SULFATED STEROIDS OF SPONGES OF THE FAMILY HALICHONDRIIDAE — NATURAL INHIBITORS OF ENDO-(1 → 3)-β-D-GLUCANASES

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A source of natural inhibitors of endo-(1 → 3)-β-D glucanases has been found — sponges of the family Halichondriidae. It has been shown that the inhibitors are sulfated steroids such as halistanol sulfate and sokotrasterol sulfate. The link between the elements of the structure of an inhibitor and its inhibiting action has been investigated. The ratio between the hydrophobic and the hydrophilic parts of the inhibitor molecule play a deciding role in the manifestation of the inhibiting effect. The action of the inhibitor is irreversible. The compositions of the enzyme-inhibitor complexes (EIs) formed on the interaction of halistanol sulfate (I) and sokotrasterol sulfate (II) with the endo-(1 → 3)-β-D-glucanase from Chlamys albidus (E) have been determined: E:I = 1:4, E:II = 1:6.

At the present time, a large number of carbohydrase inhibitors isolated from various natural sources is known [1, 2]. The most numerous among them are natural amylase inhibitors [1]. Carbohydrase inhibitors can be divided into two large groups: substances of low molecular weight (peptides [3], oligosaccharides [4, 5], glycopeptides [6], antibiotics [7-9], etc.) and high-molecular-weight substances (proteins [1] and glycoproteins [10-12]). There is no information in the literature on natural inhibitors of endo-(1 → 3)-β-D-glucanases.

In a systematic search for such inhibitors we have found that among the large number of marine organisms studied only extracts of sponges from the family Halichondriidae possess an inhibiting action with respect to the endo-(1 → 3)-β-D-glucanases of marine molluscs: L-O from Chlamys albidus [13], L-III and L-IV from Spisula sachalinensis [14], and L-V from Patinopecen sp., although amylases are distributed fairly widely in marine organisms.

A total of 90 specimens of tropical animals was tested for the presence of inhibitors of endo-(1 → 3)-β-D-glucanases, of the exo-(1 → 3)-β-D-glucanase L-II from Eulota maakii [15] and the α-amylase from Ch. albidus:

1. Echinodermata: Holothurioidea (16 specimens), Asteroidea (5 specimnes), Echinodea (2 specimens); Crinoidea (1 specimen);

2. Spongia (16 specimens);

3. Mollusca: Loricata (2 specimens); Gastropoda (11 specimens); Cephalopoda-Octopodidae (2 specimens); Ommastrephidae (1 specimen); Sepiidae (1 specimen);

4. Colenterata: Actiniaria (11 specimens);

5. Annelida: Polychaeta (3 specimens); Amphinomidae (1 specimen);

6. Arthropoda: Crustacea (2 specimens), Xantidae (11 specimens);

and 11 specimens of animals from the Sea of Japan — Coelenterata: Actinaria (3 species); Spongia: Myxillidae (1 species); Suberitidae (4 species); Halichondriidae (3 species).

The extracts from 20 specimens of tropical animals and 3 species of animals from the Sea of Japan possessed the capacity for inhibiting amylase, 6 specimens of tropical animals and 3 specimens of animals from the Sea of Japan were capable of inhibiting exolaminarinase, and only extracts of tropical sponges of the family Halichondriidae inhibited endolaminarinases. It is interesting to note that extracts of sponges of the same family from the Sea of Japan did not suppress the activity of endolaminarinases.

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TABLE 1. Action on Carbohydrases and Trypsin of Compounds (I) and (II) and Sodium Dodecyl Sulfate (SDS)

Enzyme	Act. units of $E \times 10^6$	Conc. of (I), $M \cdot 10^{-6}$	Inhibition, %	Conc. of (II), $M \cdot 10^{-6}$	Inhibition, %	Conc. of SDS, $M \cdot 10^{-6}$	Inhibition, %
Endolaminarinase L-O* Ch. albidus [13]	3	1	70	1,5	70	250	70
Endolaminarinase L-IV of S. sachalinensis [14]	3	1	30	1,5	50	250	50
Endolaminarinase L-III of S. sachalinensis [14]	3	1	25				
Endolaminarinase L-V of Patinopecten sp. [13]	3	1	70				
Takadiastase	3	35	50				
Pancreatic α-amylase	4	50	50				
β-Amylase	5	35	50				
Endocellulose [18] of Littorina mandshurica	3	50	50				
Endopustulanase PU* of Patinopecten sp.	3	30	50				
Trypsin	3	100	50				
Exolaminarinase L-II* of E. maakii	3	400	0			250	70

*Highly purified preparations of the carbohydrases, the other enzymes being commercial preparations.

