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Brown seaweed protein as an inhibitor of marine mollusk endo-(1→3)-β-D-glucanases

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Abstract

Aqueous ethanol extracts from brown seaweed were found to contain substances inhibiting *endo*-(1→3)-β-D-glucanases, the digestive enzymes of marine mollusks. The inhibitors were detected in 70% of the brown seaweeds investigated. An irreversible protein inhibitor with high specificity for *endo*-(1→3)-β-D-glucanases of marine mollusks was isolated from the brown seaweed, *Laminaria cichorioides*. As determined by gel filtration, the molecular weight of the inhibitor was 46 kDa. The value of $[I]_{50}$ (10^{-8} M) for the inhibitor was comparable with the corresponding value for natural α-amylase inhibitors from terrestrial plants. Chemical modification results indicated that tryptophan, dicarboxylic acid, histidine and probably tyrosine residues of inhibitor molecule are important for interaction of the inhibitor with the enzyme. © 2002 Published by Elsevier Science Ltd.

Keywords: Protein; Inhibitor; Brown seaweeds; *Laminaria cichorioides*; *endo*-(1→3)-β-D-Glucanase; Marine mollusk

1. Introduction

The inhibition of enzymatic processes is the basic mechanism of action of numerous toxic and medicinal agents on living organisms. Inhibitors possessing high specificity are used to establish the mechanism of action of enzymes and to determine the structure of the active centers. They have wide application in medicine, pharmacology and toxicology. Natural inhibitors of carbohydrases are of major interest. Natural inhibitors of amylases and glycosidases have been the ones mostly studied.[1–6] 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 plants and insects, and herbivores or fungal pathogens have also been studied.[4,6]

Laminarans ((1→3) and (1→6)-β-D-glucans) are reserve polysaccharides of brown seaweeds widely distributed in marine organisms. They fulfill functions analogous to those of amylose in terrestrial plants.[7] It would be 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 that inhibit 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 found in some species of marine invertebrates[8] and brown seaweeds.[9] A glyceroglycolipid that potently inhibits yeast β-glucosidase was isolated from the brown seaweed, *Hizikia fusiforme*.[3]

The purpose 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*.

2. Results and discussion

The inhibiting action of aqueous ethanol extracts from brown seaweeds was determined by measuring the

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ability to suppress the hydrolysis of laminaran by *endo*-(1→3)-β-D-glucanase (LIV) from the crystalline style of the marine bivalve mollusk, *Spisula sachalinensis*. Inhibitors were detected in 14 species of brown seaweeds, i.e., in about 70% of the investigated seaweeds (Table 1).

To isolate and study the inhibiting substances, we used the brown seaweed, *L. cichorioides*, which is widely distributed in the Sea of Japan. The species accumulates large amounts of laminaran in autumn and is characterized by its high inhibiting action. Experiments were carried out with seaweeds collected in au-

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

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

[a] The concentration of inhibitor (calculated per dry substance of extract) in the incubation mixtures was 100 μg/mL.
[b] Seaweed collected in the Sea of Japan (1998, 1999).
[c] Seaweed collected in the Okhotsk Sea (1996, 1999).
[d] Seaweed collected in the Indian Ocean (region of Seychelles; 1992).

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

Step 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		
I. Extraction by water ethanol	35	25	1
II. Purification by chloroform	25	15 ± 1	1.7
III. Precipitation by ethanol	17		2.5
IV. Hydrophobic chromatography on Polychrome-1			
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 chromotography of fraction IV-II on Polychrome-1 after treatment by *exo*-(1→3)-β-D-glucanase from *C. indicum*	1	1 ± 0.5	12.5
Gel filtration on Biogel P-30 of fraction IV-II after chromatography on Polychrome-1	0.4	0.5	25

tumn because the inhibiting substances are absent in seaweeds collected in the springtime (Table 1).

