Studies on β-1,3-Xylanase and β-Agarase from Two Strains of Marine Bacteria

青木, 恭彦

https://doi.org/10.11501/3054242

出版情報：九州大学, 1990, 農学博士, 論文博士
バージョン：
権利関係：

Studies on β-1,3-Xylanase and β-Agarase from Two Strains of Marine Bacteria

Takahiko Aoki

1990

(1)

Studies on β-1,3-Xylanase and β-Agarase from Two Strains of Marine Bacteria

Takahiko Aoki

1990

CONTENTS

Chapter I Introduction

The cell fusion is one of the new biotechnologies, and has been developing markedly in recent years. By using the cell fusion, it is expected to produce new plant species in varieties having a good harvest yield, resistance to diseases, or to harsh climates. The most of works in the literature on the cell fusion, up to date, have been done with terrestrial plants[1-4] but seaweeds.[5-10]

It is necessary to release protoplasts from plant cell wall by enzymic degradation of the structural polysaccharides, for the study on the cell fusion. That is, the availability of the enzymes capable of degrading cell wall polysaccharides may be one of the most important factors to advance the study of cell fusion. The release of protoplasts from terrestrial plants is accomplished with cellulase and pectinase, because the main polysaccharides of the cell wall are cellulose and pectin. In seaweeds, however, the polysaccharides of the cell wall differ from those of terrestrial plants except chlorophyceae(cellulose), that is, those of rhodophyceae are β-1,3-xylan, β-1,4-mannan, and porphyran, and those of phaeophyceae are alginate and fucoidan.[11] Little work has been done on the seaweed polysaccharide-degrading enzymes, and in consequence the lack of knowledge on the enzymes may lead to a delay in the study of cell fusion for seaweeds.

On the other hand, various oligosaccharides with physiological activity have been obtained from indigestible polysaccharides of terrestrial plants in recent years.[12,13] Since most of polysaccharides

of seaweeds are indigestible, they are expected to be promising materials for the oligosaccharides with physiological activity. Recently, an oligosaccharide from alginate has been reported to be effective on the human body.[14] Therefore, seaweed polysaccharides-degrading enzymes also may be beneficial to the preparation of the active oligosaccharides.

The author noticed the enzymes capable of degrading cell wall polysaccharides of the seaweeds of the genus *Porphyra*, which were cultivated abundantly and used as food stuff (nori) in Japan. Main polysaccharides of the cell wall in the seaweeds are β-1,3-xylan, porphyran, and β-1,4-mannan.[15,16] In this study, firstly, an attempt was made to obtain the three polysaccharide-degrading enzymes before the preparation of protoplasts and oligosaccharides.

β-1,3-Xylan-degrading enzyme, β-1,3-xylanase, has been reported to be produced by several strains of bacteria from marine environments (e.g. sea water, bottom mud of coastal sea, and green, brown and red algae).[17] In the report of Fujisawa *et al.*[18], of the 64 strains of β-1,3-xylan-degrading marine bacteria (unidentified) examined, at least 40 strains possessed β-1,3-xylanase activity, and 36 strains were capable of hydrolyzing β-1,4-xylan. β-1,3-Xylan-degrading enzymes also have been found in terrestrial fungi, *Aspergillus batatae*, *Chaetomium globosum*[19], and *Irpex lacteus*.[20] These organisms produced the enzymes constitutively in addition to β-1,4-xylanase when cultured on wheat bran (*Asp. batatae* and *Cha. globosum*), glucose (*Cha. globosum*), and cellulose (*Irp. lacteus*).[19] However, the β-1,3-xylanases from these organisms were not purified, and the properties were unknown yet.

Recently, Chen *et al.* purified six different β-1,3-xylanases from a fungus *Aspergillus terreus* A-07, and characterized the enzymes.[21] All of the six enzymes were endo-type β-1,3-xylanase (EC 3.2.1.32). However, these enzymes are not expected to be used for the preparation of viable protoplasts from seaweeds because they hardly acted on the substrate in neutral condition (the pH optima of these β-1,3-xylanases were from 4.0 to 5.5). Exo-type β-1,3-xylanase (EC 3.2.1.72) was also reported by Fukui *et al.* in a fungus *Chaetomium globosum*[19], but the optimal pH of this enzyme was not reported (the enzyme activty was determined at pH 3.5 in their study). In 1990, another endo-type β-1,3-xylanase was purified and characterized by Yamaura *et al.* from *Pseudomonas* sp. PT-5.[22] The purified enzyme had a pH optimum of 7.5, but the action pattern of the enzyme had not been fully investigated.

Porphyran-degrading enzyme (β-agarase, EC 3.2.1.81) has been found in several strains of bacteria isolated from marine environments, such as *Cytophaga* sp.[23] and *Pseudomonas atlantica*.[24,25] The enzymes from these bacteria hydrolyzed agar and porphyran to give neoagarotetraose as the predominant product. However, the enzymes degraded only a defined part of porphyran structure and exhibited a low hydrolyzing activity against porphyran as compared with agar. In the case of the β-agarase from *P. atlantica*, decomposing ratio of porphyran was only 23% after exhaustive digestion.[24] An extracellular β-agarase (β-agarase I) from *P. atlantica* was purified by Morrice *et al.*, and the enzymatic property was investigated.[24,25] The purified β-agarase I had a pH optimum of 7.0, and hydrolyzed agar to give neoagarotetraose as

the predominant product. The existence of another type of intracellular agarase (β-neoagarotetraose hydrolase[26] or β-agarase II, EC 3.2.1.-) from *P. atlantica* was suggested by Morrice *et al.*[24,25] The β-agarase II was likely to be an endo-type enzyme the same as β-agarase I, and hydrolyzed neoagarotetraose and agar to give neoagarobiose as the predominant product. However, the property of β-agarase II has not been fully investigated.

As regards β-1,4-mannanase, the isolation of bacteria with high productivity of the enzyme from natural habitats, and the purification and characterization of the enzymes from *Vibrio* sp. F-25 have been reported by Araki and Kitamikado[27] in the Laboratory of Fisheries Technology, Department of Fisheries, Faculty of Agriculture, Kyushu University. The author, therefore, prepared the endo-β-1,4-mannanase from the bacterial strain in this study.

Little has been reported on the search for microorganisms which produce the potent β-1,3-xylanase and agarase as mentioned above. Therefore, the author focused the object of this study on the isolation of bacteria with high productivity of the two enzymes, and on the examination of properties of the enzymes.

This dissertation describes the above states divided into seven chapters. Chapter II describes the isolation of β-1,3-xylanase-producing bacteria from natural habitats, and the identification of the bacterium with high productivity of the enzyme (AX-4 strain). Chapters III and IV describe the purification of β-1,3-xylanase from the AX-4 strain, and the characterization of the purified enzyme. Chapter V describes the

isolation of porphyran-degrading bacteria, and the identification of the bacterium with high productivity of porphyran-degrading enzyme (AP-2 strain). Chapters Ⅵ and Ⅶ describe the purification of β-agarases from the AP-2 strain, and the characterization of the enzymes. Chapter Ⅷ describes the use of β-1,3-xylanase and agarases for the preparation of protoplasts from the thalli of *Porphyra yezoensis* var. *narawaensis* and green type of *P. tenera* var. *tamatsuensis*.

Chapter II Isolation of β-1,3-Xylanase-producing Bacteria

The main polysaccharides in the cell wall of seaweeds of the genus *Porphyra* are β-1,3-xylan, β-1,4-mannan, and porphyran.[12,13] In these polysaccharides, β-1,3-xylan is a homo-polysaccharide which consists of D-xylopyranoses united to each other by β-1,3-linkage like a chain (Fig. II-1). β-1,3-Xylan found in seaweeds differs from β-1,4-xylan which is mainly found in terrestrial plants. β-1,3-Xylan is also found in the cell wall from other groups of seaweeds, such as genera *Caulerpa*[28] and *Halimeda*[11] of chlorophyceae.

The β-1,3-xylan-hydrolyzing enzyme, β-1,3-xylanase, has been reported to be produced by several strains of bacteria from marine environments[17], but no further biochemical studies on the purified bacterial enzyme were made until 1988. The enzyme has also been found from fungi.[19,20] There have been some reports on the properties of β-1,3-xylanases from fungi, but the pH optima of these xylanases were from 4.0 to 5.5.[21] They are not adequate to prepare protoplasts from seaweeds, because these enzymes hardly hydrolyzed β-1,3-xylan in a neutral condition. Therefore, the author searched for bacteria with a high productivity of β-1,3-xylanase which can degrade the substrate in a nearly neutral condition.

This chapter describes the isolation, culture conditions, and identification of a bacterium which is able to produce a large amount of β-1,3-xylanase.

Ⅱ - 1. Materials and Methods

Ⅱ - 1 - 1. Polysaccharides

β -1,3-Xylan was prepared by the method of Iriki *et al.*[29] from a green alga *Caulerpa racemosa* collected from the coastal sea in Kagoshima Prefecture. Thalli of the seaweed were dried, and powdered with a mill (mesh 80). The powder was suspended in deionized water, and sufficiently washed with water. The suspension was filtered through a cloth sack. The residue obtained was treated with 1% HCl at room temperature for 45 min, and then washed as above mentioned. The residue was treated successively with 1.25% NaOH and 1.25% H_2SO_4 at 100℃ under N_2 gas for 1 h. The residue was filtered through a cloth sack, suspended again in water, and then bleached with 1% sodium chlorite. The bleached residue was thoroughly washed with water. The crude fibre obtained was extracted with 10% NaCl at room temperature. To the extract was added 2 volumes of ethanol, and the mixture was centrifuged at 6,000 r.p.m. for 20 min, and then the precipitate was washed with ethanol containing 1% HCl followed by ethanol, and ether, and dried *in vacuo*. Finally, 8.8 g of the white powder of β -1,3-xylan was obtained from 100 g of the air-dried powder of the seaweed.

Structural saccharide of the β -1,3-xylan preparation was examined. The white powder , 0.1 g , was put into a test tube, and 2 m*l* of trifluoroacetic acid was added. The tube was sealed by heating, and the

powder was hydrolyzed at 100℃ for 3 h. After cooling, the hydrolyzed solution was dried *in vacuo*. The hydrolyzates were analyzed by thin layer chromatography (see IV-1-2). As the result, D-xylose was recognized on the chromatogram together with a trace amount of D-glucose, showing the high quality of the β-1,3-xylan preparation.

β-1,4-Mannan was prepared by the method of Love and Percival[30] from a green alga *Codium fragile* collected from the coastal sea in Fukuoka Prefecture. Porphyran was prepared by the method of Su and Hassid[15] from the dry powder of *Porphyra yezoensis* (see V-1-1).

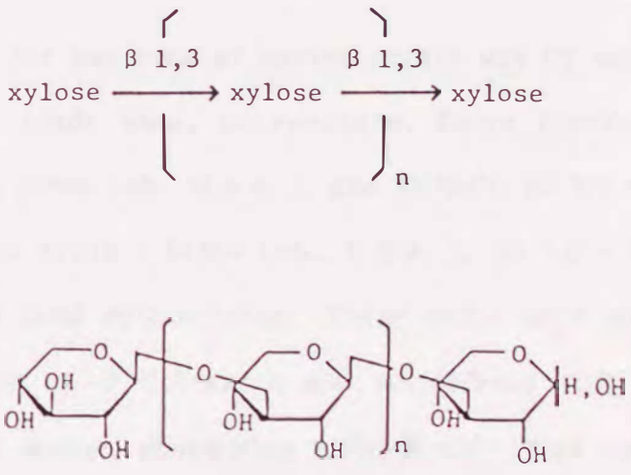


Fig.II - 1. Structure of β-1,3-xylan.

II - 1 - 2. Sources of β-1,3-xylanase-producing bacteria

Seventy-eight samples, collected during 1985 in Fukuoka Prefecture, were examined. Those of marine origin included: 4 from coastal sea water, 21 from the bottom mud of the coastal sea, 22 from algae (green, brown, and red), 5 from gills of marine fish, and 6 from intestinal contents of marine fish. Those from land and freshwater origins included: 10 from the soil, 4 from river water, and 6 from pond water.

II - 1 - 3. Method of screening for β-1,3-xylanase-producing bacteria

The medium for bacteria of marine origin was PY medium composed of 0.5% peptone (trade name, polypeptone, Daigo Eiyokagaku Co.), 0.1% yeast extract (Difco Lab., U.S.A.), and 3% NaCl, pH 7.6 - 7.8. The author used a nutrient broth (Difco Lab., U.S.A.), pH 7.0 - 7.2, for those of freshwater and land soil origins. These media were used after adding suitable amounts of β-1,3-xylan and solidifying with 1.5% agar, where required. Agar medium containing 1.0% β-1,3-xylan was whitish turbid because of the insolubility of β-1,3-xylan in water.

Before screening, each solid sample was blended with a small volume of either sterilized 3% NaCl solution (for samples of marine origin) or 0.9% NaCl solution (for those of land and freshwater origins). A small

quantity of the sample was streaked on the surface of agar medium containing 1.0% β-1,3-xylan in a petri dish, and the seeded medium was incubated aerobically at 25℃ for 4 - 7 days. Formation of the clear zone around the colony indicated the colony to be a β-1,3-xylanase-producer. The β-1,3-xylanase-producing bacteria were then purified by repeated single-colony isolations. Finally, isolated β-1,3-xylanase-producing bacteria were inoculated on agar slant media containing 0.3% β-1,3-xylan, stored in a refrigerator, and transferred monthly in homologous agar media to maintain viability.

Ⅱ-1-4. Assay of β-1,3-xylanase

Enzyme solution, 0.5 m*l*, was added to 2.0 m*l* of 50 mM sodium acetate buffer, pH 6.0, containing 0.5% β-1,3-xylan and 2 mM $CaCl_2$. After incubation at 37℃ for an appropriate period, the reaction was halted by heating the mixture in a boiling water bath for 5 min. The reducing sugar produced was then determined by Somogyi-Nelson's method[31] and expressed as D-xylose. One unit of the enzyme was defined as the activity which liberated 1 μmol of D-xylose per min, under the above condition.

Ⅱ-1-5. β-1,3-Xylanase productivity of isolated bacteria

Cells taken from agar slant culture of each isolate were transferred

to 20 m*l* of sterile medium in a 100 m*l* flask. The preparation was then incubated with shaking by a reciprocal shaker (8 cm amplitude, 90 strokes/min) at 25℃ for 4 days. The medium used was composed of 0.1% peptone, 0.1% yeast extract, 3% NaCl, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.3% β-1,3-xylan, pH 7.8 (see II-2-3-(d)). Each preparation was centrifuged, and the supernatant obtained was assayed for β-1,3-xylanase activity.

II - 1 - 6. Identification methods for AX-4 strain

Identification of the AX-4 strain was made according to the criteria given in Bergey's Manual of Systematic Bacteriology, Vol.1.[32] The medium used was PY medium, and the incubation temperature was 25℃ except for special cases. Motility, morphology, and Gram-staining characteristics were determined by light microscopy. The Gram-staining was carried out by the modification of Hucker's method.[33] Oxidase test was made by the method of Kovacs.[34] O-F test was by the Hugh-Leifson's method[35], and the formation of acid from D-glucose was determined by color-transition of BTB, and the formation of gas was observed using Durham tube. The arginine test was performed by the method of Thornley.[36] The mol% G + C of the DNA range was measured by the method of Marmur[37,38], with the DNA from *Escherichia coli* K12 used as a standard.

Other identification methods were used, where required.[39-41]

Ⅱ - 1 - 7. Examination for culture conditions of AX-4 strain

The following conditions were examined: (a) initial pH of culture medium; (b) addition of β -1,3-xylan, β -1,4-xylan, porphyran, β -1,4-mannan, and cellulose etc. into medium as the inducer of β -1,3-xylanase; (c) concentration of β -1,3-xylan in medium; (d) concentration of peptone in medium; (e) addition of inorganic salts, culture temperature and period, and shaking by a reciprocal shaker.

Ⅱ - 1 - 8. Preparation of β -1,3-xylanase from AX-4 strain

The strain was inoculated into 20 m*l* of liquid medium described in the paragraph on β -1,3-xylanase productivity of isolated bacteria (see II-1-5), and incubated with shaking (8 cm amplitude, 52 strokes/min) at 25 ℃ for 3 days. The culture was transferred to 1,000 m*l* of the same liquid medium in a 3 liter flask and incubated with shaking at the same temperature for 4 days. The culture obtained was centrifuged, and the supernatant fluid was subjected to the following precipitation procedure. All the following operations were conducted at 4 ℃. Solid ammonium sulfate was added to the supernatant fluid up to a final concentration of 75% saturation for ammonium sulfate precipitation. The mixture was kept overnight and centrifuged. The precipitate formed was dissolved in a small volume of 20 mM sodium acetate buffer, pH 6.0,

containing 2 mM $CaCl_2$, and the solution was poured into a cellulose tube and dialyzed overnight against several changes of the same buffer. The liquid inside the tube was used as β-1,3-xylanase solution (crude enzyme).

Ⅱ - 2. Results

Ⅱ - 2 - 1. Screening of β-1,3-xylanase-producing bacteria

Four colonies formed a clear zone on the plate media, thereby showing the ability to decompose β-1,3-xylan; two from the bottom mud of the coastal sea, and one each from a seaweed and from the intestinal contents of a marine fish (Table Ⅱ-1). A bacterium (AX-4 strain) originated from the bottom mud exhibited the highest β-1,3-xylanase productivity, exhibiting 48 m units per m*l* after 4-day incubation. The enzyme productivities from all the other isolates were within the range of 7.9 to 32 m units per m*l*. Therefore, the AX-4 strain was used for the following experiments.

Ⅱ - 2 - 2. Identification of AX-4 strain

The morphological and biochemical characteristics of the AX-4 strain

Table II - 1. Sources of β-1,3-xylanase-producing bacteria

Places	Samples	Number of samples	Number of isolates
Coastal sea	Sea water	4	0
	Sea bottom mud	21	2
	Sea weed	22	1
	Marine fish		
	gills	5	0
	intestine	6	1
	(Total	58	4)
River and pond	River water	4	0
	Pond water	6	0
	(Total	10	0)
Land	Land soil	10	0
	(Total	10	0)

are shown in Table II-2. The bacterium was a Gram-negative and facultatively anaerobic rod with a single polar flagellum, it did not form endo-spores, formed acid but no gas from glucose, and was sensitive to 2,4-diamino-6,7-diisopropyl pteridine. Therefore, it was assigned to the genus *Vibrio*, according to the criteria given in Bergey's Manual of Systematic Bacteriology. All species of the genus listed in the Manual, however, differed from the AX-4 strain in some characteristics. The species of the AX-4 strain was not determined.

Table II - 2. Morphological, cultural, and biochemical characteristics of AX-4 strain

Form	rod	Na^+ required for organic growth factors	+
Size	0.7-0.8 μm x 1.2-3.2 μm	Growth at:	
		4°C	-
Gram stain	Negative	30°C	+
Motility	+	35°C	+
		40°C	-
Flagellation	Single polar		
Growth in air	+	Production of:	
Unaerobic growth	+	Amylase	+
		Gelatinase	+
O-F test	Fermentation	Lipase	+
		Alginase	-
Catalase	+	Chitinase	+
Vibriostatic agent sensitivity	+	Utilization of:	
Swarming on solid complex media	-	D-Xylose	+
		L-Arabinose	+
PHB-accumulation	-	D-Mannose	+
		D-Galactose	+
Pigmentation		Sucrose	+
Yellow-orange	-	Trehalose	+
Blue-black	-	Cellobiose	+
Red	-	Lactose	-
		Salicin	+
Arginine dihydrolase	+	Citrate	±
Oxidase	+	D-Mannitol	+
		D-Sorbitol	-
Reduction of NO_3^- to NO_2^-	+	meso-Inositol	-
Luminescence	-	Ethanol	±
		L-α-Alanine	+
Gas from D-glucose	-	β-Alanine	-
		L-Serine	+
Production of acetoin and/or diacetyl	-	L-Leucine	-
		L-Histidine	+
Voges-Proskauer test(24 h)	-	L-Proline	+
Methyl red test	+	L-Tyrosine	+
Thornley arginine	+	Mol% G + C of DNA	46

II - 2 - 3. Culture conditions for β-1,3-xylanase production in AX-4 strain

(a) Initial pH of culture medium

PY media of various initial pHs (pH 6.3, 6.8, 7.3, 7.8, 8.3, and 8.8) were prepared, and 20 m*l* of each medium in a 50 m*l* flask was inoculated with the AX-4 strain, and incubated with shaking at 25℃ for 3 days. The culture was centrifuged, and the reducing sugar was determined for the supernatant solution. As shown in Table II-3, the maximal activity of β-1,3-xylanase was recognized in the medium of initial pH 7.8.

Table II - 3. Effect of medium pH on β-1,3-xylanase production of AX-4 strain

Initial pH	Final pH	β-1,3-xylanase activity (m units/ml of culture fluid)
6.3	4.5	2.3
6.8	4.7	8.1
7.3	4.7	46
7.8	7.8	48
8.3	7.9	47
8.8	8.0	46

(b) Influence of carbohydrates in medium

The influence of various kinds of carbohydrates on the enzyme production was measured using PY medium. β-1,3-Xylan (0.1 g), β-1,4-xylan (0.1 g), porphyran (0.1 g), β-1,4-mannan (0.1 g), xylose (0.1 g), cellulose (0.1 g), powder(0.2 g) and crude fibre (0.2 g) of *Caulerpa racemosa*, or powder of *Porphyra yezoensis* (0.2 g) was added to 20 m*l* of the medium as an inducer of β-1,3-xylanase. After inoculation with the AX-4 strain, the medium was incubated with shaking at 25℃ for 4 days. The culture was centrifuged, and the enzyme activity of the supernatant was measured. As shown in Fig. II-2, the enzyme activity was found evidently in the medium with β-1,3-xylan, and the trace of activity was found in medium with powder of *Caulerpa racemosa*. In all the media without β-1,3-xylan and the powder of *C. racemosa*, no β-1,3-xylanase activity was recognized.

(c) Influence of β-1,3-xylan concentration in medium

PY media containing various concentrations of β-1,3-xylan were prepared, and then incubated with shaking at 25℃ for 4 days after inoculation with the AX-4 strain. The enzyme activity was measured for the culture fluids obtained by the centrifugation of cultures. As shown in Fig.II-3, β-1,3-xylanase activity reached a maximal level when the organism grew in the media containing 0.2 to 0.5% concentration of β-1,3-xylan.

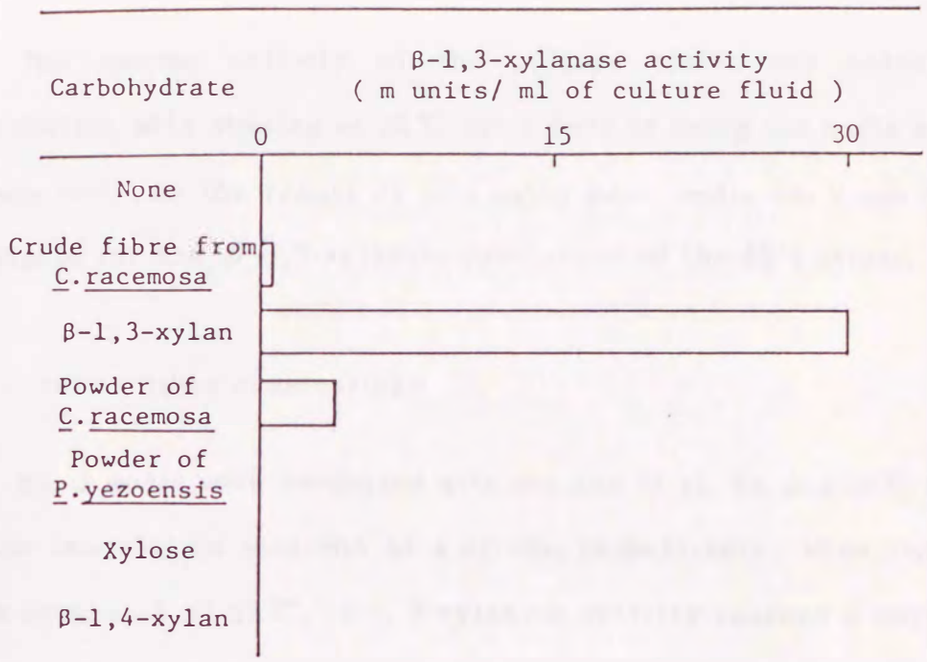


Fig.II - 2. Induction of β-1,3-xylanase in AX-4 strain by different carbohydrates.
PY medium containing 0.5% of different carbohydrate was used as culture medium.

