases are practically absent. It has earlier been shown by Japanese investigators that a partially purified sulfatase preparation from the marine mollusk *Charonia lampas* effectively desulfated only cellulose sulfate and weakly, sulfates of amylose, glycogen, sulfated polysaccharides of algae, sea urchin ovicells, etc. [17]. Later, in some species of marine mollusks collected at shore of England, a sulfatase was identified that exhibited activity toward sulfated carbohydrates of both low and high molecular masses, including fucoidan [3].

We continue to investigate the enzymes from the liver of a Gastropoda mollusk *Littorina kurila*, an easily accessible source of hydrolytic enzymes. The goal of this study is the characterization of properties and specificity of the aryl sulfatase from *L. kurila*.

RESULTS AND DISCUSSION

We previously identified two forms of aryl sulfatase in the liver of *L. kurila* that differ in their molecular masses [18]. In this study, we isolated aryl sulfatase by

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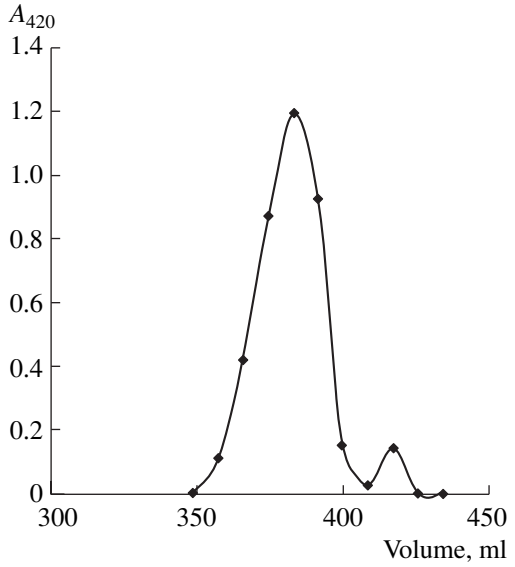


Gel filtration of arylsulfatase on a Sepharose CL 6B column (2.5 × 90 cm) under elution with 0.025 M acetate buffer, pH 5.4; activity monitoring.

a scheme that altered from that described in [18] in that it did involve the stage of hydrophobic chromatography. This modification allowed us to substantially reduce the isolation time and to obtain the most active form of the enzyme by gel filtration on Sepharose CL 6B (figure). The resulting sulfatase was homogeneous by SDS-PAGE and effectively catalyzed the cleavage of *p*-nitrophenyl sulfate.

The properties of enzyme were examined at pH 5.4. The enzyme activity at 50°C was found to retain completely over a period of 20 min at 50°C, whereas at 60°C, the enzyme lost 50% of its activity for the same time. The molecular mass of sulfatase, as indicated by PAGE, was 45 kDa, and the K_m value for *p*-nitrophenyl sulfate was 8.7 ± 1 mM.

The specificity of sulfatase from *L. kurila* was examined using sulfated polyhydroxysteroids of different structure as substrates (Table 1). After incubation with the enzyme, the chromatographic mobility of sulfated polyhydroxy steroids remained unchanged, indicating that no desulfatation of natural substances took place.

It has earlier been shown that sulfated polyhydroxysteroids are the inhibitors of 1⟶3-β-*D*-glycanases from marine mollusks [19, 20]. It was found with the use of natural sulfated polyhydroxysteroids and their synthetic derivatives that the inhibitory effect of these steroids is largely determined by their biophilicity, which is created by polar substituents in the polycyclic nucleus and the presence of hydrophobic side chain. Spectral investigations showed that chalistanol sulfate (a natural sulfated polyoxysteroid, I_{50} 10^{-6} M) induces conformational changes in the 1⟶3-β-*D*-glycanase molecule [21]. It was presumed that sulfated polyhydroxysteroids at the concentration used in the study of the enzyme specificity (50 μg/ml) can inhibit activity of the sulfatase.

