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Study on the proteases of marine bacteria

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Summary

Of 34 strains of marine isolates the greater part of them could produce the extracellular protease and half of them had amylase activity. All of them divided into two groups by means of calcium necessity on protease production. The relationship between Ca^{2+} ion and protease activity was studied by using two representative strains I–6 and C–18 for the reason of their high calcium sensitivity among test organisms.

The results were as follows: In I–6 strain, the inducible effect on protease activity was observed whenever and immediately after calcium ion supplement. To the contrary, in C–18 strain the effect of Ca^{2+} addition was only observed in logarithmic phase. The protease production of I–6 strain was, if anything, dependent on non-dialysable fractions of polypeptone or yeast extract in medium and that of C–18 strain severely on dialysed fraction of yeast extract.

Protease of C–18 strain was heat-labile and had optimal activity at 30 C and at pH 10, while that of I–6 strain was relatively stable below 60 C and having optimal temperature and pH at 50 C and at 10.5, respectively. Metal ions such as Hg^{2+}, $KMnO_4$ and DFP, specific inhibitor for serine enzymes, strongly inhibited the activities of both enzymes. The metal chelator, EDTA inhibited strongly the enzyme of C–18 strain but partly that of I–6 strain.

There are many publications on the extracellular enzymes of microorganisms. In Japan the studies of proteases in mold and bacillus sp. have been carrying on abundantly in many food industries.

It is assumed that the decomposition of macromolecule is proceeding gradually in ocean environment by marine bacteria. Marine bacteria live not only in the sea water, but also on the surface of macromolecules which serve nutrient for bacteria. But bacteria are incapable of engulfing macromolecular substances directly. In such environment bacteria may excrete hydrolytic enzymes from their cells, probably for the decomposition of complex food compounds.

Kato et al[1] studied on the proteolytic enzyme of psychrophilic marine pseudomonads, and concluded that mineral salts in sea water level were necessary for protease production. Tojo et al[2] added much contribution on the data, and they proposed that some amino acids were stimulative, while the others were inhibitive on protease production. It is well known that calcium ion may maintain the stability for protease activity, but whether it promotes the enzyme production or not is uncertained.

This paper describes effective agents, and characteristics of proteases of marine bacteria.

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Materials and methods

Bacteria. The production of extracellular protease was determined on 34 isolates; 5 strains isolated from the Kinko Bay in 1970 by Nagatomi and Kakimoto[3], 25 strains for far east 10 miles from Sata peninsila in 1974 by Kakimoto et al[4], 2 strains YEG–1 and HS–6 from the Kinko Bay in 1974 by Sakata and Nakaji, 2 strains 1055–1 and *Vibrio parahaemolyticus* STO–5 from Hokkaido University, *Bacillus subtilis* ATCC 6051 and *Vibrio metchinikovii* were used. The last two terrestrial bacteria were used to compare them with the marine isolates. The main examinations for enzyme characteristics were carried out by using both strains I–6 and C–18.

Culture media. In order to ascertain the relation between cultural media and protease production, various media were prepared. The kind and its compositions were as follows: 1) ZoBell 2216E modified medium; polypeptone 5 g, yeast extract 1 g, and artificial sea water of Herbst formula 1000 ml. 2) Medium A; 0.5% polypeptone. 0.1% yeast extract, 3% NaCl, and 50 mM $MgCl_26H_2O$. 3) Medium B; 0.5% polypeptone, 0.1% yeast extract, 3% NaCl, 50 mM $MgCl_26H_2O$ and 10 mM $CaCl_22H_2O$. 4) Medium C; 0.5% polypeptone, 0.1% yeast extract, 3% NaCl and 20 mM $CaCl_22H_2O$. pH of all media were adjusted to 7.6. For terrestrial bacteria, the concentrations of NaCl and $CaCl_22H_2O$ in the medium were 0.5% and 0.5 or 2 mM, respectively.

Determination of protease and amylase producibilities. All of the strains mentioned above were used for this experiment. The methods depended mainly upon the text book "Laboratory methods in Microbiology"[5]. The media containing casein or starch were made by modification of ZoBell 2216E medium. The assessment of protease was carried out by accounting the size of clear zone which might be observable after covering $HgCl_2$ solution on the agar plate after incubated at 25 C. The amylase activity was also qualitatively determined by the same procedure as above on the medium containing 0.5% soluble starch. The assessment of amylase depended upon the covering Lugol solution.