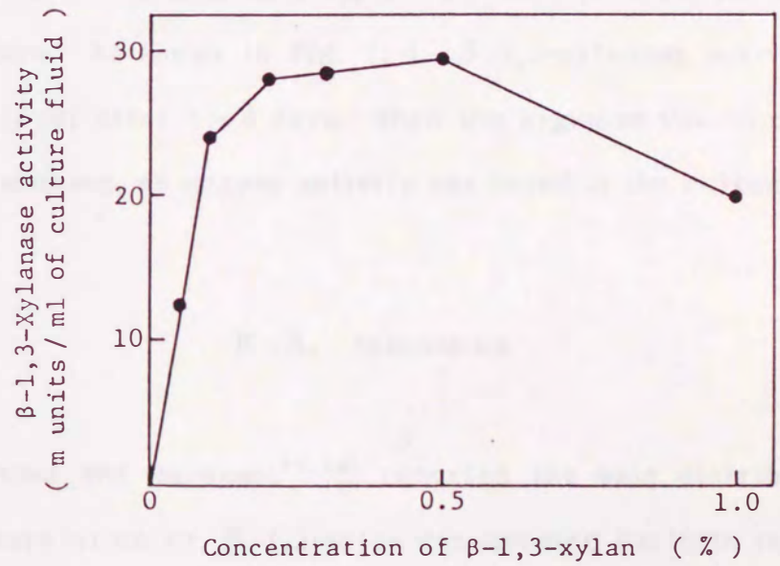


Fig.II - 3. Influence of β-1,3-xylan concentration on β-1,3-xylanase production of AX-4 strain.

(d) Composition of medium

The enzyme activity of the culture fluids was measured after incubation with shaking at 25℃ for 3 days by using the media as shown in Table II-4. As the result of this experiment, media No. 2 and No. 3 were suitable for the β-1,3-xylanase production of the AX-4 strain.

(e) Other examinations

No. 2 media were incubated with shaking at 15, 20, and 25℃ for 3 days after inoculation with the AX-4 strain, respectively. When the organism was incubated at 25℃, β-1,3-xylanase activity reached a maximal level in the culture fluid.

No. 2 medium, 1,000 *ml*, in a 3 liter flask was incubated with shaking (8 cm amplitude, 52 strokes/min) at 25℃ after inoculation with the AX-4 strain, and the enzyme activity of the culture fluid was measured daily for 7 days. As shown in Fig. II-4, β-1,3-xylanase activity reached a maximal level after 4 - 6 days. When the organism was incubated at 25℃ without shaking, no enzyme activity was found in the culture fluid.

II - 3. Discussion

Fujisawa and Murakami[17,18] reported the wide distribution and the sparse population of β-1,3-xylan-decomposing bacteria in the sea area near Shimonoseki. In this study, such bacteria were searched for in sea

Table II - 4. β-1,3-Xylanase production of AX-4 strain in various media

Medium No.	Medium composition (%)	Activity (m units/ml of culture fluid)
1	Peptone 0.1%, Yeast extract 0.05%, NaCl 3.0%, K_2HPO_4 0.2%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, β-1,3-xylan 0.3%	30.5
2	Peptone 0.1%, Yeast extract 0.1%, NaCl 3.0%, K_2HPO_4 0.2%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, β-1,3-xylan 0.3%	38.3
3	Peptone 0.5%, Yeast extract 0.1%, NaCl 3.0%, K_2HPO_4 0.2%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, β-1,3-xylan 0.3%	36.0
4	Peptone 1.0%, Yeast extract 0.1%, NaCl 3.0%, K_2HPO_4 0.2%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, β-1,3-xylan 0.3%	10.7
5	Peptone 0.5%, Yeast extract 0.1%, NaCl 3.0%, β-1,3-xylan 0.3%	33.7

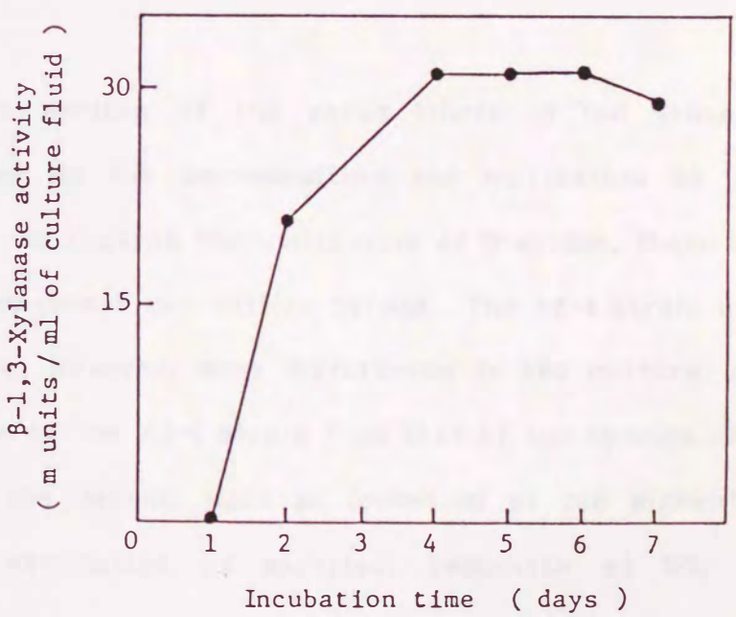


Fig.II - 4. Effect of incubation time on β-1,3-xylanase production of AX-4 strain.

water, sea bottom mud, and seaweeds collected from the coastal sea in Fukuoka Prefecture, and 4 isolates were obtained. A distinctly clear zone was detected around the colonies on plate media. Among the 4 isolates, the AX-4 strain showed the highest productivity of β-1,3-xylanase. Some other bacteria showed a doubtful clear zone on the plate media, but no more attention was given to them.

The AX-4 strain seemed to belong to the genus *Vibrio*, according to the Bergey's Manual of Systematic Bacteriology. Among all the 20 species of the genus given in the Manual, the characteristics of *V. proteolyticus*, *V. anguillarum* I, *V. splendidus* I, and *V. fluvialis* I had resemblance to that of the AX-4 strain. However, *V. proteolyticus* and *V. anguillarum* I differed from the AX-4 strain in the production of acetoin and/or diacetyl. *V. splendidus* I differed from the AX-4 strain in the having of luminescence. *V. fluvialis* I had close resemblance to the AX-4 strain on many characteristics, but it differed from the AX-4 strain in the growth at 40℃.

In the section of the genus *Vibrio* in the Manual, there is no description on the decomposition and utilization of β-1,3-xylan by bacteria. As regards the utilization of D-xylose, there is a description that *V. gazogenes* can utilize xylose. The AX-4 strain utilized xylose. There were, however, many differences in the cultural and biochemical properties of the AX-4 strain from that of the species of *V. gazogenes* given in the Manual, such as formation of red pigment and gas from glucose, utilization of sorbitol, reduction of NO_3^- to NO_2^-, and production of oxidase and arginine dihydrolase.

Ⅱ-4. Summary

A screening was undertaken to acquire bacteria with a high rate of β-1,3-xylanase productivity. Such a strain was obtained from the bottom mud of the coastal sea in Fukuoka Prefecture. The organism needed β-1,3-xylan as an inducer to produce β-1,3-xylanase, and released a large amount of the enzyme into the culture fluid, when incubated with shaking at 25℃ for 4 to 6 days in the following liquid medium: 0.1% or 0.5% peptone, 0.1% yeast extract, 3.0% NaCl, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.3% β-1,3-xylan, pH 7.8. The bacterium was assigned to the genus *Vibrio*, according to the criteria given in the Bergey's Manual of Systematic Bacteriology, Vol.1. Species name was not determined for the organism, because some differences were recognized between the characteristic of the AX-4 strain and that of all the 20 species given in the Manual.

Chapter Ⅲ Purification of β-1,3-Xylanase from *Vibrio* sp. AX-4

The AX-4 strain was assigned to the genus *Vibrio*, in the preceding chapter. Therefore, this strain is named *Vibrio* sp. AX-4 in the following chapters.

There have been reported purified β-1,3-xylanases from *Aspergillus terreus* A-07 by Chen *et al.*[21] In their study, six β-1,3-xylanases were purified by ammonium sulfate precipitation, successive column chromatography and isoelectric focusing. The specific activities of final enzyme preparations were 6.4 - 15.2 times more to the culture fluid, and the total recovery was 10.7%. These enzymes were not adequate for the preparation of protoplasts from seaweed, because they hardly acted on the substrate in neutral condition.

This chapter describes the purification of the enzyme from *Vibrio* sp. AX-4 by ammonium sulfate precipitation and successive column chromatography.

The properties determined with the crude enzyme preparation (see II-1-8) were as follows : the enzyme was likely to exhibit a high activity in the range of pH 6.0 to 7.5, and was stable in a pH region from 4.5 to 10.0, and had a molecular weight of ca. 50 kDa. The enzyme split β-1,3-xylan but not β-1,4-xylan, β-1,4-mannan, porphyran, and cellulose. These properties were considered in the purification of the enzyme.

Ⅲ-1. Materials and Methods

Ⅲ-1-1. Materials

β-1,3-Xylan and β-1,4-mannan were prepared from green algae *Caulerpa racemosa* and *Codium fragile*, respectively (see Ⅱ-1-1). Porphyran was prepared from a red alga *Porphyra yezoensis* (see Ⅴ-1-1). DEAE-Sephadex CL-6B, SP-Sephadex C-25, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals (Sweden). Polyethylene glycol was purchased from Wako Pure Chem. Ind., and hydroxyapatite was from Seikagaku Kogyo Co.

Ⅲ-1-2. Assay of enzymes

β-1,3-Xylanase was assayed by the determination of increased reducing sugar as described in Ⅱ-1-4. Protease activity was determined according to the method of Hagiwara *et al.*[42]

Ⅲ-1-3. Analytical methods

Protein was determined by the method of Lowry *et al.*[43] with bovine serum albumin as the standard. Protein was also measured with a spectrophotometer by the absorption at 280 nm. Polyacrylamide disc gel electrophoresis was performed by the Davis's method[44] on a 7% gel in a Tris-glycine buffer, pH 8.3. Protein developed on the gel was stained

with Coomassie brilliant blue R-250.

Ⅲ-2. Results

Ⅲ-2-1. Preparation of culture fluid

Vibrio sp. AX-4 was grown for 4 days in liquid medium (medium No.2; see Ⅱ-2-3), pH 7.8, composed of 0.1% peptone, 0.1% yeast extract, 3% NaCl, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.3% β-1,3-xylan, with shaking at 25℃. The culture was centrifuged at 10,000 r.p.m. for 30 min. From 7,200 m*l* of the culture, 6,840 m*l* of clear culture fluid was obtained.

All the following procedures were carried out at 4℃ unless otherwise indicated.

Ⅲ-2-2. Ammonium sulfate precipitation

The culture fluid was adjusted to 75% saturation with solid ammonium sulfate, then left to stand overnight. The precipitate formed was collected by centrifugation, and dissolved in 190 m*l* of 50 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$. The enzyme solution was dialyzed against the same buffer. This enzyme preparation showed a little activity of protease (0.26 unit/m*l*), but the enzyme preparations from the following purification procedures were free from protease.

Ⅲ-2-3. DEAE-Sepharose CL-6B chromatography

The dialyzed enzyme solution was applied to a column (2.0 × 15 cm) of DEAE-Sepharose CL-6B equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$. After being washed with 10 bed volumes of the same buffer, the column was eluted with a linear gradient of 0 - 0.8 M NaCl in 50 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$ (total vol. 600 *ml*). The enzyme activity was detected in tubes No. 74 - 80, as shown in Fig. III-1, and these fractions were pooled and concentrated to 40 *ml* with polyethylene glycol (M.W. 20 kDa).

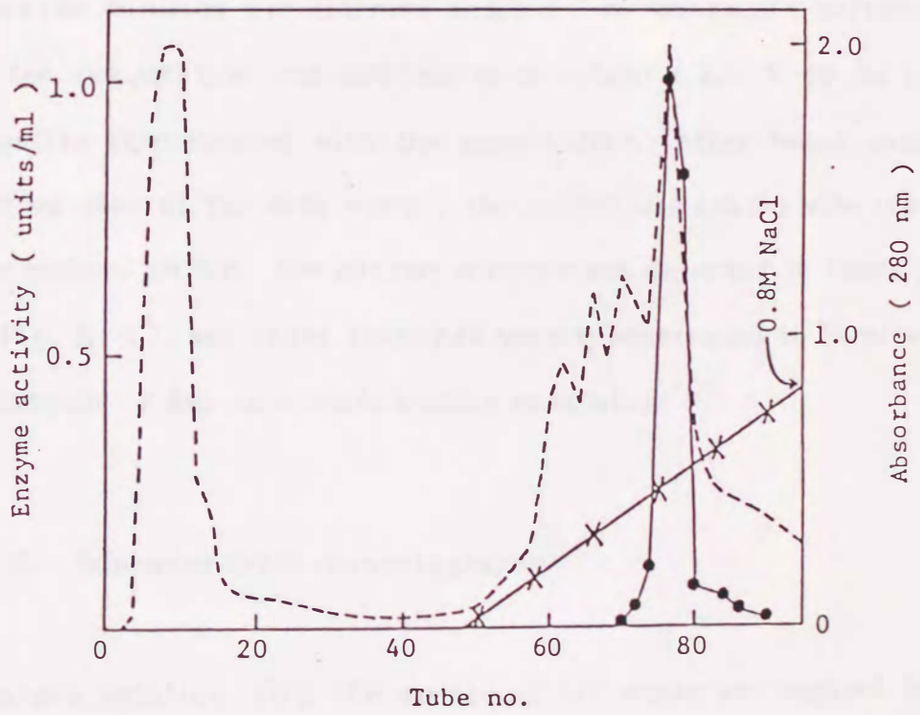


Fig.III - 1. Chromatography of β-1,3-xylanase on a DEAE-Sepharose CL-6B column. ——●——, enzyme activity; – – – – , absorbance at 280nm; ——×—— , concentration of NaCl.
tubes no. 1 to 40, 15 ml/tube; tubes no. 41 to 95, 6 ml/tube.

Ⅲ-2-4. SP-Sephadex C-25 chromatography

The enzyme solution from DEAE-Sepharose CL-6B chromatography was dialyzed against 50 mM sodium acetate buffer, pH 4.5, and then applied to a column (2.0 × 6.0 cm) of SP-Sephadex C-25 equilibrated with the same buffer. Elution was performed with the same buffer. The enzyme activity was detected in tubes No. 3 - 20 (Fig. III-2), and these fractions were concentrated to 15 m*l* with polyethylene glycol.

Ⅲ-2-5. Hydroxyapatite chromatography

The enzyme solution was dialyzed against 2 mM phosphate buffer, pH 6.8, and the preparation was applied to a column (2.0 × 20 cm) of hydroxyapatite equilibrated with the same buffer. After being washed with 6 bed volumes of the same buffer, the column was eluted with 100 mM phosphate buffer, pH 6.8. The enzyme activity was detected in tubes No. 49 - 62 (Fig. III-3), and these fractions were concentrated to 10 m*l* with a UM-10 membrane of Amicon ultrafiltration apparatus.

Ⅲ-2-6. Sephadex G-200 chromatography

The enzyme solution from the preceding procedure was applied to a column (2.5 × 80 cm) of Sephadex G-200 equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$. Elution was performed

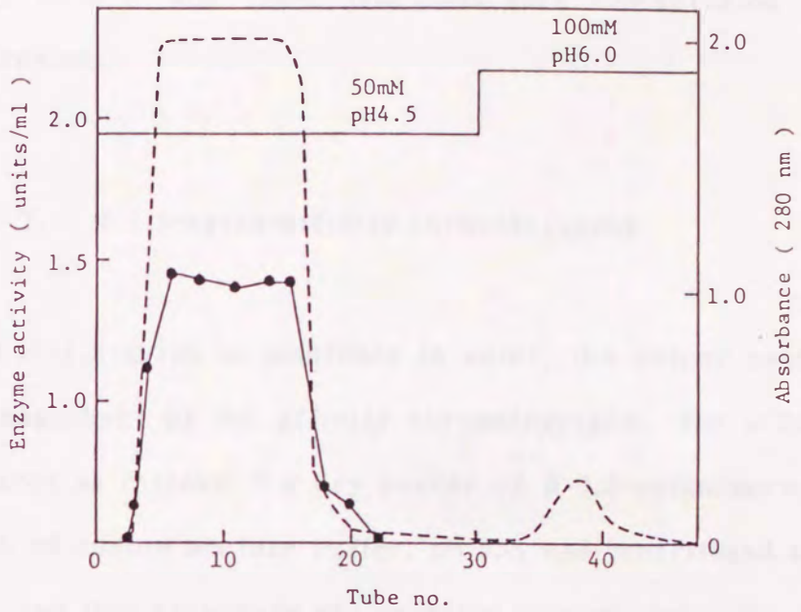


Fig.III - 2. Chromatography of β-1,3-xylanase on a SP-Sephadex C-25 column. —●—, enzyme activity; - - - - -, absorbance at 280 nm; ———, concentration and pH of acetic acid buffer.
tubes no. 1 to 30, 3.5 ml/tube; tubes no. 31 to 50, 3.0 ml/tube.

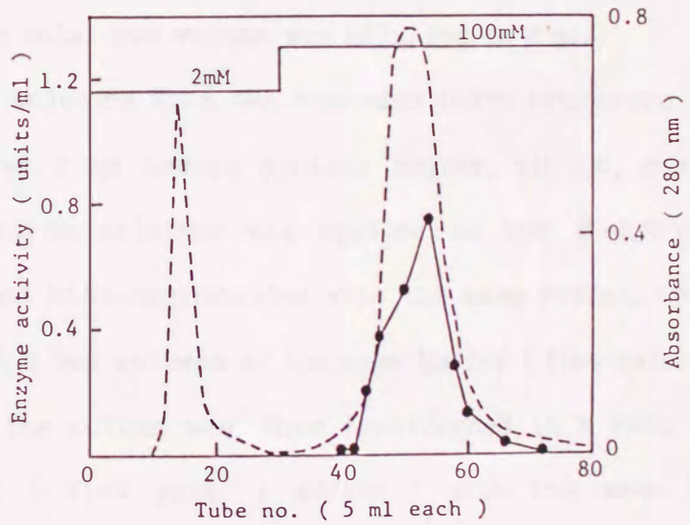


Fig.III - 3. Chromatography of β-1,3-xylanase on a hydroxyapatite column. —●—, enzyme activity; - - - - -, absorbance at 280 nm; ———, concencentration of phosphate buffer.

with the same buffer. The enzyme activity was detected in tubes No. 63 - 70 (Fig. III-4), and these fractions were concentrated to 5 m*l* by ultrafiltration.

III-2-7. β-1,3-Xylan-affinity chromatography

Since β-1,3-xylan is insoluble in water, the author used it for the specific adsorbent of the affinity chromatography. The affinity column was prepared as follows: 3 *g* dry powder of β-1,3-xylan was suspended in 30 m*l* of 5 mM sodium acetate buffer, pH 5.0, and centrifuged at 750 r.p.m. for 5 min, and this procedure was repeated several times. The precipitate obtained was mixed with Celite (2 : 3, v/v), and then put into a column (1.0 × 5.5 cm) which had been loaded with Celite (ca. 0.2 m*l*) at the bottom. A small amount of β-1,3-xylan (ca. 0.2 m*l*) was put at the top of the column, and total bed volume was adjusted to 4 m*l*.

The enzyme solution from the Sephadex G-200 procedure (III-2-6) was dialyzed against 5 mM sodium acetate buffer, pH 5.0, containing 2 mM $CaCl_2$. The enzyme solution was applied to the β-1,3-xylan-affinity column which had been equilibrated with the same buffer, and the column was washed with 5 bed volumes of the same buffer (flow rate: 1 m*l*/h) in a room at 4℃. The column was then transferred to a room at 37℃, and eluted rapidly (flow rate: 1 m*l*/min) with the same buffer after preincubation for 20 min (Fig. III-5). Active fractions (tubes No. 36 - 42) were pooled as the purified β-1,3-xylanase, and stored in a

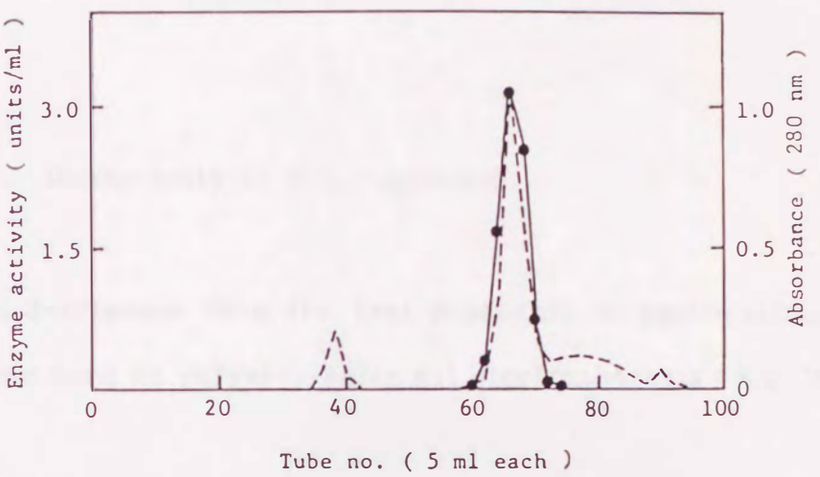


Fig.III - 4. Chromatography of β-1,3-xylanase on a Sephadex G-200 column.
—●—, enzyme activity; – – – – –, absorbance at 280 nm.

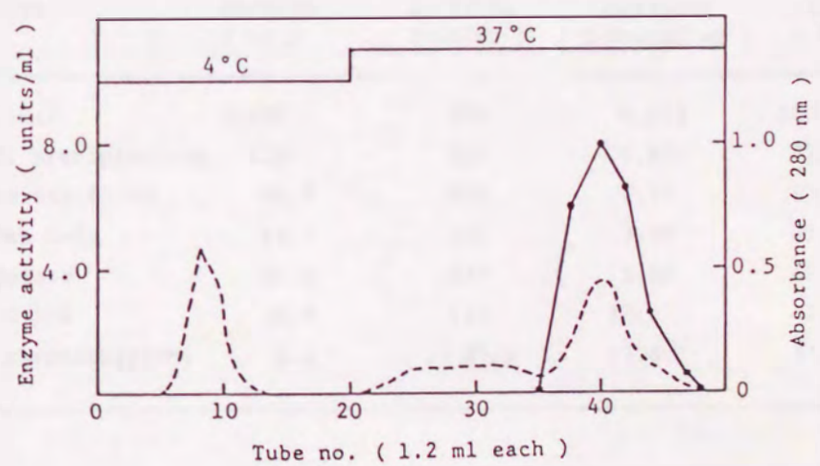


Fig.III - 5. Chromatography of β-1,3-xylanase on a affinity column.
—●—, enzyme activity; – – – – –, absorbance at 280 nm ;
———, temperature of a column.

refrigerator.

The purification and yield of the β-1,3-xylanase are summarized in Table III-1.

III-2-8. Homogeneity of β-1,3-xylanase

The β-1,3-xylanase from the last procedure of purification gave a single protein band on polyacrylamide gel electrophoresis (Fig. III-6).

