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# NATURAL EFFECTORS OF β-1,3-GLUCANASES

## ACTIVATOR OF THE β-1,3-GLUCANASES OF MARINE MOLLUSKS

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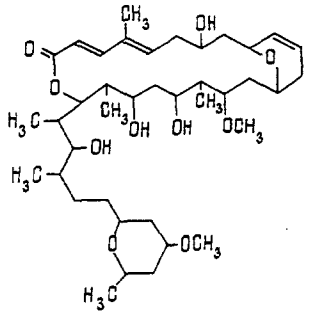
The capacity of certain extracts of marine invertebrates of the Indian and Pacific Oceans for activating β-1,3-glucanases has been detected. It has been shown that an individual compound – swinholide A from the sponge Theonella swinhoei – specifically activates β-1,3-glucanases of bivalve mollusks. In the presence of swinholide A both the hydrolysis of laminarin by β-1,3-glucanase L-IV from Spisula sachalinensis and the transglycosylation reaction are accelerated.

The search for effectors of β-1,3-glucanases in extracts of marine invertebrates of the Indian and Pacific Oceans has shown that, together with an inhibiting action [1], some metabolites bring about an activation of the enzymes. More than 300 extracts of invertebrates belonging to the most important systematic groups have been tested. It has been established that the most promising sources of activators are soft corals, sponges, and ascidians. Thus, 12 extracts out of 100 samples of soft corals, one out of 125 samples of sponges, and five out of 44 samples of ascidians activated β-1,3-glucanase L-IV from Spisula sachalinensis.

An individual compound of natural origin accelerating the enzymatic reaction of β-1,3-glucanases has been found for the first time. The substance, isolated from the sponge Theonella swinhoei has been identified as a representative of the macrolides – swinholide A [2, 3]. Macrolides from sponges form a new group of physiologically active compounds that have no structural analogues among related substances of microbial origin. It is known that swinholide A possesses antifungal activity [3].



Swinholide A

We have found that swinholide A is an activator of two isoenzymes: endo-β-1,3-glucanases L-III and L-IV from the crystalline style of the bivalve mollusk S. sachalinensis. Activation by swinholide A is expressed to a smaller degree in relation to the endo-β-1,3-glucanase L-O from the marine mollusk Chlamys albidus. Under the same condition, no changes

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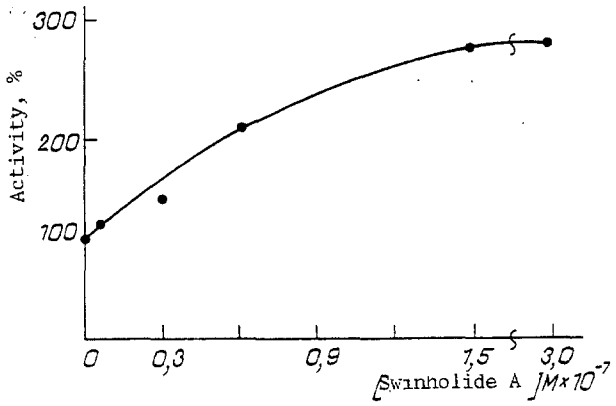


Fig. 1. Influence of swinholide A on the activity of β-1,3-glucanase L-IV from Spisula sachalinensis.

TABLE 1. Action of Swinholide A on β-1,3-Glucanases

| Enzyme, source of the enzyme | Activity in the presence of $3 \times 10^{-7}$ M swinholide A, % |
|---|---|
| β-1,3-Glucanase from potato leaves | 100 |
| β-1,3-Glucanase from tobacco leaves (total preparation) | 100 |
| endo-β-1,3-Glucanase from the Antarctic krill Euphausia superba | 100 |
| β-1,3-Glucanase from the Chiton Acanthopleura sp. | 100 |
| endo-β-1,3-Glucanse L-O-from Chlamys albidus | 130 |
| endo-β-1,3-Glucanase L-IV from Spisula sachalinensis | 210 |
| endo-β-1,3-Glucanase L-III from S. sachalinensis | 205 |

in the activities of β-1,3-glucanases from other sources were observed (Table 1). Swinholide A is probably specific for the endo-β-1,3-glucanases of bivalve mollusks. It belongs to the so called inessential activators, which are distinguished from the essential ones by the fact that the enzymes possess activity even in their absence.

