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Hideki Kobayashi · Yoshihiro Takaki · Kuniko Kobata
Hideto Takami · Akira Inoue

Characterization of α-maltotetraohydrolase produced by *Pseudomonas* sp. MS300 isolated from the deepest site of the Mariana Trench

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Abstract We have isolated a *Pseudomonas*-like amylase producer, the strain MS300, which displayed a large halo on starch medium, from the deepest site of the Mariana Trench. The strain MS300 produced two major and two minor α-maltotetraohydrolases (G4-amylase). The two major G4-amylases share the same molecular weight of 55000 but had different pI values, 5.0 and 4.7, respectively. The optimum temperature for activity of both major G4-amylases is 40°C, and the optimum pH is 6.8 for one and 8.9 for the other. MS300 produced more amylase under high hydrostatic pressure than under atmospheric pressure. Strain MS300 may be active in the deep sea at a depth of 10897 m.

Key words α-Maltotetraohydrolase · Mariana Trench · High hydrostatic pressure · Deep sea · Amylase

Introduction

There are various environments that support life on the earth. Some bacteria adapt to their surroundings and produce useful enzymes such as amylases, proteases and cellulases. Most microorganisms known at present were isolated from the continents, which occupy only 30% of the earth. We expect that the novel microorganisms will be isolated from the mud of the ocean, which occupies another 70% of the earth. The deep sea has an ecosystem different from that of the land, because there is no sunlight, a low temperature, and high hydrostatic pressure. Thus, we expect that the deep sea will be a resource of novel enzyme producers.

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K. Kobayashi (✉) · Y. Takaki · K. Kobata · H. Takami · A. Inoue
Japan Marine Science and Technology Center, Deep-Sea Microorganism Research Group, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan
Tel. +81-468-67-5556; Fax +81-468-66-6364
e-mail: hidekik@jamstec.go.jp

We have collected mud samples from the Challenger Deep (11°21.111′ N, 142°25.949′ E) region of the Mariana Trench at a depth of 10897 m by using the unmanned submersible *Kaiko*, on March 2, 1996. The Challenger Deep of Mariana Trench is the deepest point on the earth. We have isolated various non-extremophiles and extremophiles such as alkaliphiles, thermophiles, barophiles and psychrophiles from these mud samples (Kato et al. 1997a; Takami et al. 1997). What kinds of enzymes would be produced by these bacteria? Our interest has been focused on amylase. Several hydrolysis profiles of amylases are known to be obtained with starch as substrate, and this is one of the enzymes with uses in various industrial fields. In this study, we isolated *Pseudomonas* sp. MS300, the strain that produced the largest halo around the colony among the amylase producers. We characterized the amylases produced and examined the effect of hydrostatic pressure on amylase production by strain MS300.

Materials and methods

Bacterial strain and media

The isolation of the amylase producer was described previously (Takami et al. 1997). Among all amylase producers, strain MS300 formed the largest halo around the colony. Strain MS300 was grown at 25°C in a medium containing 10 g potato starch (Difco, Detroit, MI, USA), 10 g yeast extract (Difco), 10 g polypeptone (Wako, Osaka, Japan), and 10 g NaCl per liter (PYP medium) or Marine broth 2216 (Difco). For cultivation under high hydrostatic pressure, YP medium, which contains 10 g yeast extract (Difco), 10 g polypeptone (Wako), and 10 g of NaCl per liter or Marine broth 2216 (Difco) was++ used.

Amylase assay

Amylase activity was measured by the amyloclastic method (Thoma et al. 1971) or the 3,5-dinitrosalitiric acid reaction

(Sumner 1925). The amylase substrate was prepared as follows. Potato starch was solubilized in 2 N NaOH solution at 4°C for 12 h and then neutralized by addition of HCl. The amylase reaction was performed on 0.2% (wt/vol) potato starch solution containing 50 mM MOPS-NaOH (pH 7.0) as substrate for 20 min at 40°C, unless otherwise stated. One unit of enzyme was defined as the activity-forming reducing sugar corresponding to 1 μmol of glucose per 1 min in the 3,5-dinitrosalitiric acid reaction.

