NATURAL INHIBITORS OF β-1,3-GLUCANASES.

HIGH-MOLECULAR-WEIGHT INHIBITOR FROM THE TROPICAL SPONGE *Myrmekioderma granulata*

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UDC 547.964

A systematic search has been made for natural inhibitors of β-1,3-glucanases among marine invertebrates of the world ocean. It has been shown that sponges, alcyonarians, and ascidians are promising sources of inhibitors. A high-molecular-weight inhibitor specific to the β-1,3-glucanases of marine molluscs has been isolated from the tropical sponge M. granulata.

In many cases, the action of various chemical substances – toxins, drugs, etc. – on living systems is explained by the inhibition of enzymes. Inhibitors possessing a high specificity are being used more and more widely for the study of the mechanism of the action of enzymes and for determining the structure of their active centers.

Great interest is being aroused by carbohydrase inhibitors isolated from natural sources. The best-investigated are natural amylase inhibitors [1]. All known carbohydrase inhibitors can be divided into two groups: low-molecular-weight substances, which include peptides [2], oligosaccharides [3, 4], glycopeptides [5], and antibiotics; and high-molecular-weight compounds – proteins [1] and glycoproteins [7]. There are no reports in the literature on natural inhibitors of β-1,3-glucanases.

A source of natural inhibitors of these enzymes has recently been discovered: sponges of the family Halichondriidae. Substances inactivating β-1,3-glucanases have been identified: halistanol sulfate and sokotrasterol sulfate [8], and a number of derivatives of these compounds have been obtained for investigating the influence of elements of their structure on the efficacy of inhibition [9].

We have continued a systematic investigation of the inhibiting capacity of extracts of marine invertebrates of the Indian and Pacific Oceans. A high-molecular-weight inhibitor of β-1,3-glucanases has been isolated from the tropical sponge M. granulata and characterized.

The presence of inhibitors of endo-β-1,3-glucanases (Lo, L-IV, and L-V) in extracts of marine invertebrates was established from their capacity for inhibiting the interaction of the enzymes with their substrate – laminarin. Their inhibiting capacity was characterized by the residual activity. Aqueous and aqueous ethanolic extracts of the tissues and organs of marine animals have been tested. A comparison of the results obtained on two types of extracts permitted a preliminary assignment of an inhibitor to high- or low-molecular-weight compounds (in the case of inhibition by an aqueous extract or by an ethanolic extract, respectively). More than 300 specimens of tropical animals were tested for the presence of inhibitors:

Spongia (125 specimens);

Coelenterata: Alcyonacea (100 specimens), Stolonifera (4 specimens), Gorgonacea (5 specimens), Pennatulacea (2 specimens), Scleractinia (2 specimens), Actinaria (9 specimens);

Mollusca: Gastropoda (13 specimens), Bivalvia (6 specimens);

Echinodermata: Asteroidea (6 specimens), Holothuroidea (3 specimens), Crinoidea (1 specimen);

Annelida (5 specimens);

Pacific Ocean Institute of Bioorganic Chemistry [TIBOKh], Far Eastern Branch, Academy of Sciences of the USSR, Vladivostok. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 566-572, July-August, 1988. Original article submitted October 27, 1987; revision submitted January 19, 1988.

0009-3130/88/2404-0481 $12.50

Chordata: Ascidiae (44 specimens).

A comparative investigation has shown that the most promising sources of inhibitors of β-1,3-glucanases are sponges. Out of 125 species, 56 – i.e., ~45% – lowered the activity of β-1,3-glucanases to 0 or 50%. In the Ascidiae, of a comparatively small number of specimens (44 species), 17 (38%) exhibited an inhibiting action. The Alcyonacea were analyzed fairly widely – 100 species, 27% of which exhibited an inhibiting effect. Extracts of five species of Gorgonacea had no influence on the enzymatic activity – this number of species is clearly insufficient for any conclusions whatever to be drawn. Likewise, only a small number of Actinaria – nine – was analyzed, although three of them exhibited an inhibiting capacity. One of the species of Scleractinia contained substances causing a considerable decrease in the activity of the glucanase Lo. An extract of a scleractinians collected on the reefs of Mozambique completely inhibited all three β-1,3-glucanases. Extracts of three species of holothurians investigated were inactive. Individual glycosides from the holothurian Bohadschia argus [bohadschioside A (3) and bohadschioside B (4), 50 μg in a sample] did not affect the activity of glucanases.