Even in a concentration of $1.5 \times 10^{-7}$ M, swinholide A accelerated by a factor of 2.7 the hydrolysis of laminarin catalyzed by β-1,3-glucanase L-IV. With a further increase in the concentration the degree of activation scarcely changed, which indicated good complex-formation of the enzyme with the activator (Fig. 1). Dilution of the incubation mixture did not cause dissociation of the complex. An analogous activation effect was observed in the action of β-1,3-glucanase L-IV on other substrates: pachyman and yeast glucan.

An analysis of the kinetics of the accumulation of the products of the hydrolysis of laminarin by β-1,3-glucanase L-IV with the aid of liquid chromatography showed that in the presence of the activator the nature of the distribution of the products did not change. To illustrate the experiments performed, from a series of chromatograms we give two that characterize compositions of the products at one kinetic point in the presence and in the absence of swinholide A (Fig. 2).

It has been established that in the presence of swinholide A not only the hydrolysis reaction but also the transglycosylation reaction effected by β-1,3-glucanase L-IV is accelerated. To characterize the transglycosylating capacity of β-1,3-glucanase L-IV the reaction was performed with the use of laminarin as donor and p-nitrophenyl (Np) glucoside as acceptor. The p-Np oligosides formed were detected by HPLC (Fig. 3).

It may be assumed that the binding of swinholide A, which is not an analogue of the substrate, takes place in a center of the enzyme different from its active center, and the change in activity results from conformational changes in the enzyme molecule.

## EXPERIMENTAL

Enzymes. endo-β-1,3-Glucanases L-III and L-IV from S. sachalinensis, endo-β-1,3-glucanase L-O from Ch. albidus, and the β-1,3-glucanases from the chiton Acanthopleura sp., from the Antarctic krill E. superba, and from potatoes were isolated by published methods [4-8].

