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Proteins of Brown Seaweeds as Inhibitors of Endo-1→3-β-D-glucanases of Marine Invertebrates

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Abstract—It has been found that aqueous–ethanol extracts of brown seaweeds contain substances inhibiting endo-1→3-β-D-glucanases, the digestive enzymes of marine mollusks. The inhibitors were detected in 14 of 21 brown seaweeds investigated. An irreversible protein inhibitor possessing high specificity toward endo-1→3-β-D-glucanases of marine mollusks was isolated from the brown seaweed *Laminaria cichorioides*. As determined by gel filtration, the molecular mass of the inhibitor is 46 kD. The value of $[I]_{50}$ (10^{-8} M) for the inhibitor is comparable with the corresponding value for natural inhibitors of amylases from terrestrial plants. The results of chemical modification indicated that tryptophan, glutamic acid, aspartic acid, histidine, and probably tyrosine residues are important for the interaction of the inhibitor with the enzyme.

Key words: brown seaweeds, *Laminaria cichorioides*, endo-1→3-β-D-glucanase, amylase, protein inhibitor, glutamic acid

The basis for the action of many of toxic agents and medicines in living organisms is the inhibition of enzymatic processes. Inhibitors possessing high specificity are used for establishing the mechanism of action of enzymes and the structure of the active sites. Such inhibitors are of wide use in medicine, pharmacology, and toxicology.

Of special interest are natural inhibitors of carbohydrases. Natural inhibitors of amylases and glycosidases have received the most study [1-5]. Inhibitors of amylases are used in the treatment of obesity, diabetes, caries, and diseases of the gastrointestinal tract [5]. The role of inhibitors isolated from cereals in relationships between plant and insects or herbivores is studied [4]. A glycoreglycolipid that potently inhibits yeast β-glucosidase was isolated from the brown seaweed *Hizikia fusiforme* [3].

Laminarans (1→3;1→6-β-D-glucans) are storage polysaccharides of brown seaweeds that are widely distributed in marine organisms; they fulfill functions analogous to those of amylose in terrestrial plants [6]. It is logical to suppose that seaweeds, like terrestrial plants [4], possess defenses against attack by organisms that forage upon them. The defense could involve the synthesis of substances inhibiting the enzymes of the digestive tract of marine animals (for example, 1→3-β-D-glucanases hydrolyzing laminaran).

Some inhibitors of endo-1→3-β-D-glucanases have been detected in some species of marine invertebrates [7] and brown seaweeds [8]. The content of substances with such properties in green and red seaweeds and water plants was found to be very low [8].

The goal of the present work was to study the distribution of inhibitors of endo-1→3-β-D-glucanases in far-eastern and tropical brown seaweeds as well as the composition and properties of inhibitors isolated from *Laminaria cichorioides*.

MATERIALS AND METHODS

Enzymes. Endo-1→3-β-D-glucanases LIV and L_0 from the marine mollusks *Spisula sachalinensis* and *Chlamys albidus*, respectively, endo-1→6-β-D-glucanase PIV from *S. sachalinensis*, exo-1→3-β-D-glucanase LII from the terrestrial mollusk *Eulota maakii*, exo-1→3-β-D-glucanase from eggs of the sea urchin *Strongylocentrotus intermedius*, endo-1→3-β-D-glucanase from the marine bacterium *Alteromonas* sp. [9], exo-1→3-β-D-glucanase, amylase, and cellulase of the marine fungus *Chaetomium indicum*, β-D-glucosidase from the marine fungus *Aspergillus flavipes* [10], α-D-mannosidase and 1→3-β-D-glucanase from soybean leaves, and α-D-galactosidase from the marine bac-

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terium *Pseudoalteromonas* KMM [11] were from the collection of the Enzyme Chemistry Laboratory of the Pacific Institute of Bioorganic Chemistry. Yeast β-D-glucosidase, trypsin, and pronase were purchased from Sigma (USA).