The effect of sulfated polyhydroxysteroids on the activity of sulfatase from *L. kurila* was tested using *p*-nitrophenyl sulfate as a substrate. The polyhydroxysteroids studied can be tentatively divided into three structural groups: (A) sulfated polyhydroxysteroids from brittle stars (family Ophiuroidea), (B) sulfated aglycons of asterosaponins, and (C) sulfated glycosides of starfish [not glycosylated compound (4, C)] (Table 1). Compounds 1–5 of group A exert the strongest inhibitory effect on sulfatase ($I_{50} \sim 10^{-5}$ M). Compounds 4 and 5 of group B had the least inhibitory activity ($I_{50} \sim 10^{-3}$ M), and substances of group C exhibited the intermediate values of I_{50} (~10^{-4} M).

Interestingly, the introduction of a hydroxy group into the hydrophobic side chain of compound (5, A) considerably increased the inhibitory activity compared with compound (6, A). Altered positions of substituents and an introduction of a double bond into the steroid nucleus had no effect on the inhibitory capacity of compounds (6, A) and (7, A). An analysis of the data on the inhibitory effect of compounds (1, C) and (3, C) suggests that the sulfatase has affinity for the xylose that has a sulfate at C4, since the inhibitory activity of compound (1, C) is two times higher than that of compound (3, C) that contains a residue of glucose sulfated at C6 in place of xylose residue (Table 1). The desulfatation of holostane triterpene glycosides by the *L. kurila* sulfatase was detected by TLC. The compounds affected by sulfatase had tetra- and pentasaccharide carbohydrate chains in which xylose attached to the triterpene aglycon contained a sulfate group at C4 [22].

Triterpene glycosides also could inhibit the sulfatase with I_{50} values of ~10^{-4} M (Table 2). Therefore, the enzymatic desulfatation of these compounds was carried out in solutions where their concentration was less than I_{50} (<80 μg/ml). Under these conditions, sulfatase desulfated the 4-sulfate group in the xylose residues within the triterpene glycosides of sea cucumbers. The reaction led to the formation of desulfated derivatives of frondoside A, pseudostichoposide A, and cucumarioside G_1 in about 50% yields. The products were isolated by chromatography on a hydrophobic sorbent Polychrom-1 and silica gel. The desulfatation was monitored by TLC, and structures of the products were determined by ^{13}C NMR spectra. It is known that, in ^{13}C NMR spectra, the α- and β-effects of sulfate groups makes the carbon atom of the xylose residue to which the sulfate group is attached to resonate at a weaker field relative to the nonsulfated carbon atoms of this residue, whereas the signals of adjacent carbon atoms are shifted by 2–3 ppm toward a stronger field.

A comparison of ^{13}C NMR spectra of native and sulfatase-treated triterpene glycosides showed the splitting off of the sulfate group, which in native compounds is attached to C4 of the xylose residue (Table 3).

Table 1. Inhibitory effect of polyhydroxysteroids on the activity of sulfatase

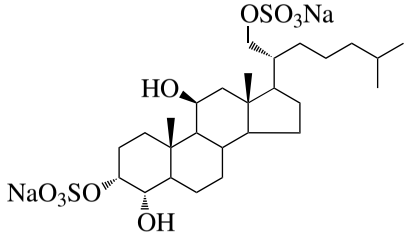
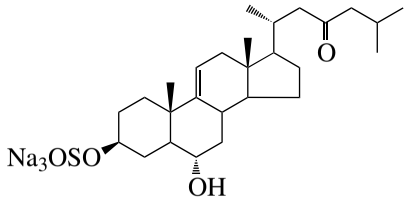
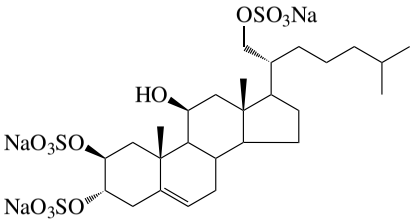
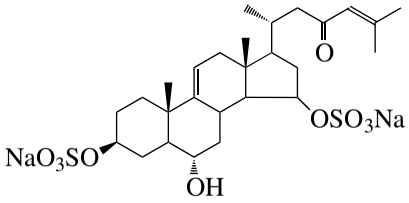
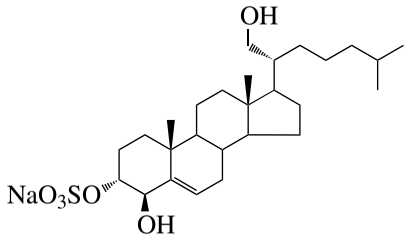
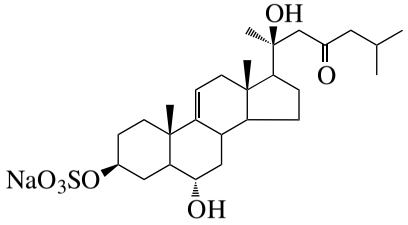
Compound no., group	Compound structure	I_{50}, M	Compound no., group	Compound structure	I_{50}, M
(1, A)		2×10^{-5}	(1, B)		6×10^{-5}
(2, A)		3×10^{-5}	(2, B)		10^{-4}
(3, A)		3×10^{-5}	(3, B)		8×10^{-4}