Table III - 1. Purification of β-1,3-xylanase from the culture fluid (6,840 ml) of AX-4 strain

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/ mg)	Yield (%)	Purification (fold)
Culture fluid	2,630	324	0.123	100	1
$(NH_4)_2SO_4$ precipitation	415	250	0.602	77.2	4.88
DEAE-Sepharose Cl-6B	88.9	205	2.30	63.2	18.7
SP-Sephadex C-25	48.7	159	3.26	49.0	26.4
Hydroxyapatite	24.0	139	5.80	42.8	47.0
Sephadex G-200	10.7	137	12.9	42.4	105
Affinity chromatography	6.1	83.4	13.6	25.7	111

Fig.III - 6. Polyacrylamide gel electrophoresis of final preparation of β-1,3-xylanase. The purified enzyme was subjected to disc electrophoresis in 7.0% polyacrylamide gel at pH 8.3. Apporoximately 50μg of the enzyme was applied per tube. A current of 3 mA was supplied per tube at room temperature. (+), anode; (-), cathode.

Ⅲ-3. Discussion

β -1,3-Xylanase was purified from the culture fluid of a marine bacterium, *Vibrio* sp. AX-4, by ammonium sulfate precipitation, and successive chromatography. Each procedure of purification improved effectively the enzyme purity, and the specific activity of each procedure was higher than that of the preceding procedure. However,

the specific activity of the affinity chromatography was not much higher than that of the preceding procedure. This reason was supposed to be that the enzyme activity was decreased in case of the elution at high temperature. This procedure was effective to purify the enzyme, though there was this shortcoming of the affinity chromatography.

Recently, another endo-type β-1,3-xylanase from a marine bacterium (*Pseudomonas* sp.) was purified by Yamaura *et al.*[22], and the recovery of final purification step was 6.0% to the culture fluid, while that of the enzyme from *Vibrio* sp. AX-4 was 23%. The low recovery of the enzyme from *Pseudomonas* sp. resulted from the coexistence of other β-1,3-xylanase in the culture fluid of the bacterium.

Ⅲ-4. Summary

β-1,3-Xylanase was purified from the culture fluid of a marine bacterium, *Vibrio* sp. AX-4, by ammonium sulfate precipitation and successive use of DEAE-Sepharose CL-6B chromatography, SP-Sephadex C-25 chromatography, hydroxyapatite chromatography, Sephadex G-200 chromatography and β-1,3-xylan-affinity chromatography. The specific activity of the final purification procedure was 111 times higher than that of the culture fluid, and the recovery was 23%. The final enzyme preparation was homogeneous on polyacrylamide gel electrophoresis.

Chapter Ⅳ Characterization of β-1,3-Xylanase from *Vibrio* sp. AX-4

There have been reported two types of β-1,3-xylanases, one type of the enzymes hydrolyzes β-1,3-xylan to D-xylose (exo-type enzyme, exo-β-1,3-xylanase, EC 3.2.1.72)[45], and another type hydrolyzes to D-xylose and D-xylooligosaccharides (endo-type enzyme, endo-β-1,3-xylanase, EC 3.2.1.32). Although β-1,3-xylanase was found in marine bacteria[17,18], fungi[19,20], and land snail[46], the property of these enzymes has not been fully investigated. The mode of action had not been studied until 1986 except the β-1,3-xylanase from *Chaetomium globosum*[19], and the unpurified enzyme proved to be an exo-type β-1,3-xylanase. In 1986, six β-1,3-xylanases were isolated from *Aspergillus terreus* A-07[21], and all of the enzymes were reported to be endo-type β-1,3-xylanase. However, the property and mode of action of bacterial β-1,3-xylanase have not been investigated. This chapter describes the characterization of the β-1,3-xylanase purified from the culture fluid of *Vibrio* sp. AX-4.

Ⅳ-1. Materials and Methods

Ⅳ-1-1. Substrates

β-1,3-Xylan was prepared from a green alga *Caulerpa racemosa* (see Ⅱ-1-1). β-1,3-Xylooligosaccharides (xylobiose to xylopentaose) were

prepared as follows: the enzyme solution (10 units) from the AX-4 strain was added to 200 *ml* of 2 mM sodium acetate buffer, pH 6.0, containing 1.0% β-1,3-xylan and 2 mM $CaCl_2$, and the mixture was incubated at 37℃ for 12 h. The mixture was heated at 100℃ for 5 min, and was concentrated *in vacuo*. The hydrolysis product obtained was applied to a column (3.5 × 25 cm) of charcoal equilibrated with water. After being washed with 2 bed volumes of water, the column was eluted with 4, 9, 11, 30, and 50% ethanol solution successively. The xylooligosaccharides obtained were spotted on a TLC-plastic sheet of Silica Gel 60. The chromatography was carried out by an ascending method (see IV-1-2). After developing, the part of silica gel containing each oligosaccharide was torn off from the sheet, gathered up, and put into water. The solution was centrifuged, and the supernatant solution was obtained, and concentrated. Each oligosaccharide preparation was examined for structural saccharide (see II-1-1) and for polymerization degree by the method of French and Wild.[47] As the result of these examinations, the preparations were evident to be purified xylooligosaccharides (xylobiose to xylopentaose).

β-1,4-Xylan, cellulose, D-xylose, and various *p*-nitrophenyl(PNP) -glycosides were obtained from Sigma Chemical Co. (U.S.A.).

IV-1-2. Thin layer chromatography of hydrolysis products

Thin layer chromatography (TLC) of saccharide was performed on a plastic sheet of Silica Gel 60 (Merck & Co., Inc.) by an ascending method,

and the saccharide was developed with a solvent of n-butanol - acetic acid - water (10 : 5 : 1, v/v), and visualized by spraying the plate with a diphenylamine-aniline-phosphate reagent.

Ⅳ-1-3. Assay of enzymes

β -1,3-Xylanase was assayed by the determination of increased reducing sugar as described in II-1-4. Activities of various glycosidases were determined as follows: the reaction mixture of 500 μl consisted of 50 μl of 6 mM PNP-glycoside, 350 μl of 2 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$, and 100 μl of the enzyme solution (ca. 0.3 unit). After incubation at 37℃ for 20 h, 3 m*l* of 200 mM glycine-NaCl-NaOH buffer, pH 10.5, was added to the reaction mixture. The quantity of *p*-nitrophenol liberated in the mixture was measured with a spectrophotometer at 400 nm.[48]

Ⅳ-1-4. Iso-electric focusing

A 110 m*l* column and Ampholines (LKB Producter, Sweden) of pH range from 3.5 to 10 or from 2.5 to 4.0 and the method of Vesterberg and Svensson[49] were used for iso-electric focusing. The electrophoresis was carried out at 2℃ for 48 h, and then the solution in the column was fractionized (flow rate: 2 m*l*/min). The fraction obtained (1.5 m*l* each) was measured at 2℃ for the pH and the enzyme activity.

Ⅳ-1-5. Molecular weight of β-1,3-xylanase

The purified enzyme solution (2.0 m*l*) was applied to a column (2.2 × 100 cm) of Sephadex G-100 equilibrated with 20 mM sodium acetate buffer, pH 6.0, containing 0.2 M NaCl and 2 mM $CaCl_2$. Standard proteins (Myoglobin, 17.8 kDa; Chymotrypsinogen A, 25 kDa; Ovalbumin, 45 kDa; Bovine albumin, 67 kDa) were also applied to the column in the same condition. Elution was performed with the same buffer. The absorption of each fraction was measured at 280 nm for the protein assay.

Ⅳ-2. Results

Ⅳ-2-1. Action pattern of β-1,3-xylanase

(a) Action of β-1,3-xylanase on β-1,3-xylan

To 10 m*l* of 10 mM sodium acetate buffer, pH 6.0, containing 50 mg β-1,3-xylan, 2 m*l* of the β-1,3-xylanase solution (2 units) was added, and the mixture was incubated at 37℃. A part of the mixture was withdrawn at various intervals, boiled to inactivate the enzyme, and spotted on a TLC plate. The enzyme degraded β-1,3-xylan to form xylose, xylobiose, xylotriose, and larger oligosaccharides, as shown in

Fig. IV-1. The result showed that the enzyme from *Vibrio* sp. AX-4 was an endo-β-1,3-xylanase.

(b) Action of β-1,3-xylanase on xylooligosaccharides

To 0.2 m*l* of 10 mM sodium acetate buffer, pH 6.0, containing 2.0% each oligosaccharide, 0.2 m*l* of the enzyme solution (0.2 unit) was added, and the mixture was incubated at 37℃ for 20 h. The reaction mixture was concentrated *in vacuo* followed by spotting on a TLC plate. The enzyme acted on xylotriose and larger xylooligosaccharides, but not on xylobiose (Fig. IV-2). The hydrolysis products from xylotriose were xylose and xylobiose. Xylotetraose was degraded to xylose, xylotriose, and a small amount of xylobiose. The hydrolysis products from xylopentaose were mainly xylose and xylotetraose, in addition to small amounts of xylobiose and xylotriose.

(c) Action of β-1,3-xylanase on PNP-glycosides

Various glycosidase activities of the enzyme preparation were examined by using PNP-α- and -β-D-galactosides, PNP-β-D-N-acetyl-galactosaminide, PNP-α- and -β-D-glucosides, PNP-β-D-N-acetyl-glucosaminide, PNP-α-L-fucoside, PNP-β-D-fucoside, PNP-α- and -β-D-mannosides, PNP-β-D-xyloside, PNP-sulfate, and PNP-β-D-glucuronide. The purified enzyme preparation was free from these glycosidases.

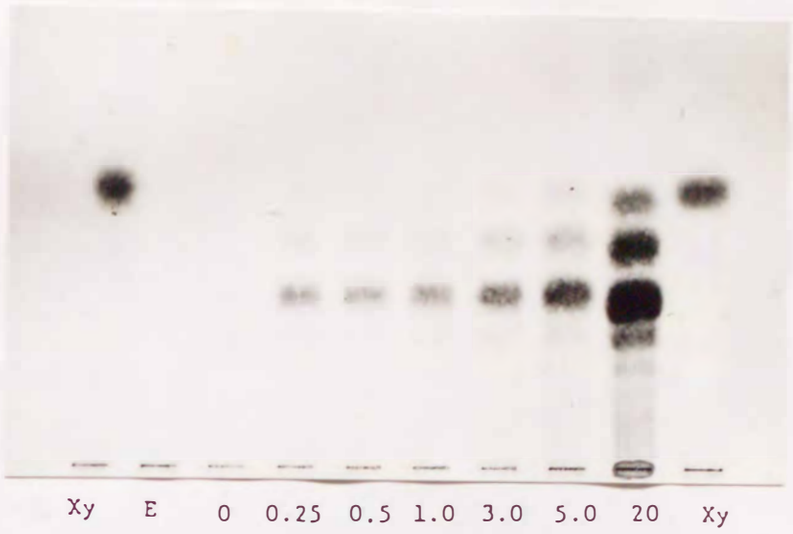


Fig.IV - 1. Thin layer chromatogram of hydrolysis products of β-1,3-xylan with β-1,3-xylanase.
Xy, xylose; E, enzyme.

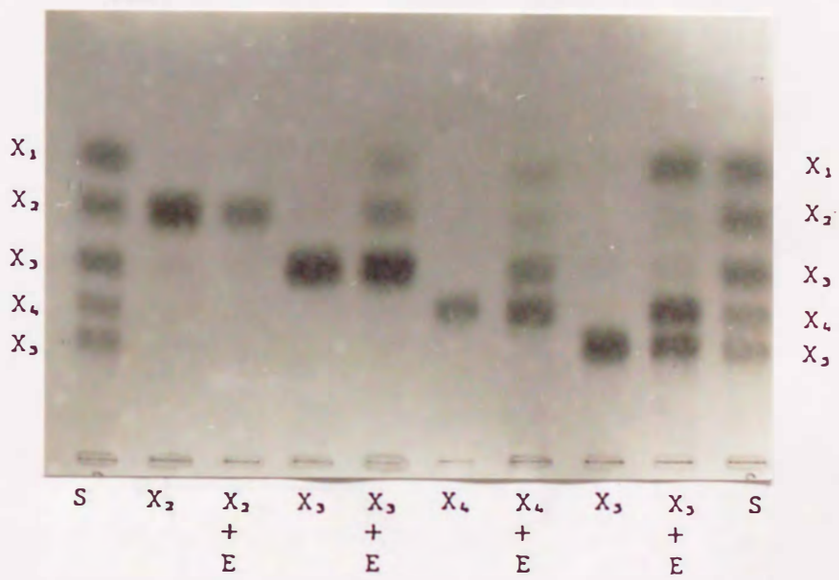


Fig.IV - 2. Thin layer chromatogram of hydrolysis products of several xylooligosaccharides with β-1,3-xylanase.
S, standard; E, enzyme; X_1, xylose; X_2-X_5, xylobiose to xylopentaose.

IV-2-2. Optimal pH of β-1,3-xylanase

The reaction mixture of 80 μl of purified enzyme solution (5 units), 1,420 μl of buffer [M/10 sodium acetate, pH 3.2 - 6.0; M/10 2-(n-morpholino) ethane sulfonic acid (MES), pH 5.0-8.0; M/15 phosphate, pH 6.0 - 8.0; M/20 Tris-HCl, pH 7.2 - 9.0], and 1.0 ml of 1.0% β-1,3-xylan aqueous suspension containing 4 mM $CaCl_2$ were incubated at 37℃ for 10 min. The enzyme exhibited a high activity in the range of pH 6.0 to 7.5 as shown in Fig. IV-3.

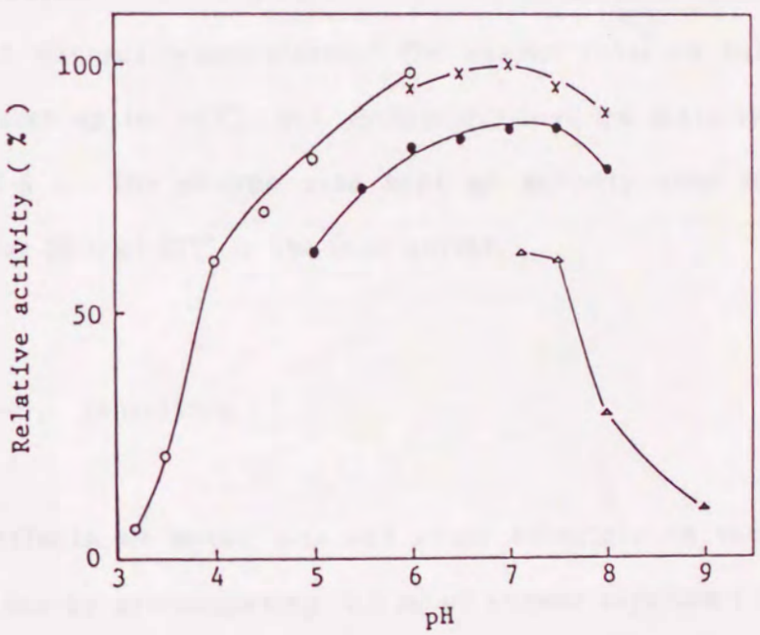


Fig.IV - 3. pH-activity curve of β-1,3-xylanase.
o , sodium acetate buffer (pH 3.2-6.0); ● , MES buffer (pH 5.0-8.0); × , phosphate buffer (pH 6.0-8.0); Δ, Tris-HCl buffer (pH 7.2-9.0).

Ⅳ-2-3. Stability of β-1,3-xylanase

The stability of the enzyme at various pHs was examined by incubating it in 50 mM sodium acetate (pH 3.2 - 6.0), phosphate (pH 5.5 - 8.0), Tris-HCl (pH 7.2 - 9.0), and glycine-NaOH (pH 9.0 - 11.0) buffers at 4℃ for 20 h. The remaining enzyme activity was assayed under the standard condition. The enzyme was stable between pH 4.5 - 10.0 (Fig. Ⅳ-4).

Ⅳ-2-4. Effect of temperature

The enzyme was incubated in 50 mM sodium acetate buffer, pH 6.0, for 15 min at various temperatures. The enzyme retained full activity at temperatures up to 40℃, but completely lost the activity above 60℃ (Fig. Ⅳ-5). The enzyme also kept an activity over 95% even after leaving for 20 h at 37℃ in the same buffer.

Ⅳ-2-5. Inhibitors

The effects of metal ions and other materials on the enzyme were investigated by preincubating 0.5 m*l* of enzyme solution (0.5 unit) with 0.25 m*l* of 3 mM test compound for 15 min at 20℃ before the assay of enzyme activity. Fe^{3+} and Hg^{2+} almost completely inhibited the activity at 1 mM concentration, while Mn^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Ag^{+}, and PCMB(*p*-chloromercuribenzoic acid) gave 30 to 70% inhibition at the same

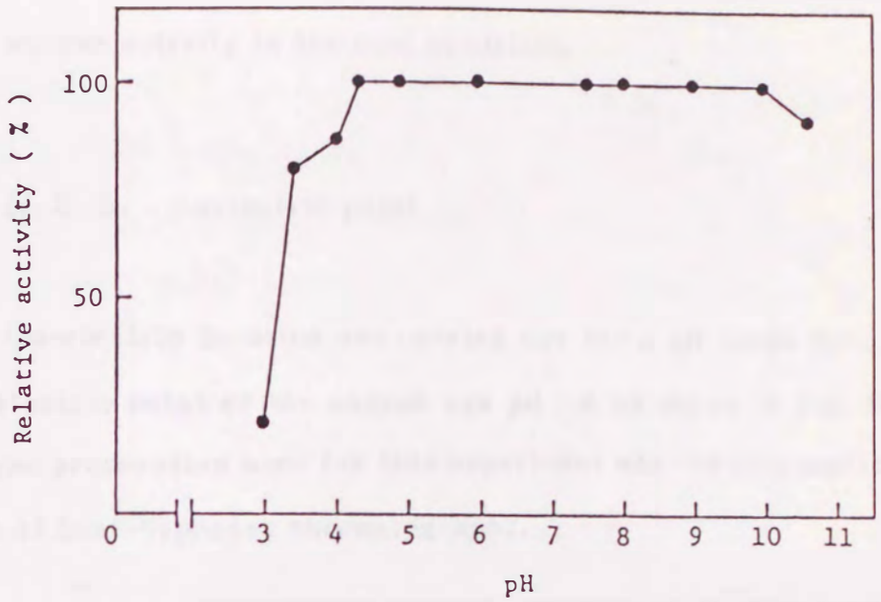


Fig.IV - 4. pH-stability curve of β-1,3-xylanase. pH 3.2 — 6.0, acetate buffer; pH 7.5 — 8.9, Tris-HCl buffer; pH 9.0 — 10.6, glycine-NaOH buffer.

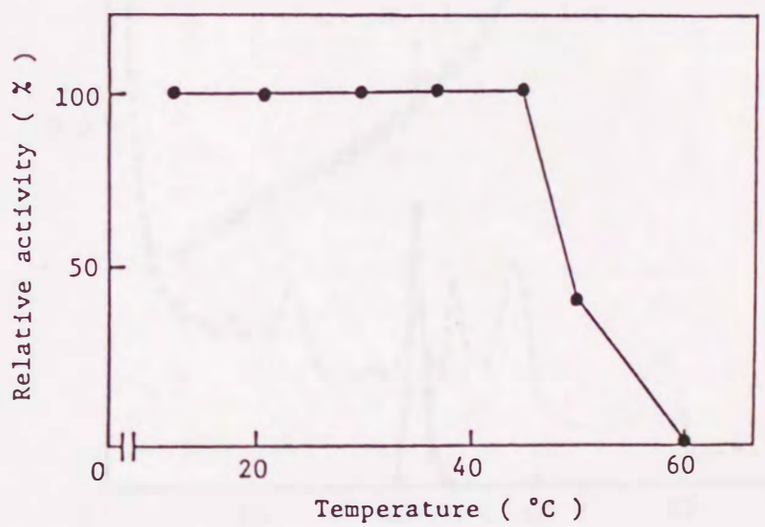


Fig.IV - 5. Temperature-stability curve of β-1,3-xylanase.

concentration. Mg^{2+}, Ca^{2+}, Na^{+}, and EDTA had no significant effects on the enzyme activity in the same condition.

IV-2-6. Isoelectric point

Iso-electric focusing was carried out for a pH range from 2.5 to 4.0. Isoelectric point of the enzyme was pH 3.6 as shown in Fig. IV-6. The enzyme preparation used for this experiment was the preparation from the step of DEAE-Sephadex chromatography.

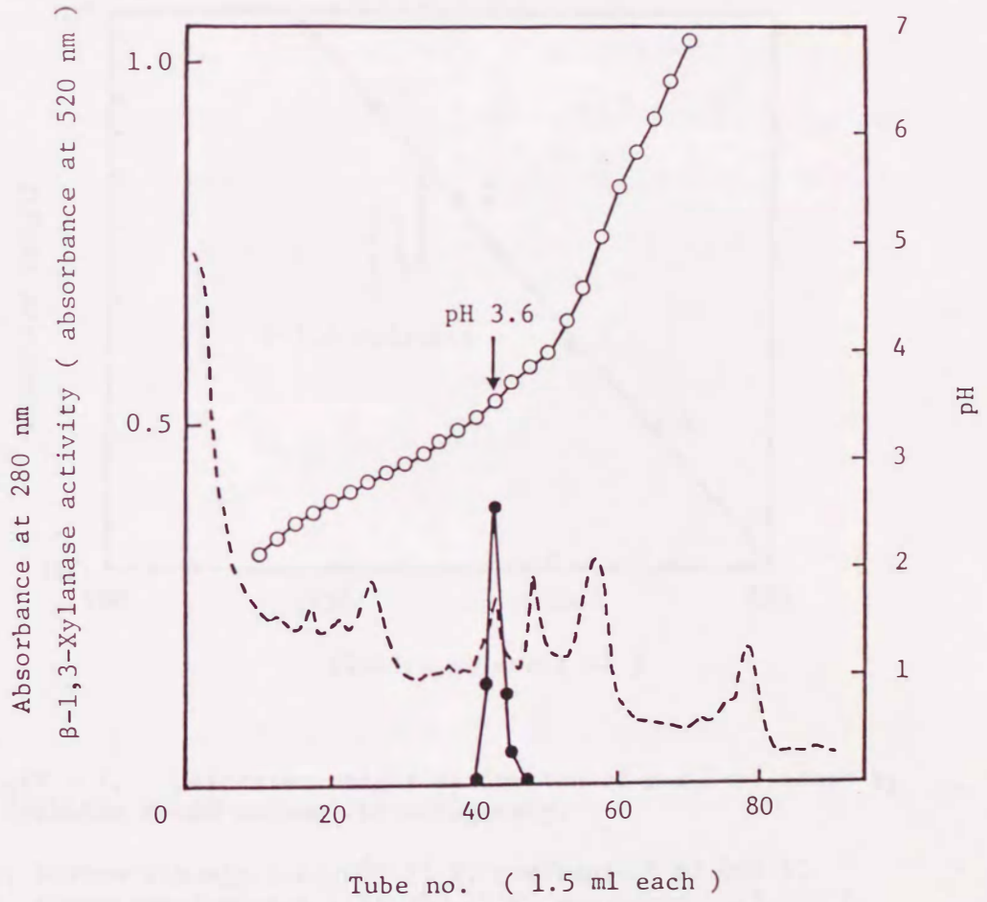


Fig.IV - 6. Isoelectric focusing of β-1,3-xylanase.
—●—, enzyme activity; - - - , absorbance at 280 nm; —o— , pH.

IV-2-7. Molecular weight of β-1,3-xylanase

The molecular weight of the enzyme was estimated on a column of Sephadex G-100 which had been calibrated with standard proteins. From logarithmic plots of the molecular weights versus the elution volumes of the proteins, the molecular weight of the enzyme was estimated to be 53,000 Da (Fig. IV-7).

