アミラーゼインヒビター生産放線菌の海洋からの分離とその分類学的諸性状

誌名	日本水産學會誌
ISSN	00215392
著者	今田, 千秋 清水, 潮
巻/号	54巻10号
掲載ページ	p. 1839-1845
発行年月	1988年10月

Nippon Suisan Gakkaishi 54(10), 1839–1845 (1988)

Isolation and characterization of an α-amylase inhibitor producing actinomycete from marine environment

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(Received May 11, 1988)

A marine actinomycete which produced an extracellular α-amylase inhibitor was isolated and identified. A new and simple starch agar plate method was employed for a quick screening of the inhibitor producer. Out of 5,158 isolates from various sea areas, only one strain which was isolated from the sediment of neritic sea area was found to produce an amylase inhibitor. The strain showed abundant to moderate growth covered with aerial mycelium of light gray to light brownish color on all the media except for glucose-asparagine agar. The spores were cylindrical and the surface was smooth. Sporangia and flagellated spores were not observed. The strain showed taxonomic features closely identical to the strain type of *Streptomyces corchorusii* except for the temperature range for growth, NaCl tolerance and intracellular pigment production in the presence of seawater. From these differences, the strain is named *Streptomyces corchorusii* subsp. *rhodomarinus* subsp. nov.

This is the first report of an amylase inhibitor producing organism from marine environment.

Amylase is widely distributed among various microorganisms as well as plants and animals. So far many investigations on this enzyme were carried out and much information has been obtained. However, studies on the inhibitor of this enzyme have been limited. Amylase inhibitors have received considerable attention in recent years because they are helpful tools for the determination of activities of amylase isozyme[1] and purification of amylases[2] as well as control of some carbohydrate-dependent diseases such as diabetes, obesity or hyperlipemia.[3,4] For these purposes, amylase inhibitors have been isolated from plants and microorganisms. These inhibitors were composed either oligosaccharides[5-7] or protein.[8-11]

To date, most amylase inhibitors of microbial origin were isolated from terrestrial actinomycetes. With regard to antibiotics, when purified, most of them are similar in structure. Thus, the exploitation of novel bioactive products of terrestrial origin seems to be difficult. Compared with terrestrial environment, marine environment has several peculiar features. It is represented by a high salinity and low concentration of organic matter and, at deeper depth, constant low temperatures and high hydrostatic pressure. Microorganisms living in this environment produce a variety of bioactive compounds which have never been found in terrestrial organisms.[12-15] This shows that marine environment has large potential to be explored and exploited for new biological products. As reported previously, the present authors screened a large number of marine bacteria, and isolated marine *Alteromonas* species which produced protease inhibitors.[16] Cultivation conditions and purifications of the inhibitors are also reported.[17-19] These substances, named "Marinostatins" had a characteristic structure distinct from that inhibitors to be isolated from a series of terrestrial organisms.[20] However, no report is yet available concerning amylase inhibitor-producing marine microorganisms, taxonomical position, and the ecological role of the amylase inhibitor-producers in their habitat. Consequently, an attempt was made to isolate marine microorganisms which produced amylase inhibitor. This paper describes the isolation of an amylase inhibitor-producing microbe from marine environment and taxonomical characteristics of the strain.

Materials and Methods

Collection of Samples

Seawater and sediment samples were collected

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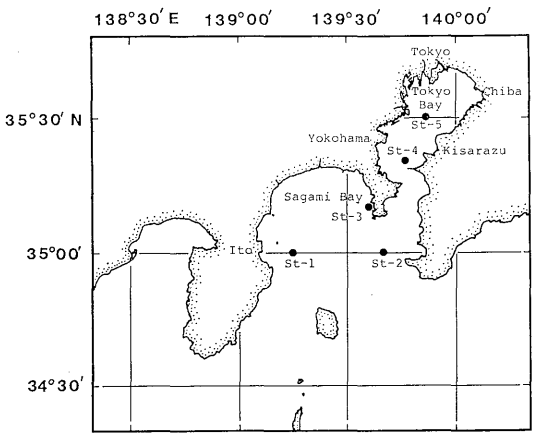

Fig. 1. Sampling stations.

from Tokyo and Sagami Bays during the KT-87-1 cruise (March 1987) on board the research vessel "Tansei-maru" of the Ocean Research Institute, University of Tokyo. Seawater and sediment sampling was also carried out using a small boat in a small inlet near Sagami Bay (St-3, September 1986). These sampling stations are shown in Fig. 1. Surface seawater samples were collected in sterile glass bottles; for deeper-water samples, a Niskin bacteriological water sampler was used (the "butterfly" type, General Oceanics Miami). Sediment samples were obtained with a box-corer sediment sampler or a Phlegar-type gravity corer.

