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β-1,3-Glucanase from Unfertilized Eggs of the Sea Urchin *Strongylocentrotus intermedius*. Comparison with β-1,3-Glucanases of Marine and Terrestrial Mollusks

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Abstract—β-1,3-Glucanase (Lu) was isolated from unfertilized eggs of the sea urchin *Strongylocentrotus intermedius*. A comparative study of some properties of β-1,3-glucanase Lu and β-1,3-glucanases with different action types—endo-β-1,3-glucanase from crystalline style of the marine mollusk *Spisula sachalinensis* (LIV) and exo-β-1,3-glucanase from the terrestrial snail *Eulota maakii* (LII)—was performed. It was found that β-1,3-glucanase Lu hydrolyzes laminaran with a high yield of glucose in the reaction products. The enzyme hydrolyzes substrates with retention of the glycosidic bond configuration, is able to cleave modified substrates, and exhibits transglycosylating activity. All properties of β-1,3-glucanase from *S. intermedius* were more similar to those of the endo-β-1,3-glucanase from the marine mollusk (LIV) than exo-β-1,3-glucanase LII from the terrestrial snail. The differences in the effect of LIV and Lu on laminaran are probably related to the functions of β-1,3-glucanase Lu from sea urchin eggs (which, in contrast to LIV, is not a digestive enzyme).

Key words: β-1,3-glucanase, action type, sea urchin eggs, *Strongylocentrotus intermedius*, *Spisula sachalinensis*, *Eulota maakii*

β-1,3-Glucanases from plants, yeast, bacteria, and fungi have been studied rather well. However, the enzyme in invertebrates is poorly studied. It is known that β-1,3-glucanase is contained in unfertilized eggs of most sea urchin species [1]. It is located in the cortical granules. After fertilization, the major portion of the enzyme is released to the perivitelline space and surrounding seawater [2]. In some species, β-1,3-glucanase appears again during gastrulation and larval development and functions as a digestive enzyme. There is evidence that these two enzymes are immunologically and functionally distinct forms [3]. The role of β-1,3-glucanase in unfertilized eggs remains obscure. Possibly, the eggs and embryos at the early developmental stages secrete β-1,3-glucanase to destroy extracellular matrix (similar to the autolytic cycle characteristic of the fungal cell wall development). However, the endogenous β-glucan substrate in the fertilization envelope of sea urchins has not been identified.

According to the International Enzyme Classification (1992), which is based on the type of the reaction catalyzed and the substrate specificity, glycanases are divided into two groups: endoglycanases (which cleave the internal bonds in the polysaccharide molecule, yielding a mixture of mono- and oligosaccharides) and exoglycanases (which sequentially cleave mono- or disaccharides from the non-reducing end of the substrate molecule).

Earlier, we showed that β-1,3-glucanases (laminarinases) are widespread in marine invertebrates [4, 5]. A thorough study of the properties, specificity, and mechanism of action of β-1,3-glucanases from crystalline styles of marine mollusks allowed us to classify them as endoenzymes. We discovered a characteristic feature of the effect of endo-β-1,3-glucanases from marine mollusks—the ability to hydrolyze laminaran with a much greater yield of the monosaccharide (glucose) compared to the endoenzymes from plants, fungi, and other sources [6].

Abbreviations: Lu) β-1,3-glucanase from *Strongylocentrotus intermedius*; LIV) β-1,3-glucanase from *Spisula sachalinensis*; LII) β-1,3-glucanase from *Eulota maakii*.

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β-1,3-Glucanases from unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* [7] and blastula-stage embryos of the sea urchin *Strongylocentrotus intermedius* [8] were classified as exo-β-1,3-glucanases based on the results of analysis of the enzymatic reaction products.

In this work, we performed a comparative analysis of some properties of β-1,3-glucanase from unfertilized eggs of the sea urchin *S. intermedius* and digestive enzymes of two action types—endo-β-1,3-glucanase from the crystalline style of the marine bivalve *Spisula sachalinensis* [6] and exo-β-1,3-glucanase from the terrestrial snail *Eulota maakii* [9]—which had been studied in our laboratory earlier.

MATERIALS AND METHODS

Reagents. Endo-β-1,3-glucanase LIV was isolated from the crystalline style of the marine bivalve *S. sachalinensis* as described in [10]; exo-β-1,3-glucanase LII, from the digestive organs of the terrestrial snail *E. maakii* as described in [9]; and β-1,3-glucanase Lu, from unfertilized eggs of the sea urchin *S. intermedius*.