Substances possessing inhibiting action were extracted from the seaweed with ethanol. The extract was then treated with chloroform (Table 2). Chromatography of the resulting extract on Polychrome-1 (Teflon, Russia) gave three fractions capable of inhibiting LIV: IV-I, IV-II, and IV-III (Table 2). The inhibiting action of fraction IV-I eluted with water from a column with Polychrome-1 disappears over a period of 3–4 h. The fraction substances were probably labile under these conditions.

The characteristics of fractions possessing inhibiting action at different stages of the purification process are shown in Table 3.

Fractions IV-II and IV-III with $[I]_{50} = 2.0 \pm 0.5$ μg/mL, $[I]_{50} = 3.0 \pm 0.5$ μg/mL, respectively, were basically mixtures of substances of protein nature and carbohydrates (Table 3). A number of methods were used to identify the active fractions IV-II and IV-III of substances (Table 3). For example, carbohydrates were detected in the fractions and quantitatively determined by the phenol–sulfuric acid method.[10] Analyses carried out using a carbohydrate analyzer and ^{13}C NMR spectroscopy showed that the single component of the carbohydrate part of fractions IV-II and IV-III was glucose. The carbohydrates were identified as a mixture of (1→3) and (1→6)-β-D-glucooligosaccharides, since the ^{13}C NMR spectra of the inhibiting fractions IV-II and IV-III contained signals typical of those oligosaccharides: C-1 (103–104 ppm), C-3 (β-1,3; 85–87 ppm), C-6 (β-1,6; 69.5 ppm) and free C-6 atoms (61–62 ppm). The degree of polymerization (DP) for the (1→3) and (1→6)-β-D-glucooligosaccharides was determined by gel filtration on Biogel P-2. The DP was 4–8 for fraction IV-II and 7–12 for fraction IV-III.

The protein content in the active fractions IV-II and IV-III was determined by the Lowry method and was found to be 20 and 28%, respectively. An absorption band with λ_{max} equal to about 280 nm, typical of proteins or peptides containing aromatic amino acids (tyrosine and/or tryptophan) was observed in the UV spectra of fractions IV-II and IV-III. The main amino acids of proteins in the fractions IV-II and IV-III are glutamic and aspartic acids (possibly glutamine and asparagine) (Table 3).

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 the marine mollusks and marine bacteria. 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 high specificity toward *endo*-(1→3)-β-D-glucanases from marine mollusks and a lesser specificity towards *endo*-(1→3)-β-D-glucanase from marine bacteria.

As is shown in Table 3, the inhibiting fraction IV-II generally consists of carbohydrates ((1→3) and (1→6)-β-D-glucooligosacchrides) and protein. Hence, to elucidate the role of each component, we performed degradation of several components using enzymes resis-

Table 3
Characteristics of inhibiting fraction obtained at various stages of purification

Step of purification	Content % (w/w)		Composition		(1,3)-β:(1,6)-β	DP [e]
	Proteins [a], % by weight	Carbohydrates [b], % by weight	Amino acids, relative % [c] Asp; Thr; Ser; Glu; Gly; Ala; Val; Ile; Leu; Pha	Mono saccharides [d]		
I. Extraction by water ethanol	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.
[a] Protein was determined by the Lowry method.
[b] Carbohydrates were determined by the phenol–sulfuric acid method.
[c] Amino acid composition was determined after hydrolysis by 6 N HCl for 48 h using a Biotronik amino acid analyzer.
[d] Carbohydrate composition was determined after hydrolysis by 2 N HCl for 2 h using a Biotronik carbohydrate analyzer.
[e] Degree of polymerization (DP) of the carbohydrate component of fractions was determined by gel filtration on Biogel P-2 using a JEOL carbohydrate analyzer.