Assay of protease activity. The substrate was made by dissolving 1% of casein (Hammerstein milk casein) in either 0.05 M Tris-HCl buffer, pH 7.6, or 0.05 M Na-carbonate buffer, pH 10.0. 2 ml of substrate solution and 0.5 ml of enzyme preparation were mixed and incubated at 30 C for 2 hr or 30 min in the case of cultural filtrate or crude enzyme preparation, respectively. After incubated, 2.5 ml of 0.4 M TCA solution was added into the reaction mixture to stop the enzyme reaction. After 30 min the precipitate was removed by passing through the filter paper Toyo No. 5C. The absorbance at 280 nm of the filtrate was measured by spectrophotometer.

Relation between protease production and supplement of Ca^{2+} ion. Prior to the examination, the bacteria were incubated in Medium A at 25 C for indicated period by shaking, then $CaCl_2$ was added into the culture until reached 10 mM finally with or without chloramphenicol (10 μg/ml). Immediately after addition of

Ca^{2+}, the activity measurement proceeded at one hour interval for 6 hr. In the case of C–18 strain, supplement of Ca^{2+} was done in the middle logarithmic phase, O. D. at 540 nm being identical to 0.45. The control test was done by comparing with the same culture without Ca^{2+} ion.

Partial purification of the enzyme. The bacterial culture grown in ZoBell modified medium, 200 ml for 24 hr was centrifuged at 7,500 rpm for 35 min. To the supernatant, was added sodium ammonium sulfate to 57% saturation. After standing at 4 C for 5 hr, the precipitate was collected by centrifugation, dissolved in 0.05 M Tris-HCl buffer (pH 7.6), and the solution was submitted to dialysis against the same buffer. The dialyzed solution was applied to the column of Sephadex G–75 (2.6×85.0 cm), previously washed with 0.05 M Tris-HCl buffer plus 0.1 M NaCl. The enzyme protein was eluted with same buffer solution.

pH optimum and pH stability. The pH optimum and pH stability for protease activity were determined by using various different buffer solutions. The buffer solutions were as follows: 0.1 M $Na_2HPO_412H_2O$–0.1 M KH_2PO_4 (pH 6.0–7.0); 0.1 M Tris-HCl (7.0–9.0); 0.1 M $NaHCO_3$–0.1 M Na_2CO_3 (9.0–11.0); 0.1 M glycine-NaOH (11.0–13.0); 0.05 N NaOH (13.8). The reaction was done at 30 C for 30 min. The pH stability of enzyme was measured after kept in various pH for 17 hr at 4 C.

Heat stability. The crude enzyme preparation was made by exposing different temperatures for 10 min and then the residual activity was determined.

Effect of chemicals on the activities of crude proteases. 0.5 ml of crude protease solution and 0.5 ml of 2×10^{-3} M chemicals solution were incubated for one hour at 30 C, then the residual activity was measured.

Results

The protease production of marine isolates

The hydrolytic activities of various bacteria on both starch and casein were shown in Table 1. As shown in the table, caseinolytic response was observable in almost all of marine isolates, while amylolytic response was only half in the same isolates. Protease activity and growth behavior during incubation were shown in Fig. 1. The activity increase of marine isolates demonstrated two types, and one was quick increase at logarithmic phase, another being slow increase irrelative to growth phase. Some strains such as in C–16, C–26 and C–32 demonstrated strong activity on the agar plate, in contrast the activity was not so strong in broth. The difference obtained in both media may be due to casein content during incubation, that is, in agar medium the casein is previously included at beginning of incubation, while in broth is not.

Protease production affected by various media is shown in Fig. 2. In some strains Na^+ and Mg^{2+} were not effective while Ca^{2+} was effective on protease production. The strains I–6, C–12, C–18 and C–26 were markedly affected by Ca^{2+}, while C–16

Table 1. Determination of protease and amylase activities of marine isolates.