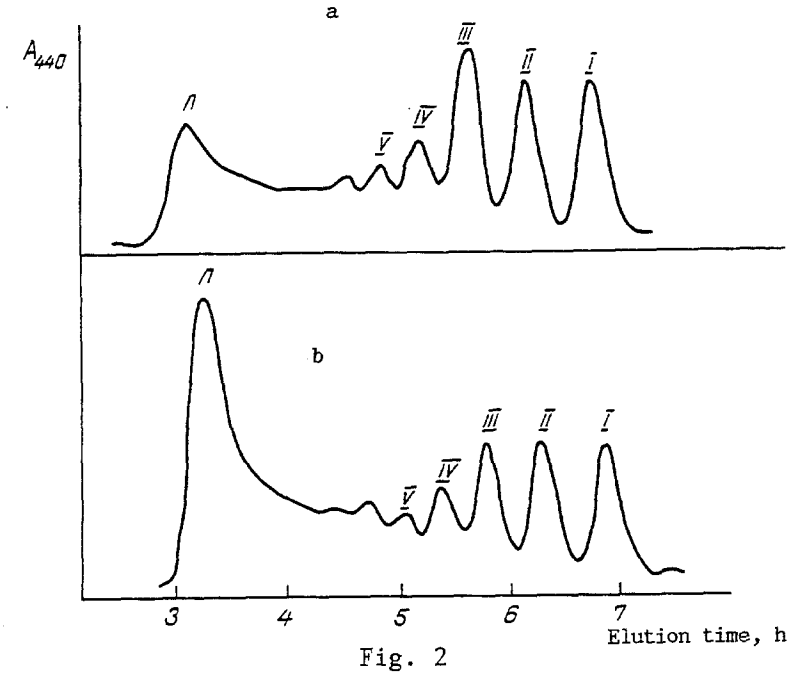


Fig. 2

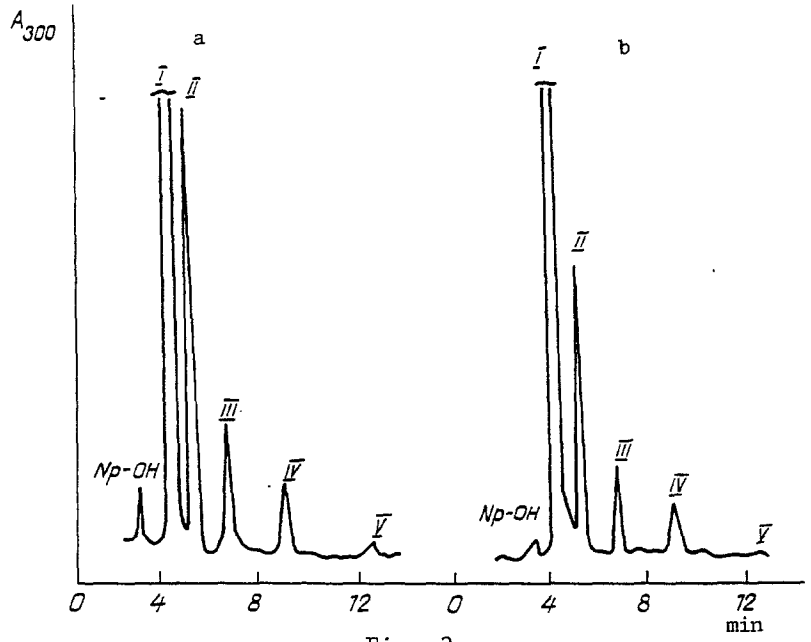


Fig. 3

Fig. 2. Chromatograms of the products of the hydrolysis of laminarin by β-1,3-glucanase L-IV: a) in the presence of swinholide A (75% hydrolysis of the substrate); b) in the absence of swinholide A (45% hydrolysis of the substrate). Reaction time 60 min: I) glucose; II) laminaribiose, etc.; L) laminarin.

Fig. 3. HPLC of the products of a transglycosylation reaction catalyzed by β-1,3-glucanase L-IV: a) in the presence of swinholide A; b) in the absence of swinholide A. Reaction time 60 min. The numerals denote the fractions of Np oligosides with degrees of polymerization equal to the numbers of the peaks.

A total preparation from tobacco was prepared by the homogenization of leaves that had previously been frozen in liquid nitrogen, followed by centrifugation at 10,000 rpm for 10 min.

Substrates. Laminarin from Laminaria cichorioides and pachyman from Poria cocos were obtained by known methods [9, 10]. Yeast glucan was provided by N. I. Shirokova (TIBOKh).

Effectors. Marine invertebrates were collected during voyage No. 20 of the Scientific Research Vessel "Professor Bogorov." The animals were homogenized, and two types of extracts were prepared: aqueous and alcoholic (ratio of material to solvent by weight 1:3). The homogenates were centrifuged at 10,000 rpm for 10 min, the alcoholic extracts were evaporated, and aqueous solutions of the necessary concentration were prepared from the dry residues.

The sponge T. swinhoei was collected in February, 1984, at a depth of 1-2 m in the Tanzanian littoral (Mante Reef, Indian Ocean) during voyage No. 17 of the Scientific Research Vessel "Professor Bogorov." Swinholide A was isolated from an ethanolic extract of the freeze-dried sponge by chromatography on columns of silica gel, Sephadex LH-20, and Ultrasil-$NH_2$. The formyl derivative was obtained by treating the substance with 96% formic acid. The UV and the $^1H$ and $^{13}C$ NMR spectra of the compound isolated and its formyl derivative were identical with the corresponding spectra given in the literature [2, 3].

Influence of Extracts on the Activities of β-1,3-Glucanases. A mixture of 50 μl of the solution under investigation and 50 μl of a solution of the enzyme in 0.05 M succinate buffer, pH 5.2, containing about $2 \times 10^{-2}$ units of glucanase activity (1 unit = 1 μmole of glucose/min) was kept at 25°C for 10 min, and then 400 μl of a solution of laminarin (1 mg/ml) was added. The samples were incubated at 37°C for 15 min, and then their β-1,3-glucanase activities were determined from the change in the amount of reducing sugars found by Nelson's method [11].

Action of Swinholide A. a) Influence on the Activities of the Enzymes. The necessary amount of swinholide A in 10 μl of 30% aqueous ethanol was added to 100 μl of a solution of a β-1,3-glucanase ($2\text{-}3 \times 10^{-2}$ activity units) in 0.05 M succinate buffer, pH 5.2, and this was followed after 10 min by 400 μl of a solution of the substrate (laminarin, pachyman, or yeast glucan). The samples were incubated at 37°C for 15 min, and the activities were determined. In all the experiments the influence of the amounts of ethanol introduced into the reaction were taken into account in a control experiment.