Isolation of Microorganisms

For the isolation of microbes, the spread plate method and filter method using a Nuclepore filter (pore size 0.2 μm) were employed. Seawater and sediment collected from Tokyo Bay and sediment from Sagami Bay were serially diluted and the 0.1 m*l* of the portions were spread on 3 different media given in Table 1. The seawater samples from Sagami Bay were concentrated on the filters. The filters which retained microorganisms were placed on the media, and they were incubated for 1 week at 25°C. The colonies appeared on the plates were isolated randomly after incubation.

Screening Method of Amylase Inhibitor Producing Microorganisms

Microorganisms producing amylase inhibitors were screened using following the plate method. This method is based on the hydrolyzation of starch in the medium by added amylase. When an iodine reagent is poured on the plate, inhibitor-producing strain formed a purple color inhibitory zone (halo) around the colony, showing the

Table 1. Composition of 3 media for the isolation of microorganisms

Medium A (for bacteria)	
Polypepton (Daigo)	6.0 g
Glucose	0.5 g
Bacto-yeast extract	0.1 g
Bacto-agar	15.0 g
Seawater (full strength)	1.0 *l*
pH	7.0
Medium B (for actinomycetes)	
Glucose	10.0 g
L-asparagine	0.5 g
KH_2PO_4	0.5 g
Bacto-agar	15.0 g
Seawater (full strength)	1.0 *l*
pH	7.0
Medium C (for yeasts)	
Glucose	20.0 g
Polypepton (Daigo)	10.0 g
Bacto-yeast extract	5.0
Bacto-agar	15.0 g
Seawater (full strength)	1.0 *l*
pH	4.0*

* pH was adjusted 4.0 by the addition of 10% lactic acid after autoclave.

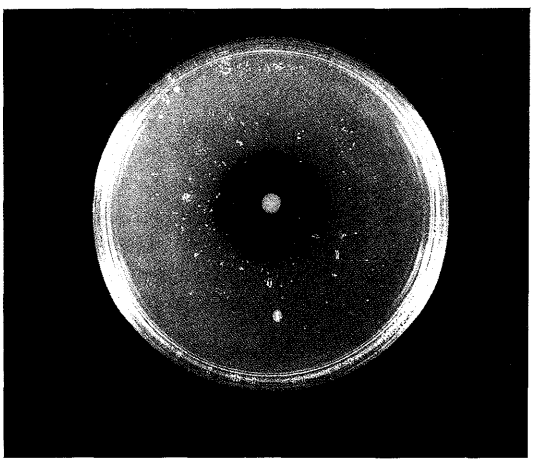

Fig. 2. Inhibitory zone of strain No. 178.

production of the inhibitor. The screening agar medium was composed of 0.6% Polypepton, 0.1% Bacto-yeast extract, 1.0% soluble starch and 1.5% Bacto-agar in full strength seawater. The pH of the medium was adjusted to 7.0. The isolates were stab inoculated on the plates and incubated at 25°C for 5 days. Five strains were tested on one plate. After incubation, all the colonies on the plate were killed by the addition of ethanol. To these plates, an α-amylase (from Human saliva, Sigma type 3) solution (500 μg/m*l* in 50 mM Tris-HCl buffer pH 7.0) was sprayed and the plates

were incubated at 37°C for 12 h followed by the flooding with an iodine solution. Colonies which formed a purple color inhibitory zone were considered to be a strain that produced an α-amylase inhibitor (Fig. 2).