Aqueous extracts of all species of starfish contained a large amount of reducing sugars and in comparison with a control they exhibited an inhibiting effect. The sums of the asterosaponins from these species had no influence on the activity of the enzymes in concentrations of up to 50 μg per sample. Of the five species of Annelida, one exhibited inhibiting activity relative to Lo.

It is difficult to expect the presence of inhibitors in molluscs having their own β-1,3-glucanases as digestive enzymes. In actual fact, in extracts of five species of Gastropoda and five bivalve molluscs no inhibiting action was detected. It is interesting that extracts of three nudibranch molluscs (out of the eight analyzed) lowered the activity of β-1,3-glucanases. The intrinsic β-1,3-glucanase activity in these species was considerably lower than in the others.

To characterize the most effective inhibitors from their molecular weights, we used gel filtration on a calibrated column of Sephadex G-75. An inhibitor was detected from the inactivation of the β-1,3-glucanase L-IV. A wide set of inhibitors with different molecular weights was detected in the materials investigated.

A high-molecular-weight inhibitor from the tropical sponge Myrmekioderma granulata, an extract of which completely inactivated the β-1,3-glucanases Lo, L-IV, and L-V was studied. The inhibitor, which was isolated by extracting the sponge with water and by gel filtration of the preparation on Sephadex S-300, was eluted as a single symmetrical peak on other types of supports (Bio-Gel P-150, Sepharose 4B). The preparation was stored in the lyophilized form. The specificity of the action of the preparation was checked on a number of enzymes (Table 1).

The inhibitor revealed a specificity for endo-β-1,3-glucanases from marine molluscs. It acted most effectively on Lo. As investigations on extracts and individual compounds showed, this enzyme is the one among the β-1,3-glucanases that is most susceptible to the action of inhibitors. It is probable that features of the structure of the enzyme – lower content of disulfide bonds and carbohydrates than in L-IV – make the molecules less resistent to various actions. The concentration of inhibitor causing 50% decrease in activity for two isoenzymes – the endo-β-1,3-glucanases L-III and L-IV – not agreeing in certain features of the mechanism of their action differed by a factor of 10 (see Table 1). The inhibitor did not act on other carbohydrases in concentrations up to 50 μg or on the proteinases and DNase in concentrations up to 100 μg. The influence of higher concentrations was of no interest, since it could be connected with nonspecific interactions. The fact that it activated the exo-β-1,3-glucanase L-II from the terrestrial mollusc E. maaki was observed.

The inhibitor that we had isolated was a glycoprotein with a molecular weight of more than 100,000 containing ~40% of carbohydrates. The carbohydrate component consisted of residues of arabinose, galactose, and glucose in various ratios. A deficiency of certain amino acids is not infrequently found in protein carbohydrase inhibitors: In the α-amylase from Streptomyces griseosporus VM-25 there are no lysine and methionine residues [10], and in the basic protein inhibitor of α-amylases from wheat there are no phenylalanine and histidine [11]. The high content of carbohydrates in the high-molecular-weight inhibitor of β-1,3-glucanases made it difficult to perform amino acid analysis and to calculate the