Strain	Caseinolytic activity 24 hr	Caseinolytic activity 48 hr	Amylolytic activity 24 hr	Amylolytic activity 48 hr
1055–1	+	++	−	−
I–4	+	++	+−	+++
I–6	+	+++	+	+++
II–4	+	++	−	+
II–5	−	−	−	−
II–8	+	+	−	−
YEG–1	+	++	++	++
HS–6	++	++	++	+++
V. parahemolyticus	+	?	+++	+++
C–2	++	+++	++	++
C–4	++	+++	−	±
C–6	++	++	++	+++
C–7	+	++	−	−
C–8	++	+++	−	−
C–9	+++	+++	++	++
C–11	+	++	−	−
C–12	++	+++	++	++
C–13	−	−	−	−
C–15	+	++	−	−
C–16	++	+++	−	−
C–17	−	+	+++	+++
C–18	++	+++	−	−
C–19	++	+++	−	−
C–20	+	++	++	+++
C–21	+	+	−	−
C–22	++	+++	−	±
C–25	++	+++	++	+++
C–26	++	+++	+	+++
C–27	++	+++	−	±
C–29	+	+	++	+++
C–30	+	++	−	−
C–32	++	+++	−	−
C–33	+	++	−	−

Size of clear zone;			Size of clear zone;	
	0 mm	−	0 mm	−
	0–1 mm	±	0–1	±
	1–4	+	1–3	+
	4–7	++	3–5	++
	7–	+++	5–	+++

was not. Among two terrestrial bacteria, *V. metchinikovii* was not affected, while *Bacillus subtilis* was similar as observed in C–18. In order to get more details on the effect of Ca ion on protease production, representative strains I–6 and C–18 were selected because of their high protease activity.

The characteristics of both strains were shown in Table 2. I–6 was identified for *Pseudomonas* sp., C–18 being *Vibrio* sp. according to the methods of Shewan[6] and Shimidu[7]: Both isolates were halophilic moderately. The latter required much more bivalent cations than the former for their growth.

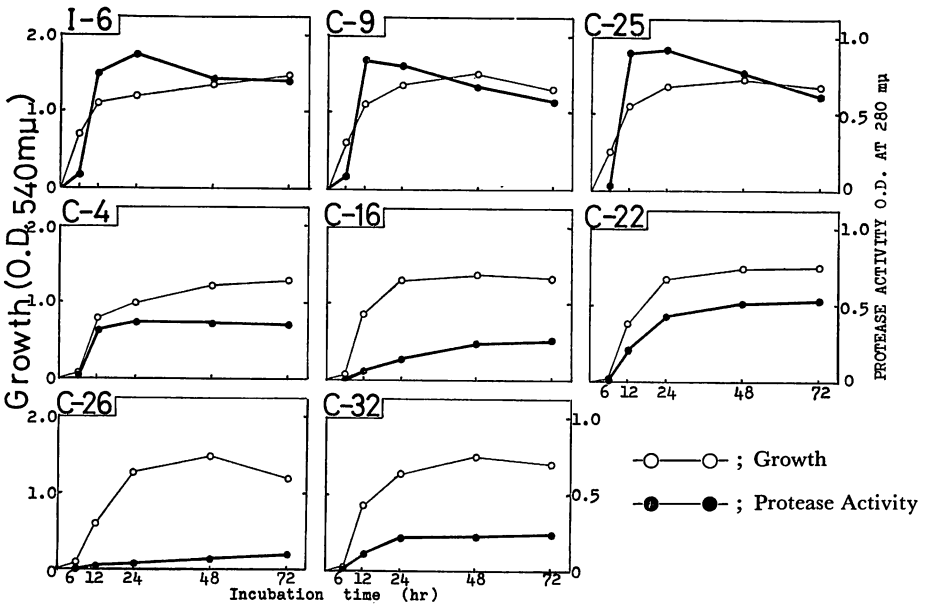

Fig. 1. Time course of protease production of marine isolates in Zobell modified medium.