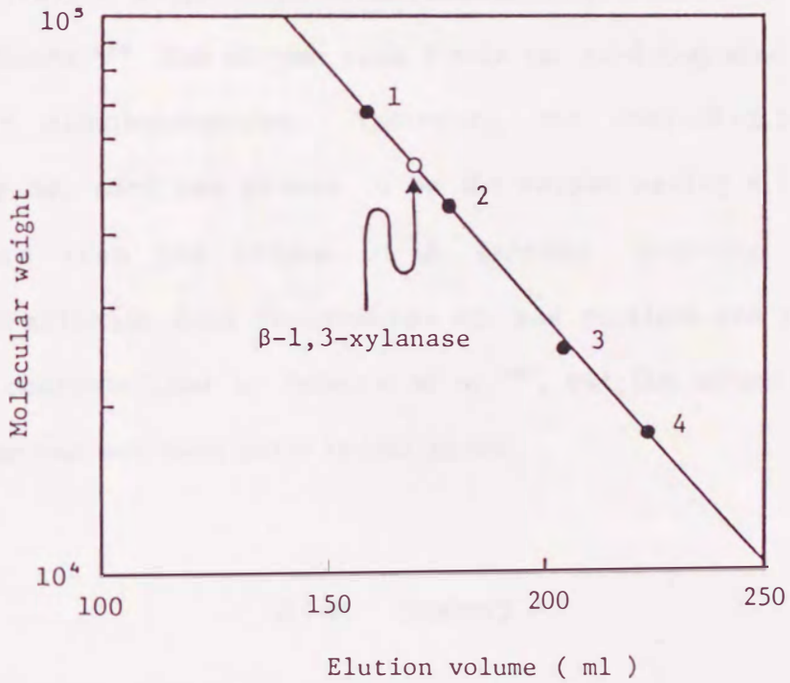


Fig.IV - 7. Molecular weight estimation of β-1,3-xylanase by Sephadex G-100 column chromatography.

1, bovine albumin (67,000); 2, ovalbumin (45,000);
3, chymotrypsinogen-A (25,000); 4, myoglobin (17,800).

Ⅳ-3. Discussion

The purified β-1,3-xylanase hydrolyzed xylotriose and larger xylooligosaccharides to give various sizes of xylooligosaccharides, but did not hydrolyze xylobiose and *p*-nitrophenyl-β-D-xyloside. By these action patterns and substrate specificities, the β-1,3-xylanase was proved to be an endo-type enzyme (endo-β-1,3-xylanase, EC 3.2.1.32) which splits at random inner xylosidic bonds in the β-1,3-xylan molecule.

The endo-type β-1,3-xylanase from *Aspergillus terreus* A-07 was able to hydrolyze larger oligosaccharides than xylotriose, but not xylotriose and -biose.[21] The enzyme from *Vibrio* sp. AX-4 degraded xylotriose and larger oligosaccharides. Therefore, the endo-β-1,3-xylanase from *Vibrio* sp. AX-4 was proved to be the enzyme having a different action pattern from the enzyme of *A. terreus*. Recently, another endo-β-1,3-xylanase from *Pseudomonas* sp. was purified and some properties were characterized by Yamaura *et al.*[22], but the action pattern of the enzyme had not been fully investigated.

Ⅳ-4. Summary

β-1,3-Xylanase from a marine bacterium, *Vibrio* sp. AX-4, was investigated for the enzymatic properties. The enzyme had a molecular weight of 53 kDa, a pI of 3.6, a pH optimum of 6.0 to 7.5, and was stable in a pH region from 4.5 to 10.0 and at temperatures below 40℃. The β-1,3-

xylanase was an endo-type enzyme which degraded β-1,3-xylan to yield xylose and various sizes of xylooligosaccharides. The hydrolysis products from xylotriose were xylose and xylobiose. Xylotetraose wasw degraded to xylose, xylotriose, and a small amount of xylobiose. The hydrolysis products from xylopentaose were mainly xylose and xylotetraose, in addition to small amounts of xylobiose and xylotriose. The enzyme did not act on xylobiose, *p*-nitrophenyl-β-D-xyloside, and β-1,4-xylan. The enzyme was completely inhibited by 1 mM Fe^{3+} and Hg^{2+}.

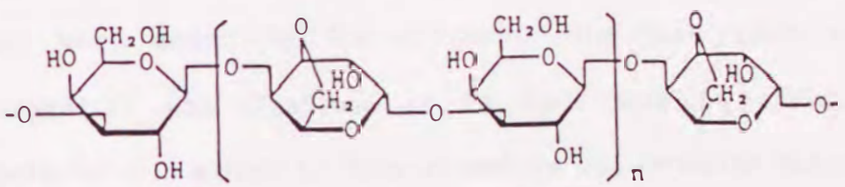
Chapter V Isolation of Porphyran-degrading Bacteria

Porphyran, a water soluble polysaccharide, is found in the cell wall from red algae of the genera *Porphyra*[50,51] and *Bangia*.[52] The other main polysaccharides of the cell wall are β-1,3-xylan and β-1,4-mannan (β-1,3-xylan was described in Chapter II.). The predominant structure of porphyran is the same as that of agarose, which is composed of repeated disaccharide units, a D-galactose and a 3,6-anhydro-L-galactose linked by β-1,3-linkage (3,6-anhydro-α-L-galactopyranosyl-(1→3)-β-D-galactopyranose).[53] Porphyran comprises this unit (neoagarobiose) linked by β-1,4-linkage like a chain, and is highly substituted by 6-O-sulfation of the L-galactose and 6-O-methylation of the D-galactose[54] (Fig. V-1). Therefore, porphyran resembles agarose in containing 3,6-anhydro-L-galactose and 6-O-methyl-D-galactose and resembles carrageenan in containing L-galactose-6-sulfate. Although there is a wide variability in the composition of the polysaccharides depending on the species of seaweeds, season and environment, the sum of L-galactose-6-sulfate and 3,6-anhydro-L-galactose was always approximately equal to the sum of D-galactose and 6-O-methyl-D-galactose.[55] Porphyran was found to comprise 49% sulfated disaccharide units and these were calculated to occur in stretches averaging 2.0 - 2.5 contiguous units.[56]

There have been some reports on porphyran-degrading enzymes from bacteria, especially on the extracellular β-agarase (EC 3.2.1.81) from *Cytophaga* sp.[23] and *Pseudomonas atlantica*.[24,25] The enzymes from

these marine bacteria hydrolyzed agar and porphyran to give neoagarotetraose as the predominant product. However, these enzymes degraded only a defined part of porphyran structure and exhibited low hydrolyzing activity on porphyran as compared with agar. In the case of the β-agarase from *P. atlantica*, the decomposing ratio of porphyran was 23% as mentioned above.[24]

The author tried to search for bacteria which produced enzymes having higher activity against porphyran than reported β-agarases. This chapter describes the isolation, culture conditions, and identification of a bacterium which is able to produce a large amount of porphyran-degrading enzyme.



Neoagarobiose

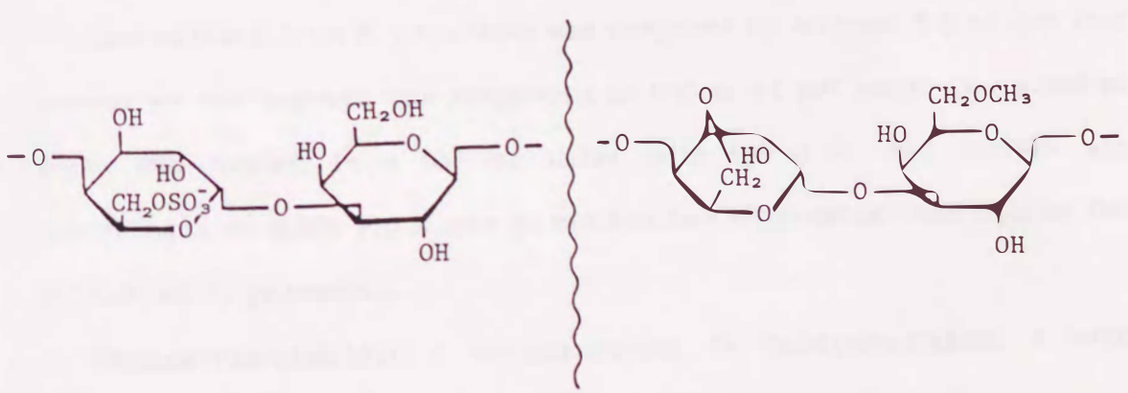


Fig.V - 1. Structure of porphyran.

V-1. Materials and Methods

V-1-1. Polysaccharides

Porphyran was prepared by the method of Su and Hassid[15] from the dry leaf powder of *Porphyra yezoensis* var. *narawaensis* collected from Ariake Bay in Fukuoka Prefecture. The powder of the seaweed was suspended in water, and sufficiently washed with water followed by filtration through a cloth sack. The residue obtained was treated with hot water at 100℃ for 1 h, and then the preparation was centrifuged at 6,000 r.p.m. for 20 min. Cetylpyridinium chloride (CPC) was added to the supernatant solution up to 10%, and the mixture was centrifuged. The precipitate obtained was dissolved in 2% NaCl solution, and 2 volumes of ethanol were added to the solution. The precipitate obtained by centrifugation was dissolved in 2% NaCl solution again, and then precipitated with ethanol. This procedure was repeated three times, and the precipitate obtained was dried *in vacuo*. Finally, 4.0 *g* white powder of porphyran was obtained from 100 *g* powder of *P. yezoensis*.

The extract from *P. yezoensis* was prepared as follows: 5 *g* of dry leaf powder of the seaweed was suspended in 600 *ml* of hot water in a 2,000 *ml* flask and heated in a boiling water bath for 3 h. The mixture was centrifuged at 6,000 r.p.m. for 20 min and the supernatant was used as the extract of *P. yezoensis*.

Neoagarosaccharides (neoagarobiose to neoagarooctaose) were prepared by the method of Groleau and Yaphe[26] (see VII-1-1).

κ-Carrageenan was purchased from Wako Pure Chem. Ind.

V-1-2. Sources of porphyran-degrading bacteria

Seventy-two samples, collected during 1985 in Fukuoka Prefecture, were examined. Those of marine origin included: 4 from coastal sea water, 18 from the bottom mud of coastal sea, 19 from algae (green, brown, and red), 5 from gills and 6 from intestinal contents of marine fish. Those from land and freshwater origins included: 10 from land soil, 4 from river water, and 6 from pond water.

V-1-3. Media used for screening of bacteria

The medium for the bacteria of marine origin was PY medium composed of 0.5% peptone, 0.1% yeast extract (Daigo Eiyokagaku Co.), and 3% NaCl, pH 7.6 - 7.8. The author used a nutrient broth (Difco Lab.), pH 7.0 - 7.2, for those of freshwater and land soil origins. These media were used after adding 0.2% porphyran and solidifying with 1.5% agar (Difco Lab.), where required.

Before screening, each solid sample was blended with a small volume of either sterilized 3% NaCl solution (for samples of marine origin) or 0.9% NaCl solution (for those of land and freshwater origins). A small quantity of the sample was streaked on the surface of agar medium in a petri dish, and incubated aerobically at 25℃ for 4 - 7 days. Since porphyran-degrading bacteria were presumed to be able to hydrolyze agar, colonies capable of liquefying agar were isolated. The isolated

bacteria were inoculated into 5 m*l* of PY medium containing 0.2% porphyran. After incubation with shaking at 25℃ for 4 days, 0.5 m*l* of each culture was added to 2 m*l* of acid albumin solution (3.26 g of sodium acetate, 4.56 m*l* of glacial acetic acid, and 1.0 g of bovine albumin fraction V were dissolved in 1 liter of water, pH adjusted to 3.72 to 3.78 with 1 : 1 concentration of HCl).[57] Albumin combined with porphyran so that it formed white turbidity in acidic solution, but not when porphyran was degraded by enzyme to smaller masses. The porphyran-degrading culture was evident by the transparency or by the significantly faint turbidity of the test solution. The porphyran-degrading bacteria selected by the acid albumin test were inoculated on agar slant medium containing 0.2% porphyran, stored in a refrigerator, and transferred monthly in homologous agar media to maintain viability.

V-1-4. Assay of porphyran-degrading enzyme

To 1 m*l* of 100 mM sodium acetate buffer, pH 6.0, containing 3.0% of porphyran and 2 mM $CaCl_2$, 0.5 m*l* of enzyme solution was added. After incubation at 37℃ for an appropriate period, the reaction was halted by heating the mixture in a boiling water bath for 5 min. The reducing sugar produced was then determined by Somogyi-Nelson's method[31] and expressed as D-galactose. One unit of the enzyme was defined as activity which liberated 1 μ mol of D-galactose per min, under the condition described above.

V-1-5. Porphyran-degrading enzyme productivity of isolated bacteria

Cells taken from the agar slant culture of each isolate were transferred to 20 *ml* of sterile PY medium containing 0.2% porphyran in a 100 *ml* flask. The preparation was then incubated with shaking by a reciprocal shaker (8 cm amplitude, 90 strokes/min) at 25℃ for 4 days. Each culture was centrifuged, and the enzyme activity of the supernatant obtained was determined.

V-1-6. Identification method for AP-2 strain

Identification of the AP-2 strain was made according to the criteria described in Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984).[32] Motility, morphology, and Gram-staining characteristics were determined by light microscopy. The Gram-staining was carried out by the modification of Hucker's method.[33] Oxidase test was carried out by the method of Kovacs,[34] and O-F test was by the Hugh-Leifson's method.[35] The formation of acid from D-glucose was determined by color-transition of BTB, and of gas was by using the Durham tube. The arginine test was made by the method of Thornley.[36] BM (basal medium) given in the Bergey's Manual was used for the test of ability to utilize organic compounds. The mol% G + C of the DNA range was measured by the method

of Marmur[37,38], with the DNA from *Escherichia coli* K12 used as a standard. Other identification methods were used, where required.[39-41]

V-1-7. Examination for culture conditions of AP-2 strain

The following conditions were examined: (a) addition of porphyran, agar, κ-carrageenan, and the extract from *P. yezoensis* var. *narawaensis* to medium as the inducer of porphyran-degrading enzyme; (b) initial pH of culture medium; (c) culture temperature and period; (d) shaking by a reciprocal shaker.

V-1-8. Preparation of porphyran-degrading enzyme from AP-2 strain

The strain was inoculated into 20 m*l* of PY medium composed of 0.5% peptone, 0.1% yeast extract, 3.0% NaCl and 0.3% porphyran, pH 7.4, and incubated with shaking at 25℃ for 1 day. The culture was transferred to 1,000 m*l* of the same PY medium, and incubated with shaking by a reciprocal shaker (8 cm amplitude, 90 strokes/min) at 25℃ for 4 days. The culture was centrifuged, and the supernatant obtained was subjected to the following procedure. All the following operations were conducted at 4℃. Solid ammonium sulfate was added to the supernatant up to a final concentration of 75% saturation for ammonium sulfate precipitation. The mixture was kept overnight and centrifuged. The precipitate obtained

was dissolved in a small volume of 20 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$, and the solution was poured into a cellulose tube and dialyzed overnight against several changes of the same buffer. The liquid inside the tube was used as porphyran-degrading enzyme solution (crude enzyme).

V-1-9. Thin layer chromatography of hydrolysis products

TLC of saccharide was performed on a plastic sheet of Silica Gel 60 (Merck & Co., Inc.) by an ascending method, and the saccharide was developed with a solvent of n-butanol - acetic acid - water (2 : 1 : 1 , v/v), and visualized by spraying the plate with a naphthoresorcinol reagent.[58]

V-1-10. Decomposing ratio of substrate

Decomposing ratio of porphyran and agar was determined as follows: to 2 m*l* of substrate solution (1.0 mg in 100 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$), 0.5 m*l* of enzyme solution (10 units in the same buffer) was added. After incubation at 37℃ for 12 h, the mixture was dialyzed for 12 h (Spectra/Por tubing, size 3) against 100 m*l* of water, and total carbohydrates were determined for inner and outer solutions by phenol-H_2SO_4 method of Dubois *et al.*[59] The ratio between dialysable and starting carbohydrates was calculated.

V-2. Results

V-2-1. Screening of porphyran-degrading bacteria

One bacterium was obtained from the bottom mud of the coastal sea, and four from seaweeds (Table V-1). An isolate (AP-2 strain) from a seaweed exhibited the highest enzyme productivity among them, and the enzyme activity of culture fluid reached 178 m units per m*l* after 4-day incubation. The enzyme productivities from all the other isolates were within the range of 70 to 157 m units per m*l*. Therefore, the AP-2 strain was used for the following experiments.

Table V - 1. Sources of porphyranase-producing bacteria

Places	Samples	Number of samples	Number of isolates
Coastal sea	Sea water	4	0
	Sea bottom mud	18	1
	Sea weed	19	4
	Marine fish		
	gills	5	0
	intestine	6	0
	(Total	52	5)
River and pond	River water	4	0
	Pond water	6	0
	(Total	10	0)
Land	Land soil	10	0
	(Total	10	0)

V-2-2. Identification of AP-2 strain

The morphological and biochemical characteristics of the AP-2 strain are shown in Table V-2. The bacterium was a Gram-negative and facultatively anaerobic rod with a single polar flagellum, it did not form endo-spores, formed acid but no gas from glucose, and was sensitive to 2,4-diamino-6,7-diisopropyl pteridine. Therefore, it was assigned to the genus *Vibrio*, according to the criteria given in Bergey's Manual of Systematic Bacteriology. However, the AP-2 strain differed from all the species of the genus listed in the Manual in some characteristics. Therefore, the species of the strain was not determined.

V-2-3. Culture conditions for the production of porphyran-degrading enzyme in *Vibrio* sp. AP-2

(a) Influence of the carbohydrates in medium

The influence of various kinds of carbohydrates on the enzyme production was measured using the PY medium without porphyran. To 20 m*l* of the medium, 0.1 *g* each of porphyran, agar, and κ-carrageenan was added as the inducer. In the case of the extract from *P. yezoensis*, the extract was substituted for the water of the medium. After inoculation with the AP-2 strain, each carbohydrate-containing medium was incubated with shaking at 25℃ for 4 days. The culture was centrifuged, and the activity of porphyran-degrading enzyme was determined for the

Table V - 1. Morphological, cultural, and biochemical characteristics of AP-2 strain

Form	rod
Size	0.8-1.2 μm x 1.8-2.2 μm
Gram strain	Negative
Motility	+
Flagellation	Single polar
Growth in air	+
Unaerobic growth	+
O-F test	Fermentation
Catalase	+
Vibriostatic agent sensitivity	+
Swarming on solid complex media	-
PHB-accumulation	-
Pigmentation	
Yellow-orange	-
Blue-black	-
Red	-
Arginine dihydrolase	-
Oxidase	+
Reduction of NO_3^- to NO_2^-	+
Luminescence	-
Gas from D-glucose	-
Indol production	-
Production of acetoin and/or diacetyl	-
Voges-Proskauer test(24 h)	-
Methyl red test	+
Thornley arginine	-
Na^+ required for growth	+
Requirement for organic growth factors	-
Growth at:	
4°C	-
30°C	+
35°C	+
40°C	-
Production of:	
Amylase	+
Gelatinase	+
Lipase	+
Alginase	-
Chitinase	+
Utilization of:	
D-Xylose	-
L-Arabinose	-
D-Mannose	+
D-Galactose	-
Sucrose	+
Trehalose	+
Cellobiose	+
Lactose	-
Salicin	+
Citrate	+
D-Mannitol	+
D-Sorbitol	+
meso-Inositol	+
Ethanol	-
L-α-Alanine	-
L-Serine	-
L-Leucine	-
L-Histidine	-
L-Proline	+
L-Tyrosine	+
D-Gluconate	+
D-Glucuronate	-
Mol% G + C of DNA	46

supernatant. The strain AP-2 was not able to produce the enzyme without an inducer as shown in Fig. V-2. The extract from *P. yezoensis* was an excellent inducer as well as porphyran, and better than agar and κ-carrageenan. From the result of this experiment, the medium containing the extract from *P. yezoensis* was selected for the following experiments.

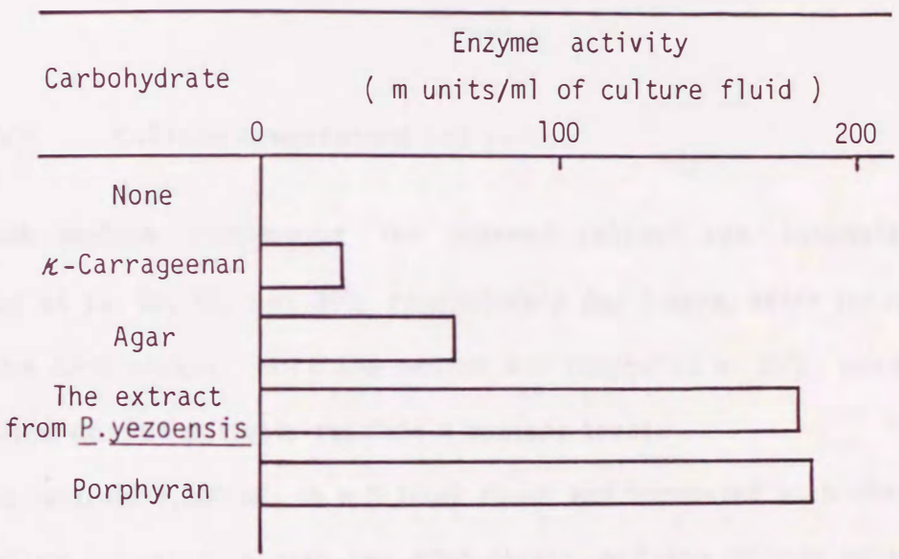


Fig.V - 2. Productivity of porphyran-degrading enzyme of AP-2 strain grown on different carbohydrates.

PY medium containing 0.2% of each carbohydrate was used as culture medium, except the case of the extract from *P. yezoensis*.

(b) Initial pH of medium

The media containing the extract from *P. yezoensis* of various pHs (pHs 6.4, 6.9, 7.4, 7.9, and 8.4) were prepared, and 20 m*l* of each medium was poured into a 50 m*l* flask. The medium was incubated with shaking at 25℃ for 3 days after inoculation with the AP-2 strain. The culture was centrifuged, and the enzyme activity of the supernatant was measured. The maximal activity of porphyran-degrading enzyme was observed at pH 7.4.

(c) Culture temperature and period

Each medium containing the seaweed extract was incubated with shaking at 15, 20, 25, and 30℃ respectively for 3 days, after inoculation with the AP-2 strain. When the medium was incubated at 25℃, porphyran-degrading enzyme activity reached a maximal level.

The medium, 1,000 m*l*, in a 3 liter flask was incubated with shaking at 25℃ after inoculation with the AP-2 strain, and the enzyme activity of the culture was measured daily for 7 days. The enzyme activity reached a maximal level after 5-day incubation as shown in Fig. V-3.

(d) Shaking of culture

When the organism was incubated at 25℃ without shaking, no enzyme activity was found in the culture. A maximal activity of the enzyme in

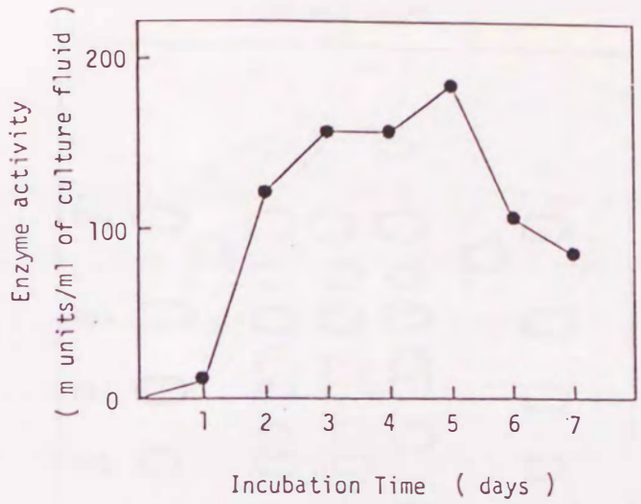


Fig.V - 3. Effect of incubation time on porphyran-degrading enzyme production of AP-2 strain.

the culture fluid was observed when 1,000 m*l* of culture was incubated with shaking under the condition of 8 cm amplitude and 90 strokes/min.