Substrates. The sources of laminaran and pustulan were the brown seaweed *L. cichorioides* and the lichen *Umbillicaria russica*, respectively. Polysaccharides were isolated as described in [12, 13]. *p*-Nitrophenyl-β-D-glucopyranoside (*p*NO_2Ph-Glc), *p*-nitrophenyl-α-D-mannanopyranoside (*p*NO_2Ph-α-D-Man), *p*-nitrophenyl-α-D-galactopyranoside (*p*NO_2Ph-α-D-Gal), and N-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) were purchased from Sigma.

Reagents. N-Bromosuccinimide, acetylimidazole, diethylpyrocarbonate, CME-carbodiimide, glycine methyl ester, *p*-chloromercuribenzoate, EDTA, sodium azide, BSA, lysozyme, ovalbumin, and carriers for chromatography were from Sigma.

Methods. Neutral sugars were determined by the phenol–sulfuric acid method [14]. Reducing sugars were determined by the Nelson method [15]. ^{13}C-NMR spectra were recorded using a WM-250 NMR-spectrometer (Bruker) in D_2O at 40°C. Methanol (δ = 50.15 ppm in reference to tetramethyl silicane) was used as an internal standard.

The monosaccharide composition of the fractions was determined after hydrolysis by 2 N HCl (100°C, 2 h) using a IC-5000 Biotronik carbohydrate analyzer (Germany). A column (385 × 3.2 mm) with Durrum DA-X8-11 resin was used for the analysis. The 2,2′-bicinchoninate method used for detection [16]. The amino acid composition of the fractions after hydrolysis by 6 N HCl (100°C, 48 h) was determined using a Biotronik amino acid analyzer (Alha Plus LKB 4151; 4.5 ± 0.5 μ, 200 × 4.5 mm column with Ultrapac). A Jeol-JLC-6AH carbohydrate analyzer (Biogel P-2; 1 × 100 cm) was used for determination of oligosaccharide composition; the orcin–sulfuric acid method was used for detection.

Obtaining extracts of seaweeds. Seaweeds were collected during expeditions of the research ship "Akademik Oparin" (Okhotsk Sea, Sea of Japan, and Indian Ocean) or at the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry (Khasan Region of the Primorsky Krai) in August–September 1992, 1996-1999 (specimens of *L. cichorioides* were collected in different seasons). Fresh seaweeds were milled and extracted with ethanol (1 liter ethanol per kg seaweed) at room temperature for 2 weeks. The extracts were evaporated under vacuum, and the dry residue was used for further analysis.

Standard procedure of inhibition. The solution of inhibitor (10 μl) in 0.05 M acetate buffer, pH 5.4, containing 0.5-100 μg of the substances and 10 μl of the enzyme solution (0.01 U) were incubated at 37°C for 10 min. After the addition of 480 μl of the substrate (1 mg/ml) and 30-min incubation, the residual activity of the enzyme was determined by the corresponding method.

Enzyme assay. The activity of the enzymes hydrolyzing polysaccharides (amylopectin, CM-cellulose, laminaran, pustulan) was determined based on the increase in the content of reducing sugars by the Nelson method [15]. The activity of proteinases (with BAPA as a substrate) and glycosidases (with *p*-nitrophenyl derivatives of the corresponding sugars as substrates) was calculated from the amount of *p*-nitrophenol released [18]. The amount of the enzyme catalyzing the formation of 1 μmole of glucose or *p*-nitrophenol per min under the assay conditions was taken to be unit of catalytic activity.