Protein content

Protein content was measured by the method of Lowry (Lowry et al. 1951) or the Bradford method (Bradford 1976), using bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on 15% polyacrylamide gel by the method described by Laemmli (Laemmli 1970). Proteins were dissolved in 16 mM Tris-HCl buffer (pH 6.8), 1% (wt/vol) SDS, 2.5% (vol/vol) β-mercaptoethanol, 20% (wt/vol) sucrose, and heat-treated at 100°C for 5 min. Protein in the gel was stained with Coomassie brilliant blue R-250. A zymogram was prepared by transferring the gel onto 1% agarose gel containing 0.1% soluble starch (Difco) and 50 mM MOPS-Na (pH 7.0). The gels were then incubated for 1 h at 30°C. Amylase activity was visualized by staining the agarose gel in a solution containing 0.2% (wt/vol) I_2–2% (wt/vol) KI (Kobayashi et al. 1992).

Isoelectric equilibrium electrophoresis of protein

The isoelectric equilibrium electrophoresis was performed on a polyacrylamide gel column containing ampholine pH 3.5–9.5 as described by Catsimpoolas (Catsimpoolas and Kenney 1972). Myoglobin basic band (pI 7.35), myoglobin acidic band (pI 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase B (pI 5.85), β-lactoglobulin A (pI 5.20), soybean tyrosine inhibitor (pI 4.55), and amyloglucosidase (pI 4.55) were used as the pI standard.

Thin-layer chromatography of the amylase product

After the enzymatic reaction with 0.2% potato starch solution, 1 μl of the reaction mixture was spotted onto a 0.2-mm silica gel 60 plate (Merck, Germany) and the plate was developed with 1-butanol : methanol : water (4 : 2 : 1). A solution containing a mixture of 0.1% glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose was used as the standard. The oligosaccharides were detected by spraying 1% diphenylamine dissolved in acetone containing 10% phosphoric acid on the TLC plate, then heating it at 160°C for 10 min.

Results

Isolation of amylase producers from the Mariana Trench

The collected sea mud was spread onto Marine broth plates containing 1% potato starch and incubated at 4°, 25°, or 55°C. The amylase producers were detected as those that showed the formation of a halo around the colony. We could isolate 39 amylase producers and, among these, the strain MS300 formed the largest halo. The strain MS300 was found to be an aerobic, gram-negative, motile, rod-shaped bacterium. Tests for oxidase and denitrification were positive. We identified the MS300 as a species of *Pseudomonas* on the basis of these data.

Purification of the amylase produced by *Pseudomonas* sp. MS300

MS300 was grown aerobically in the medium described in Materials and methods for 2 days at 25°C. SDS-PAGE of the culture supernatant showed only one prominent protein band, and its size was estimated as 55 kDa (data not shown). However, a zymogram prepared using the culture supernatant showed four bands of amylase activity of which two were major amylases (A, B) and the other two were minor amylases (C, D) (Fig. 1). The pI of the major amylases A and B indicated 5.0 and 4.7, respectively. Purification of the two major amylases (A, B) was performed as follows. After removing cells by centrifugation, ammonium sulfate was added to the culture supernatant at 40% of saturation and the mixture was held at 4°C for 16 h. After centrifugation (10 000 × *g*, 30 min, 4°C) of the mixture, the precipitation was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) at 4°C and applied to a DEAE-Toyopearl column (ID, 2.5 × 6 cm, 30 ml) equilibrated with 10 mM Tris-HCl (pH 7.5).

The amylases were eluted with a linear gradient of 0 to 0.1 M NaCl in the same buffer. The fractions containing amylase activity were collected. These fractions were applied to a DEAE-Toyopearl column (ID, 1.7 × 7.4 cm, 13 ml) equilibrated with 10 mM Tris-HCl (pH 8.5). The amylases were eluted with a stepwise gradient of NaCl concentration of 40, 80 and 120 mM. The fractions containing amylase activity were collected. The amylase-containing fraction was then applied to a 2 × Protein-Pak300 column (Millipore, MA, USA) and eluted with 25 mM MOPS-NaOH buffer (pH 7.0). The purity of each fraction was checked by means of a zymogram. We purified the two major amylases to such an extent that a single band was obtained by SDS-PAGE in each instance. We determined the N-terminal amino acid sequences of two major amylases, and both were found to be ADLAGKSPAGVRYHGGDEII. The N-terminal amino acid sequences of the two minor amylases were same as those of the major amylases.

