海水中の懸濁態プロテアーゼ活性の鉛直分布および季節変動

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Vertical Distribution and Seasonal Fluctuation of Particulate Protease (PPRase) Activity in Seawater

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The seasonal and spatial distribution of particulate protease (PPRase) activity in seawater from near to the shore to offshore areas around Japan were investigated.

The activities in Aburatsubo Inlet and Tokyo Bay were from one to two orders of magnitude higher than other offshore areas, suggesting that the active biological production causes high PPRase activity in the areas. The highest activity was observed in the upper layer of all the areas (Tokyo Bay, Sagami Bay, Off-Tateyama, and Oyashio regions) and decreased with depth.

In Aburatsubo Inlet, PPRase activity increased subsequently with the standing stock of primary producers from March to August and declined in the other months.

The population of protease-producing bacteria ranged from 10^4 to 10^7 per *l*, being approximately the same order of magnitude as that of the heterotrophic bacteria. Small populations of protease-producers were observed in Sagami Bay and Off-Tateyama, whereas considerably large populations of protease-producers were found in Aburatsubo Inlet and Tokyo Bay.

It is well-known that natural seawater contains various organic matter composed of protein, carbohydrates, nucleic acids, lipids, and so on. The amount of these substances increases with the primary producers *in situ*, whereas these material degrades through the action of enzymes excreted by microorganisms. HARVEY[1] was one of the first scientists who suspected the presence of naturally occurring enzymes in seawater. He observed that seawater samples had the ability to reduce H_2O_2, and heating such seawater to 100°C or treating it with an enzyme inhibitor repressed its ability to reduce H_2O_2. KREPS[2] found that organic catalysts diffusing out of dead microorganisms had the ability to reduce nitrate during storage of seawater. Recently, many papers concerning the enzymes in natural water such as amylase,[3] deoxyribonuclease,[4,5] and alkaline phosphatase[6-8] were reported. The quantity and ecological significance of these enzymes have been elucidated. However in the marine environment, there have not been enough studies on protease activity.[9] Estimation of the enzyme activity is necessary to clarify biological processes and biochemical cycles in the sea. During the ecological studies on heterotrophic activities of marine microbes, the present authors found that both particulate protease (PPRase) activity bound to particulate matter and protease-producing microbes were widely distributed in seawater. The purpose of the present work is to assess the method used to measure PPRase activity of seawater, the distribution of the activity in seawater samples from various areas, and some environmental factors which influence the activity in seawater.

Materials and Methods

Seawater Samples

Seawater samples were collected from Tokyo Bay, Sagami Bay, and Off-Tateyama during the KT-83-1 (February 1983) cruise and from Oyashio regions during the KT-83-10 (July 1983) cruise of the research vessel "Tansei-maru" of the Ocean Research Institute, University of Tokyo. Seawater sampling was also carried out in Aburatsubo Inlet from October 1982 to September 1983. Sampling stations are shown in Fig. 1. Surface water samples were collected in sterile glass bottles; for deeper-water samples, a Niskin bacteriological sampler was used (General Oceanics). These samples were used for microbiological analysis. Seawater samples for PPRase activity and other chemical analysis were obtained with a Van Dorn water sampler. For the measurement of PPRase activity and other environmental factors, the samples were immediately filtered through Whatman GF/F glass fibre filters that had been

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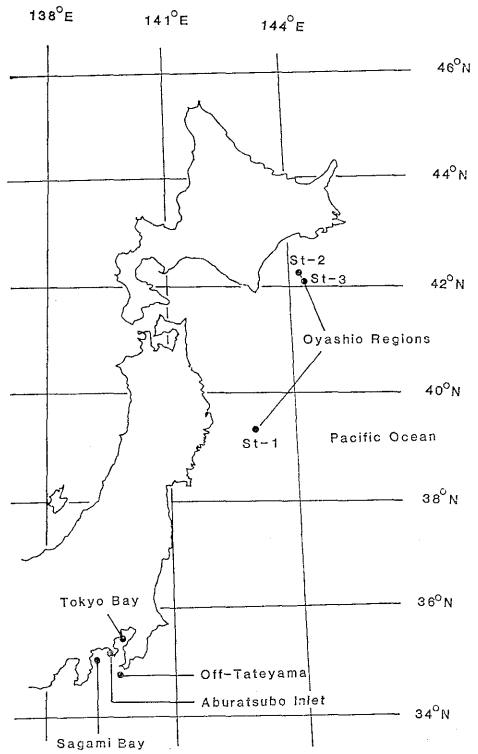