As appeared in the process of isolating the active substance from them the inhibiting activity in sponges resided in the sulfated steroids — halistanol sulfate (I, Halichondriidae sp.) and sokotrasterol sulfate (II, Halichondiidae gen. sp.), the structures of which have been established [16, 17].

To elucidate the details of the process of inhibition, we used halistanol sulfate, the concentration of which in the sponges reaches 1% [17]. We first investigated the dependence of the degree of inhibition of endo-(1 → 3)-β-D-glucanases on the time of preincubation of the enzyme with the inhibitor (I). It was found that to reach 100% inhibition of the enzyme the period of its preincubation with the inhibitor should be not less than 15 min.

The study of the specificity of the action of halistanol sulfate was performed on a series of different carbohydrases (Table 1). Halistanol sulfate acts most effectively on endolaminarinases (in concentrations 30-50 times smaller than on amylases, pustulinase, and cellulase). Trypsin is inhibited by (I) to an even smaller degree and, finally, halistanol sulfate has no action whatever on the exo-(1 → 3)-β-D-glucanose L-II from the snail E. maakii [15]. Like other carbohydrases, the endo- and exo-(1 → 3)-β-D-glucanases frequently exist in natural materials in a complex with one another [19] and, therefore, the possibility of suppressing the activity of one of the laminarinases without their separation appears very promising.

Table 1 includes sodium dodecyl sulfate (SDS) — a known protein-denaturing agent. In its most general features, SDS somewhat resembles the natural inhibitors that we have found: like compounds (I) and (II) it has hydrophobic and hydrophilic sections in its molecule. It appeared of interest to compare the action of SDS and (I) on various laminarinases. Thus, while a 50% inhibition of endolaminarinases (L-O, L-IV) and an exolaminarinase (L-II) was caused by approximately the same concentration of SDS ($250 \cdot 10^{-6}$ M), halistanol sulfate, acting on L-O and L-IV as an inhibitor in exceptionally low concentrations ($1 \cdot 10^{-6}$ M) did not inactivate L-II (the exo enzyme) over a wide range of concentrations (up to $400 \cdot 10^{-6}$). From this it may be concluded that in relation to an exolaminarinase (I) does not exhibit the properties of the detergent. The basis of the interaction of endolaminarinases with (I) and (II) obviously consists of some more specific factors such as, for example, a correspondence of the structure of the inhibitors to a section in the enzyme molecules that is common for the endolaminarinases studied and is perhaps located close to their active centers [20].

TABLE 2. Dependence of the Efficiency of Inhibition of L-O on Elements of the Structure of the Inhibitors

R_1=SO_3Na/K, 1:1; R_2=SO_3Na; R_3=H	Mol. wt. of the inhibitor	μg (I)/ml (50% inhib.) $3 \cdot 10^{-2}$ act. units	Moles of (I) per 1 mole of E (50% inhib.)	μmole of (I) per 1 act. u. of E (50% inhib.)
I	778	1	2	0,04
II	780	1,5	3	0,06
III	686	76	200	3,69
IV	924	19	37	0,68
V	550	15,8	52	0,96
VI	528	105	360	6,6
$CH_3(CH_2)_{10}CH_2OSO_3Na$	302	65	390	7,2

TABLE 3. Action of Protecting Agents (M) on the Inhibition of LIV by Halistanol Sultate*

M	Activity of the L-IV, %			
	S*+L-IV	S+LIV+M	S+LIV+I	S+LIV+I+M
Glucose	100	100	16	16
N-Acetylglucosamine	100	100	14	14
p-Nitrophenyl D-glucoside	100	100	10	10
Laminarin	100	25	8	8

*A colored lichenin was used as the substrate (S).