The products of amylases from potato starch

Amylase A produced maltotetraose from potato starch as the main initial product (Fig. 2A). Amylase B also produced

A

Fraction	Volume (ml)	Protein (mg)	Activity (u)	Specific activity (u/mg)
Culture supernatant	1800	286	1.6×10^5	550
$(NH_4)_2SO_4$ precipitation	152	176	9.1×10^4	439
DEAE-Toyopearl I	132	110	9.1×10^4	824
DEAE-Toyopearl II	8	26	2.8×10^4	1074
HPLC (Protein-Pak300 × 2)				
F-1 (amylase A)	1.2	0.30	463	1544
F-2 (amylase B)	1.6	0.31	550	1775

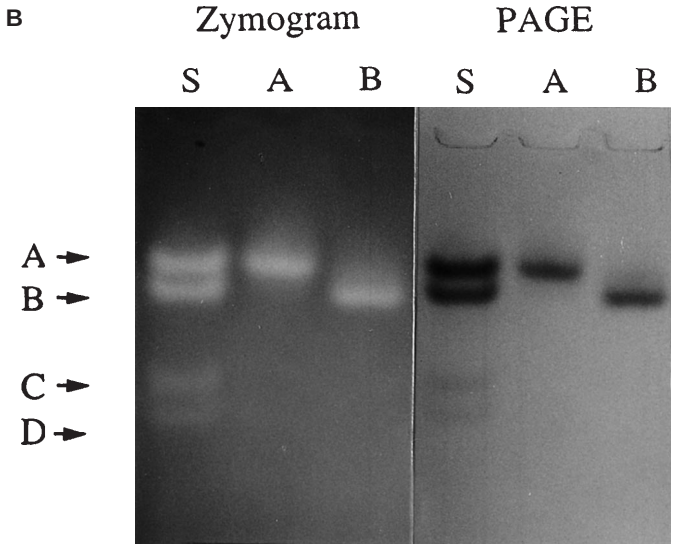

Fig. 1A,B. Purification of the amylases produced by MS300. **A** Steps employed in purification of the amylases. **B** Results of PAGE and the zymogram of each amylase fraction. Electrophoresis and staining were performed as described in Materials and methods. Lanes: *S*, culture supernatant; *A*, amylase A; *B*, amylase B. *Arrows* indicate bands of amylase activity from the culture supernatant