V-2-4. Detection of hydrolysis products

To 12 m*l* of 2 mM sodium acetate buffer, pH 6.0, containing 3% porphyran and 2 mM $CaCl_2$, 2 m*l* of the salting-out enzyme (ca. 2.5 units) from the culture fluid of the AP-2 strain was added, and the mixture was incubated at 37℃. At various intervals, a part of the reaction mixture was withdrawn and spotted on TLC-plate. Porphyran was cleaved to various neoagarooligosaccharides and other oligosaccharides (Fig. V-4). Therefore, it was suggested that β-agarase was contained in the crude enzyme solution.

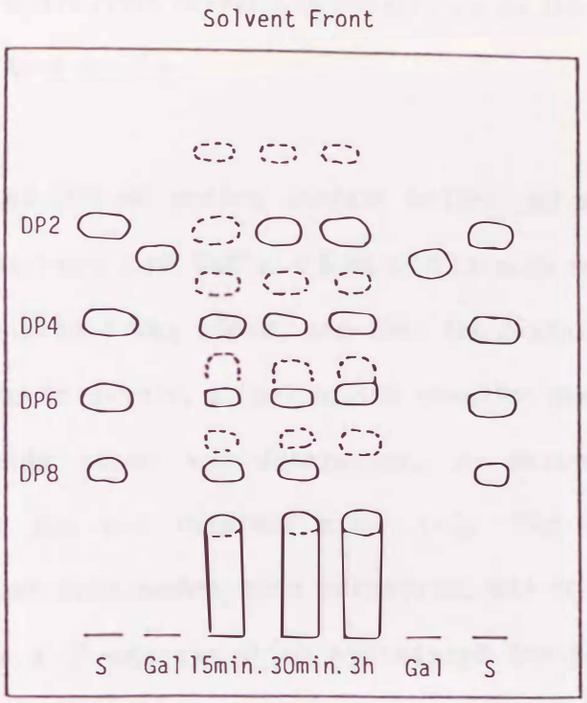


Fig.V - 4. Thin layer chromatogram of hydrolyzates of porphyran with the enzyme from AP-2 strain.

S, neoagarobiose to neoagarooctaose (DP 2,4,6,8; degree of polymerization); Gal, galactose; 15 min, 30 min, 3 h; incubation time.

V-2-5. Decomposing ratio of substrate

The crude enzyme of the AP-2 strain hydrolyzed not only porphyran but also agar, and the decomposing ratio was 52% and 84%, respectively. It was suggested that the enzyme hydrolyzed the common structure in the both substrates.

V-2-6. Hydrolysis of various substrates by the enzyme from AP-2 strain

To 1.5 m*l* of 100 mM sodium acetate buffer, pH 6.0, containing the enzyme (0.3 unit) and 2 mM $CaCl_2$, 0.5 m*l* of 0.5% each substrate (agarose, agar, and porphyran) was added, and then the mixture was incubated at 38℃. At various intervals, a part of the reaction mixture was withdrawn and the reducing sugar was determined. As shown in Fig. V-5, the reducing sugar did not increase after 1 h. The enzyme hydrolyzed agarose and agar much easier than porphyran, and it was suggested that the enzyme was a β-agarase which hydrolyzed the common structure in agarose, agar, and porphyran.

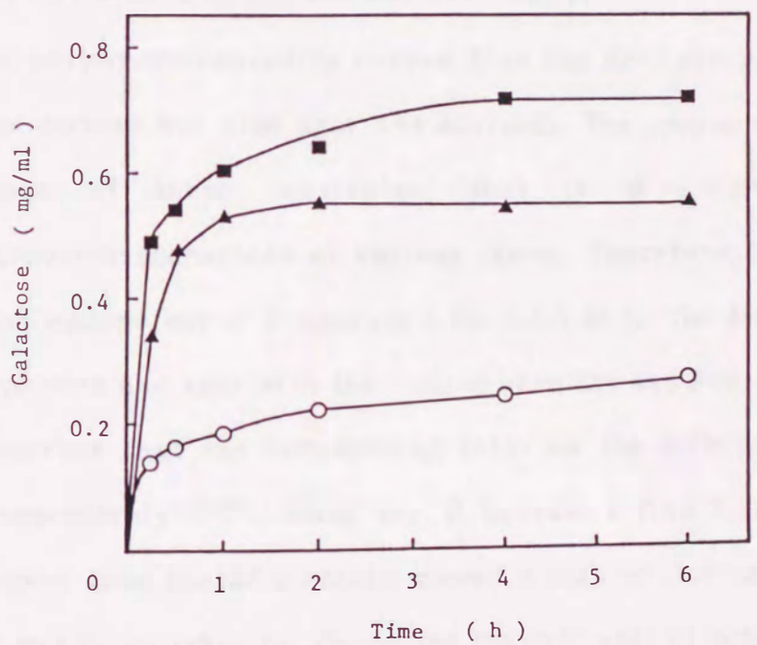


Fig.V - 5. Hydrolysis of various substrates by the enzyme from AP-2 strain.
The reaction was followed by measuring the formation of reducing sugar, expressed as galactose equivalents/ml. ■ , agarose; ▲ , agar; ○ , porphyran.

V-3. Discussion

The AP-2 strain seemed to belong to the genus *Vibrio*, according to the Bergey's Manual of Systematic Bacteriology. Among all 20 species of the genus listed in the Manual, the characteristics of *V. harveyi*, *V. parahaemolyticus*, *V. campbellii*, and *V. vulnificus* had resemblance to that of the AP-2 strain. However, *V. parahaemolyticus* and *V. vulnificus* differed from the AP-2 strain in the growth at 40℃. *V. harveyi* and *V. campbellii* had close resemblance to the AP-2 strain in many characteristics, however, they differed in the utilization ability of some organic compounds (e.g. D-glucuronate and L-serine). Therefore, the species of the AP-2 strain was not determined.

The porphyran-degrading enzyme from the AP-2 strain hydrolyzed not only porphyran but also agar and agarose. The enzyme split the common structure of these substrates, that is β-1,4-linkage, to give neoagarooligosaccharides of various sizes. Therefore, it was suggested that the enzyme was a β-agarase (EC 3.2.1.81). The decomposing ratios of porphyran and agar with the enzyme were 52% and 84%, respectively. It was reported that the decomposing ratio on the substrates was 23% and 75%, respectively[24,25], using the β-agarase I from *P. atlantica*. Since the enzyme from the AP-2 strain showed a high activity at nearly neutral pH, it may be suitable for degrading the cell wall of seaweeds.

V-4. Summary

A screening was undertaken to acquire bacteria with porphyran-degrading enzyme productivity. Several strains were obtained from the marine environments in Fukuoka Prefecture. Among them, the AP-2 strain from a seaweed showed the highest productivity of the enzyme. The organism released a large amount of the enzyme into the culture fluid, when incubated with shaking at 25℃ for 5 days in the following liquid medium: 0.5% peptone, 0.1% yeast extract, 3% NaCl in the extract from *P. yezoensis*, pH 7.4. The enzyme seemed to be a β-agarase which cleaved not only porphyran but also agar.

According to the criteria given in the Bergey's Manual of Systematic Bacteriology, Vol. 1, the bacterium was assigned to the genus *Vibrio*, but the species name was not determined for the organism.

Chapter VI Purification of Agarases from *Vibrio* sp. AP-2

The AP-2 strain was assigned to the genus *Vibrio* in Chapter V. Therefore, this strain is named *Vibrio* sp. AP-2 in the following chapters.

Two types of enzymes have been reported in endo-type agarases. One of them splits α-1,3-linkages in agar to give agarooligosaccharides of various sizes (α-agarase, EC 3.2.1.-)[60], and another enzyme splits β-1,4-linkages in agar to give neoagarooligosaccharides of various sizes (β-agarase, EC 3.2.1.81).[61] While little is reported on the enzymatic properties of α-agarase, there have been some reports on β-agarases from *Cytophaga* sp.[23], *Pseudomonas atlantica*[24], *Cytophaga flevensis*[62], luminous *Vibrio harveyi*[63], and *Pseudomonas*-like bacterium.[64] All these β-agarase-producing bacteria were originated from marine environments.

The author isolated a porphyran-degrading bacterium (*Vibrio* sp. AP-2) from a seaweed collected in the coastal sea of Fukuoka Prefecture in 1985 as described in Chapter V. Furthermore, it was suggested that the porphyran-degrading enzyme from *Vibrio* sp. AP-2 was the β-agarase which hydrolyzed agar to give various sizes of neoagarooligosaccharides.

This chapter describes the purification of the enzyme from *Vibrio* sp. AP-2 by ammonium sulfate precipitation, successive column chromatography and nuclease treatment.

The properties determined by the crude enzyme preparation (see V-1-8) were as follows: the enzyme was likely to exhibit a maximal activity at pH 6.5, and needed 3.0% porphyran in the reaction mixture to

show the maximal activity. These properties were considered in the purification of the enzyme.

Ⅵ-1. Materials and Methods

Ⅵ-1-1. Materials

The extract from *Porphyra yezoensis* was prepared as described in V-1-1. Agar (Agar Noble) was purchased from Difco Laboratories (U.S.A.). Deoxyribonuclease I (EC 3.1.21.1) and ribonuclease A (EC 3.1.27.5) from bovine pancreas were obtained from Sigma Chemical Co.(U.S.A.). CM-Sephadex C-50 and Sepharose CL-6B were purchased from Pharmacia Fine Chemicals. Toyopearl HW-55 and DEAE-Toyopearl 650 M were purchased from Tosoh Co., polyethylene glycol (M.W. 20 kDa) was from Wako Pure Chem. Ind.

Ⅵ-1-2. Assay of enzyme

Agarase activity was estimated as follows: agar solution (0.1% agar in 100 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$) was heated (100℃, 5 min) and cooled to 38℃ immediately before use. The buffered enzyme solution (0.5 m*l* of the sodium acetate buffer) was added to 1.5 m*l* of the agar solution. After incubation at 38℃ for an

appropriate period, the reaction was stopped by placing the mixture in a boiling water bath for 5 min. Reducing sugar produced was then determined by Somogyi-Nelson's method[31] and expressed as D-galactose. One unit of the enzyme was defined as the amount which liberated 1 μmol D-galactose per min under the above conditions.

Ⅵ-1-3. Polyacrylamide disc gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed as described in III-1-3.

Ⅵ-2. Results

Ⅵ-2-1. Preparation of culture fluid

Vibrio sp. AP-2 was grown for 4 days in the medium composed of 0.5% peptone, 0.1% yeast extract, and 3% NaCl in the extract from *P. yezoensis*, pH 7.4 (see V-1-1), with shaking by a reciprocal shaker (8 cm amplitude, 90 strokes/min) at 25℃. The culture was centrifuged at 10,000 r.p.m. for 30 min. From 6,000 m*l* of the culture, 5,700 m*l* of clear fluid was obtained. All the following procedures were carried out at 4℃ unless otherwise indicated.

VI-2-2. Ammonium sulfate precipitation

The culture fluid was adjusted to 75% saturation with solid ammonium sulfate, then left to stand overnight. The precipitate formed was collected by centrifugation, and dissolved in 100 m*l* of 20 mM sodium acetate buffer, pH 5.0. The solution was dialyzed against the same buffer.

VI-2-3. CM-Sephadex C-50 chromatography

A column (2.2 × 18 cm) of CM-Sephadex C-50 was washed with 50 mM Tris-HCl buffer, pH 7.2, and then equilibrated with 20 mM sodium acetate buffer, pH 5.0. The dialyzed enzyme solution was applied to the column, and the column was washed with the sodium acetate buffer. The column was eluted with a linear gradient of 0 - 0.8 M NaCl in 20 mM sodium acetate buffer, pH 5.0 (total vol. 460 m*l*). Two active fractions (non-adsorbed CM-I, tubes No. 5-10 ; adsorbed CM-II, tubes No. 66-74, which was displaced by the linear gradient) appeared as shown in Fig. VI-1. Active fractions were pooled and concentrated to 30 m*l* (CM-I) and 17 m*l* (CM-II) with polyethylene glycol, respectively.

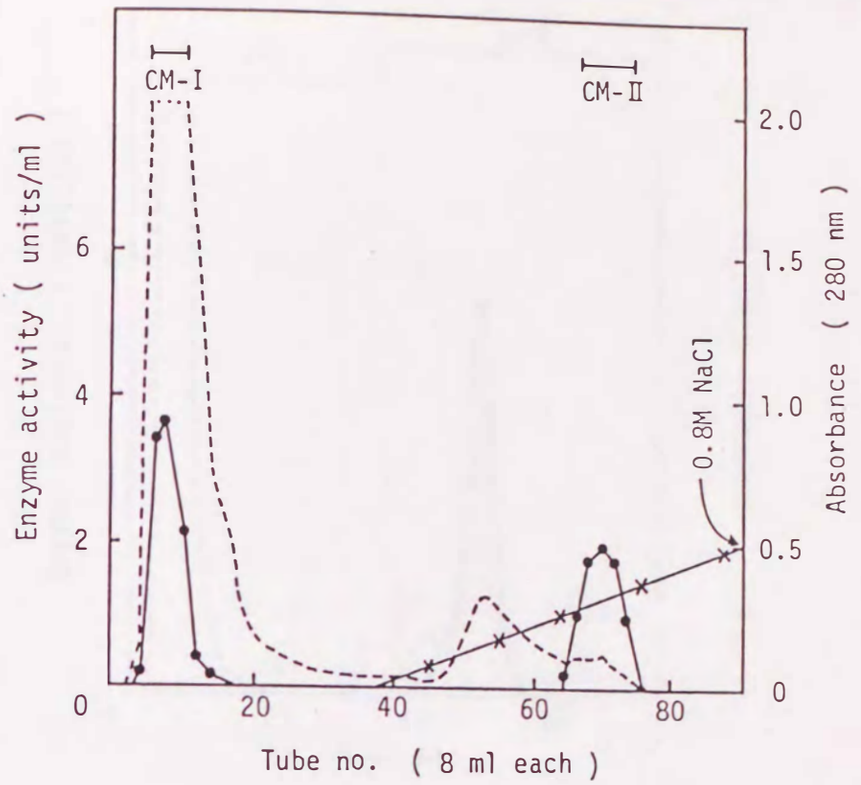


Fig.VI - 1. Chromatography of the enzyme solution on a CM-Sephadex C-50 column.
———, enzyme activity; ------, absorbance at 280 nm;
—×—, concentration of NaCl.

VI-2-4. Affinity chromatography

CM-I fraction from the CM-Sephadex C-50 procedure was dialyzed against 100 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$. The enzyme solution was applied to a column (1.3 × 13 cm) loaded with a mixture of Sepharose CL-6B agarose gel and Celite (1 : 1, v/v), which had been equilibrated with the same buffer. The column was washed with 5 bed volumes of the same buffer at the temperature of 4℃, and then eluted with 20 mM Tris-HCl buffer of pH 8.0 at 37℃ (Fig. VI-2). Active fractions (tubes No.46 - 53) obtained by the elution at 37℃ were pooled and concentrated to 11 ml with polyethylene glycol.

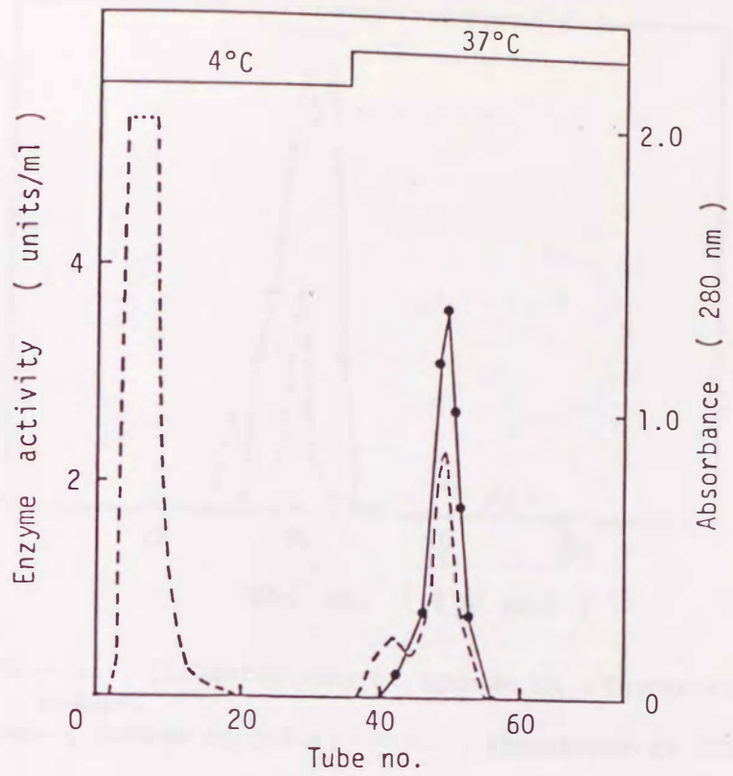


Fig.VI - 2. Chromatography of CM-I enzyme (see Fig.VI-1) on an affinity column.
—●—, enzyme activity; - - - - - , absorbance at 280 nm; ———, temperature of column; tubes 1 to 35, 6 ml/tube and N/10 acetate buffer, pH 6.0, containing 2 mM $CaCl_2$; tubes 36 to 40, 2.0 ml/tube and M/50 Tris-HCl buffer, pH 8.0.

VI-2-5. Toyopearl HW-55 chromatography

The concentrated enzyme solution was dialyzed against 20 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$, and then applied to a column (2.0 × 95 cm) of Toyopearl HW-55 equilibrated with the same buffer. Elution was performed with the same buffer. The enzyme activity was detected in tubes No. 36 - 44 (Fig. VI-3), and these fractions were concentrated to 22.5 m*l* with polyethylene glycol.

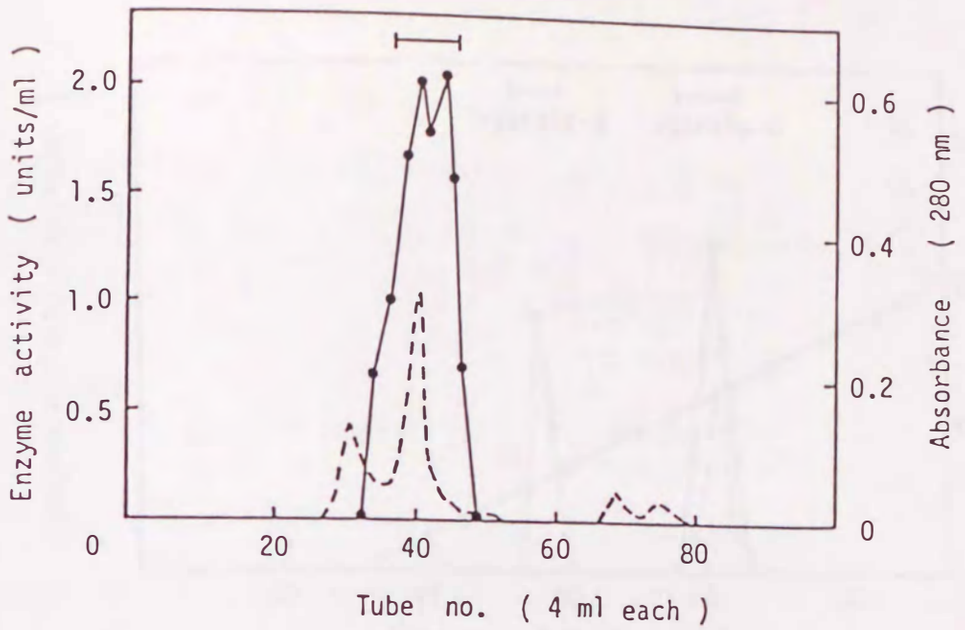


Fig.VI - 3. Chromatography of agarase on a Toyopearl HW-55 column.
—●—, enzyme activity; ----- , absorbance at 280 nm.

VI-2-6. DEAE-Toyopearl 650M chromatography

The enzyme solution from the preceding chromatography was dialyzed against 20 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$, and applied to a column (2.0 × 7.0 cm) of DEAE-Toyopearl 650M equilibrated with the same buffer. After washing with 5 bed volumes of the same buffer, the column was eluted with a linear gradient of 0 - 1.0 M NaCl in 20 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$ (total vol. 460 m*l*). Two active fractions appeared (agarase-a, tubes No. 55-60 ; agarase-b, tubes No. 78-86 ; as shown in Fig. VI-4) were pooled separately, and concentrated to 8.2 m*l*(agarase-a) and 10 m*l*(agarase-b) with polyethylene glycol. One portion of each solution was stored in the refrigerator at 4℃ to maintain, and used in the study of some properties.

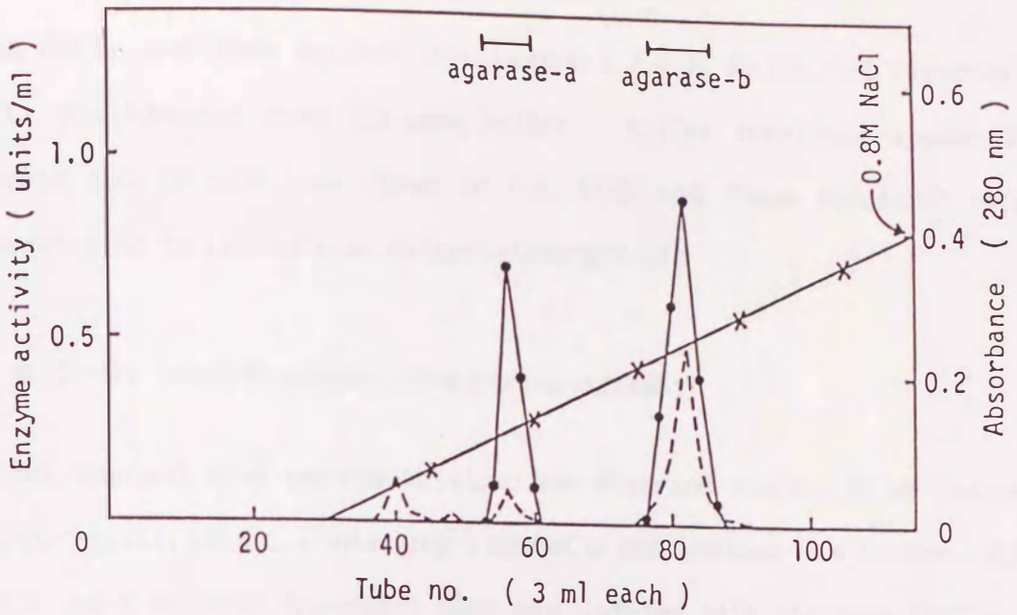


Fig.VI - 4. Chromatography of agarases on a DEAE-Toyopearl 650M column.

—●— , enzyme activity; ----- , absorbance at 280 nm; —×— , concentration of NaCl.

VI-2-7. DNase I and RNase A treatment

Ten milliliters of agarase-b solution (ca. 10 mg protein) from the previous step was dialyzed against 20 mM sodium acetate buffer, pH 6.0, and added to 50 μl of 20 mM sodium acetate buffer, pH 6.0, containing 25 μg DNase I, 10 μg RNase A, 5 mM $MgSO_4$, and 0.15 M NaCl. After incubation at 25℃ for 12 h, the solution was concentrated to 2 m*l*.

VI-2-8. Toyopearl HW-55 chromatography

CM-II fraction from the CM-Sephadex C-50 chromatography (see Fig. VI-1) was dialyzed against 20 mM sodium acetate buffer, pH 6.0, containing

2 mM $CaCl_2$, and then applied to a column (2.0 × 95 cm) of Toyopearl HW-55 equilibrated with the same buffer. Active fractions appeared (tubes No. 42 - 46) as shown in Fig. VI-5, and these fractions were concentrated to 11.5 m*l* with polyethylene glycol.

VI-2-9. DEAE-Toyopearl 650M chromatography

The concentrated enzyme solution was dialyzed against 20 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$ and applied to a column (2.0 × 7.0 cm) of DEAE-Toyopearl 650M equilibrated with the same buffer. After being washed with 5 bed volumes of the same buffer, the column was eluted with a linear gradient of 0 - 0.6 M NaCl in 20 mM sodium acetate buffer, pH 6.0, containing 2 mM $CaCl_2$. Active fractions appeared (tubes No. 88 - 94) as shown in Fig. VI-6, and these fractions were pooled, and concentrated to 7.0 m*l* (agarase-c) with polyethylene glycol.