Fig. 1. Sampling stations.

precombusted at 450°C to remove traces of organic matter. These filters were stored at −20°C until analyzed. The filtrate was discarded because the activity in the filtrate was below detection level.

Counting of Viable Bacteria

For the enumeration of heterotrophic bacteria, the spread plate method[10] and filter method using Nuclepore filter (pore size 0.2 μm) were employed. Seawater collected from Aburatsubo Inlet and Tokyo Bay were serially diluted and the 0.1 m*l* portions were spread on PPES-II agar plates without the addition of ferric phosphate and marine mud extract.[11] This modified medium is referred to as "MP" medium. The seawater samples from offshore areas were concentrated on Nuclepore filters. The filters which retained microbes were placed on the medium, and they were incubated for 2 weeks at 20°C. After incubation, the bacterial colonies that appeared on the plates were counted, and the numbers were expressed in colony forming units (cfu). The colonies were isolated randomly from the plates and purified before being stored in 1/5-strength MP stabs.

Proteolytic Activity of Isolated Bacteria

The hydrolysis of casein by isolated bacteria was examined by stab inoculation on plates of MP medium containing 1% casein. Judgement of the proteolytic activity was based on the clear zone formed around the colony on the casein agar plates after incubation for 5 days at 20°C.

Measurement of PPRase Activity

PPRase activity of seawater was measured by modified ANSON's method.[12] The filter was homogenized in 10 m*l* of 50 mM Tris-HCl buffer (pH 8.0). To the homogenate, 5 m*l* of 1% Harmmarsten casein (Wako Chemical Co. Osaka) in the same buffer and 0.5 m*l* of chloroform were added. These assay samples were incubated at 25°C for 1 or 2 days. After incubation, 1.5 m*l* of 40% trichloroacetic acid was added, and the mixture was kept for 20 min at 25°C followed by filtration through a paper filter (Toyo Roshi type No. 1). To 1 m*l* of the filtrate were added 5 m*l* of 1% Na_2CO_3 in 1 N NaOH and then, 1 m*l* of phenol reagent (Wako Chemical Co.). The mixture was kept at 25°C for 20 min and the optical density was measured at 660 nm. Autoclaved samples (121°C, 15 min) were used for reagent controls. Proteolytic activity was expressed in the amount of L-tyrosine liberated from particulate matter in 1 *l* of seawater per day under these conditions.

Measurement of Other Factors

Amounts of particulate protein (PP) were measured by the method of IWAMURA *et al.*[13] Bovine serum albumin fraction V was used as standard. Amount of seston and chrolophyll *a*

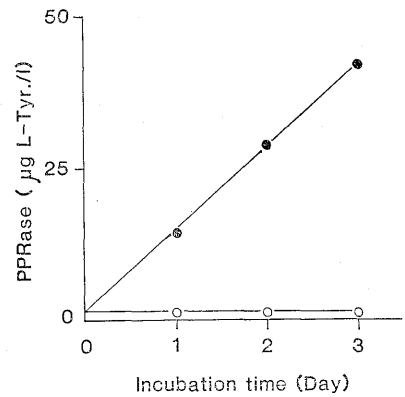

Fig. 2. Time course of PPRase activity of seawater collected from Aburatsubo Inlet. ●—●, untreated sample; ○—○, autoclaved samples.

(Chl. *a*) were measured by the methods of PARSONS and STRICKLAND[14] and STRICKLAND and PARSONS,[15] respectively.