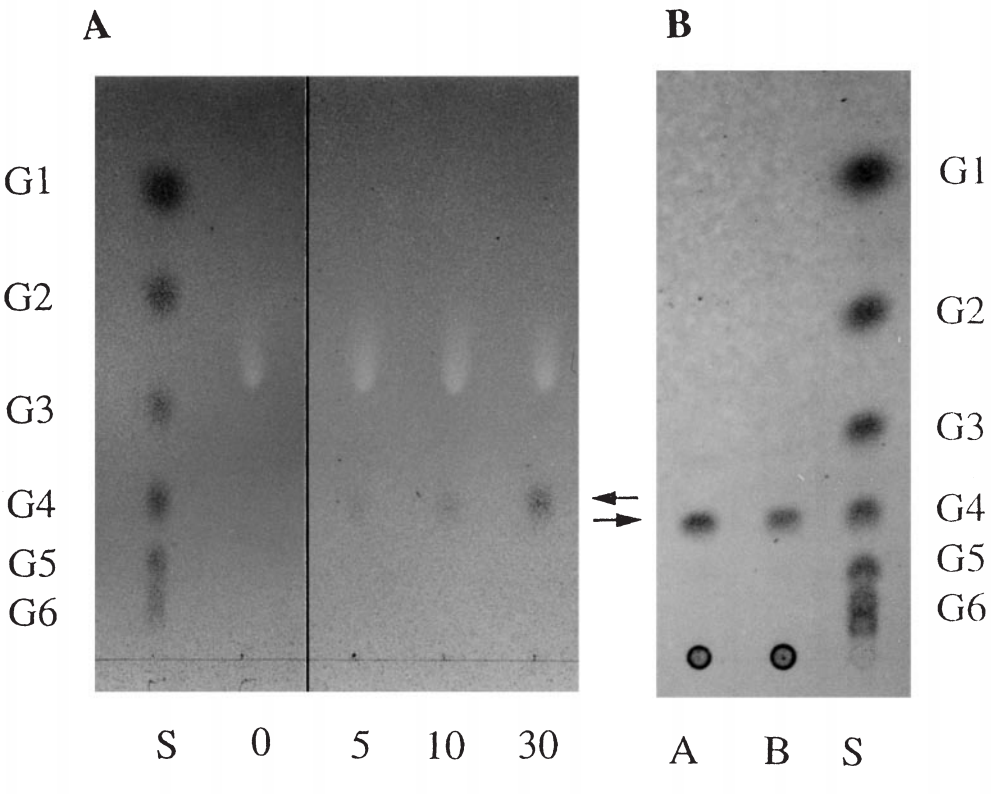

Fig. 2A,B. Thin-layer chromatography (TLC) analysis of amylase products generated from potato starch. **A** Products generated by amylase A from potato starch after various reaction times. The reaction mixture contained 50 mM MOPS-NaOH buffer (pH 7.0), 0.1% potato starch, and 10 U/ml amylase A. The reaction was performed at 40°C. Samples (1 µl) were removed after various periods of time and applied to TLC analysis. **B** Products generated by amylases A and B from potato starch. The reaction mixture was the same as described for **A** except that the potato starch concentration was changed from 0.1% to 1.0%. An *arrow* indicates the product of amylase A or amylase B. Lanes: *S*, standard oligosaccharides ranging from G1 (glucose) to G6 (maltohexaose); *0*, 0 min; *5*, 5 min, *10*, 10 min; *30*, 30 min

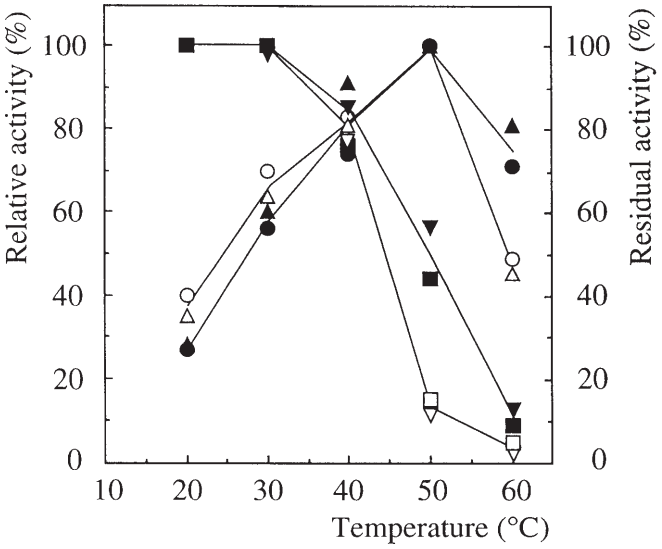

Fig. 3. Effects of temperature on the activity of amylases A and B. The relative activity of amylase A (*circles*) and amylase B (*triangles*) at each temperature in the presence or absence of 5 mM $CaCl_2$ was calculated with the activity observed at 50°C taken as 100%. The residual activity of amylase A (*inverted triangles*) and amylase B (*squares*) in the presence or absence of 5 mM $CaCl_2$ was measured after incubation for 30 min at each temperature. All *solid symbols* indicate the relative or residual activity of enzymes in the presence of 5 mM $CaCl_2$; *open symbols*, absence of $CaCl_2$

maltotetraose from potato starch (Fig. 2B). Both amylases showed no reaction with α-, β-, γ-cyclodextrin (CD) (data not shown). The maltotetraoses produced by these amylases were the α-anomer, as indicated by their mutarotation (data not shown).

Effects of temperature and pH on the activity and stability of the amylases

The optimum temperature for activity of amylase A and B was 50°C in each instance, and these enzymes were inactivated by 10-min incubation at 60°C (pH 7.0) (Fig. 3). Both the enzymes maintained their full activity after incubation for 30 min at temperatures below 40°C at pH 7.0 (Fig. 3). In the presence of 5 mM $CaCl_2$, the activity at 60°C was about 1.3 fold (amylase A) and about 1.7 fold (amylase B) higher than that in the absence of calcium ions. The optimum pH for activity at 40°C was pH 6.3 for amylase A and pH 7.8 for amylase B (Fig. 4A). Amylase A was stable over the pH range 7.0–8.0 and amylase B was stable over the pH range 7.0–9.0 on incubation at 40°C for 30 min (Fig. 4B).