TABLE 1. Action of the Inhibitor on Enzymes

Enzyme, source of the enzyme	Substrate	Conc. of inhibitor causing 50% inhibition, μg per sample	Activation, %
Endo-β-1,3-glucanase Lo *Chlamys albidus*	Laminarin	0.5	
Endo-β-1,3-glucanase L-IV *Spisula sachalinensis*	Laminarin	2.0	
Endo-β-1,3-glucanase L-III *Spisula sachalinensis*	Laminarin	20	
Endo-β-1,3-glucanase L-V *Patinopecten* sp.	Laminarin	2-3	
Endo-β-1,3-glucanase from antarctic krill	Laminarin	No inhibition up to 50 μg	
Endo-β-1,6-glucanase R-IV *Spisula sachalinensis*	Pustulan	No inhibition up to 50 μg	
Exo-β-1,3-glucanase L-II *Eulota maakii*			5 μg – 120% 20 μg – 133% 50 μg – 171%
α-Amylase from porcine pancreas	Amylopectin	No inhibition up to 50 μg	
β-Amylase from barley	Amylopectin	No inhibition up to 50 μg	
Cellulase from *Spisula sachalinensis*	CM-Cellulose	No inhibition up to 100 μg	
Trypsin	Dyed casein	No inhibition up to 100 μg	
Chymotrypsin	Dyed casein	No inhibition up to 100 μg	
DNase from crab hepatopancreas	DNA	No inhibition up to 100 μg	

amino acid composition, but it was established that the inhibitor contained a complete set of amino acids.

The inhibitor had a typical protein spectrum with a maximum at 276 nm which changed when the tryptophan residues were oxidized with N-bromosuccinimide. In this process, the inhibiting activity was retained. The inhibitor formed a strong complex with the β-1,3-glucanase L-IV, the gel filtration of which on Bio-Gel P-150 in 0.05 M succinate buffer, pH 5.2, with increased ionic strength (0.5 M NaCl), did not restore the enzymatic activity.

Incubation of the inhibitor with trypsin, chymotrypsin, and pronase for 10 h did not lead to a loss of inhibiting capacity. A quantitative experiment was performed. The inhibitor was treated with pronase for 18 h, cleavage being monitored by the ninhydrin method. The reaction products were separated by gel filtration, and the inhibiting capacity and the protein and carbohydrate contents in a sample were determined. The experiment showed that the elimination of 30% of the protein scarcely changed the inhibiting capacity of the glycoprotein. However, after lyophilization such a preparation lost its activity, and its stability in solution was considerably lowered. Its inhibiting capacity was halved when the carbohydrate moiety of the inhibitor was oxidized with sodium periodate under mild conditions (0.02 M sodium periodate, 25°C, 30 min). This fact confirmed the great importance of the carbohydrate component of the inhibitor in interaction with the enzyme. A similar result was obtained for a glycoprotein inhibitor of α-amylase isolated from beans. Oxidation of the carbohydrate moiety of the inhibitor with sodium periodate completely deprived it of the capacity for inhibiting α-amylase [12].

The inhibitor withstood heating at 70°C for 10 min without loss of activity and was stable in the pH interval from 3.5 to 6.5 (Fig. 1). It exhibited lectin activity. No specificity toward readily available commercial monosaccharides was detected. Furthermore, the monosaccharides from the components of the L-IV molecule (glucose, mannose, and galactose, N-acetylglucosamine, N-acetylgalactosamine) did not protect the L-IV from the inactivating

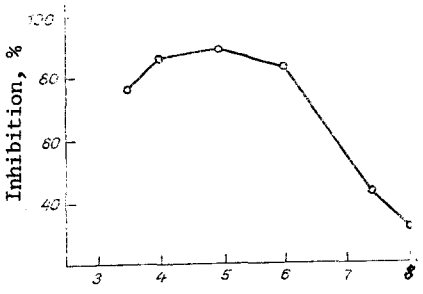

Fig. 1

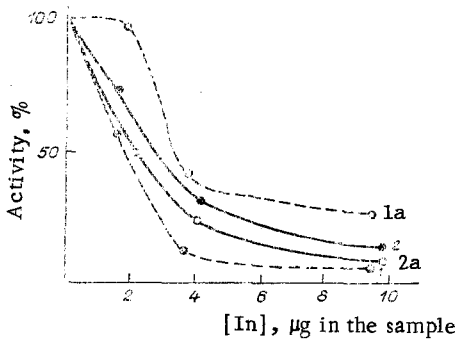

Fig. 2

Fig. 1. Influence of the pH on the activity of the inhibitor.