Table 4
Specificity of inhibitor action

Enzymes	Substrates	$[I]_{50}$ (μg/mL)
endo-(1→3)-β-D-Glucanase from *Spisula sachalinensis* (LIV)	laminaran	2 ± 0.5
endo-(1→3)-β-D-Glucanase from marine mollusc *Chlamus albidus* (LO)	laminaran	2 ± 0.5
(1→3)-β-D-Glucanase from marine bacterium *Alteromonas* sp.	laminaran	18
endo-(1→6)-β-D-Glucanase from *Spisula sachalinensis* (P IV)	pustulan	n.i. [a]
exo-(1→3)-β-D-Glucanase from *Eulota maakii* (L II)	laminaran	n.i. [a]
exo-(1→3)-β-D-Glucanase from *Strongilocentrotus intermedius*	laminaran	n.i. [a]
exo-(1→3)-β-D-Glucanase from *Chaetomium indicum*	laminaran	n.i. [a]
β-Glucosidase from yeast	*p*-NO_2Ph-Glc [b]	n.i. [a]
β-Glucosidase from *Aspergillus flavipes*	*p*-NO_2Ph-Glc [b]	n.i. [a]
α-D-Mannosidase from soybean leaves	*p*-NO_2Ph-α-D-Man [c]	n.i. [a]
(1→3)-β-D-Glucanase from soybean leaves	laminaran	n.i. [a]
α-D-Galactosidase from marine bacterium *Pseudoalteromonas* KMM-706	*p*-NO_2Ph-α-D-Gal [d]	n.i. [a]
Amylase from *C. indicum*	amylopectin	n.i. [a]
Cellulase from *C. indicum*	KM-cellulose	n.i. [a]
Trypsin	BAPA [e]	n.i. [a]
Pronase E (proteinase E from *Streptomyces griseus*)	BAPA [e]	n.i. [a]

[a] Inhibition of the enzymes by fraction IV-II was not found up to 100 μg/mL.
[b] *p*-NO_2Ph-β-D-Glc, *p*-nitrophenyl-β-D-glucopyranoside; activity was measured by release of *p*-nitrophenol.
[c] *p*-NO_2Ph-α-D-Man, *p*-nitrophenyl-α-D-mannopyranoside; activity was measured by release of *p*-nitrophenol.
[d] *p*-NO_2Ph-α-D-Gal, *p*-nitrophenyl-α-D-galactopyranoside; activity was measured by release of *p*-nitrophenol.
[e] BAPA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; activity was measured by release of *p*-nitroaniline.

Table 5
Effect of group-specific modifiers on the inhibitor (fraction IV-II)

Reagent	Concentration of reagent, M	Group being modified	Inhibition [a] (%)
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}	Hys, Tyr	0
CME-carbodiimide	10^{-2}	–COOH	0
CME-carbodiimide and glycine methyl ester	10^{-2}	–COOH	0
Diethylpyrocarbonate	10^{-2}	Hys	0

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

tant to the inhibitor. So an *exo*-(1→3)-β-D-glucanase from the marine fungus, *Chaetomium indicum*, and a yeast β-D-glucosidase were used to hydrolyze the carbohydrate component. The protein component was degraded with trypsin and pronase. The inhibitor had no effect on these enzymes. Treatment of fraction IV-II with *exo*-(1→3)-β-D-glucanase from *C. indicum* or yeast β-D-glucosidase resulted in almost complete cleavage of (1→3) and (1→6)-β-D-glucooligosaccharides to glucose. Such treatment was not accompanied by a change in the inhibiting action of fraction IV-II. In contrast, treatment with trypsin or pronase causes full inactivation of the inhibitor. It is therefore highly probable that the inhibitor is a protein or peptide.

We performed chemical modification of the amino acid residues of fraction IV-II using group-specific reagents (Table 5). As can be seen from Table 5, modification of tryptophan, dicarboxylic acids, histidine and probably tyrosine resulted in complete loss of the inhibiting action of fraction IV-II which suggests that these amino acids participate in an inhibitor–enzyme interaction.