Results

The time course of PPRase activity measured in seawater samples collected from Aburatsubo Inlet is shown in Fig. 2. No proteolytic activity was detected in the autoclaved samples whereas the activity increased linearly with time for 3 days in untreated samples. This indicates that hydrolysis of the substrate is due to heat labile enzymatic action.

Table 1. Regional distribution of bacteria, PP, and PPRase activity in various samples*

Area (Depth m)	Date of sampling	Viable counts of bacteria (cfu/*l*)		PP (μg/*l*)	PPRase activity (μg L-Tyr/*l*·day)
		Heterotrophs	Protease-producer		
Aburatsubo Inlet (0–5)	Oct., 1982 ~ Sep., 1983	2.7×10^{7}	1.3×10^{7}	939	141
Tokyo Bay (0–10)	Feb., 1983	1.1×10^{7}	2.0×10^{6}	583	276
Sagami Bay (0–200)	Feb., 1983	6.8×10^{4}	2.2×10^{4}	61	3
Off-Tateyama (0–200)	Feb., 1983	8.3×10^{4}	4.8×10^{4}	67	2
Oyashio Regions (0–1000)	Jun., 1983	5.0×10^{5}	2.0×10^{5}	129	11

* All values in this table are showed as averaged ones.

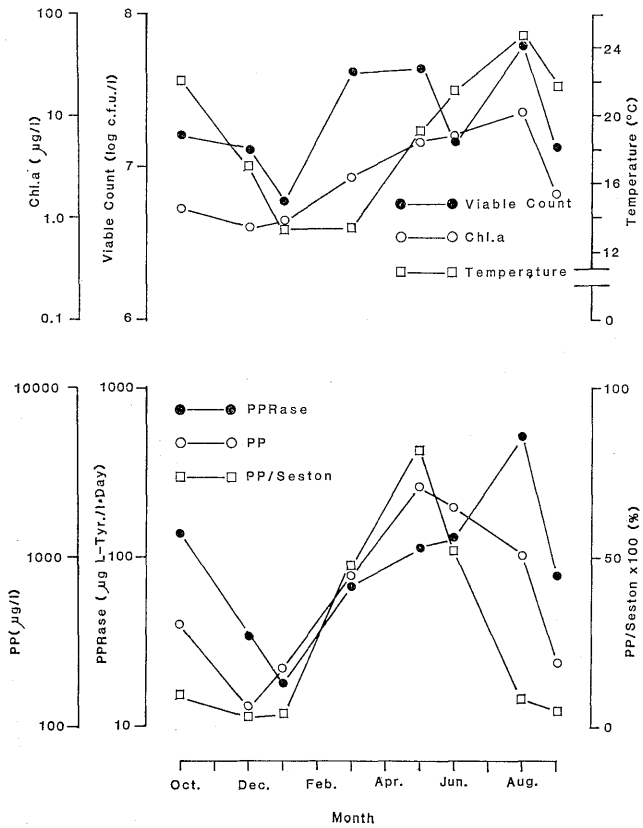

Fig. 3. Seasonal fluctuation of PPRase activity and other environmental factors in Aburatsubo Inlet.

Table 1 summarizes the distribution of bacteria, PP, and PPRase activity in the various seawater samples. Protease-producing bacteria were widely distributed in various areas. The population ranged from 10^4 to 10^7 per *l* of seawater, being approximately the same order of magnitude as that of heterotrophic bacteria. Considerably large populations of protease-producers and high PPRase activity were found in eutrophic Aburatsubo Inlet and Tokyo Bay, whereas small populations of protease-producers and low PPRase activity were observed in Sagami Bay and Off-Tateyama. However, no relationship was found between the activity and the number of protease-producer in seawater.