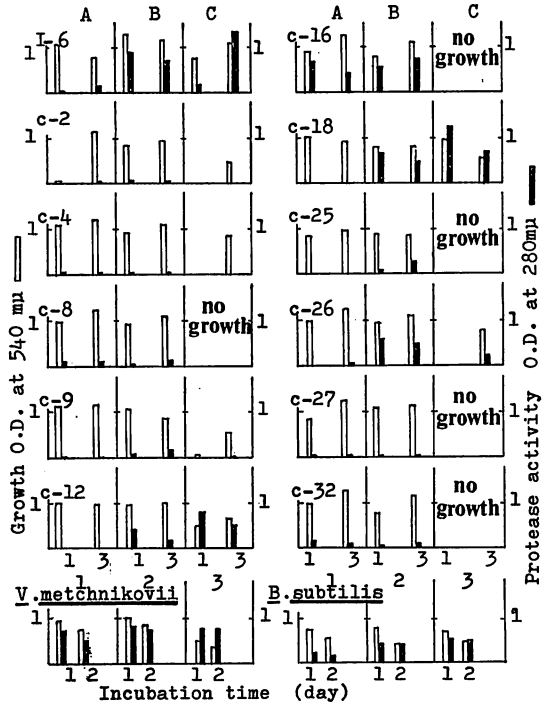

Fig. 2. Effect fo Ca^{2+} ion on protease production.

Table 2. Bacteriological characteristics of I–6 and C–18 strains.

Character	I–6	C–18
Cell form	Rod	Rod
Gram's stain	Negative	Negative
Motility	+	+
Flagellation	Monotrichous	Monotrichous
Endospore	−	−
Casein hydrolysis	+	+
Gelatin hydrolysis	+	+
Starch hydrolysis	+	−
Hugh & Leifson test	Oxidative	Fermentative
Oxidase test	+	+
Catalase test	+	±
Pigmentation	Yellow	−
0/129 sensitivity	−	±
Typing for mineral requirement	H type	H type
Optimal of NaCl for growth	5.5%	3.5%

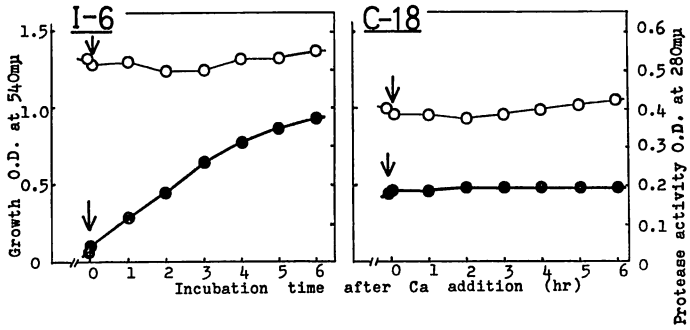

Fig. 3. Effect of Ca^{2+} ion addition on protease production
The arrow indicates the time of Ca^{2+} addition.
-○——○- ; Growth, -●——●- ; Activity

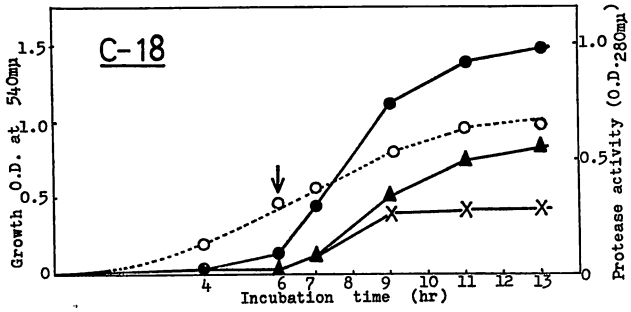

Fig. 4. Effect of Ca^{2+} ion addition on protease production
··○·····○·· ; Growth, Activity -×——×- ; No addition
-●——●- ; at 0 time
-▲——▲- ; at 6 hrs.

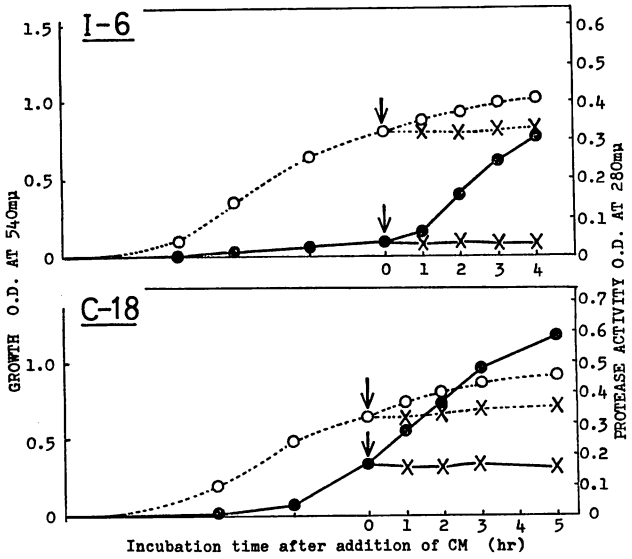

Fig. 5. Effect of chloramphenicol on protease production in ZoBell modified medium.
Growth: ··○·····○·· ; control, ··×·····×·· ; after addition of CM
Activity: -●——●- ; control, -×——×- ; after addition of CM.