Fig. 2. Inhibition of the β-1,3-glucanase L-IV modified at a lysine residue (1) and modified at two histidine residues (2) in comparison with corresponding samples of the native enzyme (1a, 2a).

action of the inhibitor. Preincubation of the inhibitor with laminarin did not influence the effectiveness of inhibition. The inhibiting capacity is probably not connected with the lectin properties of the inhibitor.

To determine the participation of the individual amino acid residues of the β-1,3-glucanase L-IV in binding with the natural inhibitor we used the method of chemical modification. β-1,3-Glucanase L-IV was modified at one lysine residue with pentane-2,4-dione. We had shown previously that under these conditions the most accessible free amino group of the enzyme is modified with no change in its capacity for the hydrolysis and transglycosylation reactions [13]. Since at pH 7.0 the stability of the inhibitor falls appreciably, the β-1,3-glucanase L-IV modified at this pH was transferred into 0.05 M acetate buffer, pH 5.2, by gel filtration in Bio-Gel P-6 with the simultaneous elimination of the excess of modifying agent. An examination showed that the spectrum of the modified enzyme did not change at pH 5.2 in the course of an hour – the time for performing the inhibition experiment. Inhibition was carried out by the native L-IV and that modified at a lysine residue (Fig. 2). Modification partially protected the enzyme from the inactivating action of the inhibitor. It may be assumed that the pentane-2,4-dione-modified lysine residue takes part in the binding of the L-IV molecule with the inhibitor. We also investigated the behavior of the enzyme in the molecule of which two histidine residues had been alkylated with diethyl pyrocarbonate, with the retention of 40% of the initial activity. The inhibitor inactivated the modified L-IV at the same rate as the native enzyme (see Fig. 2). Apparently, the histidine residues that were modified do not take part in the interaction of the L-IV with the inhibitor.

EXPERIMENTAL

Enzymes. The endo-β-1,3-glucanases L-III and L-IV, the endo-β-1,6-glucanase P-IV, and the cellulase from S. sachalinensis, the endo-β-1,3-glucanase from Ch. albidus, the endo-β-1,3-glucanase from antarctic krill, and the exo-β-1,3-glucanase from E. maakii were obtained by known methods [14-16]. Crab hepatopancreas DNase was supplied by N. I. Menzorova (TIBOKh), and the α- and β-amylases, trypsin, chymotrypsin, and pronase were commercial preparations.

Substrates. Laminarin from Laminaria cichorioides and pustulan from Umbillicaria rustica were isolated by published methods [17, 18]. Amylopectin, dyed casein, and CM-cellulose were commercial preparations.

Inhibitors. Marine invertebrates were collected during cruise No. 20 of the Scientific Research Vessel Professor Bogorov. The animals were homogenized and extracted with water (ratio by weight 1:3). The homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatant was used for the investigations. For prolonged storage, the extracts were lyophilized, and the sponges and alcyonarians were freeze-dried in comminuted form. Individual glycosides from holothurians and the total asterosaponins from starfish were supplied by É. V. Levina (TIBOKh).

Inhibition Procedure. A mixture of 50 µl of the solution to be investigated for its inhibiting capacity and 50 µl of a solution of the enzyme containing ~$2 \cdot 10^{-2}$ units of carbohydrase activity (1 unit = 1 µmole of glucose/min) was kept at 25°C for 10 min, and then 400 µl of a solution of the appropriate substrate (1 mg/ml) was added. The sample was incubated at 25°C for 15 min, and its residual activity was then determined. The activities of the carbohydrases were determined from the increase in the amount of reducing sugars by Nelson's method [19]. Proteolytic activity was determined spectrophotometrically (λ 400 nm) from the liberation of the dye from the dyed casein, and the DNase activity as described in [20].

The molecular weights of the inhibitors in the extracts were evaluated by the gel filtration of 50 mg of freeze-dried total preparation through a calibrated column of Sephadex G-75 (1.5 × 30)* equilibrated with 0.05 M acetate buffer containing 0.1 M NaCl. The inhibitor was detected from its effect on the activity of L-IV.