To separate the carbohydrates from the active protein component, we used Biogel P-30 (Table 2). Gel filtration of fraction IV-II on Biogel P-30 allowed us to separate carbohydrates and to obtain two protein peaks: a main peak (component I) eluted with free volume $[I]_{50}=1.0\pm0.5$ μg/mL) and a minor peak (component II). But in that case carbohydrates were not fully separated. When (1→3) and (1→6)-β-D-glucooligosaccharides of the fraction were split with *exo*-(1→3)-β-D-glucanase from *C. indicum*, and the inhibitor was purified by chromatography on Polychrome-1 and Biogel P-30, the protein inhibitor yield was increased and the carbohydrate content diminished. As a result, the protein fraction (component I) with $[I]_{50}=0.5$ μg/mL was obtained (Table 2). According to the data for the gel filtration on Sephadex G-75, the molecular weight of the protein was about 46 kDa (Fig. 1). Consequently, the value of $[I]_{50}$ for component I was 10^{-8} M.

Component I obtained after Biogel P-30 was homogeneous on SDS–PAGE electrophoresis, but was composed of three components (component I, II and III) on SDS–PAGE after heating (Fig. 2). The M_w of component I was at 60 kDa, and that of component II was 30 kDa. Probably inhibitor I isolated from the brown alga, *L. cichorioides*, consisted of two polypeptide chains of molecular weight of about 30 kDa. It is also known that the molecular weights of proteins determined by gel filtration (46 kDa) might be less than that obtained by electrophoresis (60 kDa).[11] The appearance of component III may be elucidated by incomplete unfolding of component I (Fig. 2) under insufficient denaturing conditions.[12]

But the highly purified component I lost its inhibitory activity. Hence, some properties of the inhibitor were studied using the inhibitor solution at an earlier purification stage. To study some properties of inhibitory substances, their stability and optimal storage conditions, we used the highly active fraction IV-II ($[I]_{50}=2.0\pm0.5$ μg/mL). The activity of the fraction as a function of pH, temperature, and duration of storage were studied. The inhibitor was stable in solution at pH 3.5–6.5 over a period of 2 days (Fig. 3). But in the pH interval from 7.0

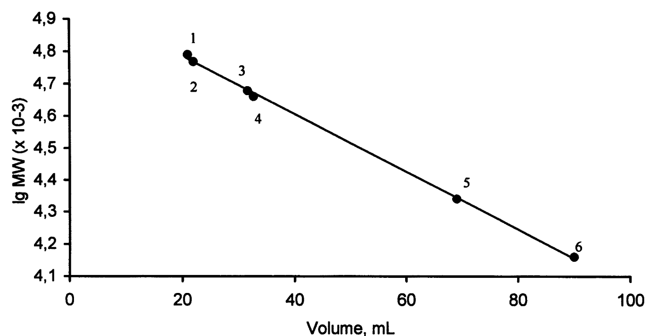

Fig. 1. Dependence of l g M_W on elution volume (Sephadex G-75; 20 × 460 mm; 3 mL/h). 1. Component I with LIV (ca. 68 kDa); 2. BSA (67 kDa); 3. ovalbumin (46 kDa); 4. component I (46 kDa); 5. *endo*-(1→3)-β-D-glucanase from *Spisula sachalinensis* (LIV) (22 kDa); 6. lysozyme (14 kDa).

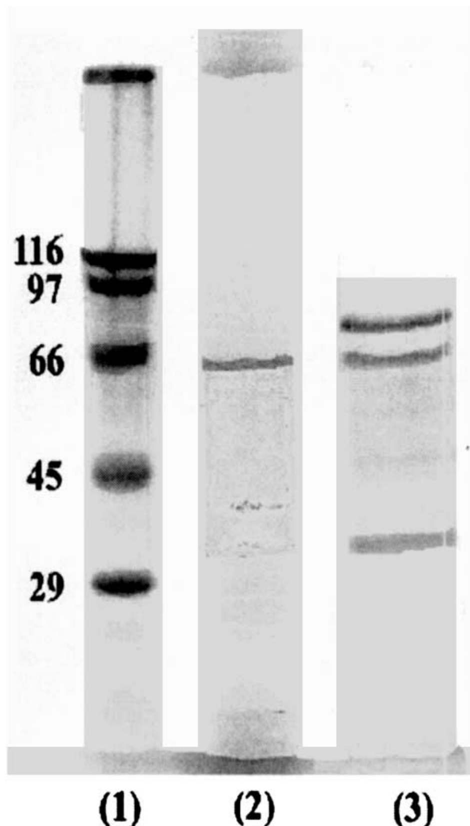

Fig. 2. SDS–PAGE electrophoresis. Lane 1: carbonic anhydrase (116 kDa), egg albumin (97 kDa), bovine albumin (66 kDa), phosphorylase B (45 kDa), β-galactosidase (29 kDa); lane 2: component I; lane 3: component I after treatment by heat.