Identification of Microorganism

Methods and media described by the International Streptomyces Project[21] (ISP) and Waksman[22] were used to determine most of the morphological and physiological characteristics of the microorganisms. Inoculated media were incubated at 28°C for 14 days before observation. Detailed observation of mycelial morphology was performed by a light microscope (XF-Ph-21, Nikon) and a transmission electron microscope (Nihon Denshi type JEM-100C) after the strain was incubated on ISP-2 medium at 28°C for 7 to 14 days. Liquefaction of gelatin was examined at 20 and 28°C for 14 days on a medium composed of gelatin 200 g, beef extract 10 g, peptone 10 g, NaCl 1 g in distilled water 1,000 m*l*. The medium was placed in an ice box after incubation to detect liquefaction. Milk peptonization and coagulation was obtained in a skim milk medium. The utilization of carbon sources was tested on Pridham and Gottlieb's medium[23] containing miscellaneous carbohydrates to a final concentration of 1%. Nitrate reduction was examined in a medium composed of peptone 10 g, NaCl 5 g, KNO_3 5 g in distilled water 1,000 m*l*. Formation of nitrite was detected with reagents consisting of 0.8% sulfanilic acid in 5 N acetic acid and 0.5% α-naphthylamine in the same solvent. Temperature range for growth (2–55°C) and NaCl tolerance (0–15%) were tested on ISP-3 and -4 medium,

Table 2. Sampling stations and number of isolates screened

Sampling station	Sampling date	Sample	No. of isolates screened		
			Medium A	Medium B	Medium C
St-1	Mar. 7, 1987	seawater	153	53	2
		sediment	621	157	30
St-2	Mar. 8, 1987	seawater	101	42	0
		sediment	629	202	39
St-3	Sep. 12, 1986	seawater	303	29	4
		sediment	980	113	43
St-4	Mar. 8, 1987	seawater	203	29	4
		sediment	524	121	29
St-5	Mar. 8, 1987	seawater	119	28	0
		sediment	429	151	20

Table 3. Cultural characteristics of strain No. 178

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Oat meal agar (ISP-3)	abundant	light gray	reddish brown	none
Yeast-malt extract agar (ISP-2)	abundant	light brownish gray	pale yellowish brown	none
Inorganic salts-starch agar (ISP-4)	abundant	light brownish gray	pale yellow	none
Glycerol-asparagine agar (ISP-5)	good	light gray	pale yellow	none
Sucrose-nitrate agar	moderate-good	light gray	pale yellow	none
Glucose-asparagine agar	poor	light gray	pale red	none
Nutrient agar	good-moderate	light gray	light brownish gray	none
Starch agar	good	light gray	pale brown	none
Skim milk agar	moderate	light gray	light reddish yellow	none
Tyrosine agar	moderate	light brownish gray	reddish brown	none
Peptone yeast extract iron agar (ISP-6)	abundant	light brownish gray	pale brown	none

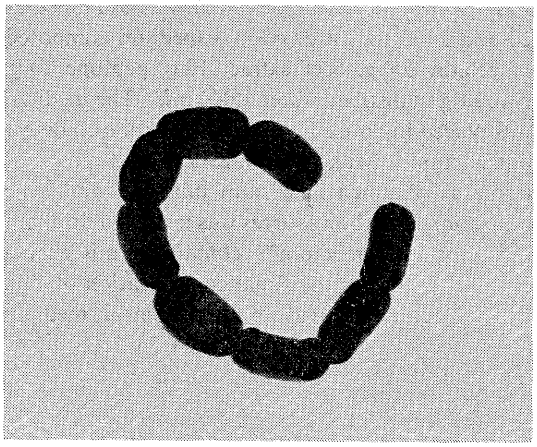

Fig. 3. Transmission electron micrograph of aerial mycelium of strain No. 178.

respectively. The chemical analyses of sugar in whole cells and diaminopimelic acid in cell wall were carried out by the method of Lechevalier and Lechevalier.[24] Intracellular pigment of the strain was extracted from the vegetative mycelium with methanol using a Nutrient broth after incubation for 3 days at 28°C with shaking followed by centrifugation of the mycelium. The absorption spectra of the pigment were measured with a spectrophotometer (Hitachi type U-3200). The type cultures (*Streptomyces corchorusii* 5340 and *S. chibaensis* 5220) used for taxonomic comparison were obtained from ISP.