Effect of metal ions on the activity

The purified enzymes were treated in the starch solution (0.1%) with 1 mM metal chloride. As shown in Table 1, both amylase A and B were inhibited by Zn^{2+}, Cu^{2+}, and Hg^{2+}, showing 1%–3%, 2%–4%, and 1%–3% of the activity of

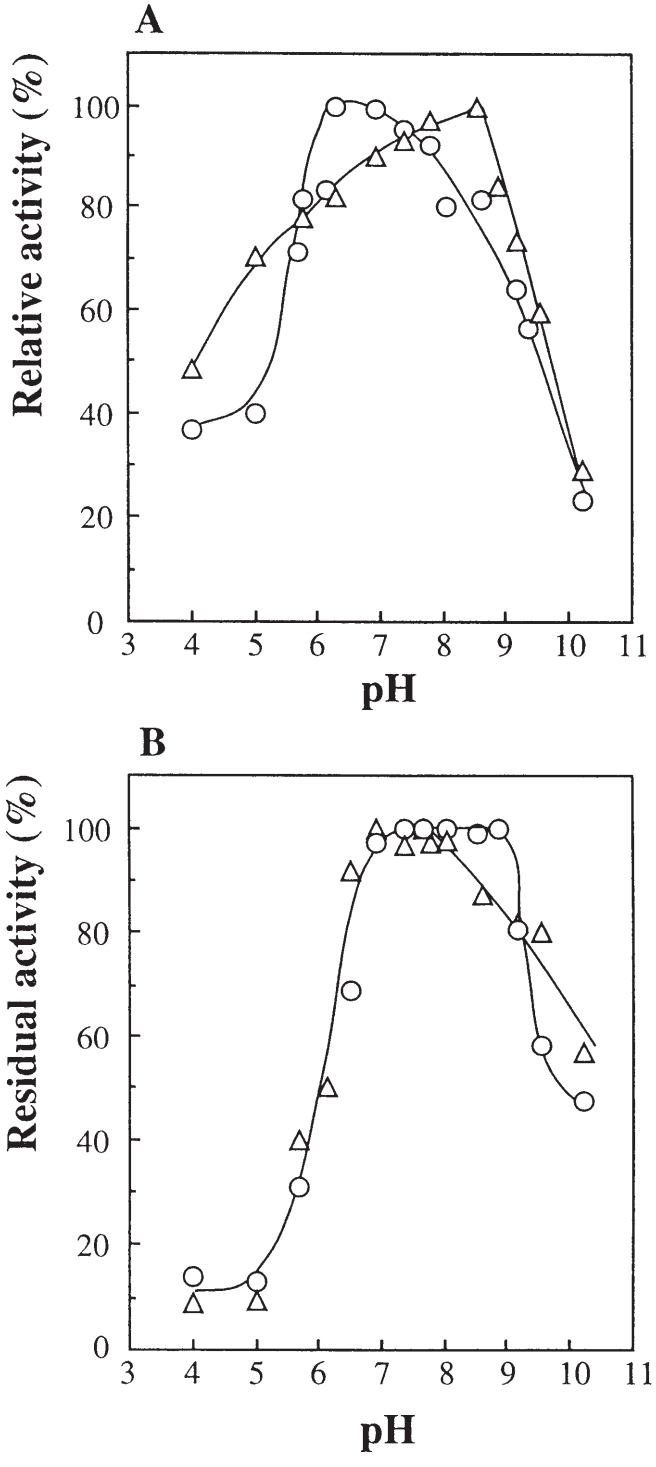

Fig. 4A,B. Effects of pH on the activity of amylases A and B. The relative activity (**A**) and residual activity (**B**) of amylase A (*circles*) and amylase B (*triangles*) are shown under various pH conditions. The relative activity was calculated with the maximum activity taken as 100%. The residual activity was measured after incubation under various pH conditions for 30 min. Reaction mixtures were buffered with 50 mM acetate/NaOH (pH 4.0–5.7), phosphate/NaOH (pH 5.7–6.3), MOPS/NaOH (pH 6.5–7.8), Tris/HCl (pH 7.8–8.6), CHES/NaOH (pH 8.7–9.5), or glycine/NaOH/NaCl buffer

Table 1. Effect of metal ions on amylase activity

Metal ions[a]	Relative activity (%)	
	Amylase A	Amylase B
Na^{+}	105	105
K^{+}	110	105
Ni^{2+}	41	38
Zn^{2+}	1	3
Co^{2+}	50	45
Fe^{2+}	90	85
Ca^{2+}	103	98
Cu^{2+}	2	0
Mn^{2+}	89	81
Hg^{2+}	1	3
Mg^{2+}	98	91
Pb^{2+}	19	24
Al^{3+}	83	82
Fe^{3+}	23	13
None	100	100

[a] All metal ions were added as the chloride salt at the concentration of 1 mM

the control, respectively. Upon addition of Mn^{2+}, Fe^{2+}, and Al^{3+}, each of the amylases was inhibited by 81%–90% as compared to the control. None of the metal ions tested in this study selectively inhibited only one of these enzymes.