to 8.0, even a 15-min incubation resulted in a 20–25% decrease in inhibitor action. Storage of the fraction in solution at pH 3.5–8.0 for 3 days was accompanied by decrease in inhibitor action of fraction IV-II with respect to LIV (the inhibiting effect disappeared completely at pH >6.5). The stability of the inhibiting action of fraction IV-II was studied at a temperature interval ranging from -15 to 100 °C. The results showed high stability of inhibitor: it maintained activity for about 3–5 min at 100 °C. The lyophilized preparation retained its inhibitory properties over 6 months when stored at 4 °C.

The extent of inhibition was found to be independent of the substrate concentration. The minimum time required for full realization of the inhibiting action of the inhibitor was about 5 min. This suggests that inhibition was irreversible. The enzyme–inhibitor complex did not dissociate during gel filtration on Sephadex G-75, and this also supports the irreversible nature of the inhibitor. The purified component I and *endo*-(1→3)-β-D-glucanase LIV were mixed in approximately a 1.2:1 molar ratio, and the enzyme inhibitor complex was separated by Sephadex G-75 gel-filtration chromatography (Fig. 1). It was shown that the molecular weight of the main protein (about 68 kDa) was very close to the sum of the molecular weights of component I (46 kDa) and enzyme LIV (22 kDa). LIV activity was not revealed after gel filtration. So component I formed a stoichiometric 1:1 stable complex with *endo*-(1→3)-β-D-glucanases (LIV) from the marine mollusks, *S. sachalinensis*.

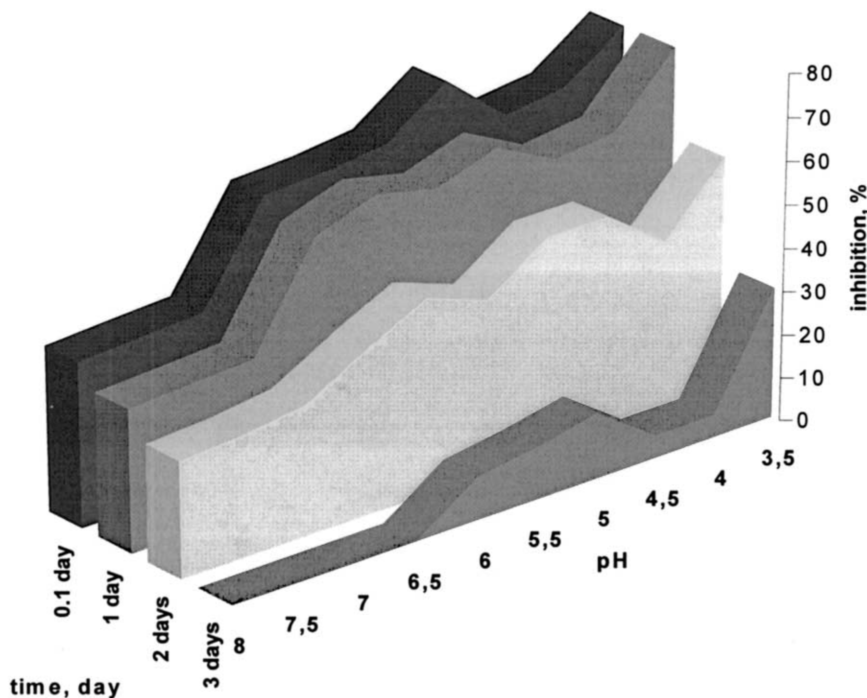

Fig. 3. The stability of inhibitor (of fraction IV-II) at different pH values (0.05 M citrate–phospate buffer, at 4 °C).

