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Some properties of partially purified protease inhibitors from a red alga, *Porphyra yezoensis*

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A plasmin inhibitor fraction was obtained from a marine red alga, *Porphyra yezoensis*. It was in partially purified by gel filtration on Sephadex G-100 and by ion exchange chromatography on DEAE-Sephacel at pH 7.4. The inhibitors fractionated on gel filtration were eluted in the region of M.W. 5000-40000, which inhibit competitively the plasmin catalyzed hydrosis of H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide (S-2251). These inhibitors seemed to be of protein property. They were also effective toward trypsin. Thus, it was found that proteinlike protease inhibitors occur also on a marine red alga, *Porphyra yezoensis*.

Key Index Words: *Plasmin inhibitor*; Porphyra yezoensis; *proteinlike protecse inhibitor*; *red alga*; *Rhodophyta*.

Proteinlike protease inhibitors are widely distributed in the plant kingdom (BIRK 1976). In cotrast to higher plants, protease inhibitors of algae have never been studied in detail except a few red algae (WATANABE 1979, 1980). In view of this fact, we investigated precisely the protease inhibitors from marine red algae, *Grateloupia livida* and *Grateloupia elliptica*, and found that some of its properties are characteristic of the red algae (WATANABE 1980).

Then we investigated the biochemical properties of plasmin inhibitor of another red alga, *Porphyra yezoensis*, to compare the results with those from the former red algae. The reason for choosing *P. yezoensis* is based mainly on the fact we have recently used this alga as the experimental material for the investigation of specific immunotherapy for cancer. This paper discribes the separation procedure of the pharmacologically active fraction of *P. yezoensis*, and these compounds to have a proteinaceous nature.

Materials and Methods

Algal material; The frond of *Porphyra yezoensis* were obtained from culture grounds Harima and Akashi, Hyōgo prefecture, in January 1980. After dried in air, the fronds were used for extraction of plasmin inhibitors.

Chemicals; Plasmin, H-D-Valyl-Leucyl-L-lysine-p-nitroanilide (S-2251), and N-Benzoyl-L-phenyalanyl-L-valyl-L-arginine-p-nitroanilide (S-2160) are the products of the Kabi diagnostica. Trypsin is the product of the Sigma Chemical Company. Coomassie Brilliant Blue G-250 (CBB G-250) was obtained from Bio-rad Laboratories. All other chemicals were purchesed from Nakarai Chemicals Ltd.

Extraction of protease inhibitors; Algal fronds (1 Kg) were ground with 2 liters of cold distilled water in a Polytron homogenizer. Homogenate was filtered through five layers of gauze. The residue was

treated again in the same way as above and the extracts were combined to centrifuge at 10000×g for 30 min. To the supernatant was added solid ammonium sulfate to a 65% saturation and the precipitate formed was collected by centrifugation at 12000×g for 30 min and dissolved in 25 m*l* of 0.01 M Tris-HCl buffer at pH 7.4. The solution was subject to gel filtration on Sephadex G-100, following by ion exchange chromatography on DEAE-Sephacel at pH 7.4.

Protein assay; Protein assay is based on the observation that the absorbance maximum is changed from 465 nm to 595 nm when the inhibitor was bound to the CBB G-250 occurs. Protein contents were determined by the methods of Bradford (1976), to obtain the plasmin specific inhibitory activity, using lyophilized preparation of bovine plasma gammaglobulin as standard.

Enzyme assay; Inhibitory activity for plasmin was assayed spectrophotometrically by measuring the degree of splitting the substrate S-2251 because the rate of p-nitroaniline formation thereby increased linearly with increasing concentrations of plasmin. Assays were performed in 10 mm quartz of 1.0 m*l* capacity. The cuvettes were placed at 37°C in a thermostatted compartment of a Shimazu model UV-200S double beam spectrophotometer and the reaction was recorded automatically. In a small test tube, 0.15 m*l* of 0.8 C. U./m*l* plasmin solution, 0.15 m*l* of 0.1 M Tris-HCl buffer, pH 7.4 and 0.9 m*l* of sample were placed and preincubated for 60 min at 37°C. The reaction was started by addition of 0.8 m*l* of the preincubation mixture to 0.2 m*l* of 3.5 mM substrate in a assay micro-cuvette (Preheated to 37°C). Increase in the absorbance at 405 nm was recorded for about 5 min. As a blank test, the activity of plasmin without inhibitor was measured under the same assay condition. Trypsin activity was measured using S-2160 as substrate.