The purification and yield of the agarases-a, -b, and -c are summarized in Table VI-1.

VI-2-10. Homogeneity of agarases

The agarases-a, -b, and -c from the last procedures (agarases-a, -b, and -c from procedures VI-2-6-I, VI-2-7-I, and VI-2-9-II, respectively) of purification gave a single protein band on polyacrylamide disc gel electrophoresis (Fig. VI-7), respectively.

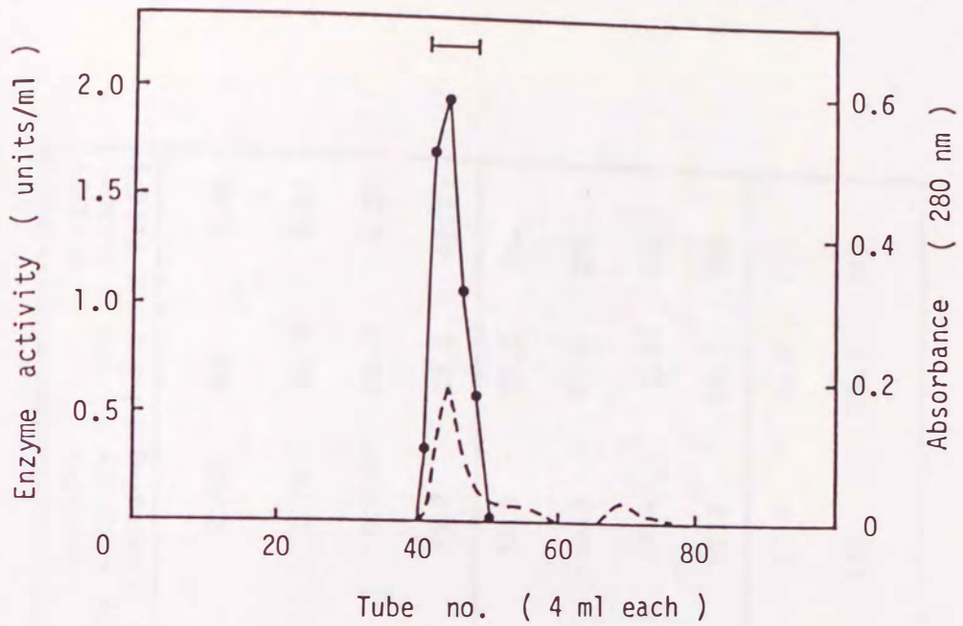


Fig.VI - 5. Chromatography of CM-II enzyme (see Fig.VI-1) on a Toyopearl HW-55 column.
—●—, enzyme activity; - - - - -, absorbance at 280 nm.

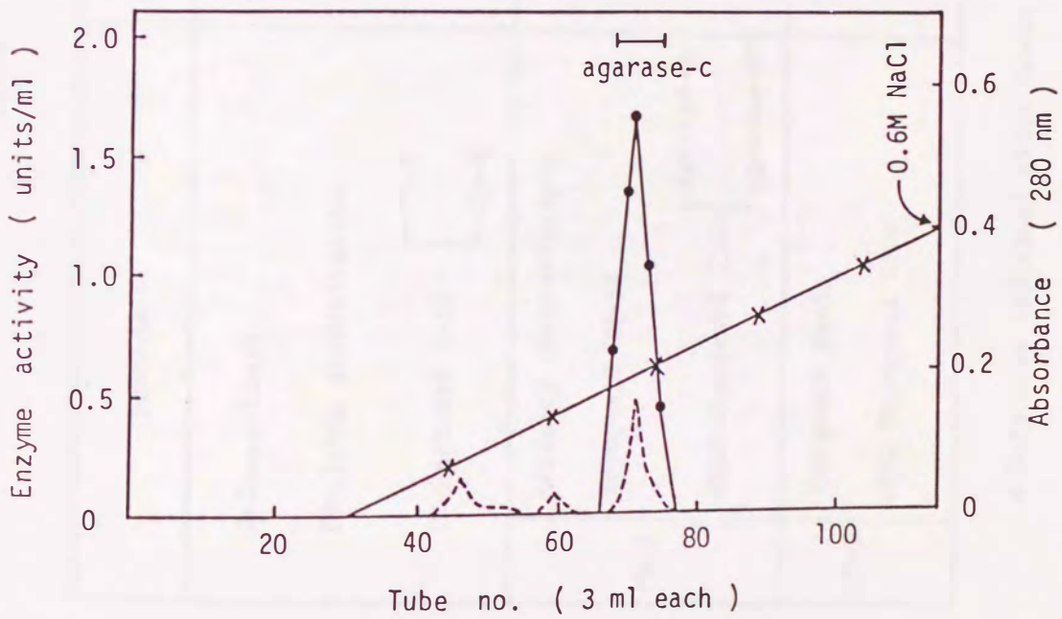


Fig.VI - 6. Chromatography of agarase on a DEAE-Toyopearl 650M column.
—●—, enzyme activity; ---- , absorbance at 280 nm;
—×— , concentration of NaCl.

Table VI - 1. Purification of agarases from the culture fluid (5,700 ml) of strain AP-2

Procedure		Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Culture fluid		13,100	2,100	0.161	100	1.00
$(NH_4)_2SO_4$ precipication		1,550	1,710	1.10	81.5	6.83
CM-Sephadex C-50	CM-I	1,040	720	0.690	34.3	4.29
	CM-II	37.7	597	15.8	28.4	98.1
CM-I	Affinity chromatography	19.6	677	34.5	32.2	214
	Toyopearl HW-55	13.0	576	44.3	27.4	275
	DEAE-Toyopearl 650M agarase-a	1.05	81.3	77.4	3.87	481
	DEAE-Toyopearl 650M agarase-b*	9.35	494	52.8	23.5	328
CM-II	Toyopearl HW-55	15.9	539	33.8	25.6	210
	DEAE-Toyopearl 650M	4.46	537	120	25.5	745

* Data were indicated after DNase and RNase treatment.

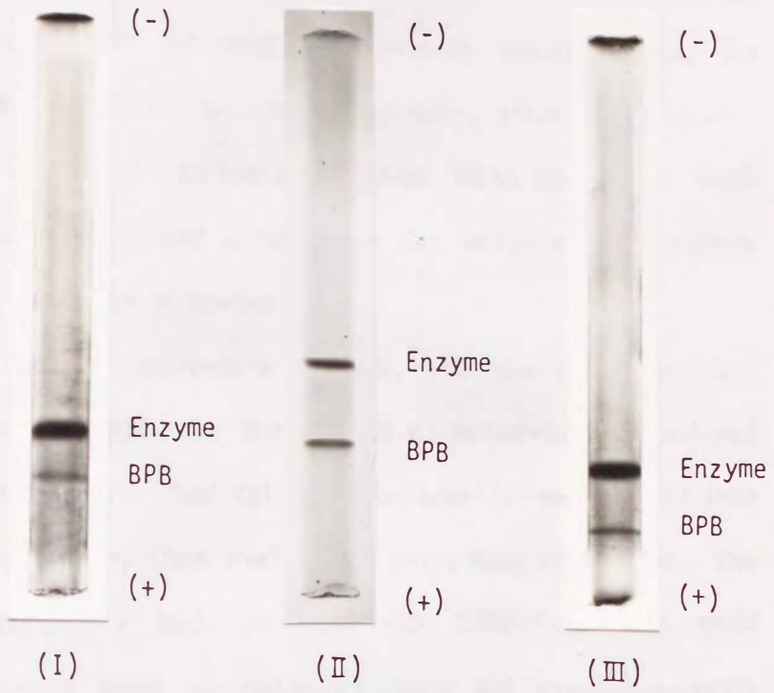


Fig.VI - 7. Polyacrylamide gel electrophoresis of the final preparation of each agarase [agarase-a (I), agarase-b (II), and agarase-c (III)]. The purified enzymes were subjected to disc erectrophoresis in 7.0% polyacrylamide gel at pH 8.3. Approximately 50 ug of each enzyme was applied per tube.
A current of 3 mA was supplied per tube at room temperature. BPB, bromophenol blue; (+), anode; (-), cathode.

VI-3. Discussion

Agarases-a and -c were purified from the culture fluid of a marine bacterium, *Vibrio* sp. AP-2, by ammonium sulfate precipitation, by successive use of CM-Sephadex C-50 chromatography, affinity chromatography, Toyopearl HW-55 chromatography, and DEAE-Toyopearl 650M chromatography. DNase and RNase treatments was performed to purify agarase-b in addition to above procedures.

In the purification of agarases-a and -b, the use of a modified agarose-affinity chromatography by the method of Malmqvist[64] improved effectively the enzyme purity. The value of the specific activity of this procedure was 50 times higher than that of the preceding procedure. The preparations of agarases-a and -c from the DEAE-Toyopearl 650M procedure gave a single band on polyacrylamide gel electrophoresis respectively, while the persistent efforts to purify agarase-b to homogeneity were unsuccessful until the author used nucleases. Agarase-b preparation before the nucleases treatment gave two bands on polyacrylamide gel electrophoresis. The main band appeared less towards the cathode side than the fainter one. When the stained gel was treated with acridine orange, another band appeared at the cathode end of the gel. Further examination on agarase-b (e.g. for heat treatment, ultraviolet absorption, carbazole reaction, and orcinol reaction) suggested that the enzyme formed a complex with some nucleic acid. Since the addition of protamine and streptomycin to the enzyme solution

was not effective in removing nucleic acid, the author used small quantities of DNase and RNase to purify the enzyme preparation. When the agarase solution was analyzed by electrophoresis after ten-fold concentration, two more very faint bands appeared nearer the anode than the main band. Compared this gel with a control one, developed with DNase and RNase, it was found that the faint bands were due to the two nucleases. The other experiments suggested that the contamination with a small amount of nucleases hardly affected not only the activity of the agarase but also properties such as optimal pH and pattern of action. Therefore, the author used the agarase-b preparation from the last procedure as the purified agarase-b.

VI-4. Summary

Agarases were purified from the culture fluid of a marine bacterium, *Vibrio* sp. AP-2, by ammonium sulfate precipitation, successive column chromatography and nucleases treatment. The specific activities of the final enzyme preparations (agarases-a, -b, and -c) were 481, 328, and 745 time higher than that of the culture fluid, and the recoveries were 3.87, 23.5, and 25.5%, respectively.

The final enzyme preparations appeared to be homogeneous on polyacrylamide gel electrophoresis.

Chapter Ⅶ Characterization of three Agarases from *Vibrio* sp. AP-2

Endo-type β-agarase splits β-1,4-linkages and acts on agar and porphyran to give neoagarooligosaccharides of various sizes.[61]

In 1983, a β-agarase (β-agarase I) from *P. atlantica* was purified and the enzymatic properties were investigated by Morrice *et al.*[24] The β-agarase I had a molecular weight of 32 kDa and a pH optimum of 7.0, and hydrolyzed agar to give neoagarotetraose as the predominant product.

The existence of another type of intracellular agarase (β-neoagarotetraose hydrolase[23] or β-agarase II) from *P. atlantica* was suggested by Morrice *et al.*[24] The β-agarase II was likely to be an endo-type enzyme the same as β-agarase I, and hydrolyzed neoagarotetraose or agar to give neoagarobiose as the predominant product. However, the properties of β-agarase II have not been fully investigated.

This chapter describes the characterization of three agarases from *Vibrio* sp. AP-2.

Ⅶ-1. Materials and Methods

Ⅶ-1-1. Substrates

Neoagarosaccharides(neoagarobiose to neoagarooctaose) were prepared

by the method of Groleau and Yaphe[26] from hydrolysis products of agar with β-agarase. Agar (Agar Noble; Sigma Chemical Co., U.S.A.), 25 g, was washed with 500 ml of 0.05 M NaCl by stirring at room temperature for 24 h, and the supernatant was removed by decantation. The washed agar was added to 500 ml of 2 mM sodium acetate buffer, pH 6.0, and dissolved at 100℃. The agar solution was cooled at 42℃ in a water bath and 4.0 ml of purified β-agarase (2,000 units/ml) from *P. atlantica* (Sigma Chemical Co.) was added. Hydrolysis of agar was performed for 3 h at 42℃. Additional β-agarase (2.5 ml) was added to the agar hydrolyzate and the mixture was stirred at room temperature for 21 h. The agar hydrolyzate was concentrated *in vacuo* to 30 ml. Ethanol (4 volumes) was added and the small oligosaccharides were recovered in the supernatant fraction after removal of the precipitate by centrifugation at 6,000 r.p.m. for 20 min. The supernatant obtained was dried *in vacuo*, and added to 15 ml of water. The small oligosaccharides solution obtained (2 ml) was applied to a column (2.2 × 93 cm) of Sephadex G-15 equilibrated with water. Elution was performed with water, and neoagarobiose was separated from other neoagarooligosaccharides. Neoagarotetraose, neoagarohexaose, and neoagarooctaose were separated by re-chromatography on Sephadex G-15 column. Each neoagaro-oligosaccharide obtained was spotted on a TLC-plastic sheet of Silica Gel 60, and the chromatography was carried out by an ascending method (see V-1-9). Each preparation was purified (neoagarobiose to neoagaro-octaose) as shown in Fig. VII-1.

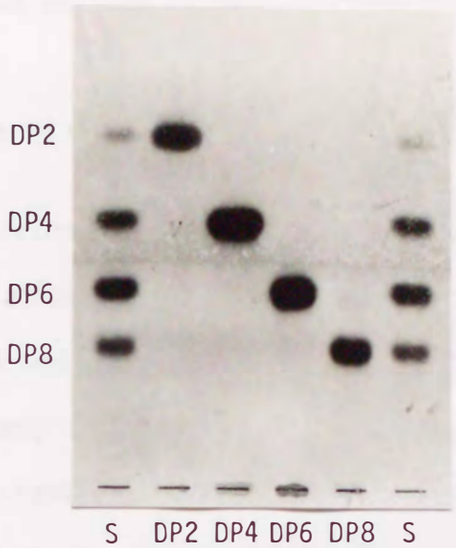


Fig.VII - 1. Thin layer chromatogram of neoagarooligosaccharide preparations.

DP 2, 4, 6, 8, degree of polymerization of neoagarosaccharides; S, standard.

Ⅶ-1-2. Molecular weight of three agarases

Each purified enzyme (2.0 m*l*) was applied to a column (2.0 × 95 cm) of Toyopearl HW-55 equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl. Standard proteins (Ribonuclease A, 13 kDa; Myoglobin, 17.8 kDa; Bovine albumin, 67 kDa) were applied to the column in the same condition. Elution was performed with the same buffer. The absorption of each fraction was measured at 280 nm for the protein assay.

Ⅶ-2. Results

Ⅶ-2-1. Action pattern of agarases

(a) Action of agarases on agar and κ-carrageenan

The reaction mixture consisted of 0.5 m*l* of 2 mM sodium acetate buffer, pH 6.0, containing 0.3% agar or κ-carrageenan and 0.1 m*l* of each enzyme solution (1 unit), and it was incubated at 38℃ for 12 h. The agarases acted on agar to give various sizes of neoagarooligosaccharides (Fig. VII-2). All enzymes did not hydrolyze κ-carrageenan.

To 1 m*l* of 2 mM sodium acetate buffer, pH 6.0, containing 0.3% agar, 0.1 m*l* of agarase-b solution (1 unit) was added, and the mixture was incubated at 38℃. A part of the reaction mixture was withdrawn at various intervals, boiled to inactivate the enzyme, and spotted on a TLC-plate. Agarase-b acted on agar to give neoagarooligosaccharides of various sizes (Fig. VII-3). The predominant product was a dimer.

(b) Action of agarases on neoagarooligosaccharides

To 0.5 m*l* of 10 mM sodium acetate buffer, pH 6.0, containing 1.0% each neoagarooligosaccharide, 0.1 m*l* of each enzyme solution (1 unit) was added, and the mixture was incubated at 38℃ for 12 h. The reaction mixture was concentrated *in vacuo* followed by spotting on a TLC plate (see V-1-9). The predominant hydrolysis products of neoagarooligosaccharides with each agarase are summarized in Table VII-1. Agarases-a and -c hydrolyzed neoagarohexaose to give neoagarobiose and neoagarotetraose, but did not act on neoagarotetraose. Agarase-b hydrolyzed

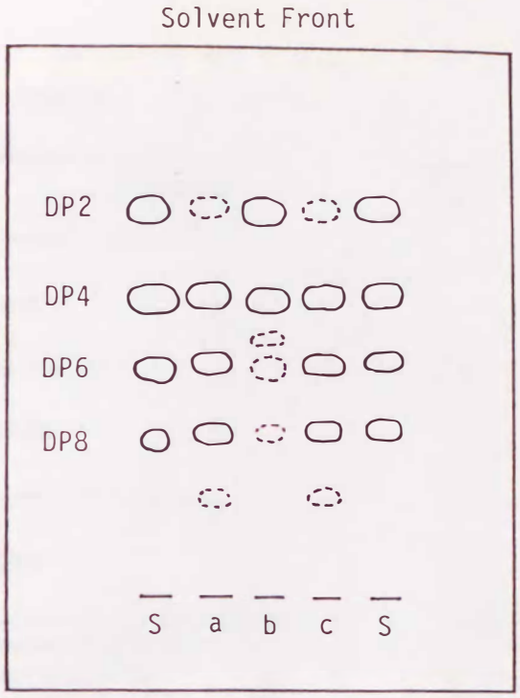


Fig.VII - 2. Enzymatic hydrolysis of agar by agarases-a, -b, and -c.
S, neoagarobiose to neoagarooctaose (DP 2,4,6,8; degree of polymerization).

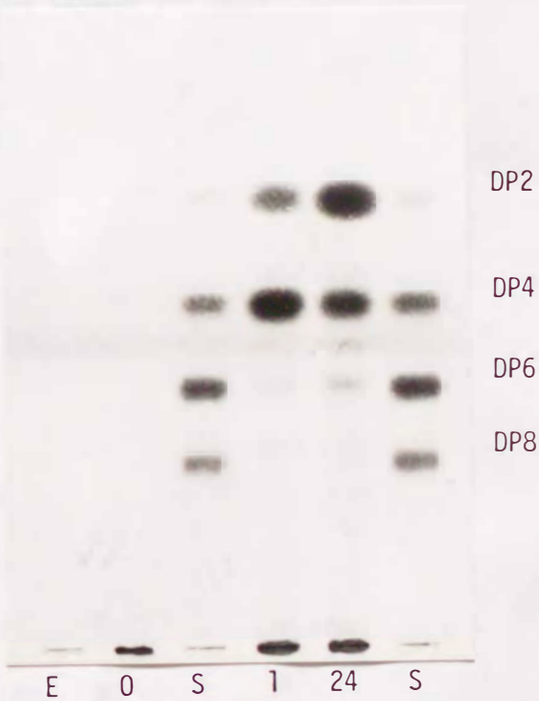


Fig.VII - 3. Thin layer chromatogram of hydrolyzate of agar with agarase-b from Vibrio sp. AP-2.
DP 2, 4, 6, 8, degree of polymerization of neoagarosaccharides; E, enzyme; S, standard; 0,1,24, reaction times(h) of the enzyme with agar.

Table VII - 1. Enzymatic hydrolysis of neoagarosaccharides by agarases-a, -b, and -c.

	Oligosaccharide DP*1	Products DP
Agarase-a	8	4
	6	4, 2
	4	—*2
Agarase-b	8	6, 4, 2
	6	4, 2
	4	2
Agarase-c	8	4
	6	4, 2
	4	—

*1 DP: degree of polymerization.
*2 —: trace of activity.

neoagarotetraose and larger neoagarooligosaccharides. The predominant hydrolysis product of agarase-b was dimer, while the products of agarases-a and -c were tetramer (Fig. VII-4).

Ⅶ-2-2. Effect of pH on activity of agarases

The reaction mixtures of 0.5 m*l* of each agarase solution (ca. 1.5 units) and each 1.5 m*l* of various buffers (M/10 sodium acetate buffer, pH 3.2 - 6.0 ; M/15 phosphate buffer, pH 6.0 - 8.0; M/10 glycine-NaOH buffer, pH 9.0 - 11.0) were incubated at 38℃ for 30 min, and the reducing sugars were determined. As shown in Fig. VII-5, the maximal activities of agarases-a, -b, and -c were obtained at pH 6.5, 5.5, and 7.0, respectively.

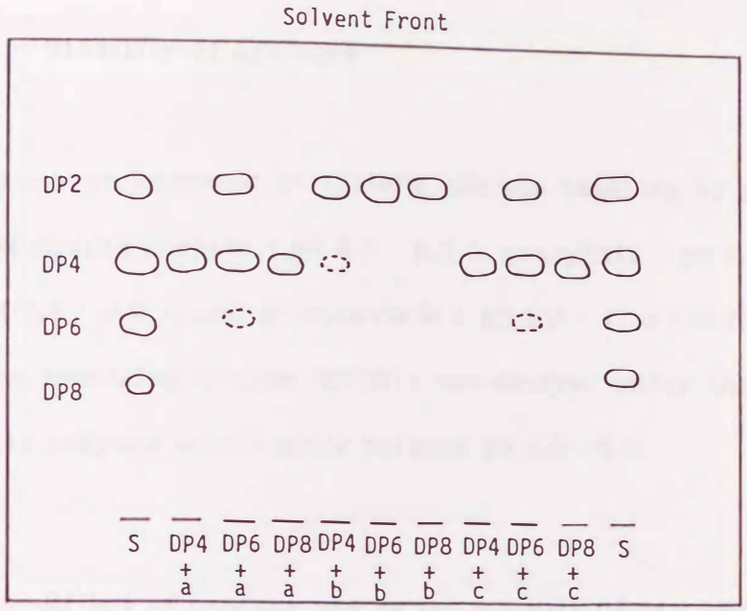


Fig.VII - 4. Enzymatic hydrolysis of neoagarooligosaccharides by agarases-a, -b, and -c.
S, neoagarobiose to neoagarooctaose (DP 2, 4, 6, 8 ; degree of polymerization).

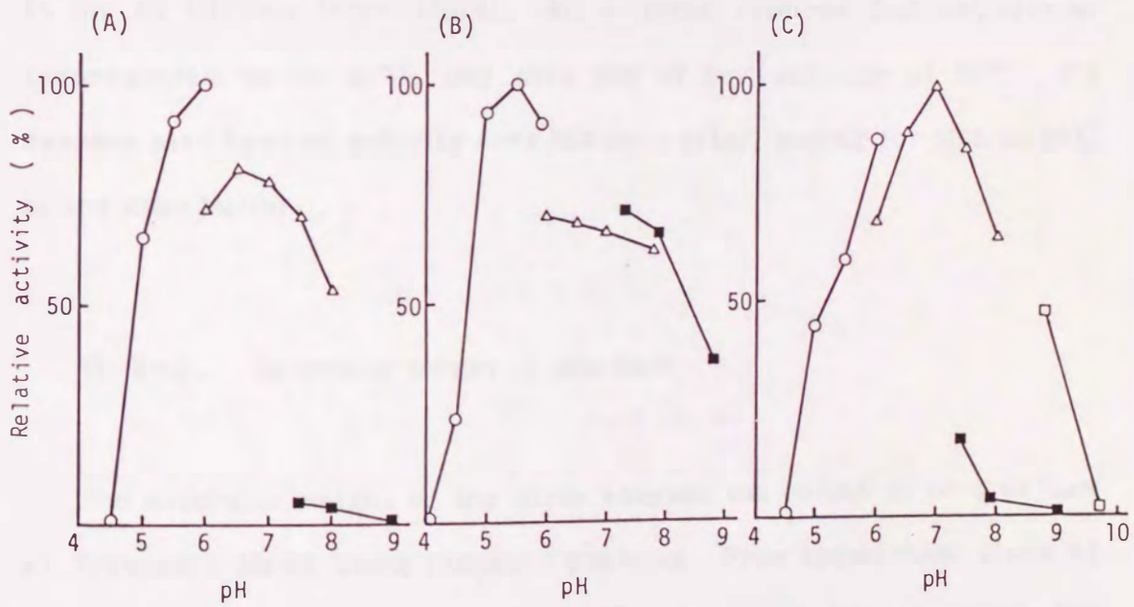


Fig.VII - 5. pH-activity curves of agarases-a(A), -b(B), and -c(C).
○, sodium acetate buffer (pH 3.2 - 6.0); △, phosphate buffer (pH 6.0 - 8.0); ■, Tris-HCl buffer (pH 7.5 - 9.0); □, glycine-NaOH buffer (pH 9.0 - 11.0).