b) Analysis of the Products of the Hydrolysis of Laminarin. To 200 μl of a solution of β-1,3-glucanase L-IV in 0.05 M succinate buffer, pH 5.2, were added 20 μl of an aqueous alcoholic solution of swinholide A (1 mg/ml) and 5 ml of a solution of laminarin (1 mg/ml). A control mixture contained no swinholide A. After 15, 30, 60, and 120 min, 1 ml alliquots were taken and the reaction was stopped by boiling. The degree of hydrolysis of the laminarin was determined from the increase in the amount of reducing sugars. Gel filtration of the samples was conducted on a column (0.6 × 100 cm) of Bio-Gel P-2 (400 mesh) using a Jeol JLC-6AH automatic analyzer, in which the amount of sugars was determined with the aid of the orcinol/sulfuric acid reagent.

c) Conditions of the Transglycosylation Reaction. The reaction mixture contained: 100 μl of a solution of β-1,3-glucanase ($2\text{-}3 \times 10^{-2}$ units) in 0.05 M succinate buffer (pH 5.2), 10 μl of a solution of swinholide A (1 mg/ml) in 30% aqueous ethanol (the control sample contained 10 μl of 30% aqueous ethanol), and 0.5 ml of a solution of laminarin (2 mg/ml) and of p-Np glucoside (2 mg/ml). Incubation was carried out at 37°C, and 50-μl aliquots were taken at 10-minute intervals. The reaction was stopped by the addition of 200 μl of acetonitrile. The HPLC analysis of the transglycosylation products was carried out on a Du Pont series 8000 liquid chromatograph with a 10 × 250 mm Ultrasil-$NH_2$ column (Beckman). The rate of elution was 4 ml/min. The eluting system was: acetonitrile–5.0 mM Na acetate buffer, pH 4.0-4.3 (80:20). The UV absorption was recorded at 300 nm.

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# COMPOSITION OF THE PHOSPHOLIPIDS OF SEEDS OF CERCIS SILIQUASTRUM

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The phospholipids of the polar lipids of the seeds of the Judas tree and the fatty-acid composition of the N-acylphosphatidylethanolamines, the phosphatidylcholines, and the phosphatidylethanolamines, and also the molecular-species types of the fatty acids of the phosphatidylcholines and the phosphatidylethanolamines have been studied. It has been established that the seeds of the Judas tree contain ten phospholipids. The main fatty acids are palmitic and oleic.

Cercis siliquastrum L. – the Judas tree (family Leguminosae) – is a species from the Mediterranean. It is cultivated as a decorative plant in the Crimean Carpathians, in the Caucasus, and in Central Asia. The Judas tree has scarcely been studied for the presence of biologically active substances. There is only information on the presence in the leaves of the plant of tanning substances of the pyrocatechin and gallotannin series [1, 2].

We have investigated the composition of the phospholipids (PLs) of the seeds of the Judas tree growing in Georgia. The comminuted seeds were extracted with n-hexane, and after the solvent had been distilled off 10-12% of neutral lipids was obtained. The polar lipids were extracted from the defatted raw material by Folch's method [3] and were freed from impurities (systems 1 and 2) [4], as a result of which a total of 2.9% of phospholipids was obtained.

By two-dimensional TLC in a layer of silica gel in systems 3 and 4 [5, 6] the total PLs revealed ten phosphorus-containing spots the quantitative amounts of which were determined spectrophotometrically (%): phosphatidylcholines (PCs) – 48.2; phosphatidylinositols (PIs) – 14.1; phosphatidylethanolamines (PEs) – 13.5; N-acyl-PEs – 7.4; phosphatidylglycerides (PGs) – 5.4; lyso-PCs – 4.5; lyso-PIs – 3.7; phosphatidic acid (PA) – 3.2. Diphenylglycerides (DPGs) and N-acyl-lyso-PEs were detected in trace amounts. As we see, the main components of total PLs of Judas tree seeds are PCs, PIs, and PEs.

To isolate the homogeneous phospholipids, the total material was first fractionated by chromatography on a column of silica gel and was then separated by preparative TLC. As a result, the PCs, PEs, and N-acyl-PEs were isolated, these being identified from their IR spectra [7-9] and the products of acid hydrolysis. The fatty acids split out were analyzed by GLC. To determine the position specificity of the distribution of the fatty acids in the phospholipid molecules we studied the products of their enzymatic hydrolysis.

Fatty acids in the PCs and PEs were represented by five components and those in the N-acyl-PEs by seven (Table 1). In all cases, among the saturated acids palmitic predominated, and among the unsaturated acids oleic. The PEs and PCs differed from one another both qualitatively and quantitatively. The total degree of saturation of the PEs (69.5%) was consid-

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