Isolation of inhibitors from *L. cichorioides*. Fresh seaweed was extracted with ethanol (1 : 1 w/w) at room temperature for 2 weeks. Aqueous–ethanol extract (I) was treated with chloroform several times. The layer containing chloroform was separated and discarded. The active aqueous–ethanol fraction (II) was concentrated under vacuum. After addition of 80%-ethanol, the supernatant, which contains inhibiting substances, was evaporated under vacuum. The dry residue (III) was dissolved in water and placed on the column with hydrophobic resin polychrome-1 (polytetrafluoro-ethylene; 160 × 70 mm) (fraction IV). The column was eluted with water (fraction IV-I) until sugar completely disappeared. Further elution was performed using a stepwise gradient of ethanol in water: 5% (fraction IV-II), 10, 20, and 40% (fraction IV-III) of ethanol. The content of inhibitor in the fraction was determined using the standard procedure. The active fractions IV-I, IV-II, and IV-III were lyophilized.

Separation of the components of fraction IV-II. The dry substances of fraction IV-II (~10 mg) were dissolved in water and placed on the column with Biogel P-30 (120 × 10 mm). The column was eluted with water with the rate of elution of 0.3 ml/min. Protein in fractions was registered spectrophotometrically at 280 nm. Carbohydrates were determined by the phenol–sulfuric acid method. The inhibiting action was characterized using the standard procedure. The active fractions P-30-1 and P-30-2 were lyophilized.

Effect of the duration of preincubation on the inhibiting action (fraction IV-II). The mixture of solutions of endo-1→3-β-D-glucanase LIV and inhibitor (100 μl each) was incubated at 37°C. Aliquots (20 μl) were withdrawn at intervals (0, 5, 10, 15, 20, 25, and 30 min) to determine the residual enzymatic activity.

Effect of the substrate concentration on the inhibiting action (fraction III). The inhibiting action was determined using the standard procedure. The concentration of the substrate (laminaran) was varied from 0.5 to 10 mg/ml.

Effect of pH on the stability of the inhibitor (fraction IV-II). The solution of inhibitor (20 μg in 100 μl) in 0.05 M citrate–phosphate buffer of certain pH (in the range from 3.5 to 8.0) was incubated at 4°C. Aliquots (10 μl) were withdrawn at intervals (15 min, 1, 2, and 3 days) to determine the degree of inhibition.

Effect of temperature on stability of the inhibitor (fraction IV-II). The solution of inhibitor (20 μg in 200 μl)

in 0.1 M acetate buffer, pH 5.2, was incubated at −15, +4, +20, +37, +50, and +100°C over a period of 1-365 days. Aliquots (10 μl) were withdrawn to determine the degree of inhibition.

Determination of specificity of the inhibitor (fraction IV-II). To determine the specificity of the inhibitor, we used the following enzymes: endo-1→3- and 1→6-β-D-glucanases LIV and RIV from *S. sachalinensis*, exo-1→3-β-D-glucanase LII from *E. maakii*, exo-1→3-β-D-glucanase from *S. intermedius*, exo-1→3-β-D-glucanase, amylase, and cellulase from marine fungus *C. indicum*, β-D-glucosidase from marine fungus *A. flavipes*, α-D-mannosidase and 1→3-β-D-glucanase from soybean leaves, α-D-galactosidase from marine bacterium *Pseudoalteromonas* KMM, trypsin, and pronase. The enzyme solution (10 μl, 0.01 U) was added to 10 μl of the solution of inhibitor in 0.1 M acetate buffer (pH 5.2 and 7.5 for pronase and pH 8.0 for trypsin). After 10-min incubation, the solution of the corresponding substrate (0.48 ml) was added and the residual activity was determined using the standard procedure.

Treatment of the inhibitor (fraction IV-II) by the enzymes. 1) Exo-1→3-β-D-glucanase from *C. indicum* or yeast β-glucosidase (0.05 U) was added to 1 ml of the solution of inhibitor (fraction IV-II; 10 mg) in 0.1 M acetate buffer, pH 5.2. After 1-h incubation, the increase in reducing sugars was determined by the Nelson method [5]. Then the products of the reaction were analyzed using a Jeol-JLC-6AH or Biotronik analyzer. The inhibitory action of fraction IV-II after the treatment by the enzyme was estimated by the standard procedure.