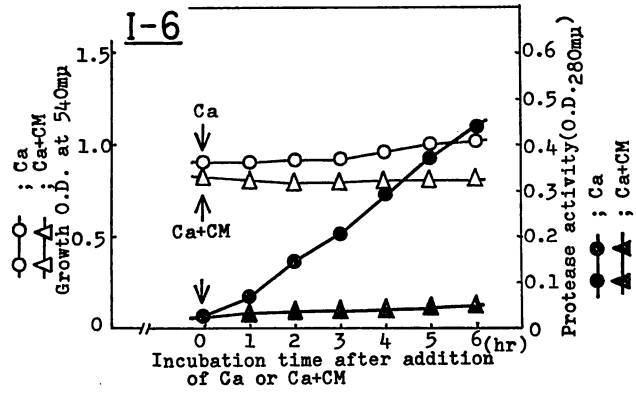

Fig. 6. Effect of Ca^{2+} addition on protease production under the inhibitive condition of protein synthesis with chloramphenicol (CM).

The activity in I–6 remarkably increased after supplementing Ca^{2-} ion during the incubation, on the other hand in C–18 strain did not as shown in Fig. 3. However, the activity of C–18 strain increased after Ca^{2+} ion supplement during the mid way of log phase (Fig. 4). The effect of calcium supplement together with chloramphenicol, the inhibitor for protein synthesis, was also observed. Immediately after the addition of chloramphenicol, the enzyme production stopped as shown in Figs. 5 and 6. These results seemed to exclude the assumptions that Ca^{2+} promotes the excretion of protease accumulated in the cells or activates the inactive precursor pro-

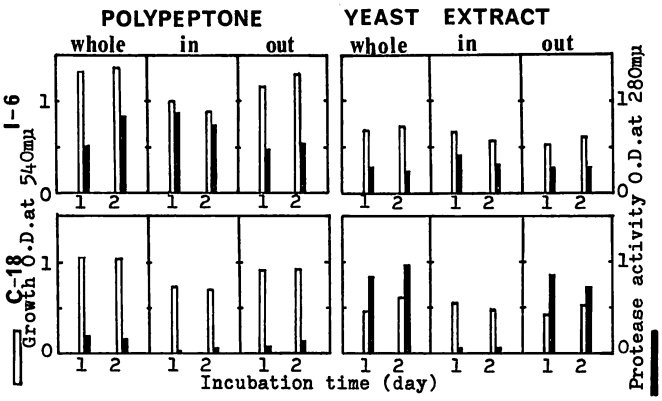

Fig. 7. Effect of dialyzed or nondialyzed components of yeast extract or polypeptone on protease production.

teins.

The effect of organic components in medium is examined in Fig. 7. The protease production of I–6 strain was, if anything, dependent on non-dialysable fractions of polypeptone or yeast extract in medium and that of C–18 strain severely on dialysed fraction of yeast extract. Among amino acids, arginine and aspartic acid were slightly stimulative while cysteine and histidine were inhibitive for the protease production of both isolates.

Some properties of proteases from marine isolates

Figs. 8 and 9 show the elution profiles of enzyme proteins on gel filtration by Sephadex G–75 column. On this condition, an active peak for protease activity was observed in both cases.

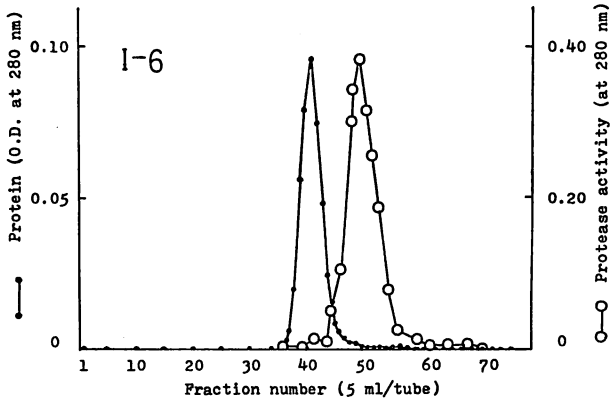

Fig. 8. Gel Filtration by Sephadex G–75 Column (3.0×75.0 cm)