Effect of hydrostatic pressure on the growth and amylase production of MS300

Strain MS300 was incubated at 4°C under various hydrostatic pressures for 2 weeks, and the number of colony-forming units (CFU) was found to increase at pressures less than 75 MPa, starting with an initial CFU of 4×10^5/ml, and amylase activity was detected in the culture supernatant at pressures below 100 MPa (Fig. 5).

Optimum growth occurred at 25 MPa, and the number of viable cells was about twice that observed at 0.1 MPa. The CFU at 100 MPa could not be accurately determined because of cell aggregation. The initial amylase activity was not detected because the cells were washed before inoculation of the medium. The amount of amylase activity in the supernatant decreased under high hydrostatic pressure. The amylase activity at 75 MPa was 73% of that at 0.1 MPa, although CFU at 75 MPa was 6% of that at 0.1 MPa. Amylase activity was also detected at 100 MPa in spite of cell aggregation.

Higher amylase productivity under high hydrostatic pressure

MS300 cells grown to the stationary phase in YP medium were harvested and washed with the same medium. Thereafter, the cells were suspended to fresh YP medium, and cell suspension was adjusted to an optical density of 2.0 at 660 nm (OD_{660}). The culture was incubated at 25°C under pressure conditions of 0, 30, and 60 MPa for 3 days. The

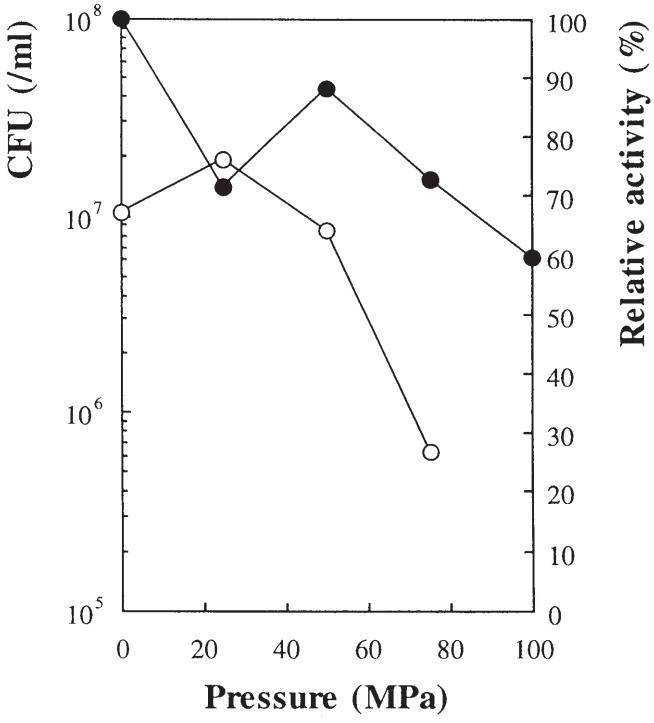

Fig. 5. Effects of pressure on growth and amylase production by strain MS300. The cells were incubated at 4°C under various pressure conditions for 2 weeks. The number of colony-forming units (CFU, *open circles*) was determined by counting the colonies that grew on Marine broth plates after incubation. Cell density before incubation was 4.0×10^4 CFU/ml. Amylase activity (*Solid circles*) was measured by the 2-dinitrosalitiric reaction described in Materials and methods. Relative activity was calculated with maximum activity (0.1 MPa) taken as 100%

Table 2. Effect of hydrostatic pressure on amylase production

Pressure (MPa)	Activity (U/ml)	OD_{660}[a]
0.1	3.6	2.0
30	4.5	2.1
60	4.8	1.9

[a] OD_{660} of the culture after incubation

amount of amylase produced by MS300 was 3.7 U/ml in 50 ml of the culture under 0.1 MPa. Much more amylase was produced under high pressure than under atmospheric pressure (Table 2). At 30 MPa and at 60 MPa, MS300 produced about 1.3 and 1.4 times as much amylase as that observed under 0.1 MPa. The OD_{660} of these cultures did not change in the course of incubation.