Table 1. (Contd.)

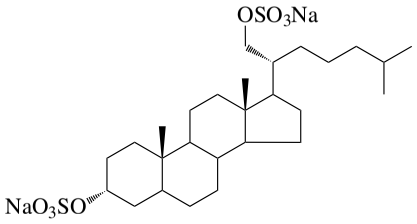
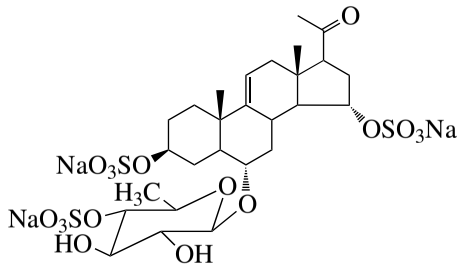
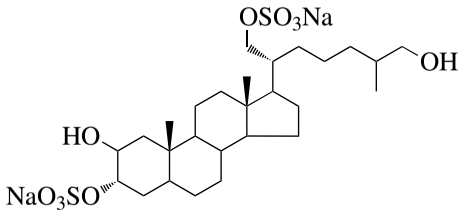
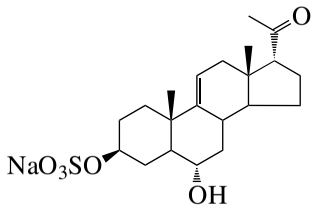
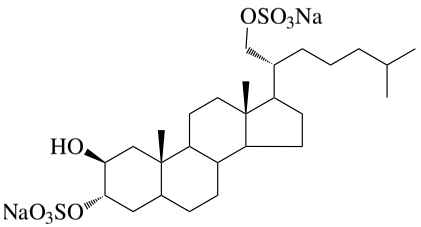
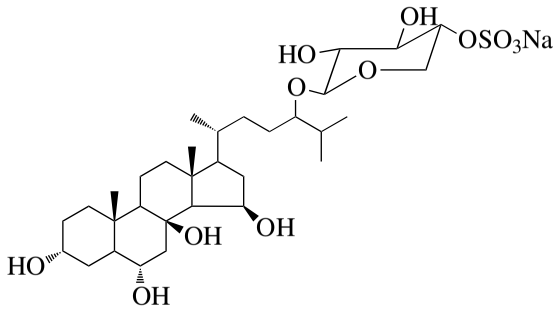
Compound no., group	Compound structure	I_{50}, M	Compound no., group	Compound structure	I_{50}, M
(4, A)		4×10^{-5}	(4, B)		10^{-3}
(5, A)		4×10^{-5}	(5, B)		3×10^{-3}
(6, A)		4×10^{-4}	(1, C)		6×10^{-5}

Table 1. (Contd.)