Laminaran from the brown alga *Laminaria cichorioides* was isolated as described in [11], periodate-oxidized laminaran was synthesized according to [12]. Pachyman, CM-pachyman, and lichenan were kindly provided by the researchers from the Pacific Institute of Bioorganic Chemistry; *p*-nitrophenyl-β-D-glucopyranoside was obtained from Sigma (USA).

Analytical methods. Neutral saccharides were determined by the phenol–sulfuric acid method [13], reducing saccharides were assayed by the method of Nelson [14], and glucose content was measured by the glucose oxidase method [15]. Liquid chromatography of oligosaccharides was performed on a Jeol-JLC-6AH automatic analyzer (Japan) equipped with a column (1 × 100 cm) packed with Biogel P-2. Saccharides were determined using the orcinol–sulfuric acid reagent. *p*-Nitrophenyllaminarioligosaccharides (the products of trans-glycosylation) were analyzed by HPLC on a DuPont chromatograph (series 8800) (USA) equipped with an Ultrasil-NH_2 column (10 × 250 mm) in the system acetonitrile–H_2O (80 : 20). The products were detected at 300 nm. Protein content in the preparations was determined by the Lowry method [16].

Determination of enzymatic activity. A. Standard reaction medium for determination of the hydrolytic activity of β-1,3-glucanase contained the enzyme (20 μl), laminaran (500 μl, 1 mg/ml), and 0.05 M succinate buffer (pH 5.2). The mixture was incubated at 37°C for 20 min. The amount of the enzyme that produced 1 μmol of glucose in 1 min under these conditions was taken to be equal to one activity unit.

B. To determine the trans-glycosylating activity, the enzyme solution (100 μl, $2 \cdot 10^{-2}$ U) in 0.05 M succinate buffer (pH 5.2) was added to solution (500 μl) containing laminaran (2 mg) and *p*-nitrophenyl-β-D-glucopyranoside (2 mg). The mixture was incubated at 37°C. At the intervals specified, 50-μl aliquots were taken and mixed with acetonitrile (200 μl). The samples were analyzed by HPLC.

Purification of β-1,3-glucanase Lu from unfertilized eggs of the sea urchin *S. intermedius*. The sea urchin eggs (2.5 g) were suspended in 0.02 M Tris-HCl buffer (200 ml, pH 7.5) and centrifuged at 15,000 rpm for 20 min. The supernatant was loaded on a column (40 × 2.5 cm) packed with DEAE-Toyopearl (Toyo Soda, Japan) equilibrated in the same buffer. The proteins were eluted with linear NaCl gradient (0-0.5 M, 1000 ml of each solution) prepared in the same buffer, at a rate of 60 ml/h.

The fractions that contained β-1,3-glucanase were concentrated using ultrafiltration on a PM-10 membrane (Amicon, Holland) to a volume of 5 ml and loaded on a column (60 × 2.5 cm) packed with Sephadex G-200 (Pharmacia, Sweden). The proteins were eluted with the same buffer at the rate of 30 ml/h. The fractions that contained β-1,3-glucanase were pooled, concentrated using ultrafiltration to a volume of 1 ml, and chromatographed in several portions on Mono-Q (AKTA FPLC, Pharmacia Biotech., Sweden). The enzyme was eluted with a linear NaCl gradient (0-0.6 M, 250 ml of each solution) prepared in 0.02 M Tris-HCl buffer (pH 7.5).

Purified β-1,3-glucanase was transferred to 0.05 M succinate buffer (pH 5.2) using gel filtration on a column (35 × 2.5 cm) packed with Sephadex G-75.

Measuring optical rotation. Polarimetric studies were conducted in a quartz cuvette on a Perkin-Elmer 141 polarimeter (USA). The enzyme solution (0.3 ml, 10^{-2} U) prepared in 0.05 M succinate buffer (pH 5.2) was added to laminaran (1.2 ml, 15 mg), and optical rotation was measured at the time intervals specified (5, 10, 15, and 30 min). Prior to the last measurement, a drop of ammonia was added to the reaction mixture.

Characteristics of the laminaran hydrolysis products. The enzyme solution (0.2 ml, $2 \cdot 10^{-2}$ U) prepared in 0.05 M succinate buffer (pH 5.2) was added to laminaran solution (11.8 ml, final concentration in the reaction mixture 15 mg/ml). At certain time intervals, aliquots (1 ml) for HPLC analysis as well as aliquots for determination of reducing saccharides and glucose were taken. The reaction was stopped by boiling.