2) Pronase or trypsin (0.05 U) was added to 1 ml of the solution of the inhibitor (fraction IV-II; 10 mg) in 0.1 M phosphate buffer, pH 7.5 (for pronase) or 8.0 (for trypsin). After 1-h incubation, the inhibitory action of fraction IV-II was estimated by the standard procedure.

Effect of group-specific reagents on the inhibitor (fraction IV-II). Aqueous solutions of the modifying agents (50 μl; concentration 0.1 M) were added to samples containing 30 μl of inhibitor (6 μg/ml) in 0.05 M succinate buffer, pH 5.2. The following modifying agents were used: N-bromosuccinimide, acetylimidazole, diethylpyrocarbonate, CME-carbodiimide, glycine methyl ester, *p*-chloromercuribenzoate, EDTA, and sodium azide. After 30-min incubation, the excess of the reagent was removed by passing the sample through a calibrated column (55 × 10 mm) with Biogel P-30. The inhibitory action of the modified inhibitor was compared with that for the native sample subjected to gel filtration under the same conditions.

Determination of molecular mass of inhibitor (P-30-1). The molecular mass of the inhibitor was determined by gel filtration on a column (100 × 20 mm) with Sepharose CL-4B. BSA (67 kD), LIV from *S. sachalinensis* (22 kD), and lysozyme (14 kD) were used as standards.

RESULTS AND DISCUSSION

The inhibiting action of the extracts from brown seaweeds was characterized on the basis of their ability to suppress the interaction of endo-1→3-β-D-glucanase (LIV) from the crystalline style of the marine bivalve mollusk *S. sachalinensis* with laminaran. Inhibitors were detected in 14 species of brown seaweeds, i.e., about 80% of the samples studied (Table 1).

To isolate and study the inhibitor, we used the brown seaweed *L. cichorioides* widely distributed in the Sea of Japan. The species accumulates large amounts of laminaran and is characterized by high inhibiting action. Experiments were carried out with seaweeds collected in autumn because for seaweeds collected in spring the content of inhibiting substances in aqueous–ethanol extract is negligible (Table 1). Accumulation of laminaran in the seaweed *L. cichorioides* occurs also in autumn in the period of the reproductive development. In terrestrial plants the highest level of the substances possessing the inhibiting action with respect to the enzymes of grass-eating animals was detected in germinating seeds [1, 2].

The substances possessing the inhibiting action were extracted from seaweeds with ethanol. Then the extract was treated with chloroform (Table 2). The characteristics of the fractions possessing the inhibiting action at the different stages of purification are presented in Table 3. The ^{13}C-NMR spectrum of the original extract contains signals of C-atoms with chemical shift values corresponding to mannitol: 64.3 ppm (C1; C6), 72.2 ppm (C2; C5), and 70.6 ppm (C3; C4). The experiments showed that the enzymatic activity of endo-1→3-β-D-glucanases from marine invertebrates was not inhibited by mannitol.

Chromatography of the extract on polychrome-1 gave three fractions capable of inhibiting LIV: IV-I, IV-II, and IV-III (Table 2). The inhibiting action of fraction IV-I eluted by water from the column with polychrome-1 disappears over a period of 3-4 h. The substances of the fraction are probably labile. For this reason, we did not study fraction IV-I. Fractions IV-II and IV-III with $[I]_{50} = 2.0 \pm 0.5$ and 3.0 ± 0.5 μg/ml, respectively, were mixtures of substances of protein nature and carbohydrates (Table 3).