Ⅶ-2-3. Stability of agarases

The stability of agarases at various pHs was examined by incubating them in 50 mM sodium acetate (pH 3.2 - 6.0), phosphate (pH 5.5 - 8.0), Tris-HCl (pH 7.2 - 9.0), and glycine-NaOH (pH 9.0 - 11.0) buffers at 4℃ for 20 h. The remaining enzyme activity was assayed under the standard condition. All enzymes were stablfe between pH 4.0 - 9.0.

Ⅶ-2-4. Effect of temperature on the activity of agarases

Each enzyme was incubated in 50 mM sodium acetate buffer, pH 6.0, for 15 min at various temperatures. All enzymes retained full activity at temperatures up to 45℃, and over 50% of full activity at 60℃. All enzymes also kept an activity over 95% even after leaving for 20 h at 25℃ in the same buffer.

Ⅶ-2-5. Molecular weight of agarases

The molecular weight of the three enzymes was estimated on a column of Toyopearl HW-55 using standard proteins. From logarithmic plots of the molecular weights versus elution volumes of the proteins, the agarases-a, -b, and -c were estimated to be 34 kDa, 20 kDa, and 18 kDa, respectively (Fig. VII-6).

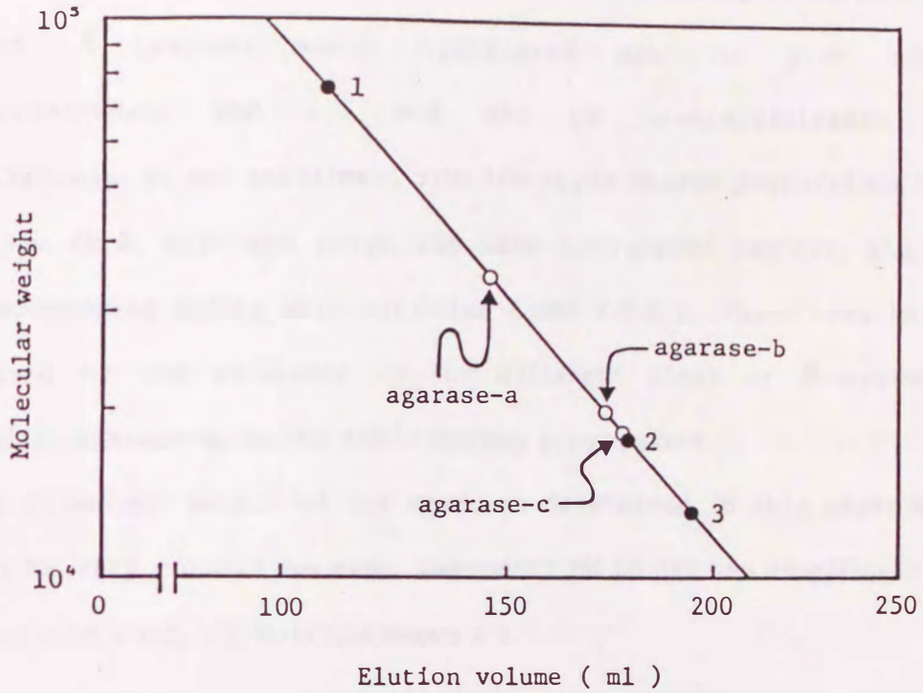


Fig.VII - 6. Molecular weight estimation for agarases-a, -b, and -c by Toyopearl HW-55 column chromatography.

1, bovine albumin (67,000); 2, myoglobin (17,800); 3, ribonuclease A (13,000).

Ⅶ-3. Discussion

With respect to the hydrolysis pattern, the purified agarase-b was indicated clearly to be a novel endo-type β-agarase which hydrolyzed neoagarotetraose, larger oligosaccharides and agar to give mainly neoagarobiose. Therefore, the existence of a novel type β-agarase was corroborated, of which the existence had been suggested in literature.[24] The agarase did not react with κ-carrageenan like the other β-agarases

reported.[25,62,65] Agarases-a and -c indicated the same pattern as other reported β-agarases which hydrolyzed agar to give mainly neoagarotetraose, and did not act on neoagarotetraose and κ-carrageenan. In the experiment with the crude enzyme preparation from *Vibrio* sp. AP-2, agar and porphyran were hydrolyzed rapidly, and the high decomposing ratios were exhibited (see V-2-5). These results may be caused by the existence of the different kinds of β-agarases, especially agarase-b, in the crude enzyme preparation.

The molecular weight of the agarases determined in this experiment may not be very correct because Toyopearl HW-55 gel has an affinity for some proteins (e.g. chymotrypsinogen A).

Ⅶ-4. Summary

The properties of three agarases from *Vibrio* sp. AP-2 were investigated. The enzymes (agarases-a, -b, and -c) had molecular weights of 34, 20, and 18 kDa, and the pH optima of 6.5, 5.5, 7.0, respectively, and were stable in a pH region from 4.0 to 9.0, and at temperatures below 45 ℃. All the agarases were β-agarase which degraded agar to yield neoagarooligosaccharides. While agarase-a and agarase-c hydrolyzed agar to give neoagarotetraose as the predominant product, agarase-b was a novel β-agarase which gave neoagarobiose as the predominant product. The three enzymes did not react with κ-carrageenan.

Chapter Ⅷ Preparation and Regeneration of Protoplasts from Thalli of Seaweeds of Genus *Porphyra*

The preparation of protoplasts from terrestrial plants was first reported in 1972,[1] and many studies of breeding by the method of cell fusion have been carried out in recent years.[2-4] However, few studies on protoplasts from seaweeds have been reported so far, and the method of protoplast preparation from red and brown algae has not been established.[5-10] Since the structural polysaccharides of seaweed cell wall differed from those of terrestrial plants[11], the availability of enzymes which degrade these polysaccharides is effective to advance the study on the cell fusion of seaweeds.

In the previous chapters, the author described the isolation and identification of two bacteria with the high productivity of β-1,3-xylanase and β-agarase (porphyran-degrading enzyme). The author also described the purification of a β-1,3-xylanase and β-agarases from the bacteria (see Chapters III and VI), and the characterization of these enzymes (see Chapters IV and VII).

In this chapter, the author describes the use of β-1,3-xylanase and β-agarases for the preparation of protoplasts from thalli of *Porphyra yezoensis* and *P. tenera*.

Ⅷ-1. Materials and Methods

Ⅷ-1-1. Vegetative thalli of seaweed

The shell-living conchocelises of reddish brown types of *Porphyra yezoensis* var. *narawaensis* and *P. tenera* var. *tamatsuensis* were obtained from Ariake Fisheries Experiment Station of Fukuoka Prefecture. The author cultured the seaweeds through the life cycle of conchocelis, conchospore, thallus, monospore, and to thallus, using Provasoli's ASP_{12} (NTA) medium.[66,67] In the culture of reddish brown thalli developed from the conchospores of *P. tenera*, the author found a green thallus which formed a lot of green monospores after maturation. The green monospores developed to green thalli in the culture medium.

The author used thalli from *P. yezoensis* var. *narawaensis* and the green type of *P. tenera* var. *tamatsuensis* in this experiment.[68]

Ⅷ-1-2. Enzymes

β-1,3-Xylanase from *Vibrio* sp. AX-4 and porphyran-degrading enzyme (β-agarase) from *Vibrio* sp. AP-2 were prepared from the culture fluids by ammonium sulfate precipitation (see II-1-8 and V-1-8). β-1,3-Xylanase and β-agarase solutions were dialyzed against 50 mM 2-(N-morpholino) ethane sulfonic acid (MES) buffer, pH 6.0, before use. β-1,4-Mannanase was prepared as follows: *Aeromonas* sp. F-25 (stored in the Laboratory of Fisheries Technology, Kyushu University) was grown at 25℃ for 3 days

in a liquid medium composed of 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% NaCl, 1.0% peptone, 0.1% yeast extract, and 0.5% konjac powder, pH 7.0. The culture obtained was centrifuged at 8,000 r.p.m. for 30 min. Solid ammonium sulfate was added to the supernatant solution up to a final concentration of 75% saturation for ammonium sulfate precipitation. After standing for 24 h, the enzyme was collected by centrifugation, dissolved in water, and dialyzed against MES buffer, pH 6.0. The dialyzed solution was used as β-1,4-mannanase solution.

Papain was obtained from Nagase Seikagaku Kogyo Co., and used to remove the surface mucos substance of the algal thalli.*

Ⅷ-1-3. Enzyme assays

The activities of β-1,3-xylanase and β-agarase were measured as described in II-1-4 and VI-1-2. The activity of β-1,4-mannanase was measured as follows: enzyme solution, 0.5 m*l*, was added to 2.0 m*l* of 1.0% β-1,4-mannan suspension in 50 mM acetate buffer, pH 6.0. After 30 min of incubation at 37℃, the reducing sugar produced was determined by Somogyi-Nelson's method.[31] One unit of the enzyme was defined as the activity that produces reducing capacity equivalent to 1 μmol of D-mannose per min under the above condition.

Ⅷ-1-4. Preparation of protoplasts

---

* O. Imada: Japan Kokai Tokkyo Koho, JP 991891, 121-123 (1979).

Basal parts of the thalli were removed in advance, and 1 ~ 5 g of the thalli (length 1 ~ 3 cm) was dipped into 30 m*l* of a papain solution (50 mM Tris-HCl buffer, pH 7.4, containing 1.0 ~ 2.5% papain and 0.7 M mannitol) at 22℃ for 15 min with shaking. The thalli treated with papain were washed 5 times with 25 mM MES buffer, pH 6.0, containing 5 mM $CaCl_2$, 2.0% NaCl, 0.5% potassium dextran, and 0.7 M mannitol, and then cut into small pieces (1 ~ 2 mm^2) with a razor blade. Adherent water of the small pieces was absorbed by a filter paper for measuring the weight.

The small pieces of thalli treated papain (ca. 50 ~ 100 mg) were suspended with 5 m*l* of cell wall-degrading enzyme solution (MES buffer, pH 6.0, containing 0.7 M mannitol, and 0.1 ~ 1.0 unit each of β-1,3-xylanase, β-agarase, and β-1,4-mannanase) in a 20 m*l* flask, and shaken at 22℃. The suspension was filtered through a nylon net, 40 μ mesh size, and the filtrate was centrifuged at 1,000 r.p.m. for 5 min. The precipitated protoplasts were washed 3 times with ASP_{12} (NTA) culture medium containing 0.5 M mannitol. The number of protoplasts was counted 5 times by using a Thoma's hemacytometer under the same condition, and the average value and standard deviation were calculated.

Ⅷ-1-5. Culture of protoplasts

A cover slip (3 × 3 cm) was placed on the center of a petri dish (dia., 9 cm) containing ASP_{12} (NTA) culture medium (50 m*l*, containing 0.7 M mannitol). The above protoplasts were transferred to the surface of

cover slip by using a sterilized pipet, and were incubated at 400 lx, 9 : 15 h LD, 16 ~ 18℃. After 2 days of incubation, the cover slip adhered to protoplasts was transferred to a petri dish containing ASP_{12} (NTA) culture medium, and incubated at 1,000 lx for several days. After regeneration of cell wall, forming of rhizoid, and beginning of cell division, the petri dish containing cultured protoplasts was incubated at 4,000 lx with shaking under the same condition. When the protoplasts developed into calli of 2 ~ 3 mm in length after several weeks, the calli adhered to the cover slip were transferred to 800 m*l* of culture medium in a 1 liter beaker covered with a wrap film, and was incubated under the same condition and aerated. The culture medium was changed to fresh medium every 10 days.

Ⅷ-2. Results

Ⅷ-2-1. Pre-treatment of seaweed thalli with papain

The thallus of *P. yezoensis* was treated with various concentrations of papain solutions, and then cut into small pieces with a razor blade. The small pieces (50 mg) were dipped into the enzyme solution containing three kinds of enzymes (0.1 unit each of β-1,3-xylanase, β-agarase, and β-1,4-mannanase), and then shaken at 22℃ for 90 min. When the thallus was pre-treated with 1.0 ~ 2.5% papain solution, the number of protoplasts released was 2.5 times larger than that of the protoplasts without pre-treatment with a papain solution (Fig. VIII-1).

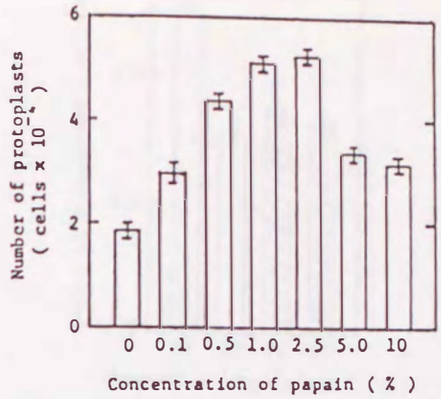


Fig.VIII - 1. Effect of papain pre-treatment on protoplast isolation from vegetative thalli of *P. yezoensis*.

Ⅷ-2-2. Effects of temperature and pH on protoplast isolation

A thallus of *P. yezoensis* was treated with 2.0% papain solution, and cut into small pieces. The small pieces (100 mg) were dipped into 5 m*l* of MES buffer, pH 6.0, containing 0.7 M mannitol, and 0.1 unit each of β-1,3-xylanase, β-agarase, and β-1,4-mannanase, and otherwise into 5 m*l* of 50 mM Tris-HCl buffer, pH 7.4, containing these enzymes, 5 mM $CaCl_2$, 2.0% NaCl, 0.5% potassium dextran sulfate, and 0.7 M mannitol. The mixtures were incubated at 22℃ or 27℃ with shaking. The largest number of protoplasts were released from *P. yezoensis* (Fig. VIII-2), when the thallus was treated with MES buffer, pH 6.0, containing three kinds of enzymes and 0.7 M mannitol, and incubated at 22℃.

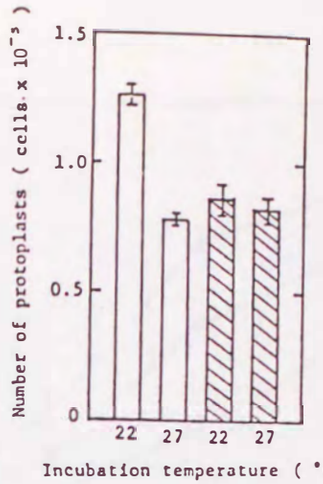


Fig.VIII - 2. Effect of pH value of the enzyme solution and incubation temperature on isolation of protoplasts from vegetative thalli of *P. yezoensis*.

□, MES buffer, pH 6.0; ▨, Tris-HCl buffer, pH 7.4.

Ⅷ-2-3. Effects of enzyme concentration and reaction time on protoplast isolation

The small pieces of *P. yezoensis* thallus (100 mg) were treated with a 2.0% papain solution, and the pieces treated were dipped into 5 m*l* of MES buffer, pH 6.0, containing various concentrations of enzymes and 0.7 M mannitol, and then shaken at 22℃ for 90 min. After the mixture was filtered through a nylon mesh, the number of protoplasts released was counted. The undegraded thalli were again dipped into 5 m*l* of newly prepared enzyme solution, followed by shaking at 16℃ for 20 h. The protoplasts released were counted. More than 4.8 × 10^5 of protoplasts were released from 100 mg of a thallus of *P. yezoensis* (Fig. VIII-3), when the mixture containing over 0.5 unit each of all enzymes was shaken for over 1.5 h, or the mixture containing 0.1 unit each of all enzymes was shaken for over 3 h.

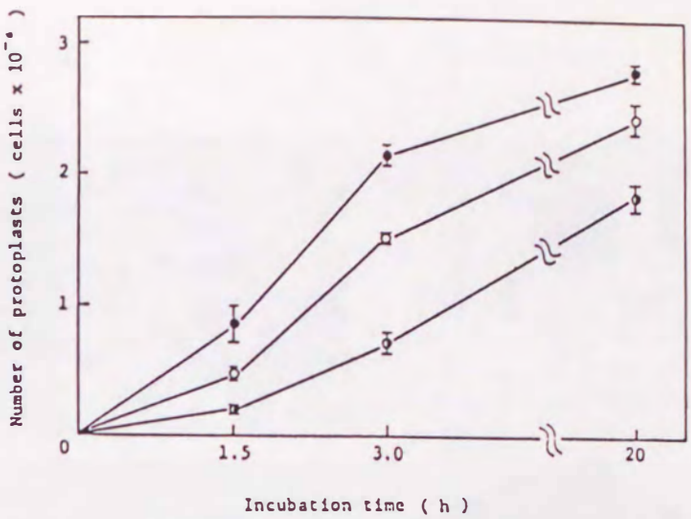


Fig.VIII - 3. Protoplast isolation as related to length of incubation period in enzyme solution.
Activities of β-agarase, β-1, 4-mannanase, and β-1,3-xylanase in enzyme solution: ◑, 0.1 unit each; ○, 0.5 unit each; ●, 1 unit each.

Ⅷ-2-4. Regeneration of protoplasts from *P. yezoensis* and *P. tenera*

The protoplasts were prepared from the thalli (1 ～ 3 cm in length) of *P. yezoensis* and *P. tenera*, and cultured as described in VIII-1-5. The protoplasts from each seaweed formed rhizoids after 5 ～ 10 day incubation (Figs. VIII-4-A and VIII-5-A), and began to divide (Figs. VIII-4-B and VIII-5-B). Some protoplasts from each seaweed divided regularly, and developed into normal thalli of *P. yezoensis* and *P. tenera*. The other protoplasts divided irregularly, and developed variably into callus after several weeks. The callus developed into fixed form thallus (Figs. VIII-4-C,D, E) or thick thallus that had a

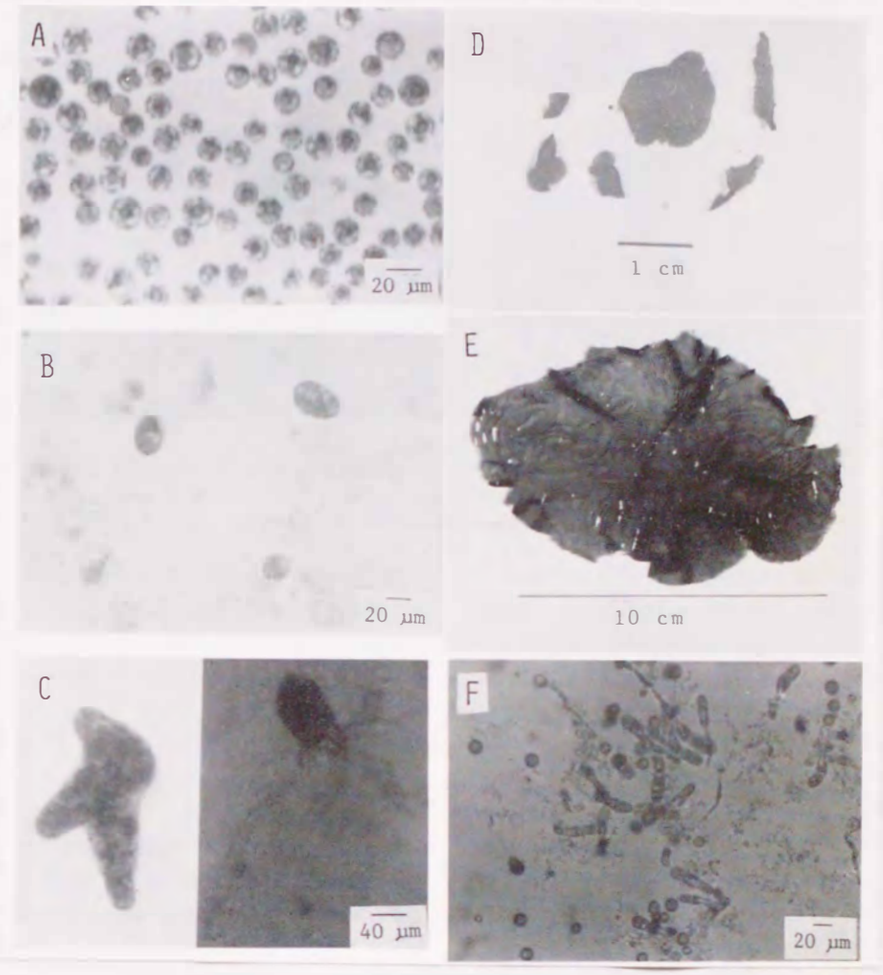


Fig.VIII - 4. Thallus regeneration from protoplast culture of *P. yezoensis*.

A, protoplasts from thalli of *P. yezoensis*; B, dividing cells after 1–2 weeks; C, calli after 6–7 weeks; D, thalli after 11 weeks; E, thallus after 13 weeks; F, monospores and germlings formed from regenerated thallus after 14 weeks.

curled edge. Some of the calli developed further (several hundred μm to several mm in length), and formed germlings. Furthermore, the germlings developed into normal thalli of *P. yezoensis* and *P. tenera* (Figs. VIII-5-C,D,E). The regenerated thalli released a lot of monospores, and these monospores formed germlings (Figs. VIII-4-F and VIII-5-F). All the germling of wild-type of *P. yezoensis* developed into reddish brown thalli, and all the germling of green variant of *P. tenera* developed into green thalli.

Ⅷ-3. Discussion

The study in this chapter indicated that the mixture solution of three kinds of enzymes (β-1,3-xylanase, β-agarase, and β-1,4-mannanase) were able to release a large amount of protoplasts having the ability to regenerate from *P. yezoensis* and *P. tenera*. In general, it is necessary to obtain the suspension containing $5 \times 10^4 \sim 10^6$ protoplasts for an experiment of cell fusion.[6,69] When thalli of the genus *Porphyra* were cut into small pieces after treatment with a 2.0% papain solution, and 100 mg of small pieces were dipped into 5 m*l* of the enzyme solution containing 0.5 unit each of the three enzymes followed by shaking at 22℃ for 1.5 ~ 3 h, $10^5 \sim 10^6$ protoplasts were released. When the enzyme solution lacked any one of the three enzymes, no protoplast (or extremely small amount of protoplasts) was released from the thalli.

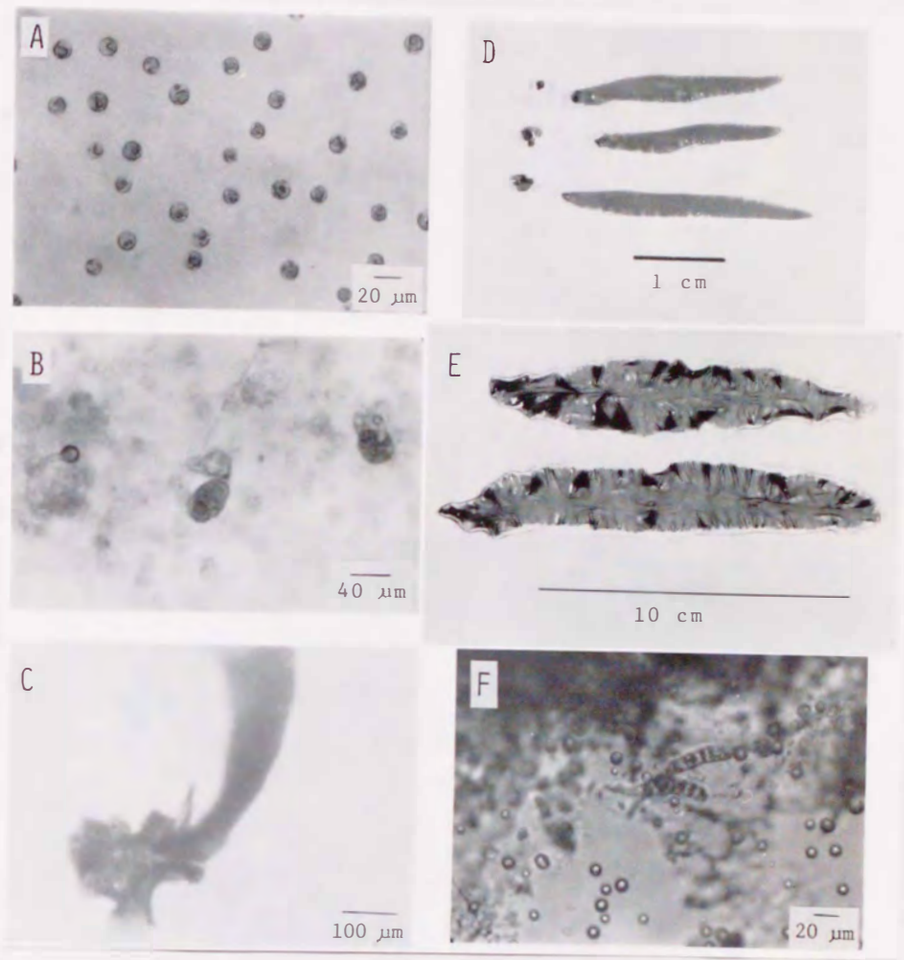


Fig.VIII - 5. Thallus regeneration from protoplast culture of *P. tenera*.