Seasonal Fluctuation of PPRase Activity and Other Environmental Factors

Figure 3 illustrates the seasonal fluctuation of PPRase activity and other environmental factors over a 12-month period in the Aburatsubo Inlet. PP and PP/Seston increased with the standing

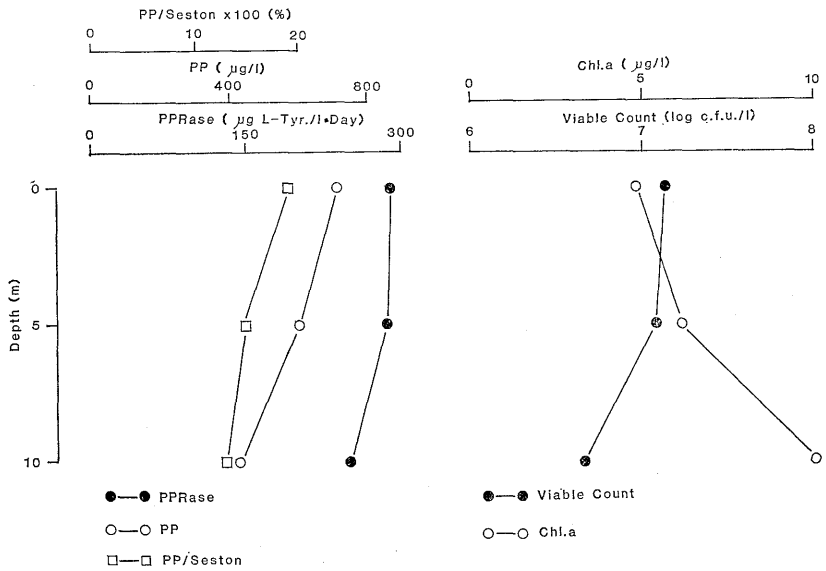

Fig. 4. Vertical profile of PPRase activity and other environmental factors in Tokyo Bay.

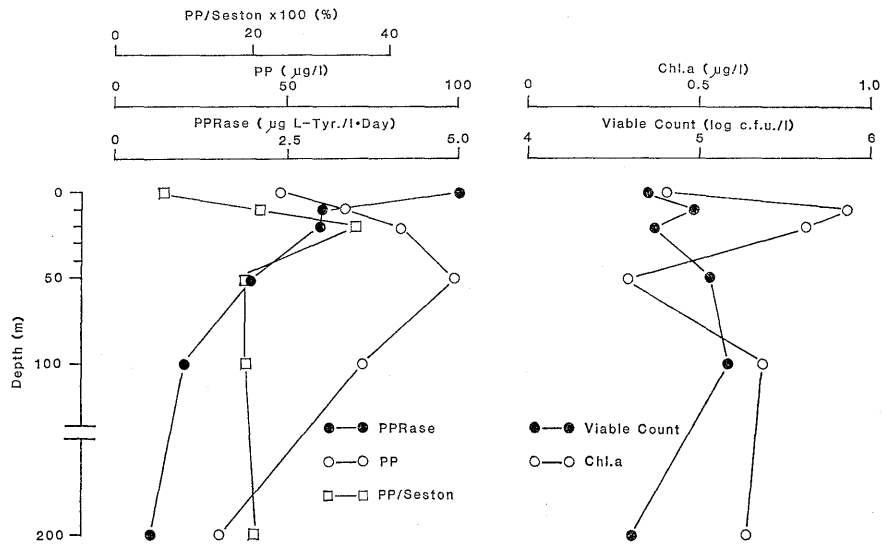

Fig. 5. Vertical profile of PPRase activity and other environmental factors in Sagami Bay.

stock of phytoplankton (Chl. *a*). The highest PP was observed in May. PPRase activity showed a seasonal fluctuation similar to that of PP. However, its maximal value was obtained in August, three months later than that of PP.