Results

An extract from *Porphyra yezoensis* prepared as described above was fractionated on a Sephadex G-100 column equilibrated with a 0.05 M Tris-HCl buffer,

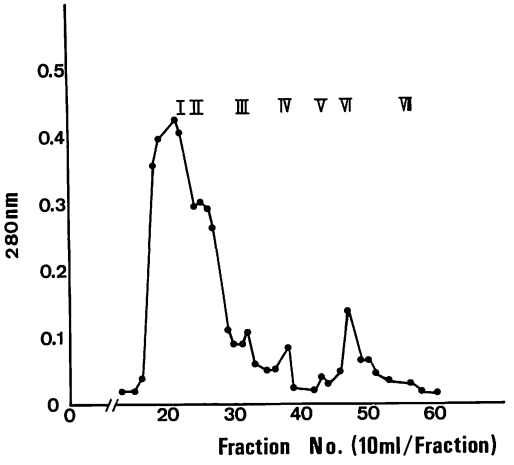

Fig. 1. Gel filtration on Sephadex G-100 of ammonium sulfate fraction from red alga, *Porphyra yezoensis*.

Table 1. Effect of the fractions on plasmin activity.

Fractions	Protein content	Residual activity of plasmin	Inhibitory activity for plasmin
	μg/ml	%	%
P-II	60	60	40
P-III	33	73	27
P-IV	12	63	37
P-V	3	42	58
P-VI	3	60	40
P-VII	5	60	40

Table 2. Thermostability of plasmin inhibitor fractions.

Fractions	Absorbance at 450 nm/min	Inhibitory activity
		%
Inhibitor II	.089	37
Inhibitor II at 95°C, 5 min	.128	5
Inhibitor V	.076	44
Inhibitor V at 95°C, 5 min	.062	54
None	.136	—

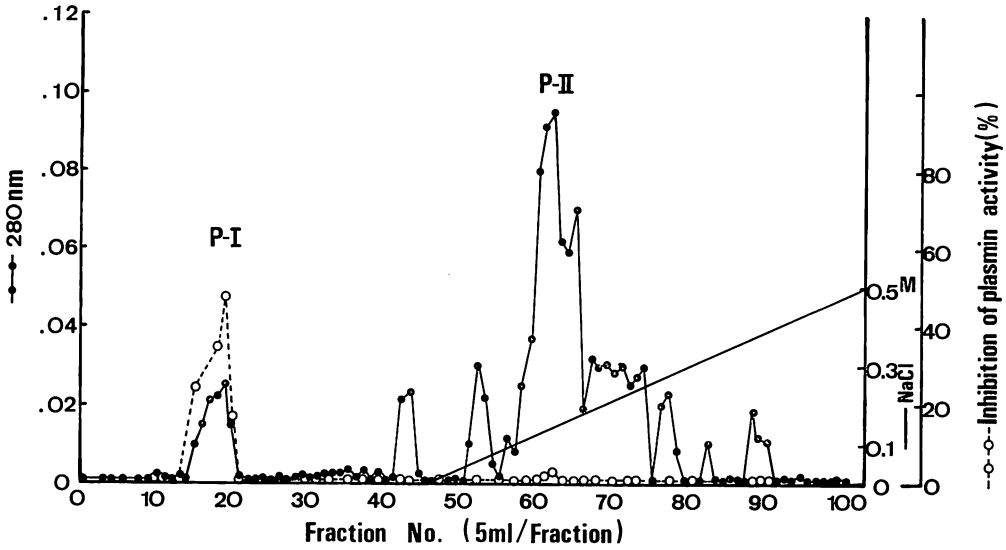

Fig. 2. Chromatography of gel-filtrated inhibitor-V on a DEAE-Sephacel column (1.0× 30 cm) equilibrated 0.05 M Tris-HCl buffer, pH 7.4 and eluted with a linear NaCl gradient, represented by the solid line (—). The column was charged with 50 μg of the preparation.