To determine the influence of the elements of the structure of the inhibitors on the efficacy of inhibition, we synthesized a series of substances (Table 2). Substances (III), (IV), and (V) were obtained by modifying (I) and (II) [21], and (VI) by sulfating a steroid alcohol from Halichondriidae sp. The greatest inhibiting capacity with respect to the endo-(1 → 3)-β-D-glucanase L-O was possessed by the natural compounds (I) and (II) (Table 2). The conversion of a hydrophobic side chain of a steroid compound into a hydrophilic one decreased the efficacy of inhibition by two orders of magnitude [compare the concentrations of (I) and (III) in Table 2]. A decrease in the number of sulfate groups in the substance (V, Table 2) and in the number of sulfate and hydroxy groups (VI, Table 2) also appreciably lowered the capacity for inactivating the endolaminarinase L-O. The inhibition action of the compounds (III) and (VI) became comparable with that of SDS. The acylation of (III) with a fatty acid increased the hydrophobicity of the side chain [see compound (IV) in Table 2] and restored the capacity of the derivative for inhibiting L-O to some degree. Thus, it can be seen that the ratio between the hydrophobic and hydrophilic moieties of the molecules of (I) and (II) plays a definite role in the manifestation of the inhibiting effect.

Special investigations showed that the enzyme-inhibitor complex (EI) formed on the interaction of (I) with endolaminarinases was irreversible, i.e., the bond of the enzyme and the inhibitor was stable under the conditions of an enzymatic reaction.

Thus, the use of laminarin [the substrate of endo-(1 → 3)-β-D-glucanases], p-nitrophenyl β-D-glucoside, N-acetyl-D-glucosamine, and glucose (acceptors in the transglycosylation reaction catalyzed by the enzymes investigated [22, 23]) for protecting L-IV from the action of

(I) was unsuccessful (Table 3). In this case, the enzymatic reaction was recorded from the appearance of the products of the hydrolysis by the laminarinase L-IV of a colored lichenin [a (1 → 3), (1 → 4)-β-D-glucan], which can also serve as a substrate for the endo-(1 → 3)-β-D-glucanases investigated [24].

Gel filtration on Sephadex G-50 and on Bio-Gel P-100 in 0.05 M acetate buffer and at increased ionic strength (0.5 M solution of sodium chloride) of a mixture of L-IV and (I) with completely suppressed enzymatic activity did not restore this activity. Consequently, under the conditions that are the optimum for the occurrence of the enzymatic reaction and under even more severe conditions, the enzyme-inhibitor complex does not break down, and inhibitors (I) and (II) can be regarded as irreversible.

Attention is attracted by the considerable increase in the molecular weight of the complex as compared with the free enzyme (mol. wt. of L-IV 22,000). On Sephadex G-50, the enzyme-inhibitor complex issues with the free volume (mol. wt. >30,000). The molecular weight of the EI was determined more accurately (as about 45,000) with the aid of gel filtration on Bio-Gel P-100.

Calculations (see the Experimental part and Table 2) have shown that 2 moles of (I) and 3 moles of (II) are sufficient for the 50% inactivation of 1 mole of L-O, and 3 moles of (I) cause 50% inhibition of 1 mole of L-IV.

If a mixture of enzyme and inhibitor in which the activity of the enzyme has not been completely suppressed is subjected to gel filtration, again a complex with a molecular weight likewise about 45,000 is formed. If, however, a threefold excess of inhibitor (I) in comparison with the amount necessary for complete suppression of the activity of the enzyme is taken, the complex formed under such conditions issues from Bio-Gel P-100 with the free volume.

It is likely that the increased concentration of the inhibitor causes the aggregation of the enzyme.

EXPERIMENTAL

Enzymes. The endo-(1 → 3)-β-D-glucanases I-III and L-IV from *S. sachalinensis*, the L-O and amylase from *Ch. albidus*, the L-V and PU from *Patinopectin* sp., the endocellulase from *L. mandshurica*, and the exo-(1 → 3)-β-D-glucanases from *E. maakii* were obtained by methods described in the literature [13-15, 18], while the α- and β-amylases, the takadiastase, and the trypsin were commercial preparations.

Substrates. Laminarin from *L. cycharioides* and pustulan – a β-(1 → 6)-glucan from *Umbillicaria rossica* – were obtained as described in [25, 26]. The colored lichenin, carboxymethylcellulose, amylopectin, and benzoylarginine p-nitroanilide were commercial preparations.