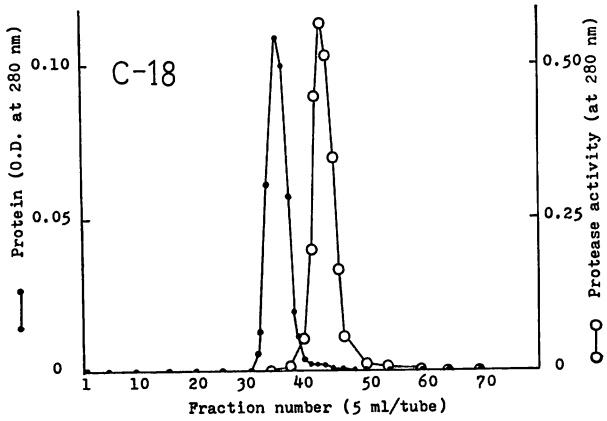

Fig. 9. Gel Filtration by Sephadex G–75 Column (2.6×85.0cm).

The optimal temperature for enzyme activity was about 50 C for the protease of I–6 strain and about 30 C for C–18 strain (Fig. 10). The enzyme of C–18 strain began to lose its activity obviously at 30 C or higher without the addition of Ca^{2+} and inactivated at 50 C completely. On the other hand, the enzyme of I–6 strain began to lose at 65 C and at 80 C completely (Fig. 11).

pH optima of crude proteases were pH 10.5 and 10.0 in the cases of I–6 and C–18, respectively as shown in Fig. 12. Stability on pH was shown in Fig. 13. The protease of I–6 was stable at alkaline pH below 11, while that of C–18 was stable at

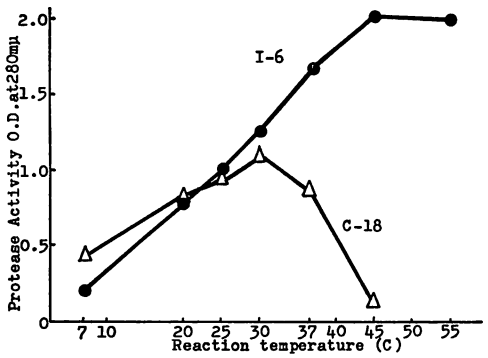

Fig. 10. Temperature-activity curve of the proteases of crude enzyme preparations.

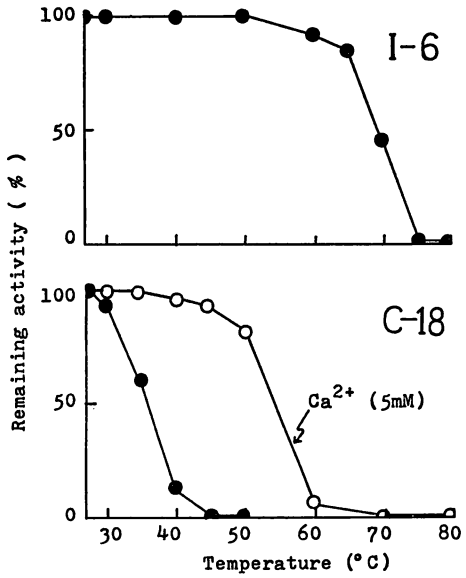

Fig. 11. Heat Stability of Protease.

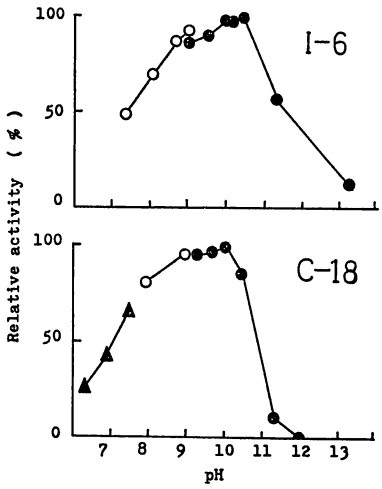

Fig. 12. Effect of pH on Protease Activity.