A number of methods was used to identify the substances in the active fractions IV-II and IV-III (Table 3). For example, carbohydrates were detected in the fractions and quantitatively determined by the phenol–sulfuric acid method [9]. They were identified as a mixture of 1,3;1,6-β-D-glucooligosaccharides, since the ^{13}C-NMR spectra of the inhibiting fractions IV-II and IV-III contained only the signals typical of these oligosaccharides: C1 (103-104 ppm), C3 (β-1,3; 85-87 ppm), C6 (β-1,6; 69.5 ppm), and free C6-atoms (61-62 ppm). The degree of polymerization of 1→3;1→6-β-D-glucooligosaccharides determined by gel filtration on Biogel P-2 was 4-8 for fraction IV-II and 7-12 for fraction IV-III. Analysis carried out

Table 1. Inhibiting action of extracts from brown seaweeds with respect to endo-1→3-β-D-glucanase from the marine mollusk *S. sachalinensis*

No.	Seaweed: type, order, family	Relative activity of LIV[1], %
	PHAEOPHYTA	
	PAEOSPOROPHICEAE	
	Laminariales	
	Alariaceae	
1	*Alaria fistulosa*[2]	100
2	*Alaria marginata*[2]	20
3	*Undaria pinnatifida*[2]	50
	Laminariaceae	
4	*Costaria costata*[2]	30
5	*Laminaria bargardianail*[2]	150
6a	*Laminaria cichorioides*[2] (April)	100
6b	*Laminaria cichorioides*[2] (September)	5
7a	*Laminaria japonica*[2]	5
7b	*Laminaria japonica*[2]	10
7c	*Laminaria japonica*[2]	20
7d	*Laminaria japonica*[2]	10
8	*Laminaria digitata*[3]	100
9	*Lessonia sp.*[3]	100
10	*Laminaria longiper*[3]	70
11	*Agarum cribrosum*[2]	15
	Desmarestiales	
	Desmarestiaceae	
12	*Desmarestia ligutata*[2]	100
13	*Dichloria viridis*[2]	100
14	*Turbinaria sp.*[4]	10
	Ralfsiales	
	Ralfsiaceae	
15	*Analipus japonicus*[2]	150
	CYCLOSPOROPHYCEAE	
	Fucales	
	Cystoseriaceae	
16	*Cystoseira crassipes*[2]	60
	Sargassumaceae	
17	*Sargassum pallidum*[2]	25
18	*Sargassum sp.*[4]	20
	Fucusaceae	
19	*Fucus evanescens*[3]	50
20	*Pelvetia sp.*[2]	55
21	*Pelvetia wrightii*[2]	50

[1] The concentration of inhibitor (calculated per dry substance of extract) in the incubation mixtures was 100 μg/ml.

[2, 3, 4] Seaweeds were collected in the Sea of Japan (1998, 1999), Okhotsk Sea (1996, 1999), and Indian Ocean (region of Seychelles; 1992), respectively.

using a carbohydrate analyzer and ^{13}C-NMR showed that the single component of carbohydrate part of fractions IV-II and IV-III was glucose; mannitol was completely removed from the active fractions by the chromatography on polychrome-1.

An absorption band with λ_{max} = 278 nm typical of proteins or peptides containing aromatic amino acids (tyrosine and/or tryptophan) is observed in the UV spectra of fractions IV-II and IV-III. The content of protein in the active fractions IV-II and IV-III determined by the Lowry method was found to be 20 and 28%, respectively. According to the data of amino acid analysis, the main amino acids in the proteins are glutamic and aspartic acids (possibly, glutamine and asparagine) (Table 3). Taken alone, aspartic acid, glutamine, and asparagine are ineffective as inhibitors of endo-1→3-β-D-glucanase from *S. sachalinensis*. In contrast, glutamic acid is a rather potent inhibitor of the enzyme: $[I]_{50} = 10^{-5}$ M.