Vertical Distribution of PPRase Activity and Other Environmental Factors

The vertical distribution of PPRase activity and other environmental factors in inner Tokyo Bay are shown in Fig. 4. PPRase activity was the

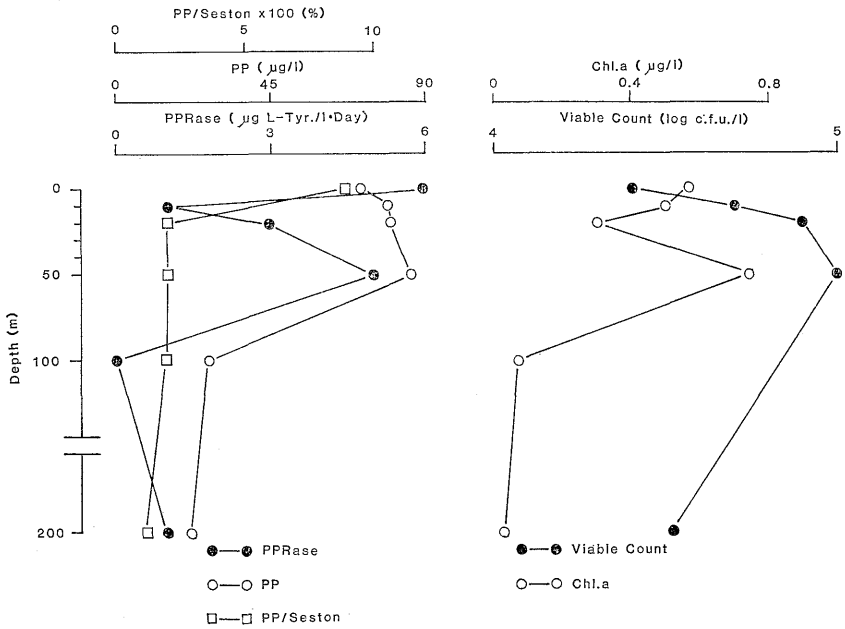

Fig. 6. Vertical profile of PPRase activity and other environmental factors in Off-Tateyama.

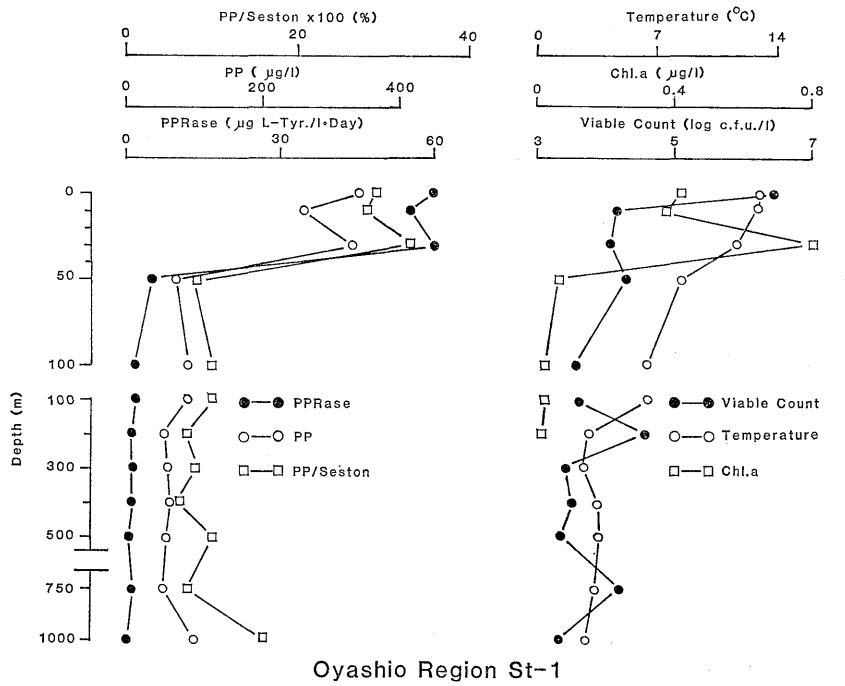

Fig. 7. Vertical profile of PPRase activity and other environmental factors in Oyashio Region (St-1).