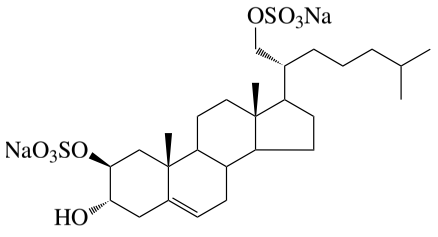
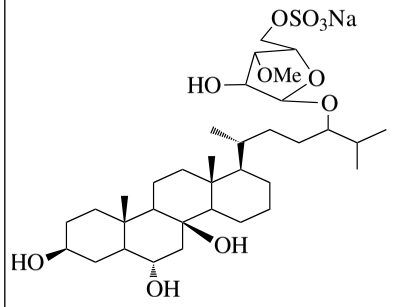
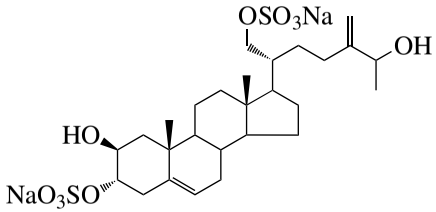
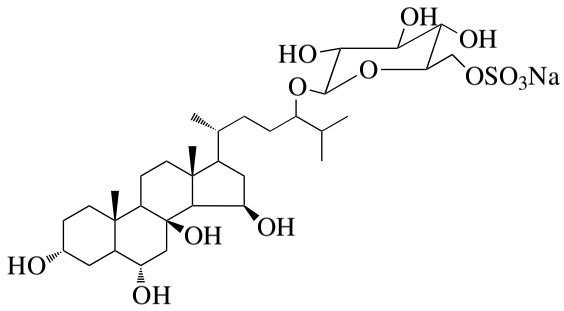
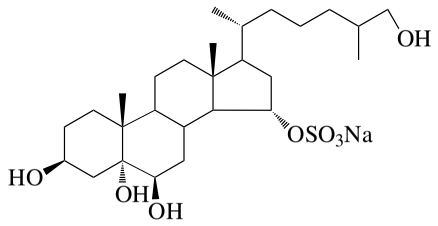
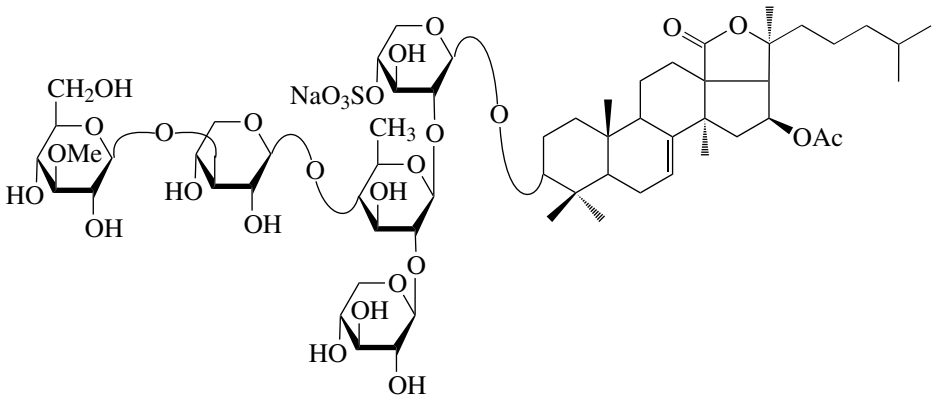
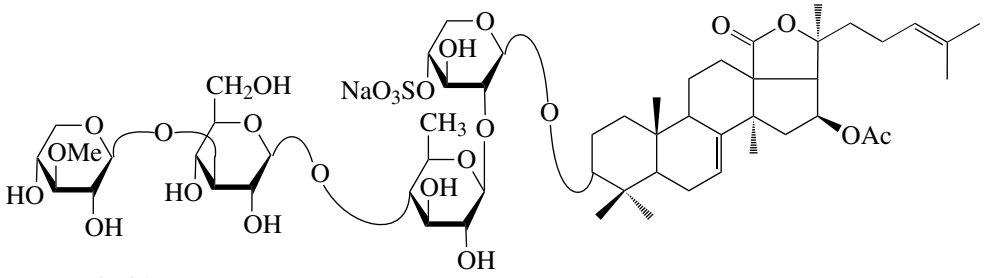
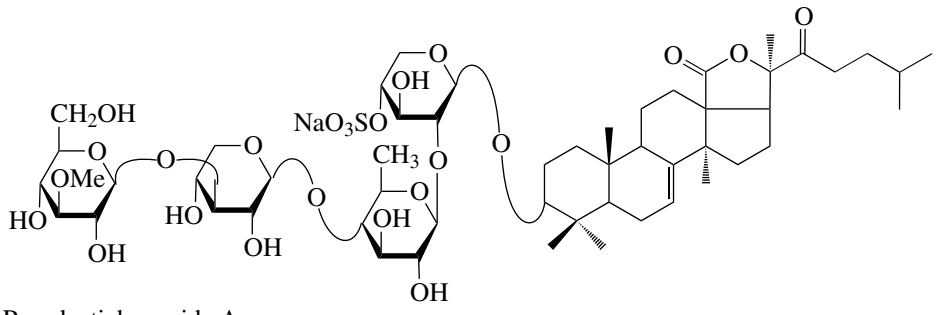
Compound no., group	Compound structure	I_{50}, M	Compound no., group	Compound structure	I_{50}, M
(7, A)		4×10^{-4}	(2, C)		10^{-4}
(8, A)		5×10^{-4}	(3, C)		10^{-4}
			(4, C)		10^{-4}