RESULTS AND DISCUSSION

The scheme of purification of β-1,3-glucanase from unfertilized eggs of the sea urchin *S. intermedius* was somewhat different from schemes reported for similar

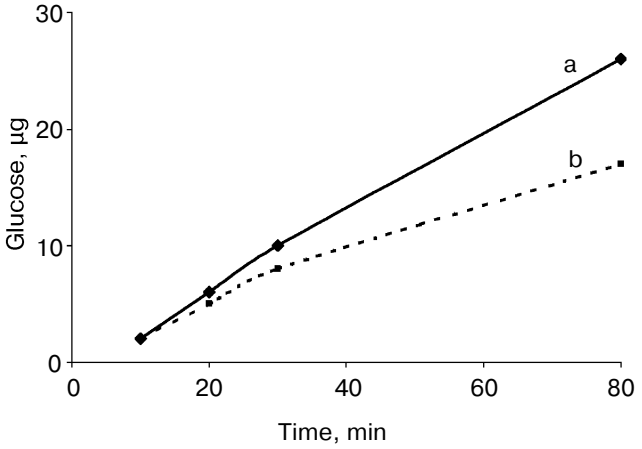

Fig. 1. Effect of β-1,3-glucanase Lu on laminaran: a) accumulation of reducing saccharides; b) increase in the glucose content.

sources and included ion-exchange chromatography on DEAE-Toyopearl, gel filtration on Sephadex G-200, and chromatography on Mono-Q.

The action type of β-1,3-glucanase from unfertilized eggs of the sea urchin *S. intermedius* was determined using several approaches. The reaction products were analyzed by two methods: all reducing saccharides were assayed by the method of Nelson [14], and the glucose content was determined by the glucose oxidase method [15]. It was shown earlier for exo-β-1,3-glucanase from *E. maakii* that the content of reducing saccharides at the initial stages of hydrolysis completely coincided with the glucose content [9]. However, in the case of endo-β-1,3-glucanase from *S. sachalinensis*, the content of reducing saccharides was considerably greater than that of glucose, which is indicative of the presence of laminarioligosaccharides among the reaction products [17]. The data obtained for β-1,3-glucanase from *S. intermedius* (Fig. 1) suggest that glucose is a predominant hydrolysis product at the initial stages of the laminaran hydrolysis, whereas oligosaccharides are accumulated at later stages.

The products formed from the treatment of laminaran with the enzymes were also analyzed on a liquid analyzer. Figure 2 shows the results of liquid chromatography of the products of the laminaran hydrolysis with three enzymes studied, at the same extent of the substrate hydrolysis (approximately 14%). The glucose content in the products of exhausting laminaran hydrolysis with these enzymes is shown in Table 1.

One of the criteria used for the determination of the action type of glycanases is stereochemistry of the hydrolysis products. Exoglycanases and endoglycanases hydrolyze the substrate with inversion and retention of the configuration of the bond cleaved, respectively. The configuration of the products of the laminaran hydrolysis with β-1,3-glucanases was determined by the change in the optical rotation of the incubation medium. The results of these measurements are summarized in Table 2.

In the course of the laminaran hydrolysis with LII, the optical rotation of the solution steadily increased to a positive value, which after addition of a drop of ammonia (a catalyst of the anomerization reaction) sharply decreased to the steady-state level. A similar picture observed in the case of exo-β-1,3-glucanase from *Helix pomatia* [18] allowed us to conclude that the laminaran hydrolysis catalyzed by this enzyme yielded α-glucose.

It was shown earlier using NMR spectroscopy that LIV hydrolyzed laminaran with retention of the configuration of the bond cleaved [19]. According to the data presented in Table 2, the optical rotation of the solution in the course of the laminaran hydrolysis with LIV not only does not reach a high positive value, but even does not change after the ammonia addition. The optical rotation is increased even more slowly in the case of the laminaran hydrolysis with β-1,3-glucanase Lu from unfertil-

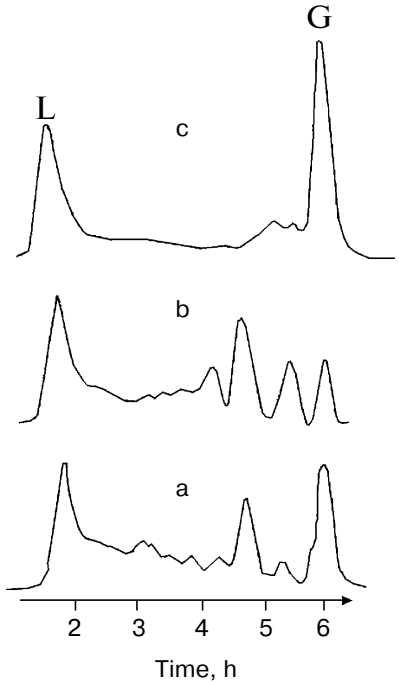

Fig. 2. Gel filtration of the products of the laminaran hydrolysis catalyzed by β-1,3-glucanases Lu (a), LIV (b), and LII (c) on a Jeol-JLC-6AH automatic liquid analyzer equipped with a column (1 × 100 cm) packed with Biogel P-2. L, laminaran; G, glucose.