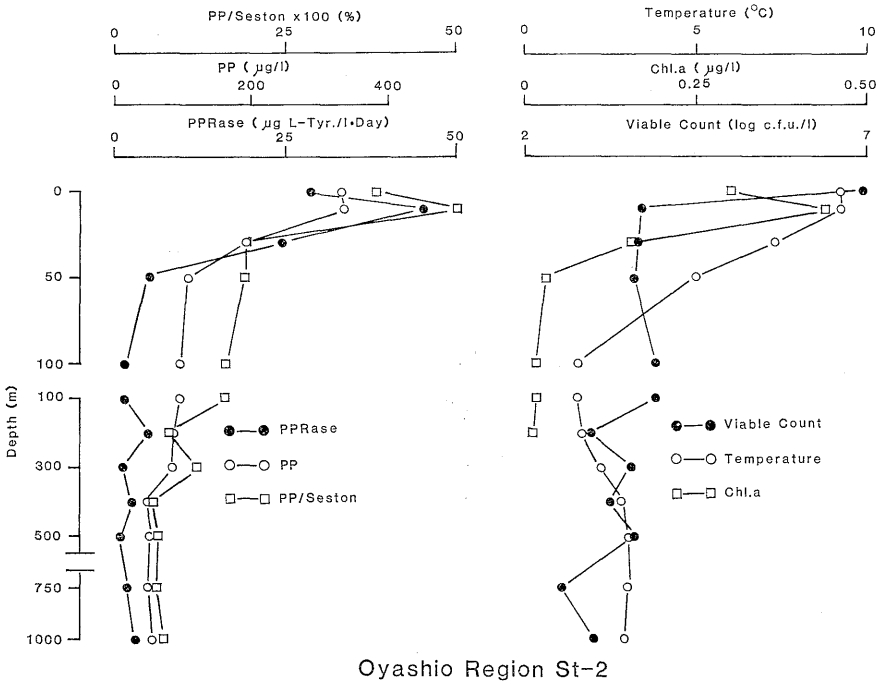

Fig. 8. Vertical profile of PPRase activity and other environmental factors in Oyashio Region (St-2).

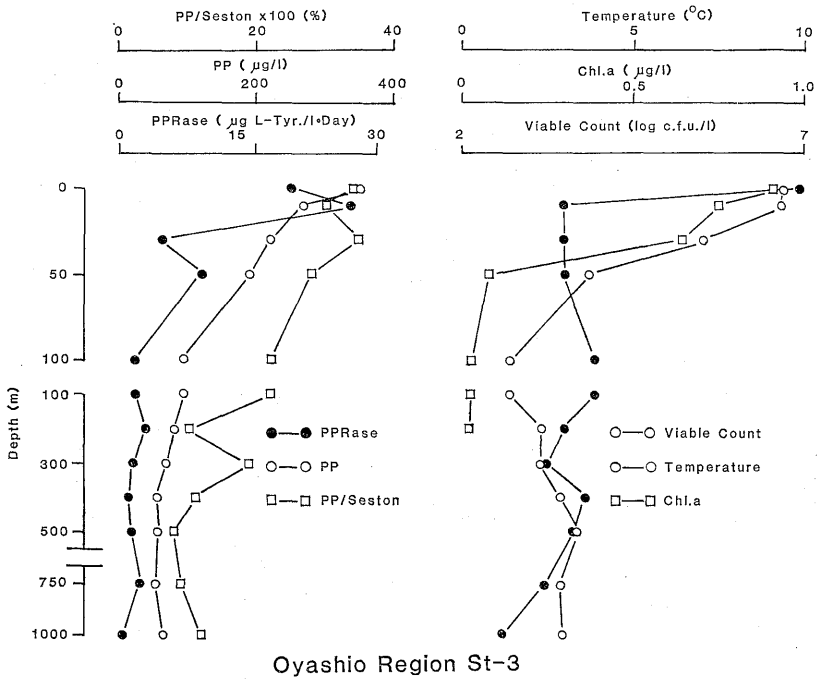

Fig. 9. Vertical profile of PPRase activity and other environmental factors in Oyashio Region (St-3).

highest at the surface layer and decreased with depth. The distribution of PPRase activity paralled closely the pattern of PP, PP/Seston, and the viable count of heterotrophic bacteria. Approximately 18% of heterotrophic bacteria were found to be protease-producers (Table 1). The amount of Chl. *a*, PP, and the viable count suggest that there is an excessive biological production in the bay.