Table 2. Inhibitory effect of holostane glycosides on sulfatase

Compound	I_{50}, M
 Frondoside A	4×10^{-4}
 Cucumarioside G_1	1×10^{-3}
 Pseudostichoposide A	1×10^{-3}

The sulfatase from *L. kurila* did not affect the native fucoidan. Fucoidan from *Fucus evanescens* and its sulfatase-treated sample on a DEAE Toyopearl column showed similar elution profiles at chromatography. The identity of the ^{13}C NMR spectra and similar electrophoretic mobilities of the samples confirmed this conclusion.

Some characteristics of enzymes similar to sulfatase from *L. kurila* have been reported. For example, a sulfatase that hydrolyzes *p*-nitrophenyl sulfate but not fucoidan was found in the liver of the marine mollusk *Haliotis* sp. [5]. On the other hand, a partially purified preparation from the mollusk *Patinopecten maximus* effectively hydrolyzed synthetic substrates *p*-nitrocatechol sulfate and sulfated *L*-fucose and partially desulfated natural fucoidan [24].

Thus, the sulfatase from *L. kurila* can be considered as aryl sulfatase that does not affect fucoidan but catalyzes the splitting off of the sulfate group at position C4 of the xylose residue incorporated into the carbohydrate chains of molecules of holostane triterpene glycosides from sea cucumbers.

The biological activity of some compounds is known to correlate with the content of sulfates. For example, it was found that the desulfated derivatives of triterpene glycosides from sea cucumbers possess stronger antifungal, antitumor, and hemolytic activities than their sulfated analogues [25]. On the other hand, the anticoagulating activity of fucoidans from *F. vesiculosus* and *Pelvetia canaliculata* increases with the growing content of sulfates [26]. The regioselective sulfatation of carbohydrate molecules can be accom-

plished chemically, using specifically protected sugars, and enzymatically. In the enzymatic approach, two types of enzymes are used: sulfotransferases for which nonsulfated sugars serve as acceptors, and glycosyltransferases that use sulfated sugar derivatives as acceptors. An original approach to obtaining sulfated sugar derivatives was recently proposed, which combines the chemical and enzymatic methods. Sulfatation is accomplished chemically, and a selective desulfatation, by means of sulfatases [27]. The sulfatase from *L. kurila* is promising for the directed desulfatation of natural glycosides aimed at design of new biologically active compounds.

Table 3. Chemical shifts (ppm) of carbon atoms (C3, C4, and C5) of the xylose residue incorporated in natural sulfated triterpene glycosides and their desulfated derivatives

Compound	C3	C4	C5
Frondoside A	75.8	76.1	64.4
Frondoside A-DS*	77.7	70.3	66.9
Cucumarioside G_1	75.8	75.3	64.0
Cucumarioside G_1-DS*	78.1	70.7	66.7
Pseudostichoposide A_1	76.2	75.3	64.0
Pseudostichoposide A_1-DS*	78.0	70.7	66.5

* DS, desulfated derivative.

EXPERIMENTAL

Reagents. Inorganic salts, acids, and Polychrom were commercial preparations from Reakhim (Russia); carriers for chromatography (DEAE cellulose, Sepharose CL 6B) and BSA were from Sigma (United States).

Substrates and inhibitors. *p*-Nitrophenyl sulfate was from Sigma (United States). Triterpene glycosides from sea cucumbers: frondoside A, pseudostichoposide A, cucumarioside G_1, and sulfated polyoxysteroids were kindly provided by the Laboratory of the Chemistry of Sea Natural Compounds (Pacific Institute of Bioorganic Chemistry, Far East Division, Russian Academy of Sciences).