Thus, inhibitors of a protein nature that possess a high specificity to *endo*-(1→3)-β-D-glucanases, the digestive enzymes of marine mollusks, were obtained from the brown seaweed, *L. cichorioides*. The value of $[I]_{50}$ for inhibitors from the brown seaweed is comparable with the corresponding value for natural amylase inhibitors from terrestrial plants.[1]

3. Experimental

Reagents.—*N*-Bromosuccinimide, *N*-acetylimidazole, diethyl pyrocarbonate, CME-carbodiimide, glycine methyl ester, *p*-chloromercuribenzoate, EDTA, sodium azide, and resins for chromatography were purchased from Sigma Chemical Co.

Methods.—Total sugars were determined by the phenol–H_2SO_4 method.[10] Reducing sugars were determined by the Nelson method.[13] Proteins were determined by the Lowry method.[14] ^{13}C NMR spectra were recorded using a Bruker WM-250 NMR spectrometer in D_2O at 40 °C (10-mm tubes, internal methanol, δ 50.15). The monosaccharide composition of the fractions was determined after hydrolysis with 2 N HCl (100 °C, 2 h) using a 1C-5000 Biotronik carbohydrate analyzer (Germany). A column (3.2 × 385 mm) with Durrum DA-X8-11 resin was used for the analysis. The 2,2′-bicinchoninate method was used for the detection.[15] The amino acid composition of the fractions after hydrolysis with 6 N HCl (100 °C, 48 h) was determined using an amino acid analyzer (Alpha Plus LKB 4151; 4.5 ± 0.5 μm, 4.5 × 200 mm column with Ultrapac). A JEOL-JLC-6AH carbohydrate analyzer (Biogel P-2; 1 × 100 cm) was used to determine oligosaccharide composition; the orcine–H_2SO_4 method was used for detection of carbohydrates.

Enzymes.—*endo*-(1→3)-β-D-Glucanases LIV and LO from the marine mollusks, *S. sachalinensis* and *Chlamus 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, *Strongilocentrotus intermedius*, *endo*-(1→3)-β-D-glucanase from the marine bacterium, *Alteromonas* sp.,[16] *exo*-(1→3)-β-D-glucanase, amylase, and cellulase of the marine fungus *C. indicum*, β-D-glucosidase from the marine fungus *Aspergillus flavipes*,[17] α-D-mannosidase and (1→3)-β-D-glucanase from soybean leaves, and α-D-galactosidase from the marine bacterium, *Pseudoalteromonas* KMM-706[18] were from the collection of the Enzyme Chemistry Laboratory, Pacific Institute of Bioorganic Chemistry. Yeast β-D-glucosidase, trypsin, pronase, BSA, lysozyme, ovalbumin, carbonic anhydrase, egg albumin, bovin albumin, phoshorylase B and β-galactosidase were purchased from Sigma Chemical Co. (USA).

Substrates.—Laminaran from the brown seaweed, *L. cichorioides*, and pustulan from the lichen, *Umbillicaria russica*, were isolated as previously described in Refs. 19 and 20. *p*-Nitrophenyl-β-D-glucopyranoside (pNO_2Ph-Glc), *p*-nitrophenyl-α-D-mannanopyranoside (pNO_2-Ph-α-D-Man), *p*-nitrophenyl-α-D-galactopyranoside (pNO_2-Ph-α-D-Gal), and *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) were purchased from Sigma Chemical Co.

Extracts of seaweeds.—Seaweeds were collected during expeditions of the research vessel 'Academic Oparin' (Okhotsk Sea, Sea of Japan, and Indian Ocean) or at the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry, Khasan District, Maritime Province in August–September 1992, and in 1996–1999 (species of *L. cichorioides* were collected in different seasons). Fresh seaweeds were milled and extracted with EtOH (1 L of EtOH per 1 kg of fresh seaweed) at rt for 2 weeks. The extracts were evaporated under vacuum, and the dry residue was used for further experiments.