Results

Sampling Stations and Number of Isolates Screened

The data on the sampling are listed in Table 2. Out of 142 isolates collected from St-3 using medium B, only one strain (No. 178) from sediment produced an α-amylase inhibitor. No inhibitor-producing strains were found among 4,956 isolates among 3 media from other samples. The results indicate that marine microorganism which produce an amylase inhibitor was very rare like terrestrial environments.

Taxonomical Characteristics of the Inhibitor-Producing Strain

Cultural characteristics of strain No. 178 with various agar media were summmarized in Table 3. The strain showed abundant to moderate growth covered with aerial mycelium of light gray to light brownish gray on all the media except for glucose-asparagine agar. The reverse side color was pale yellow to pale brown on most media. No soluble pigment was produced on all the media. Mature spores occurred generally in chain of more than 10 spores forming loose spirals or hooks. The spores were cylindrical and 0.6–0.7 $\times$ 1.2–1.3 μm in size. Spore surface was smooth. Sporangia and flagellated spores were not observed. No fragmentation of substrate mycelium was observed in agar media (Figs. 3 and 4). Chemical analysis of cell wall and whole cell sugars of strain No. 178 showed that it contained L,L-diaminopimelic acid and no

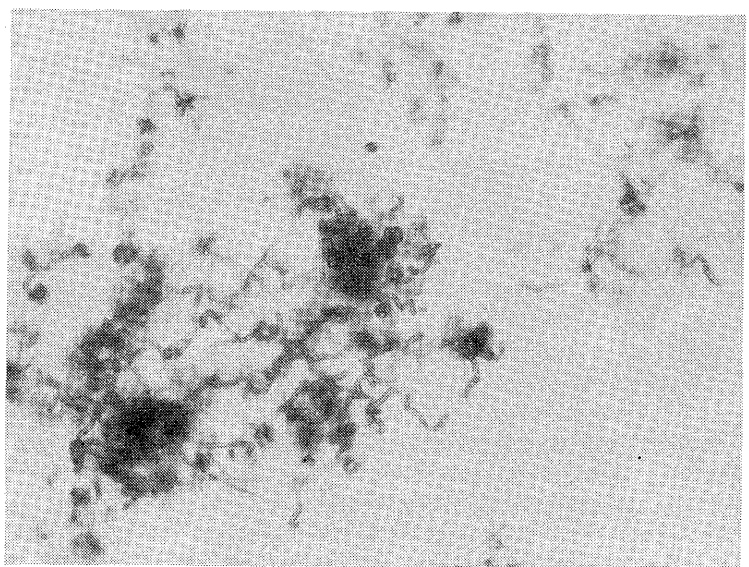

Fig. 4. Light micrograph of aerial mycelium of strain No. 178.

Table 4. Physiological properties of strain No. 178

Liquefaction of gelatin	positive
Hydrolysis of strach	positive
casein	positive
urea	positive
esculin	positive
Catalase	present
Tyrosinase activity	negative
Cellulolytic activity	negative
Nitrate reduction	positive
Action on milk	rapid peptonization weak coagulation
H_2S formation	negative
NaCl conc. range for growth	0–12%
Temperature range for growth	20–45°C

Table 5. Utilization of carbon compounds by strain No. 178

Carbon source	Utilization*1
No carbon source*2	−
D-Glucose*3	+
L-Arabinose	+
Sucrose	+
D-Xylose	+
i-Inositol	+
D-Mannitol	+
D-Fructose	+
Rhamnose	+
Raffinose	+

*1 +, positive utilization; −, no growth.
*2 negative control.
*3 positive control.