Table 2. Purification of inhibitor from the brown seaweed *L. cichorioides*

Stage of purification	Yield of the inhibiting fraction, % of weight of dry seaweed	$[I]_{50}$, μg/ml	Degree of purification with respect to the inhibiting action
Brown seaweed	100		
Extraction by aqueous–ethanol mixture (fraction I)	35	25	1
Treatment by chloroform (fraction II)	25	15 ± 1	1.7
Precipitation by ethanol (fraction III)	17		2.5
Hydrophobic chromatography			
Fraction IV-I	4	10 ± 1	2.5
Fraction IV-II	2	2 ± 0.5	12.5
Fraction IV-III	1.5	3 ± 0.5	8.3
Hydrophobic chromatography of fraction IV-II on polychrome-1 after the treatment by exo-1→3-β-D-glucanase from *C. indicum*	1	2 ± 1	12.5
Gel filtration of fraction IV-II on Biogel P-30 after chromatography on polychrome-1	0.4	1 ± 0.5	25

Table 3. Characteristics of the inhibiting fractions obtained at different stages of purification

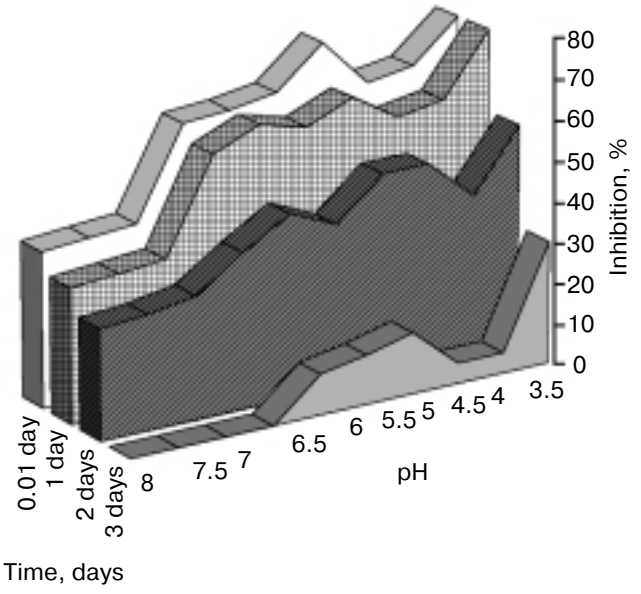
No.	Stage of purification	Content					DP[5]
		proteins[1], % by weight	carbohydrates[2], % by weight	amino acids[3], relative % Asp/Thr/Ser/Glu/ /Gly/Ala/Val/Ile/ /Leu/Phe	monosaccharies[4]	1→3-β: 1→6-β (^{13}C-NMR)	
I	Extraction by aqueous–ethanol mixture	3	9	8.4/1.6/2.0/71.6/1.8/ /11.5/3.1/0/0/0	Glc/Mn		
II	Treatment by chloroform	3	9	n.d.	Glc/Mn		
III	Precipitation by 80% ethanol	3	11	n.d.	Glc/Mn		
IV	Hydrophobic chromatography						
	Fraction IV-I	n.d.	90	n.d.	Glc/Mn		
	Fraction IV-II	20	70	4.3/2.2/3.6/68.1/ /11.3/3.6/0/0/0	Glc	80 : 20	4–8
	Fraction IV-III	28	30	12.7/6.0/4.4/38.6/ /8.0/8.4/9.0/6.2/ /12.4/7.0	Glc	80 : 15	7–12

Note: n.d., not determined.
[1] Protein was determined by the Lowry method.
[2] Carbohydrates were determined by the phenol–sulfuric acid method.
[3] Amino acid composition was determined after hydrolysis by 6 N HCl for 48 h using an amino acid analyzer Biotronik.
[4] Carbohydrate composition was determined after hydrolysis by 2 N HCl for 2 h using a Biotronik carbohydrate analyzer.
[5] Degree of polymerization (DP) of the carbohydrate component of fractions was determined by gel filtration on Biogel P-2 using a Jeol carbohydrate analyzer.