Enzyme assay.—The activities of the enzymes catalyzing the hydrolysis of polysaccharides (amylopectin, CM-cellulose, laminaran, pustulan) were determined by measuring the increase in the content of reducing sugars by the Nelson method.[13] 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. The amount of the enzyme catalyzing the formation of 1 μmol of glucose or *p*-nitrophenol per min under the assay conditions was taken to be one unit of enzymatic activity.

Standard inhibition procedure.—The inhibitor solution (10 μL) in 0.05 M acetate buffer, pH 5.4 and 10 μL of the enzyme solution (0.01 U) were incubated at 37 °C for 10 min. After 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.

Isolation of inhibitors from L. cichorioides.—Fresh seaweed was cut to pieces and extracted with EtOH (1:1 w/w) at rt for 2 days. The aq EtOH extract (I) was treated with $CHCl_3$ several times. The layer containing the $CHCl_3$ was separated and discarded. The active aq EtOH fraction (II) was concentrated under vacuum. After addition of 80%-EtOH, the supernatant, which contained inhibiting substances, was evaporated under vacuum. The dry residue (III) was dissolved in water and placed on a column with hydrophobic resin Polychrome-1 (polytetrafluoroethylene; 70 × 160 mm) (fraction IV). The column was eluted with water (fraction IV-I) until the sugar completely disappeared. Further elution was performed using a stepwise gradient of EtOH in water: 5% (fraction IV-II), 10, 20, and 40%

(fraction IV-III) of EtOH. The inhibitor content in the fraction was determined using the standard procedure. The active fractions IV-I, IV-II, and IV-III were lyophilized (Table 2).

Separation of fraction IV-II components.—Dry substances of fraction IV-II (~10 mg) were dissolved in water and placed on a column with Biogel P-30 (10 × 120 mm). The column was eluted with water, elution rate 0.3 mL/min. Protein in fractions was registered spectrophotometrically at 280 nm. Carbohydrates were determined by the phenol–H_2SO_4 method at 480 nm. The inhibiting action was characterized using the standard procedure. The active fractions (component I and component II) were lyophilized.

Effect of preincubation duration on inhibiting action (fraction IV-II).—A mixture of solutions of (1→3)-β-D-glucanase LIV and the 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 substrate concentration on inhibiting action (fraction IV-II).—The inhibiting action was determined using a standard procedure. The concentration of the substrate (laminaran) varied from 0.5 to 10 mg/mL.

Effect of pH on stability of inhibitor (fraction IV-II).—A 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 inhibition degree.

Effect of temperature on inhibitor (fraction IV-II) stability.—An inhibitor solution of (20 μg in 200 μL) in 0.1 M acetate buffer, pH 5.2, was incubated at −15, +4, +20, +37, +50 °C over a period of 1–365 days, and at 100 °C for 1–15 min. Aliquots (10 μL) were withdrawn to determine the degree of inhibition.

Determination of inhibitor (fraction IV-II) specificity.—To determine the specificity of the inhibitor, we used the following enzymes: *endo*-(1→3)-β-D-glucanase (LIV) and (1→6)-β-D-glucanase (PIV) 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 the marine fungus, *C. indicum*, β-D-glucosidase from the marine fungus, *A. flavipes*, α-D-mannosidase and (1→3)-β-D-glucanase from soybean leaves, α-D-galactosidase from the marine bacterium, *Pseudoalteromonas* KMM-706, trypsin, and pronase. The enzyme solution (10 μL, 0.01 U) was added to 10 μL (1 mg/mL) 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 a 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 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.[13] Then the products of the reaction were analyzed using a JEOL-JLC-6AH or Biotronik analyzer. The inhibitory action of fraction IV-II after treatment by enzyme was estimated by the standard procedure.

(2) Pronase or trypsin (0.05 U) was added to 1 mL of the inhibitor solution (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 standard procedure.