Table 1. Characteristics of the products of exhausting hydrolysis of laminaran from *L. cichorioides* with β-1,3-glucanases

β-1,3-Glucanase (10^{-2} U)	Degree of hydrolysis, %	Glucose content in the products, %
LII	95	90
Lu	63	33
LIV	60	40

Table 2. Change in optical rotation of incubation mixture during laminaran hydrolysis with β-1,3-glucanases

Time, sec	$[\alpha]_D$, degrees		
	LII	Lu	LIV
5	−19	−23	−18
10	−13	−22	−11
15	−8	−20	−5
20	+2	−18	−3
30	+11	−16	+3
30*	+1	−15	+3

Note: The $[\alpha]_D$ value of laminaran from *L. cichorioides* (10 mg/ml) was −23°; $[\alpha]_D$ of α-glucose, +105°; $[\alpha]_D$ of β-glucose, +20°; $[\alpha]_D$ of equilibrium mixture of α- and β-anomers of glucose, +55°.
* The volume of NH_4OH added to the mixture was 100 μl.

Table 3. Effect of β-1,3-glucanases on different substrates

Substrate	Relative hydrolysis rate, %		
	LII	Lu	LIV
Laminaran from *L. cichorioides*	100	100	100
Periodate-oxidized laminaran	0.5	85	90
Pachyman	0	13	10
CM-pachyman	7	24	14
Lichenan	16	22	20
p-Nitrophenyl-β-D-glucopyranoside	0	7	2.1

ized eggs of the sea urchin and it also does not change after addition of ammonia. It is most likely that the products with β-configuration at C1 are formed in this case (similar to LIV-catalyzed hydrolysis).

It is known that substrates with an altered nonreducing end are not cleaved by exoenzymes; however, this modification has almost no affect on the rate of hydrolysis of these products by endoenzymes. Among the glucanases studied, only LII did not cleave modified laminaran, whereas LIV and Lu hydrolyzed it at a high rate (Table 3). The rates of pachyman (a high-molecular-weight glucan) and lichenan (a mixed β-1,3; β-1,4-glucan) hydrolysis with glucanases LIV and Lu were similar.

It is known that endoglycanases are able to catalyze both hydrolysis and trans-glycosylation, whereas exoglycanases hydrolyze only the glycosidic bonds in the substrate. However, some exceptions to this rule have been discovered recently. For example, an exo-β-1,3-glucanase that can catalyze trans-glycosylation has been recently found [20]. Currently, little is known of the trans-glycosylating properties of β-1,3-glucanases. The majority of works published only report the presence or absence of the trans-glycosylating properties in this group of enzymes [21, 22]. Earlier, we showed that endo-β-1,3-glucanase from marine mollusks exhibited an increased ability to catalyze trans-glycosylation reactions compared to other carbohydrases [6]. A comparative evaluation of the trans-glycosylating activity of β-1,3-glucanases from different sources was performed by us earlier [23]. The trans-glycosylating activity was assessed by the rate of formation of *p*-nitrophenol-labeled products, with laminaran and *p*-nitrophenyl-β-D-glucopyranoside being used as donor and acceptor, respectively. It was shown that β-1,3-glucanase LII does not catalyze trans-glycosylation. At equal glycolytic activity and under similar conditions, LIV and Lu catalyzed incorporation of 11.5 and 0.4% of the acceptor into the reaction products during 1 h, respectively. The trans-glycosylating activity of Lu is much lower than that of LIV but comparable with the activity of an endo-β-1,3-glucanase of plant origin [23].

Thus, β-1,3-glucanase from unfertilized eggs of the sea urchin *S. intermedius* catalyzes laminaran hydrolysis, retaining the configuration of the bond cleaved and yielding a high glucose content in the reaction products. It can also hydrolyze modified substrates. In addition, this enzyme displays trans-glycosylating activity. Due to all these properties, it is more similar to endo-β-1,3-glucanase LIV from the marine mollusk than exo-β-1,3-glucanase LII from the terrestrial snail.

Some differences in the composition of the products of the laminaran hydrolysis with β-1,3-glucanases LIV and Lu and their trans-glycosylating ability are apparently related to their different biological roles.

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