Inhibitors. The marine invertebrates investigated for their inhibiting power were collected on expedition voyages Nos. 12 and 13 of the Scientific Research Vessel "Professor Bogorov." The freshly collected animals were completely homogenized in 0.9% sodium chloride solution (ratio by weight 1:3). The homogenates were centrifuged and the supernatant was freeze-dried. Halistanol sulfate and sokotrasterol sulfate were obtained as described previously [17] from specimens of the sponge Halichondriidae sp. (northwestern part of the island of Madagascar, December, 1981) and of Halichondriidae gen. sp. (island of Sokotra, January, 1982), respectively. Inhibitors (III), (IV), (V), and (VI) were obtained as described in [21].

Standard Procedure for the Inhibition of Carbohydrases. A mixture (0.2 ml) containing 20 μg of a freeze-dried sample under investigation for its inhibiting power or from 1-400 μg of inhibitors and from $2 \cdot 10^{-2}$ to $5 \cdot 10^{-2}$ units of carbohydrase activity in acetate buffer, pH 5.3 (1 unit = 1 μmole of glucose/min) was kept at 25°C for about 15 min and then, to detect residual carbohydrase activity, solutions of the appropriate substrates were added. The final concentration of the substrates was 1 μg/ml of incubation mixture. The mixture (1 ml) was incubated for 15 min and the activities of the enzymes were recorded by Nelson's method [27]. As the substrates for the laminarinases we used laminarin, for the amylases amylopectin, for the cellulases carboxymethylcellulose, for the pustulinases pustulan, and for trypsin benzoylarginine p-nitroanilide.

Protection of L-O from the Action of (I). Mixtures (1 ml) containing the enzyme ($6 \cdot 10^{-2}$ units), the protecting agent (4 mg), (I) (3 μg) and the substrate (2 mg) in various combinations (Table 3) were incubated at pH 5.3. The colored lichinen was used as the substrate.

The reaction was stopped by the addition of three volumes of acetone and, after the precipitate had been centrifuged off, the solutions were photometered at 490 nm.

Determination of the Type of Inhibition. Solutions (1 ml) of the inhibitor (I) (100 μg), of L-O (1 mg), and of a mixture of (I) and L-O (100 μg and 1 mg, respectively) in which the activity of the L-O had been 50% suppressed were subjected to gel filtration on a column of Sephadex G-50 (3 × 50 cm) in 0.05 M acetate buffer (pH 5.3) at the rate of 18 ml/h. The gel filtration of mixtures of (I) and L-IV with completely suppressed activity and with a threefold excess of (I) was carried out on a column (1 × 90 cm) of Bio-Gel P-100 in 0.05 M acetate buffer and 0.5 M sodium chloride (pH 5.3).

The fractions were analyzed for the presence of enzyme by the addition of a solution of laminarin to 0.01 ml of a fraction (incubation at 25°C for 30 min) and recording the enzymatic reaction by Nelson's method. The inhibiting capacities of the fractions were tested by using L-O and then performing the reaction by the standard procedure. The yield of protein was recorded from the absorption at 280 nm. As standards for determining the molecular weight of the enzyme-inhibitor complex we used bovine albumin (mol. wt. 67,000), L-IV (mole. wt. 22,000), and chymotrypsin (mol. wt. 25,000).

Calculation of the E:I Ratio in an Enzyme-Inhibitor Complex. a) Since 1 mg of L-O with a molecular weight of 20,000 was 50% inhibited by 100 μg of (I) with a molecular weight of 780, a 50% inhibition of the activity of 1 mole of L-O requires 2.5 mole of (I);

b) $3 \cdot 10^{-2}$ units of L-O with a molecular weight of 20,000 was 50% inhibited by 1 μg of (I) (Table 2). The purified enzyme preparation contained three units of specific activity per 1 mg. Consequently, the 50% suppression of one activity unit of L-O requires the addition of 33 μg of (I) [1 mg of L-O and 99 μg of (I)]. From these facts we obtain the value E:I = 1 mole of L-O: 2.5 mole of (I), agreeing with the calculation by the method of a).

SUMMARY

1. A source of natural inhibitors of endo-(1 → 3)-β-D-glucanases has been found in sponges of the family Halichondriidae. The inhibitors are sulfated steroids: halistanol sulfate and sokotrasterol sulfate.

2. The ratio between the hydrophobic and hydrophilic moieties of the inhibitor molecule plays a decisive role in the manifestation of the inhibiting effect.

3. The action of the inhibitors is irreversible.

4. The compositions of the enzyme-inhibitor complexes have been determined.

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