Effect of group-specific reagents on inhibitor (fraction IV-II).—Aqueous solutions of 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, *N*-acetylimidazole, diethyl pyrocarbonate, CME-carbodiimide, glycine methyl ester, *p*-chloromercuribenzoate, EDTA, and sodium azide. After 30 min incubation, the excess reagent was removed by passing the sample through a calibrated column (10 × 55 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 similar conditions (Table 5).

Gel-filtration experiments.—Gel-filtration experiments were carried out on a columns with Biogel P-30 (15 × 420 mm) and Sephadex G-75 (20 × 460 mm). BSA (67 kDa), ovalbumin (46 kDa), LIV from *S. sachalinensis* (22 kDa), and lysozyme (14 kDa) were used as standards.

Electrophoresis.—SDS-PAGE was done on a 13% stab gel by the method of Laemmli.[21] Reference proteins were carbonic anhydrase, egg albumin, bovine albumin, phoshorylase B and β-galactosidase of M_w 29 kDa, M_w 45 kDa, M_w 66 kDa, M_w 97 kDa, M_w 116 kDa, respectively.

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References

1. Andrzejczuk-Hybel, J. *Posteky Biochem.* **1981**, *27*, 181–196.
2. Zhang, N. Y.; Jones, B. L.; Tao, H. P. *Cereal Chem.* **1997**, *74*, 119–122.

3. Kurihara, H.; Ando, J.; Hatano, M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1241–1244.
4. Broadway, R. M. *Can. J. Plant Pathol.* **1996**, *18*, 476–481.
5. Lankisch, M.; Layer, P.; Rizza, R. A.; Dimagno, E. P. *Pancreas* **1998**, *17*, 176–181.
6. Ham, K. S.; Wu, S. C.; Darvill, A. G.; Albersheim, P. *Plant J.* **1997**, *11*, 169–179.
7. Painter, T. I. *Pure Appl. Chem.* **1983**, *55*, 677–694.
8. Zvyagintseva, T. N.; Elyakova, L. A.; Krasohin, V. B. *Comp. Biochem. Physiol.* **1992**, *102B*, 187–191.
9. Zvyagintseva, T. N.; Sova, V. V.; Yermakova, S. P.; Scobun, A. S.; Elyakova, L. A. *Biol. Sea (Vladivostok)* **1998**, *24*, 246–249.
10. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Reiber, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
11. Sova, V. V.; Svetasheva, T. G.; Kostyuk, I. O. *Biochemistry (Moscow)* **1992**, *57*, 1344–1347.
12. Carnier, M.; Vacheron, M. J.; Pellon, G.; Guinard, M.; Michel, C. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 448–453.
13. Nelson, N. *J. Biol. Chem.* **1944**, *153*, 375–381.
14. Lowri, O. N.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 269.
15. Waffenshmidt, S.; Jaenice, L. *Anal. Biochem.* **1987**, *165*, 337–340.
16. Sova, V. V.; Zvyagintseva, T. N.; Svetasheva, T. G.; Burtseva, Yu. V.; Elyakova, L. A. *Biochemistry* **1997**, *62*, 1300–1306.
17. Burtseva, Yu. V.; Sova, V. V.; Pivkin, M. V.; Zvyagintseva, T. N. *Biochemistry* **2000**, *65*, 1175–1183.
18. Bacunina, I. Yu.; Sova, V. V.; Nedashkovskaya, O. I.; Kulman, R. A.; Likhosherstov, L. M.; Martynova, M. D.; Mihailov, V. V.; Elyakova, L. A. *Biochemistry* **1998**, *63*, 1420–1427.
19. Zvyagintseva, T. N; Shevchenko, N. M.; Popivnich, I. B.; Sundukova, E. V.; Isakov, V. V.; Scobun, A. S.; Elyakova, L. A. *Carbohydr. Res.* **1999**, *322*, 32–39.
20. Zvyagintseva, T. N; Sundukova, E. V.; Elyakova, L. A. Russian Federation Pat. 1642725, 1966; *Chem. Abstr.* **1997**, *126*, 306–493.
21. Laemmli, U. K. *Nature* **1970**, *227*, 630–685.