characteristic sugars. Table 4 shows the physiological properties of strain No. 178. The strain grew in the temperature range of 20–45°C, the optimum being 25–30°C. It grew in the NaCl concentration range of 0–12%. Liquefaction of gelatin, hydrolysis of casein, starch, urea, and esculin were all positive. Rapid peptonization with weak coagulation on milk was also observed. This strain, however, had no cellulolytic or tyrosinase activities. All carbohydrates tested as the sole carbon sources were well utilized (Table 5). From these results, the strain No. 178 is considered to belong to the genus *Streptomyces*, and its group of the gray color-series with smooth spore surface and with chains of Retinaculiaperti-type and without melanoid pigment formation.[25] Based on the above features, *Streptomyces corchorusii* ISP 5340 and *S. chibaensis* ISP 5220 were chosen as candidate taxa for the strain No. 178. As shown in Table 6, a comparative examination of the two type strains revealed that strain No. 178 was similar to *S. corchorusii* with exception of some physiological differences. The type strain grew at the range of 20–50°C, while strain No. 178 at 20–45°C. The type strain had a narrow concentration range of NaCl for growth (0–6%) whereas strain No. 178 had a wide range (0–12%). Furthermore, when strain No. 178 was incubated in seawater medium, it produced a red color intracellular pigment which was extracted from the vegetative mycelium with methanol. The absorption spectra of this pigment had a peak at 530 nm, whereas the peak of alkali-denatured pigment shifted to 460 nm (Fig. 5). This may suggest that this pigment is a pH-indicator. The phenomenon described above is not seen with both the type strains. The strain No. 178 was therefore classified as a new subspecies of *S. corchorusii*, and the name *S. corchorusii* subsp. *rhodomarinus* subsp. nov. Imada and Simidu was given.

Discussion

The amylase inhibitors from both plants and microbial sources have been found valuable for the study of activity and structure of amylases,[1] and several applications for the medical purposes have appeared. Low molecular and protein inhibitors are both effective for this purpose.[3,4]

The physiological role of plant α-amylase inhibitors are generally thought to be a defensive function against animal or insect attack through the inhibition of their amylase. They do not inhibit amylases of the plants in which they are found,[26] whereas they are quite inhibitory against aminal salivary and pancreatic α-amylases.[27] The amylase inhibitors of wheat are also inhibitory against insect amylases.[28] Although there were many studies on the role of microbial amylase inhibitors, the results are far from unequivocal[27,29].

Table 6. Comparison of strain No. 178, *S. corchorusii* ISP-5340, and *S. chibaensis* ISP-5220

	Strain No. 178	*S. corchorusii*	*S. chibaensis*
Liquefaction of gelatin	positive	positive	negative
Temperature range for growth	20–45°C	20–50°C	20–50°C
NaCl conc. range for growth	0–12%	0–6%	0–3%
Intracellular pigment	red	none	none

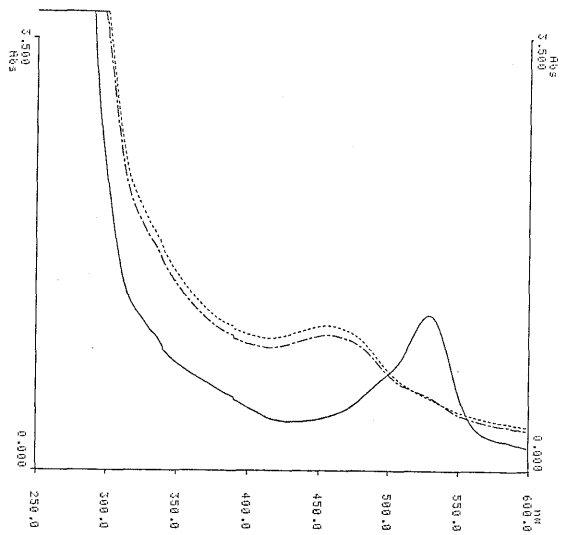

Fig. 5. Absorption spectra of intracellular pigment of strain No. 178.
——— Seawater medium
---------- Seawater medium (alkali-denatured)
—·—·— fresh water medium

So far, amylase inhibitors of microbial origin were screened by culturing each strain using a liquid medium and by the determination of the inhibitory activity in the filtrate. This method was time-consuming and hence not suitable for screening of a large number of cultures in a short period of time. The plate method developed in the present study was very effective for the screening of many cultures.

Actinomycetes are common terrestrial microorganisms. Some of them are transported to river through rain or irrigated waters finally to marine environment[30] which actinomycetes grow. These actionmycetes frequently survive in seawater or settle down to the sediment in fairly a dense population. Some of them can adapt to the conditions of marine environment. Isolation of actinomycetes from marine environment gives us another source for finding new bioactive compounds as exemplified by the present investigation.

Acknowledgements

We are grateful to Ms. K. Kita-Tsukamoto, Ocean Research Institute, University of Tokyo, for helping us with the electron microscopy. Thanks are also extended to Drs. Y. Okami and A. Takahashi, Institute of Microbial Chemistry for valuable comments for classification of the inhibitor-producing strain.

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