Table 3. Plasmin-inhibitory activities of Peak-I and II from DEAE-Sephacel ion exchange chromatography of Inhibitor II from *P. yezoensis*.

Fractions	Absorbance at 405 nm/min	Inhibitory activity
		%
Peak-I	.056	47
Peak-II	.104	2
None	.106	—

Table 4. Thermostability of Peak-I from DEAE-Sephacel ion exchange column chromatography.

Fraction	Absorbance at 405 nm/min	Inhibitory activity
		%
Plasmin+Peak-I (Inhibitor V origin) +Peak-I heated at 95°C for 5 min	.060	41
	.057	44
None	.103	—

pH 7.4. The result was shown in Fig. 1. Peaks II and VII eluted in the fractions of relatively low molecular weights showed a clear inhibitory activity for plasmin. They were found in the regions of molecular weights estimated to be 30000-40000 and 5000-10000, respectively.

The inhibitors eluted as a symmetrical peak in repeating gel filtration on Sephadex G-100 column.

Table 1 shows the effect of fractions from gel filtration on the activity of plasmin. The fraction covered by peak V which was eluted in a fairly lower molecular weight region were called Inhibitor-V. It showed a higher plasmin-inhibitory activity, but it was stable on heating at 95°C for 5 min.

It seemed to be a thermostable inhibitor. The fractions covered by peak II were called Inhibitor-II. It was almost completely inactivated by heating at 95°C for 5 min, suggesting that the inhibitor may be proteinlike nature (Table 2).

Then, Inhibitor-II fraction was applied to a 1.0×30 cm column of DEAE-Sephacel equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, and eluted by linear gradient elution with buffers containg 0-0.5 M NaCl.

Two peaks clearly separated were obtained, as shown in Fig. 2. Peak-I showed inhibitory activity for plasmin, while Peak-II eluted thereafter showed practically no inhibitory activity for plasmin (Table 3).

The peak-I also effective toward trypsin. It was, however, digestied by pepsin at 56°C although the data were not shown here. The peak-I derived from Inhibitor-V on the similar gel filtration showed remarkable stability to a high temperature (Table 4).

Discussion

Recently, the works on pharmaceutically active substances in marine algae have been increased. For examle, ZELENSKI and WORTHEN (1974) reported that they separated a compound possessing both anti-inflammatory and anti-curare activities from a crude fraction of *Eisenia bicyclis* and found that it is a laminaran-like polymer.

Furthermore, there are also some reports that extracts of the brown algae, in particular, showed larvicidal (CONOVER 1966), antiviral (BERTI 1962) and antibacterial (CONOVER 1964, SIEBURTH 1964, 1965) activities.

In this work, we also found the presence of protein-like substances showing plasmin-inhibitory activity in the extract from *Porphyra yezoensis*, a red alga commonly used as food in Japan. Purity of these inhibitors was not high, but most of them showed protein property upon the Bradford's staining reaction. Some of them were unstable to heat while others, in particular, Peak I from DEAE-Sephacel ion-exchange chromatography of Peak V obtained from gel filtration of a crude extract was very stable to heat. Since the mother fraction Peak V was eluted in a fairly lower molecular weight region, the heat-stable inhibitor may be of a small protein or a polypeptide. Further investigations of the chemical properties and the physiological functions of these inhibitors are in progress.