To study some properties of inhibitors, their stability, and the optimum conditions of storage, we used the highly active fraction IV-II that was obtained in large quantities. The activity of the fraction as a function of pH, temperature, and the duration of storage was studied. The inhibitor was stable in solution at pH 3.5-6.5 over a period of 2 days (figure). In the pH interval from 7.0 to 8.0 even 15-min incubation resulted in the 20-25% decrease in the inhibiting action of the inhibitor. Storage of the fraction in solution at pH 3.5-8.0 for 3 days was accompanied by a decrease in the inhibiting action of fraction IV-II with respect to LIV (the inhibiting effect disappears completely at pH > 6.5). Preparations corresponding to earlier stages of purification were more stable: fraction III retained its inhibiting action when kept at 4°C in the pH interval from 3.5 to 8.0 over a period of 6 months.

The stability of the inhibitor was studied in the temperature interval from –15 to 100°C. The results showed high stability of inhibitor: lyophilized preparation retained its inhibitory properties over at least half a year in storage at 4°C (pH 3.5-6.5).

The degree of inhibition was found to be independent of the substrate concentration. The minimum time



Stability of the inhibitor (fraction IV-II) at different pH values (0.05 M citrate–phosphate buffer; 4°C).

Table 4. Specificity of the inhibiting action of inhibitor

Enzymes	Substrate	$[I]_{50}$, μg/ml
Endo-1→3-β-D-glucanase from *S. sachalinensis* (LIV)	laminaran	2 ± 0.5
Endo-1→3-β-D-glucanase from marine mollusk *C. albidus* (L_0)	laminaran	2 ± 0.5
1→3-β-D-glucanase from marine bacterium *Alteromonas* sp.	laminaran	18
Endo-1→6-β-D-glucanase from *S. sachalinensis* (PIV)	laminaran	no[1]
Exo-1→3-β-D-glucanase from *Eulota maakii* (LII)	laminaran	no[1]
Exo-β-1,3-glucanase from *Echinodermata*	laminaran	no[1]
Exo-1→3-β-D-glucanase from fungus *Chaetomium indicum*	laminaran	no[1]
Yeast β-glucosidase	p-NO_2Ph-β-D-Glc[4]	no[1]
β-Glucosidase from fungus *A. flavipes*	p-NO_2Ph-β-D-Glc[4]	no[1]
β-D-Mannosidase from soy leaves	p-NO_2Ph-α-D-Man[2]	no[1]
1→3-β-D-glucanase from soy leaves	laminaran	no[1]
α-D-Galactosidase from marine bacterium *Pseudoalteromonas* KMM	p-NO_2Ph-α-D-Gal[3]	no[1]
Amylase from fungus *C. indicum*	amylopectin	no[1]
Cellulase from fungus *C. indicum*	CM-cellulose	no[1]
Trypsin	BAPA[5]	no[1]
Pronase E (proteinase E from *Streptomyces griseus*)	BAPA[5]	no[1]

[1] Inhibition of the enzymes by fraction IV-II was not found up to 100 μg/ml.
[2] *p*-Nitrophenyl-α-D-mannopyranoside; activity was measured by release of *p*-nitrophenol.
[3] *p*-Nitrophenyl-α-D-galactopyranoside; activity was measured by release of *p*-nitrophenol.
[4] *p*-Nitrophenyl-β-D-glucopyranoside; activity was measured by release of *p*-nitrophenol.
[5] N-Benzoyl-DL-arginine-*p*-nitroanilide; activity was measured by release of *p*-nitroaniline.

required for full realization of the inhibiting action of the inhibitor was about 5 min. These data suggest that the inhibition is irreversible.

To characterize the specificity of the inhibitor, we studied its action on different enzymes (Table 4). The inhibitor was able to inactivate only endo-1→3-β-D-glucanases from marine mollusks and the marine bacterium. Fraction IV-II was inert with respect to the following enzymes: endo-1→6-β-D-glucanase from the marine mollusk *S. sachalinensis*, exo-1→3-β-D-glucanases, glycosidases (gluco-, manno-, and galactosidases) from various sources, trypsin, and pronase. Thus, fraction IV-II contains inhibiting substances possessing a high specificity toward endo-1→3-β-D-glucanases from marine mollusks and a lesser specificity toward endo-1→3-β-D-glucanases from the marine bacterium.