Discussion

Takami et al. first isolated various microorganisms from mud samples obtained from the Challenger Deep (Takami et al. 1997). Some of these were amylase producers. In this study, we purified and characterized the two major amy-

lases produced by strain MS300, which made the largest halo on the starch-containing medium among all of the Mariana isolates. MS300 was identified as a member of the genus *Pseudomonas*. This strain was found to produce two major amylases (amylase A and B) and two minor amylases (Fig. 1). Both major amylases generated maltotetraose from starch (Fig. 2) and did not react with α-, β-, or γ-CD. The maltotetraose produced by these amylases from potato starch was the α-anomer (data not shown). Therefore, both amylase A and B were identified as α-maltotetraohydrase (G4-amylase).

G4-amylases from *Pseudomonas stutzeri* strains have been reported (Shmidt and John 1979). Robyt and Ackerman reported that *P. stutzeri* produced seven G4-amylases that differed in molecular mass and isoelectric point (Robyt and Ackerman 1971). Sakano et al. reported two G4-amylases that had the same molecular mass but different isoelectric points (Sakano et al. 1983). Those G4-amylase isozymes had almost the same properties. Strain MS300 was found to produce four G4-amylases. The major amylases, A and B, had greater resistance to inhibition by metals than any other maltotetraohydrase from *Pseudomonas* (see Table 1). The optimum pH for activity of amylase A and B differed. Furthermore, the pI, optimum temperature, and pH for activity of amylase A and B were different from those of the G4-amylases from *P. stutzeri* (see Figs. 3, 4AB). Thus, our findings indicate that amylase A and B are novel types of G4-amylases. The nucleotide sequence of the G4-amylase (*amy* P) from *P. stutzeri* MO-19 has been reported (Fujita et al. 1989). The first 20 amino acids of the N-terminal sequence of amylase A and B showed 80% similarity with that of G4-amylase from *P. stutzeri* MO-19. However, other properties of this G4-amylase have not been reported.

The microbial diversity in the Mariana Trench has been reported as described, but activity of the microbes there is still unknown. Mariana isolate MS300 could grow and produce amylase at pressure below 75 MPa in Marine broth (see Fig. 5). Furthermore, this strain produced much more amylase under high-pressure conditions (see Table 2). Although MS300 cells aggregated at pressure below 100 MPa, amylase activity was detected in the culture supernatant, and the turbidity of the culture started increasing when the culture was decompressed to 0.1 MPa (data not shown). MS300 was active at a very low level at pressures below 100 MPa.

Marine broth provides growth conditions far different from the in situ conditions of the Mariana Trench. Our findings suggest that MS300 may be active at the sea bottom of the Mariana Trench. Although we have not fully established ideal methods for isolation and cultivation of deep-sea microorganisms, the increase of CFU or turbidity has been monitored as an indicator of bacterial activity (Kato and Bartlett 1997). When the microorganisms grow at a very low rate, it is difficult to apply such an indicator for estimation of bacterial growth. In the case of enzyme producers, not only the CFU but also the amount of enzyme activity present in the culture supernatant can be applied as an indicator of bacterial activity. Thus, the enzyme producer is considered to be one of the good sample organisms for studying the deep-sea ecosystem.

Strain MS300 could grow and produce amylase under 75 MPa (Fig. 5). This strain produced much larger amounts of amylase under high hydrostatic pressure than at atmospheric pressure (Table 2). The amylase activity in culture at 75 MPa was 73% of that at atmospheric pressure in spite of lower cell density (Fig. 5). The mechanism responsible for this increase in amylase production at high pressure remains unknown. There are some reports that some deep-sea bacteria have a pressure-regulated operons (Kato et al. 1997b). High hydrostatic pressure influences phospholipid membrane fluidity (DeLong and Yayanos 1985) and caused a change in phospholipid composition of the membranes (Kaneshiro and Clark 1995). These changes may promote the secretion of enzymes.

The deep-sea environment is thought to support a very low level of biological activity, and the variety of microorganisms is thought to be very limited. However, we have isolated a novel type G4-amylase producer from this extreme environment. We are convinced that the deep sea is a valuable source of new microbial resources. Also, we can expect the deep-sea environment will provide many novel microbial biocatalysts.

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