A, protoplasts from thalli of *P. tenera*; B, dividing cells after 1–2 weeks; C, callus and thallus after 5–6 weeks; D, calli and thalli after 7–8 weeks; E, thalli after 10 weeks; F, monospores and germlings formed from regenerated thallus after 12 weeks.

In several reports, the preparation of protoplasts from the seaweeds of genus *Porphyra* has been experimented by using acetone power of abalone hepatopancreas purchased from Sigma Chemical Co. (U.S.A.).[70] The activities of enzymes contained in the abalone acetone powder were measured by using 50 mM sodium acetate buffer, pH 6.0, and incubation at 37℃ (see II-1-4, VI-1-2, and VIII-1-3). As the result, 0.77 unit of β-1,3-xylanase, 4.0 units of β-agarase, and 12 units of β-1,4-mannanase were recognized in 1 *g* of acetone powder. The data indicated that the extract from over 0.6 *g* of acetone powder was necessary to prepare 5 m*l* of enzyme solution containing over 0.5 unit each of all enzymes. As far as our experience, only a small amount of protoplasts were released from the thalli of genus *Porphyra* by using a 2.0% abalone acetone powder solution. Furthermore, it was required to be incubated for a longer time than using the enzyme from bacteria. When the enzymes were prepared from bacteria, 56 units of β-1,3-xylanase, 84 units of β-agarase, and 1,500 units of β-1,4-mannanase were respectively obtained from the ammonium sulfate precipitation of 1 liter culture fluids.

Fujita *et al.* reported the protoplast preparation from the seaweeds of genus *Porphyra* by using a crude enzyme preparation from the cultured supernatant of *Pseudomonas* sp. P-1 in 1987.[9] However, the activity of the enzymes contained within the enzyme preparation, and the number of protoplasts released were not described.

Ⅷ-4. Summary

β-1,3-Xylanase, β-agarase, and β-1,4-mannanase were prepared to degrade the cell wall of seaweeds of the genus *Porphyra*, from the culture fluids of *Vibrio* sp. AX-4, *Vibrio* sp. AP-2, and *Aeromonas* sp. F-25, respectively. The reddish brown thalli of *P. yezoensis* (wild type), after treatment with a 2.0% papain solution, were suspended in 5 m*l* of MES buffer, pH 6.0, containing 0.7 M mannitol and 1 unit each of β-1,3-xylanase, β-agarase, and β-1,4-mannanase. When a 100 mg weight of the thalli was shaken in the enzyme solution at 22℃ for 90 min, ca. 8.6×10^5 protoplasts were released. Protoplasts from *P. yezoensis* grew in a ASP_{12} (NTA) medium and developed into reddish brown thalli of ca. 10 cm in length after 3 months. A number of protoplasts were obtained from green thalli of a green variant of *P. tenera*. The protoplasts developed into green thalli of ca. 10 cm in length after 2 months. A lot of monospores released from the regenerated thalli of *P. yezoensis* and *P. tenera*, and developed into reddish brown thalli and green thalli, respectively.

Chapter IX Summary

The preparation of protoplasts from phaeophyceae and rhodophyceae was rarely reported. The reason why the preparation had been unsuccessful might be that the structural polysaccharides of cell wall in the seaweeds differed from those of terrestrial plants, and the polysaccharide-degrading enzymes were not available to many workers. The availability of enzymes capable of degrading the polysaccharides is to be one of the most important processes to prepare protoplasts from the seaweeds. Moreover, these enzymes may be helpful to the preparation of useful oligosaccharides. In this study, the author noticed the enzymes capable of degrading cell wall polysaccharides of the genus *Porphyra*, especially β-1,3-xylanase and porphyran-degrading enzyme.

First, a screening was undertaken to obtain β-1,3-xylan-degrading bacteria by the clear zone-discriminating method. As the result, 4 isolates were obtained from 78 samples of bacterial sources collected from the sea, freshwater, and land in Fukuoka Prefecture. A strain (AX-4 strain) obtained from the bottom mud of the coastal sea showed the highest productivity of β-1,3-xylanase among the 4 isolates.

The AX-4 strain was a Gram-negative and facultatively anaerobic rod with a single polar flagellum, did not form endo-spores, produced acid but no gas from glucose, and was sensitive to 2,4-diamino-6,7-diisopropyl pteridine. Therefore, it belonged to the genus *Vibrio*, according to the criteria of Bergey's Manual of Systematic Bacteriology. The species of the strain was not named because of some discrepancies in morphological

and biochemical characteristics between the strain and all species listed in the Manual.

The oraganism needed β-1,3-xylan as an inducer to produce β-1,3-xylanase, and released a large amount of the enzyme into the culture fluid (48 m units/m*l*), when incubated with shaking at 25℃ for 4 to 6 days in the following liquid medium containing 0.1% peptone, 0.1% yeast extract, 3.0% NaCl, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.3% β-1,3-xylan, pH 7.8.

The author purified β-1,3-xylanase from the culture fluid of the AX-4 strain, by ammonium sulfate precipitation and successive column chromatography. The specific activity of final enzyme preparation was 111 times higher than that of the culture fluid, and the recovery was 23%. The final enzyme preparation was homogeneous on polyacrylamide gel electrophoresis. The enzyme had a molecular weight of 53 kDa, and pI of 3.6. The enzyme exhibited a high activity at a range of pH 6.0 to 7.5, and was stable in a pH region from 4.5 to 10.0, and at temperatures below 40℃. The β-1,3-xylanase was an endo-type enzyme which degraded β-1,3-xylan to xylose and various sizes of xylooligosaccharides. The enzyme cleaved xylotriose to xylose and xylobiose, and xylotetraose to xylose, xylotriose and a small amount of xylobiose. The hydrolysis products from xylopentaose were mainly xylose and xylotetraose in addition to small amounts of xylobiose and xylotriose. The β-1,3-xylanase did not act on xylobiose, *p*-nitrophenyl-β-D-xyloside, and β-1,4-xylan.

Next, the author undertook a screening to obtain porphyran-

degrading bacteria by using the acid albumin test. As the result, 5 isolates were obtained from 72 samples of bacterial sources collected from the sea, freshwater, and land in Fukuoka Prefecture. A strain (AP-2 strain) obtained from a seaweed showed the highest productivity of porphyran-degrading enzyme among the 5 isolates.

The AP-2 strain was a Gram-negative and facultatively anaerobic rod with a single polar flagellum, did not form endo-spores, produced acid but no gas from glucose, and was sensitive to 2,4-diamino-6,7-diisopropyl pteridine. Therefore, it belonged to the genus *Vibrio*, according to the criteria of Bergey's Manual of Systematic Bacteriology. The main characteristics of the AP-2 strain corresponded to those of *V. harveyi* and *V. campbellii* given in the Bergey's Manual. However, the strain differed in the two species in the utilization ability of some organic compounds. Therefore, the species of the strain was not named.

The organism released a large amount of the porphyran-degrading enzyme into the culture fluid (127 m units/*ml*), when incubated with shaking at 25℃ for 5 days in the following liquid medium: 0.5% peptone, 0.1% yeast extract, 3.0% NaCl in the seaweed extract, pH 7.4. The enzyme from the AP-2 strain exhibited a high activity at pH 6.5, and cleaved not only porphyran but also agar to give various sizes of neoagaro-oligosaccharides.

The author purified three β-agarases (agarases-a, -b, and -c) from the culture fluid of the AP-2 strain, by ammonium sulfate precipitation, successive column chromatography, and nucleases treatment. The specific activities of the agarases of the final purification procedure were 481,

328, and 745 time higher than that of the culture fluid, and the recoveries were 3.87, 23.5, and 25.5%, respectively. The final enzyme preparations was homogeneous on polyacrylamide gel electrophoresis. The enzymes had molecular weights of 34 kDa, 20 kDa, and 18 kDa, and the pH optima of 6.5, 5.5, and 7.0, respectively, and were stable at a pH region from 4.0 to 9.0, and at temperatures below 45℃. With respect to the hydrolysis pattern, it was indicated that the agarase-b was a novel endo-type β-agarase which hydrolyzed neoagarotetraose, larger oligosaccharides and agar to give mainly neoagarobiose. The action patterns of agarases-a and -c were the same as prior reported β-agarases which hydrolyzed agar to give mainly neoagarotetraose, and did not act on neoagarotetraose. The decomposing ratios of porphyran and agar with the mixture of three enzymes were 52% and 84%, respectively. All the β-agarases did not hydrolyze κ-carrageenan.

Finally, the author tried to prepare the protoplasts from two red algae, *Porphyra yezoensis* var. *narawaensis* and a green type of *P. tenera* var. *tamatsuensis*, as an application of the β-1,3-xylanase and β-agarases. The thalli of seaweeds were cut into small pieces after treatment with 2.0% papain solution, and then each 100 mg of small pieces was suspended in 5 m*l* of MES buffer, pH 6.0, containing 0.7 M mannitol. To the suspension, 5 m*l* of the enzyme solution containing 0.5 unit each of β-1,3-xylanase, β-agarase, and β-1,4-mannanase was added. After shaking at 22℃ for 1.5 to 3 h, a large amount of protoplasts (10^5 - 10^6) were released from the thalli.

The protoplasts from the thallus of the wild type of *P. yezoensis*

grew in a ASP_{12} (NTA) medium and developed into reddish brown thalli of ca. 10 cm in length after 3 months. The protoplasts from the green variant of *P. tenera* developed into green thalli of ca. 10 cm in length after 2 months. A lot of monospores were also released from regenerated thalli of *P. yezoensis* and *P. tenera*, and developed into reddish brown and green thalli, respectively.

Acknowledgements

The author is sincerely grateful to Professor M. Kitamikado, Kyushu University, for his help in the preparation of this dissertation and the many useful suggestions for its improvement.

The author is particularly indebted to Dr. T. Araki and Prof. R. Ueno, Mie University, for bacteriological and enzymological experiments, and their continuing interest and encouragement.

The author is grateful for many helpful discussions from the following: Dr. T. Nakamura, Prof. K. Koga, and Dr. K. Yamaguchi.

Facilities and experiments time made available to the author by Laboratory of Fisheries Technology, Kyushu University and Laboratory of Quality and Control of Marine Products, Mie University are greatly appreciated.

References

1) P.S. Carlson, H.H. Smith, and R.D. Dearling: *Proc. Natl. Acad. Sci. U.S.A.*, 69, 2292-2294 (1972).

2) D. Aviv, S. Bleichman, P. Arzee-Ginen and E. Galun: *Theor. Appl. Genet.*, 67, 499-504 (1984).

3) K.N. Kao: *Molec. gen. Genet.*, 150, 225-230 (1977).

4) K.K. Kamo, K.L. Chang, M.E. Lynn and T.K. Hodges: *Planta*, 172, 245-251 (1987).

5) P.A. Millner, Maureen E. Callow and L.V. Evance: *Planta*, 147, 174-177 (1979).

6) D. Zhang and J. Shandong: *Coll. Oceanol.*, 13, 57-65 (1983).

7) N. Saga: *Bot. Mag. Tokyo*, 97, 423-427 (1984).

8) Y. Fujita and S. Migita: *Bull. Fac. Fish. Nagasaki Univ.*, 57, 39-45 (1985).

9) Y. Fujita and S. Migita: *Jap. J. Phycol.*, 35, 201-208 (1987).

10) N. Saga and Y. Sakai: *Nippon Suisan Gakkaishi*, 50, 1085 (1984).

11) E. Percival and R.H. McDowell: Chemistry and Enzymology of Marine Algal Polysaccharides, Academic Press, London, 1967, pp. 88-93.

12) T. Mizuno, Y. Iwasaki, H. Satoh, T. Usui, K. Shinkai, M. Arakawa and M. Tanaka: *Bull. Fac. Agr. Shizuoka Univ.*, 32, 29-40 (1982).

13) H. Kanayama, M. Togami, N. Adachi, Y. Fukai and T. Okumoto: *Yakugaku Zasshi*, 106, 307-312 (1986).

14) K. Nisizawa, H. Noda, R. Kikuchi, and T. Watanabe: *Hydrobiologia*, 151-152, 5-29 (1987).

15) J.C. Su and W.Z. Hassid: *Biochemistry*, 1, 468-474 (1962).

16) E. Frei and R.D. Preston: *Proc. Roy. Soc.*, B, 160, 314-327 (1964).

17) H. Fujisawa and M. Murakami: *Nippon Suisan Gakkaishi*, 36, 741-747 (1970).

18) H. Fujisawa and M. Murakami: *Nippon Suisan Gakkaishi*, 37, 119-123 (1971).

19) S. Fukui, T.Suzuki, K.Kitahara, and T. Miwa: *J. Gen. Appl. Microbiol.*, 6, 270-282 (1960).

20) K. Nisizawa, I. Morimoto, N. Handa, and Y. Hashimoto: *Arch. Biochem. Biophys.*, 96, 152-157 (1962).

21) W.P. Chen, M. Matsuo, and T. Yasui: *Agric. Biol. Chem.*, 50, 1183-1194 (1986).

22) I. Yamaura, T. Matsumoto, M. Funatsu and E. Mukai: *Agric. Biol. Chem.*, 54, 921-926 (1990).

23) M. Duckworth and J.R. Turvey: *Biochem. J.*, 113, 687-692 (1969).

24) L.M. Morrice, M.W. McLean, F.B. Williamson and W.F. Long: *Eur. J. Biochem.*, 135, 553-558 (1983).

25) L.M. Morrice, M.W. McLean, W.F. Long and F.B. Williamson: *Eur. J. Biochem.*, 137, 149-154 (1983).

26) D. Groleau and W. Yaphe: *Can. J. Microbiol.*, 23, 672-679 (1977).

27) T. Araki and M. Kitamikado: *Nippon Suisan Gakkaishi*, 47, 753-760 (1981).

28) I.M. Mackie and E.E. Percival: *J. Chem. Soc.*, 1151-1156 (1959).

29) Y. Iriki, T. Suzuki, K. Nishizawa, and T. Miwa: *Nature*, 187, 82-83 (1960).

30) J. Love and E. Percival: *J. Chem. Soc.*, 3345-3350 (1964).

31) M. Somogyi: *J. Biol. Chem.*, 195, 19-23 (1952).

32) P. Baumann, A.L. Furniss, and J.V. Lee: in " Bergey's Manual of Systematic Bacteriology " (ed. by N.R. Kreig and J.G. Holt), Vol. 1, Williams & Wilkins, U.S.A., 1984, pp. 518-538.

33) M. Frobisher: in " Fundamental of Microbiology ", W.B. Sanders Co., Philadelphia, 1953, pp. 106-107.

34) N. Kovacs: *Nature*, 178, 703 (1956).

35) R. Hugh and E. Leifson: *J. Bact.*, 66, 24-26 (1953).

36) M.J. Thornley: *J. Appl. Bact.*, 23 (1), 37-52 (1960).

37) J. Marmur: *J. Mol. Biol.*, 3, 208-218 (1961).

38) J. Marmur and P. Doty: *J. Mol. Biol.*, 5, 109-118 (1962).

39) M.E. Rhodes: *J. Gen. Microbiol.*, 18, 639-648 (1958).

40) R. Sakazaki and A. Balows: in " The Prokaryotes " (ed. by M.P. Starr, H. Stolp, H.G. Trüper, A. Balows and H.G. Schlegel), Springer-Verlag, New York, 1981, pp. 518-538.

41) V.B.D. Skerman: " Abstracts of Microbiological Methods ", Wiley-Interscience, New York, 1969.

42) B. Hagiwara, H. Matsubara, M. Nakai, and K. Okunuki: *J. Biochem.*, 45, 185-194 (1958).

43) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall: *J. Biol. Chem.*, 193, 265-275 (1951).

44) B.J. Davis: *Ann. N. Y. Acad. Sci.*, 121, 404-413 (1964).

45) R.F.H. Dekker and G.N. Richards: *Adv. Carbohydr. Chem. Biochem.*, 32, 317-319 (1976).

46) G. Okada: *Bull. Fac. Edu. Shizuoka Univ.*, 23, 57-66 (1972).

47) D. French and G.M. Wild: *J. Am. Chem. Soc.*, 75, 2612 (1953).

48) J. Conchie and G.A. Levvy: in " Method in Carbohydrate Chemistry " (ed. by R.L. Wolfrom), 2, Academic Press, New York, 1962, pp.345.

49) O. Vesterberg and H. Svensson: *Acta.Chem.Scand.*, 20, 820-834 (1966).

50) L.H. Villarroel and A.B.Zanlungo: *Carbohydrate Research*, 88, 139-145 (1981).

51) D.J. Brash, H.M. Chang, C.T. Chuah and L.D. Melton: *Carbohydrate Research*, 97, 113-125 (1981).

52) A. Usov, S.V. Yarotsuky and A.S. Shashkov: *Biopolymer*, 19, 977-990 (1980).

53) E. Percival and R.H. McDowell: Chemistry and Enzymology of Marine Algal Polysaccharides, Academic Press, London, 1967, pp. 129-133.

54) E. Percival and R.H. McDowell: *ibid.*, pp. 134-137.

55) D.A. Rees and E. Conway: *Biochem. J.*, 84, 411-416 (1962).

56) M. Morrice, M.W. McLean, W.F. Long and F.B. Williamson: *Eur. J. Biochem.*, 133, 673-684 (1983).

57) M.B. Mathews: in " Methods in Enzymology " (ed. by E.F. Neufeld and V. Ginsburg) Vol. 8, Academic Press Inc., New York, 1966, pp. 654-662.

58) M. Duckworth and W. Yaphe: *J. Chromatogr.*, 49, 482-487 (1970).

59) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith: *Analyt. Chem.*, 28, 350-356 (1956).

60) K.S. Young, S.S. Bhattacharjee and W. Yaphe: *Carbohydrate Research*, 66, 207-212 (1978).

61) W. Yaphe: *Can. J. Microbiol.*, 3, 987-993 (1957).

62) H.J. van der Meulen and W. Harder: *Antonie Leeuwenhoek J.Microbiol.Serol.*, 41, 431-447 (1975).

63) S. Fukusawa, P. V. Dunlap, M. Baba and M. Osumi: *Agric. Biol. Chem.*, 51, 265-270 (1987).

64) M. Malmqvist: *Biochim. Biophys. Acta.*, 537, 31-43 (1978).

65) J.R. Turvey and J. Christison: *Biochem. J.*,105, 311-316 (1967).

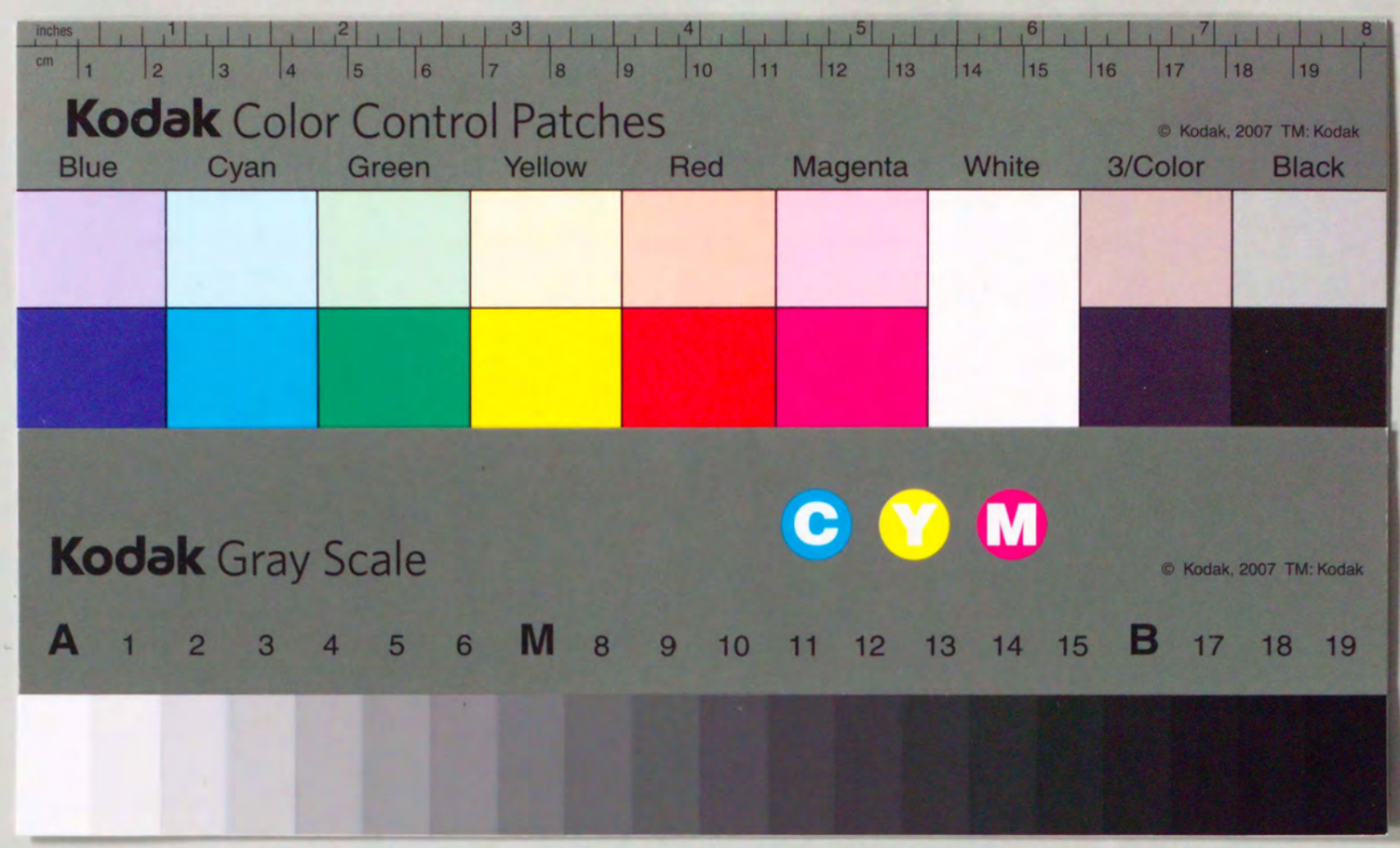
66) L. Provasoli, J.J.A. McLaughlin and M.R. Droop: *Arch. Microbiol.*, 25, 392-428 (1957).

67) H. Iwasaki: *Biol. Bull.*, 121, 173-187 (1961).

68) T. Araki, T. Aoki and M. Kitamikado: *Nippon Suisan Gakkaishi*, 53, 1623-1627 (1987).

69) P.M. Bradley, D.P. Cheney, P. Donald and N. Saga: *Plant Cell, Tissue Organ Cult.*, 12, 55-60 (1988).

70) M. Polne-Fuller and A. Gibor: *J. Phycol.*, 20, 609 (1984).


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19