The inhibiting fraction IV-II consists generally of carbohydrates (1→3;1→6-β-D-glucooligosaccharides) and protein. Therefore, to elucidate the role of each component, we performed the degradation of the components using enzymes resistant to the inhibitor. Exo-1→3-β-D-glucanase from *C. indicum* and yeast β-D-glucosidase were used for hydrolysis of the carbohydrate component. The protein component was degraded by trypsin and pronase (Table 4). Treatment of fraction IV-II by

Table 5. Effect of group-specific modifiers on the inhibitor

Reagent	Concentration of reagent, M	Group being modified	Inhibition*, %
p-Chloromercuribenzoate	5×10^{-2}	-SH	100
N-Bromosuccinimide	10^{-2}	Trp	0
EDTA	5×10^{-2}	Me^{2+}	100
Sodium azide	10^{-2}	Me^{2+}	100
Acetylimidazole	10^{-2}	His, Tyr	0
CME-carbodiimide	10^{-2}	-COOH	0
CME-carbodiimide and glycine methyl ester	10^{-2}	-COOH	0
Diethylpyrocarbonate	10^{-2}	His	0

* Suppressing the inhibiting action of inhibitor; inhibitor concentration (2 μg/ml) which yielded 50% inhibition of LIV (0.01 U) served as a control.

exo-1→3-β-D-glucanase from *C. indicum* or yeast β-D-glucosidase resulted in almost complete cleavage of 1→3;1→6-β-D-oligosaccharides to glucose. Such a treatment is not accompanied by change in the inhibiting action of fraction IV-II. In contrast, treatment by trypsin or pronase causes full inactivation of the inhibitor. It is highly probable that the inhibitor has protein nature.

To characterize the participation of certain amino acid residues of inhibitor IV-II in interaction with the enzyme, we performed chemical modification using group-specific reagents (Table 5). As can be seen from Table 5, modification of tryptophan, dicarboxylic acids, histidine, and probably tyrosine resulted in full loss of the inhibiting action of fraction IV-II, suggesting that these amino acids participate in the interaction of the inhibitor with the enzyme.

To separate the carbohydrates from the active protein component, we used Biogel P-30 (Table 2). Gel filtration on Biogel P-30 allowed us to separate carbohydrates and obtain three protein peaks: the main peak eluted with the free volume ($[I]_{50}$ = 1.0 ± 0.5 μg/ml) and two minor peaks. When 1→3;1→6-β-D-glucooligosaccharides were split by exo-1→3-β-D-glucanase from *C. indicum* into glucose and the inhibitor was purified by chromatography on polychrome-1 and Biogel P-30, the yield of the protein inhibitor increased. Protein fraction with $[I]_{50}$ = 0.5 μg/ml was obtained (Table 2). According to the data of gel filtration on Sepharose CL-4B, the molecular mass of the protein is ~46 kD. Consequently, the value of $[I]_{50}$ for inhibitor-1 is 10^{-8} M.

Thus, inhibitors of protein nature possessing a high specificity toward endo-1→3-β-D-glucanases, digestive enzymes of marine mollusks, have been obtained from a brown seaweed *L. cichorioides* (the value of $[I]_{50}$ for inhibitor-1 was found to be 10^{-8} M). It should be noted that the value of $[I]_{50}$ for inhibitors from the brown seaweed is comparable with the corresponding value for natural inhibitors of amylases from terrestrial plants [1]. Of particular interest is the capability of glutamic acid (one of the main amino acids comprising the inhibitor from *L. cichorioides*) to efficiently suppress the activity of endo-1→3-β-D-glucanase LIV.

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