Fucoidan was isolated from a brown alga *F. evanescens* by the procedure described in [28] and additionally purified to remove alginic acid as follows. Glacial acetic acid (150 ml) was added to 300 ml of a fucoidan solution (50 mg/ml), and the precipitate was immediately separated by centrifugation (9000 *g*, 10 min). The supernatant was adjusted to pH 7.0 with NaOH. The resulting salt was removed by ultrafiltration on an NMWL 1000 membrane (Millipore, United States) by successive dilution. The resulting solution was applied onto a column of DEAE cellulose (20 × 30 cm) equilibrated with 0.01 N HCl. Elution with a stepwise gradient of NaCl (0.35, 0.5, 0.75, 1, 1.5, 2, and 3 M) using 3 l of each solution led to fucoidan, whose yield was registered by the phenol sulfate method [29]. Sugar-containing fractions were dialyzed, concentrated by ultrafiltration on an NMWL 1000 membrane, and lyophilized. The fucoidan fraction that was eluted with a 2 M NaCl solution was used.

Purification of sulfatase. Sulfatase was purified by the scheme described in [18], except that the stage of hydrophobic chromatography was omitted.

Protein was determined by the Lowry method [30] with BSA as a standard.

Sulfatase activity was registered by the appearance of *p*-nitrophenol in the reaction of sulfatase with *p*-nitrophenyl sulfate. A reaction mixture containing 50 μl (10^{-2} units) of the enzyme solution in 0.05 M succinate buffer, pH 5.4, and 50 μl of an aqueous solution of the substrate (1 mg/ml) was incubated for 30 min at 37°C, then 100 μl of 1 M Na_2CO_3 solution was added, and the absorption of the solution at 420 nm was measured.

A unit of enzyme activity was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of the substrate per min.

Determination of thermostability. Aliquots of a solution of the enzyme in buffer, pH 5.4 (50 μl, 10^{-2} units) were kept for 20 min in a water thermostat (U-2, Germany) at a temperature from 20 to 70°C at an interval of 5°C. After cooling, 100 μl of a *p*-nitrophenyl sulfate solution (1 mg/ml) was added, and the residual activity was determined as described above. The stability of the enzyme that was kept at 60°C (300 μl of solution) was checked by taking 50-μl aliquots every 10 min for 1 h.

Molecular mass was determined by 10% PAG–0.1% SDS electrophoresis by the method of Laemmli [31].

Determination of the Michaelis constant. A reaction mixture containing 50 μl of the enzyme (10^{-2} units) in a buffer, pH 5.4, and 50 μl of a *p*-nitrophenyl sulfate solution (concentration 0.1–3 mg/ml) was incubated for 20 min at 37°C, and the activity was determined. The Michaelis constant (K_m) was calculated using the Lineweaver–Burk transform.

Determination of the inhibitory activity. The reaction mixture contained 50 μl of the solution of the enzyme (10^{-2} units) in buffer, pH 5.4, and 50 μl of an aqueous solution of inhibitors of different concentrations (1.5, 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05 mg/ml). The mixture was kept for 10 min, 50 μl of a *p*-nitrophenyl sulfate solution (1 mg/ml) was added, and the mixture was incubated for 20 min at 37°C. The reaction was terminated by adding 100 μl of Na_2CO_3, and the residual activity of the enzyme was determined. I_{50} was taken to be the concentration of the substance at which a 50% inhibition of the enzyme activity occurs.

Preparation of products of enzymatic transformation of triterpene glycosides. Glycosides were added to a solution of sulfatase (20 ml, 2 units) in

buffer, pH 5.4, so that their final concentrations were no higher than I_{50}, and the mixture was incubated for 72 h. The reaction was quenched by boiling. The reaction mixture was passed through a column (1 × 15 cm) of Polychrom-1 (Biolar, Latvia), washed with water, and the reaction products were eluted with 70% ethanol and evaporated to dryness. The dry residue was chromatographed on a column (1 × 15 cm) of silica gel (KSK, Russia) eluted with 100 : 50 : 4 (v/v) chloroform–ethanol–water. The yield of the compounds was monitored by TLC.

^{13}C NMR spectra were obtained in pyridine at 60°C on a Bruker DRX300 NMR spectrometer with a working frequency of 125.77 MHz.

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