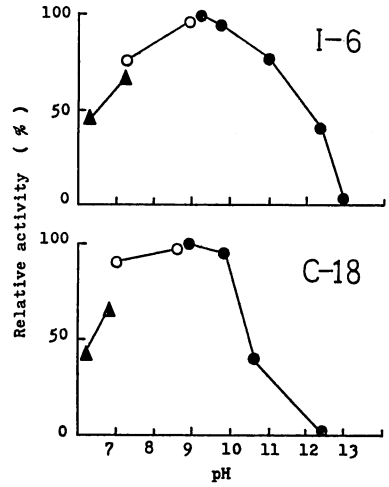

Fig. 13. Effect of pH on the Stability of Protease.

neutral.

Effect of metallic ions is shown in Table 3. The both bacterial proteases were markedly inhibited by Cu^{2+}, Zn^{2+} and Ag^{+} as well as Hg^{2+} ion. Effects of chemicals on the both proteases are shown in Table 4. According to this table, both proteases were also inhibited not only by oxidation agents $KMnO_4$ and K_2CrO_7, but

Table 3. Effects of Metal Ions on Protease Activities.

Metal ion Conc. (mM)		Remaining activity (%) I-6 Dialys.	I-6 Gel-filt.	C-18 Dialys.	C-18 Gel-filt.
Control (D. W.)		100	100	100	100
NaCl	1	105		105	
	10	90		91	
	100	74		74	
KCl	1	104		99	
LiCl	1	103		101	
$AgNO_3$	1	84		79	
$MgCl_2$	1	104		105	
$MnCl_2$	1	90		99	
$ZnCl_2$	1	64	55	68	4
$CuSO_4$	1	52	80	77	31
$HgCl_2$	1	40	47	35	38
$CaCl_2$	1	107	96	126	173
	5	95	84		222
EDTA	1	86	92	5	8
	5	85	88	4	8
	10	83	86	3	10

Table 4. Effects of Chemicals on Protease Activities.

Chemicals	Conc. (mM)	Remaining activity (%) I–6	C–18
Control (C. W.)		100	100
Ascorbate	1	101	101
KCN	1	102	96
Na_2SO_3	1	104	97
$NaHSO_3$	1	102	94
Cysteine	1	102	95
DTT	1	94	107
Hydroquinone	1	84	78
2-Mercaptoethanol	1	98	93
$K_2Cr_2O_7$	1	70	80
$KMnO_4$	1	20	9
SLS	1	95	96
	7	72	84
Monoiodoacetate	1	99	94
pCMB	1	100	100
Citrate	1	96	95
EDTA	1	86	5
DFP	1	19	5

Table 5. Reactivation of the EDTA-treated Eznyme Preparations by Ca^{2+} Ion.

Preincubation	$CaCl_2$ conc. (mM)	Relative activity (%) I–6	C–18
D. W.	0	100	100
	0.2	102	118
	1	98	119
	5	94	108
EDTA (1 mM)	0	82	34
	0.2	83	88
	1	78	85
	5	70	76

also by DFP which was specific for serine enzyme, whereas some reducing agents or SH reagents did not give the inhibitive effect on both proteases. 10 mM of EDTA by which 15–20% of protease activity in I–6 strain was inactivated gave protease of C–18 strain the complete inhibition. The reversal effect of Ca^{2+} ion on the protease activity which was inhibited by EDTA is shown in Table 5. The difference between both strains was observed in the sensitivity to EDTA, namely the C–18 strain was more sensitive than I–6 strain, and reversal effect of Ca^{2+} ion was also observable in the C–18 strain.

Discussion

There are many studies of bacterial extracellular protease with regards to an enzymological view, but there is little information on the relation between the protease production and the environment they live. The present work demonstrated the protease of marine bacteria selected in the experiment was produced strongly by supplement of Ca^{2+} ion in the culture medium. In the culture supplemeted with chloramphenicol, the protease production stimulated by Ca^{2+} was never detected. Assuming the result, it was supposed that Ca^{2+} ion may have some important role on the protease production in the bacteria. Both strains do not have a distinct precursor pool of protease in their inner cells. However, whether Ca^{2+} ion contributes to synthesis or secretion of protease protein is still unknown. The behavior of C–18 strain on Ca^{2+} requirement regarding protease production was different from I–6 strain. The period of protease production was restricted within log phase and the detection of it failed by means of calcium supplement in stationary phase. The protease of C–18 also required Ca^{2+} for maintenance of its stability unlike that of I–6. However, the possibility that Ca^{2+} affects synthesis or secretion of enzyme in the case of C–18 strain can not also be excluded.

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