The vertical distribution of PPRase activity and other environmental factors in Sagami Bay and Off-Tateyama are shown in Figs. 5 and 6, respectively. Compared with the data of Tokyo Bay, the vertical distributions of PP, PP/Seston, and the viable count of heterotrophic bacteria did not always follow the pattern of PPRase activity in these areas. The average PPRase activity in surface waters was two orders of magnitude lower than those of Aburatsubo Inlet and Tokyo Bay. Of the heterotrophic populations, 32 and 58% were found to be protease-producers (Table 1).

The vertical distribution of PPRase activity and other environmental factors in Oyashio Regions are shown in Figs. 7, 8, and 9. PPRase activity, PP, PP/Seston, and the viable count of heterotrophic bacteria were found to be maximal above 30 m depth. At 50 m, these factors declined sharply but became constant below 100 m depth. This indicates that rapid decomposition of PP occurred above a depth of 50 m. The vertical distribution pattern of PPRase activity was fairly similar to that of PP, PP/Seston, and heterotrophic bacteria in these areas as was the case in Tokyo Bay. On the average, 40% of heterotrophic populations were found to be protease-producers (Table 1). The protease-producer population of the heterotrophs was fairly constant from the surface to the bottom layers. The mean PPRase activity in surface waters of these areas was one order of magnitude lower than those of Aburatsubo Inlet and Tokyo Bay.

Discussion

Biological decomposition of PP in marine environments has been considered to be the result of bacterial action and digestion by living zooplankton. In addition, hydrolyzing enzymes excreted into the seawater may also play an important role in decomposing PP.

In the present study, PPRase activity was detected in all seawater samples examined. This indicates that seawater has a potential ability to hydrolyze PP.

As for deoxyribonuclease[4,5] and phosphatase,[6-8] the natural seawater contains detectable levels of these activities, whereas protease was not detectable. This suggests that, compared to the former enzymes, most protease occurs as particulate enzymes or binds to particulate matters in seawater.

In Aburatsubo Inlet, PPRase activity increased with a concomitant increase of phytoplankton standing crop from March to August, and declined after that period. The highest activity was observed in the upper layer at all the regions (Tokyo Bay, Sagami Bay, Off-Tateyama, and Oyashio Regions) and declined with depth. PPRase activity of Aburatsubo Inlet and Tokyo Bay were 1 or 2 orders of magnitude higher than other offshore areas.

The average depth of the sea is 3,800 m (maximum depth 10,800 m), and the temperature of most of the water mass is less than 5°C. Seawater contains fairly high concentration of cations like Na^+, K^+, Ca^{2+}, and Mg^{2+}. Considering these characteristics, it is expected that the enzymes produced by marine microorganisms are different from those by terrestrial microorganisms. HANSON and KIM[16] reported that alkaline phosphatase prepared from marine sediment samples was not inhibited by the addition of NaCl up to 6%, indicating that the enzyme could be active in the sea. KOBORI and TAGA[17] isolated marine *Pseudomonas* No. 393 from 200 m depth. Under 1,000 atm hydrostatic pressure, the phosphatase activity of this bacterial strain was 3.2 times higher than 1 atm. However, during the investigation enzyme activity was determined at a specific incubation temperature (25°C), assay pH (8.0), substrate concentration, and hydrostatic pressure (1 atm) in order to compare the relative potential for hydrolysis of different seawater samples. Therefore, rates of decomposition of PP estimated by PPRase activity do not equal the *in situ* rates.

Initially, difficulties were encountered due to bacterial contamination in the enzyme assay. However the addition of chroloform to the reaction mixture not only obviated this problem, but also gave a convenient method of assaying the activity.

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