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渡辺恒雄*，渡辺和人：紅藻スサビノリ（*Porphyra yezoensis*）から得た部分精製セリン系蛋白質分解酵素阻害物質の二，三の性質**

プロテアーゼインヒビターは，微生物，血液，臓器，高等植物種子に広く存在し，生理機能の制御および臨床的意義を有することで重要視されているが，海藻に関する知見は少ない。生体のホメオスティシスの維持に重要な役割をしているセリン系プロテアーゼ，プラスミンを特異的に阻害する物質を海藻中に探索し，スサビノリに蛋白性プラスミンインヒビターが存在することを見出した。この物質が耐熱性を有することは明らかになったが，その生理的意義は不明で現在検討中である。*(305茨城県筑波郡谷田部町小野川，国立公害研究所。生理・生化学研究室)，**(305茨城県新治郡桜村，筑波大学環境科学)

書評：「赤潮に関する近年の知見と研究の問題点」赤潮研究会編集委員会，日本水産資源保護協会発行，定価1450円

日本各地の湾や内海で沿岸性赤潮が頻発し，近年とくに漁業被害が顕著になってきた。水産庁，環境庁を中心に関係諸機関はその対策にのりだし，赤潮発生状況調査や発生機構の解明等の研究に着手した。昭和52年には赤潮研究に直接あるいは間接的に関連した研究者・専門家を集めて「赤潮研究会」を組織し，研究体制の母体を確立した。そこで 1) 赤潮発生の現状調査と発生機構の解明，2) 赤潮構成生物の実体把握と同定，3) 赤潮発生の予察．4) 赤潮の防除対策，5) 赤潮関連情報の伝達・整理，のプロジェクト研究・調査が開始された。

本書は「赤潮研究会」の報告書の性格をもち，同会が当初目標とした，これまでの研究状況と現況の把握および今後の研究方針の設定にある程度の見通しがついてまとめたものと思われる。5章からなる本書の内容は上述の5つのプロジェクト研究の各班に各章が対応している。

第1章赤潮をつくる生物，では赤潮主要構成生物および貝毒原因生物を分類群毎に概説してある。執筆者によって概説のしかたはまちまちで，ある群では同定の要領や生物体の観察方法を解説したもの，別の群ではそれらの分類学の最近の動向を紹介したもの，あるいは執筆者自身の最新のデータを混じえより具体的に説明してあるもの等がある。本章のまとめで，実用分類学の立場からこの章に関連した研究の難しさを指摘し，それを克服すべき展望が述べられているが，その論旨がユニークで興味をひく。

第2章赤潮はなぜおこるか，執筆者はいずれも我国の赤潮研究の推進者であり，それぞれの専門分野の立場から赤潮発生機構の解明への努力の跡がうかがえる章で本書の中核をなしている。また人工的な赤潮を発生させる装置の開発と外的要因の制御がより完全に出来るマイクロコズムでの解析実験は赤潮研究の新しい分野として期待される。

第3章赤潮の発生は予知できるか，この章は赤潮の発生とそれによる漁業被害に直接対応しながら研究・調査を進めている水試・水研関係の研究者が担当している。現場のデータと経験にもとづいて赤潮発生予察の方法や指標の探索が検討され，予察の可能性が示唆されている。

第4章赤潮は防止できるか，は赤潮の発生を防止する方法と発生した赤潮を除去する方法の両面から検討が加えられている。前者は水質管理規制と浚渫船によるヘドロ回収によって汚染源を断つ方策に，また後者は赤潮回収船の設計や泥・粘土の沈降とともに赤潮生物を除去する効果的な方法の開拓に力を注いでいる。

第5章は赤潮関連情報の整理で，実際の赤潮発生の観測手段とその処理法および各分野から集まってくる情報の系統的な整理・保存・伝達について言及されている。この分野の仕事はその端緒についたばかりと思われたが，すでに赤潮発生の状況の連絡や予察・防除の情報交換に各地の水試・水研にテレックスが設置され運用されている。また赤潮構成生物や貝毒原因生物などの形態や行動を正確に記録し，同定作業を容易にする目的で，VTR が取り入れられているという。

本書により我国の赤潮発生状況，それによる被害と対策，赤潮研究の進行度合が明らかにされた。しかし全体として我国の赤潮研究には基礎生物学的な知見の不足がめだつ印象をうけた。

なお本書購入は以下に申し込まれるとよい。社団法人，日本水産資源保護協会，東京都千代田区永田町1-11-35。

（筑波大・生物　原　慶明）