The molecular weight of the inhibitor from M. granulata was determined by gel filtration through a column of Bio-Gel P-150 (1.5 × 30).* As standards were used catalase (M 240,000), albumin (67,000), and DNP-alanine (255.2).

Purification of the Inhibitor from M. granulata. On a column of Sephadex S-300 (1.5 × 30)* equilibrated with 0.05 M acetate buffer, pH 5.2, was deposited 40 mg of a freeze-dried extract of M. granulata dissolved in 5 ml of buffer. The rate of elution was 15 ml/h and the fraction volume 2.5 ml. The inhibiting capacity of the fractions was detected by the standard procedure using the β-1,3-glucanase L-IV, and protein was measured from the absorption at 280 nm.

Characteristics of the Reversibility of the Complex of the Inhibitor with L-IV. A solution of inhibitor (1 mg/ml) was added to 0.5 ml of L-IV (1 mg/ml) until the activity of the enzyme had completely disappeared. The complex was deposited on a column of Bio-Gel P-150. The fractions obtained on separation were checked for the presence of β-1,3-glucanase and inhibitor.

Protein was determined by Lowry's method [21]. The total amount of sugars was estimated by the phenol/sulfuric acid method [22]. The monosaccharide composition was analyzed by the GLC of the polyolacetate derivatives on a Pye-Unicam instrument with a flame-ionization detector in glass columns containing Gas Chrom Q impregnated with 3% of QF-1.

Spectrophotometric measurements were performed on a Varian Carey-219 spectrophotometer. The reaction of the inhibitor with N-bromosuccinimide was carried out in the cell of the spectrophotometer. To a solution of the inhibitor (1 mg/ml) in acetate buffer, pH 5.2, 10-µl portions of a 10^{-2} M solution of N-bromosuccinimide were added successively. After each addition the change in the spectrum and the inhibiting capacity were checked.

The lectin activity was determined from the capacity for agglutinating erythrocytes [23].

Inhibition of L-IV in the Presence of Monosaccharides. A mixture containing 6 µg of inhibitor and 10 µg of a monosaccharide (glucose, galactose, mannose, N-acetylglucosamine, or N-acetyl galactosamine) in 50 µl was kept with 50 µl of L-IV (2×10^{-2} units) for 10 min. Then 400 µl of a laminarin solution (1 mg/ml) was added. After incubation at 25°C for 15 min, the residual activity was determined in comparison with a control containing the substrate and the monosaccharide.

Modification of the Lysine Residues in the L-IV Molecule. To 1.5 ml of a solution of L-IV in 0.1 M phosphate buffer, pH 7.0, was added 50 µl of pentane-2,4-dione. The mixture was kept in the dark at 4°C for 15 min. The excess of reagent was separated on a column of Bio-Gel P-6 (1 × 5)* equilibrated with 0.05 M acetate buffer, pH 5.2. The degree of modification was calculated from features of the UV spectrum [24].

Modification of Histidine Residues in the L-IV Molecule. To the cell of a spectrometer containing 2 ml of a solution of L-IV (4 mg/ml) in phosphate buffer pH 6.0, was added 1 mg of diethyl pyrocarbonate in 10 µl of absolute ethanol. The degree of modification was monitored spectrophotometrically [25]. After a value of the absorption at 240 nm, corresponding to the modification of two histidine residues, had been reached, the sample was

*No units given; presumably cm – Publisher.

deposited on a column of Bio-Gel P-6 to eliminate the excess of modifying reagent. Increasing amounts of inhibitor were added to samples of the native and the modified L-IV. The residual activities were determined. To each modified sample corresponded a sample of native L-IV containing an equal number of activity units.

SUMMARY

1. A systematic search for natural inhibitors of β-1,3-glucanases has been made among marine invertebrates of the world ocean. It has been found that sponges, alcyonarians and ascidians are promising sources of inhibitors.

2. A high-molecular-weight inhibitor from the tropical sponge M. granulata specific to β-1,3-glucanases of marine molluscs has been characterized.

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