

Changes in Proteolytic Activities in Stored Seawater and Bacterial Isolates

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Abstract—Organic matter in the ocean water column is produced predominantly as high molecular weight matter that cannot be directly assimilated by bacteria. Consequently, bacterial extracellular ectoenzymes act in the processing of the polymer in the sea. Proteins are important substrates for bacterial growth and sources of organic carbon and nitrogen. However, little is known about the protein degradation process and the distribution of the proteolytic enzymes among the bacterial species in natural assemblages. In this study, we evaluated the potential activities of proteolytic enzymes in seawater and the isolated bacteria from seawater. Results on seawater showed that trypsin, chymotrypsin and aminopeptidase were most active whereas the elastase activity was weak. Enzyme activities during 112 days experiment showed that microbial activity acting in proteolysis process varies in many ways in seawater. *Gammaproteobacteria*, *Alphaproteobacteria*, *Cytophaga-Flexibacter-Bacteroides* and *Betaproteobacteria* were isolated from the stored seawater. Among the 52 isolates, *Alpha-*, *Betaproteobacteria* and *Cytophaga-Flexibacter-Bacteroides* showed highest activities in aminopeptidase whereas *Gammaproteobacteria* showed aminopeptidase and chymotrypsin activities, suggesting that different bacterial species produce different types of proteases. This study suggests different enzymes produced by diverse natural bacterial communities are acting in different reactions in the protein processing in sea.

Keywords: proteolytic enzymes, bacteria, protein, seawater

INTRODUCTION

Heterotrophic bacteria play a critical role in the biogeochemical cycling in the ocean (Azam *et al.*, 1993). One of the important roles of bacteria in microbial loop is to convert the high molecular weight compounds to low molecular weight compounds. Bacteria can only take up molecules smaller than 600 Da. Therefore, high molecular weight matters should be cleaved by enzymes for bacteria to efficiently utilize dissolved organic matter (DOM), indicating the importance of the bacterial extracellular enzymes. Proteins are important sources of organic C and N for heterotrophic bacteria. In marine environments various groups of bacteria play an important role in the degradation of proteins, where many different proteases act to produce oligo peptides and amino acids. Our previous

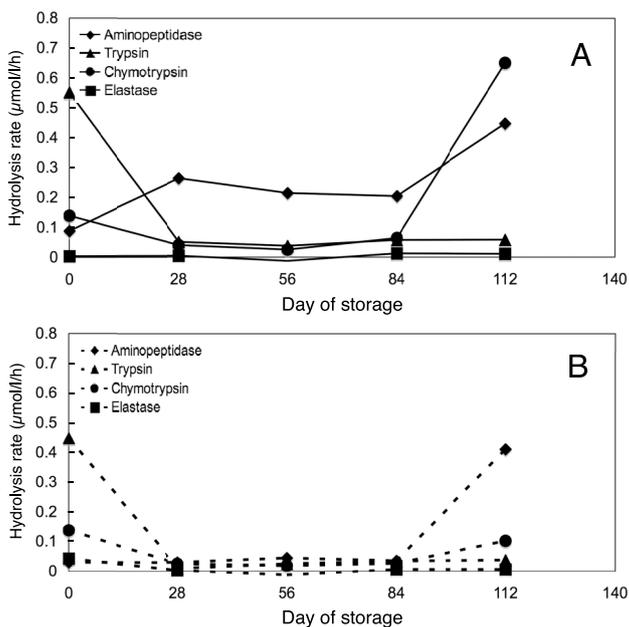


Fig. 1. Time course change of protease activities in (A) unfiltered and (B) $<0.2 \mu\text{m}$ fraction of the stored seawater.

studies indicated that proteases in seawater contain aminopeptidase, trypsin and chymotrypsin type enzymes (Obayashi and Suzuki, 2005, 2008). The enzymes are produced by various organisms; however, detailed study on isolated bacteria is limited. The isolation and enzyme studies are of great importance in understanding the protein dynamics in the organic matter cycling through microbial loop. In this study, we evaluated the potential activities and the time variation of proteolytic enzyme profiles in seawater and among the bacterial species constituting the bacterial community in natural assemblages.

MATERIALS AND METHODS

Seawater samples

Seawater samples were collected from coastal areas in Matsuyama, Japan and filtered through $150 \mu\text{m}$ plankton net. In the laboratory, seawater was fractionated to $0.2 \mu\text{m}$ -passed fraction and unfiltered fraction; both were used for protease assay.

Proteases assay

Seawater was stored at room temperature ($20\text{--}25^\circ\text{C}$) under dark and sub-

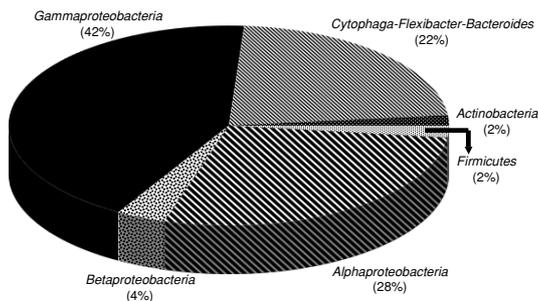


Fig. 2. The composition of isolates to phyla based upon the analysis of partial 16S rDNA gene sequence. Bacteria were isolated from 0, 56 and 84 day stored seawater.

samples were taken on days 0, 28, 56, 84 and 112. Enzyme activity was assayed using peptide analogue methylcoumarylamide (MCA) substrates (Obayashi and Suzuki, 2005). Nineteen types of MCA substrates for aminopeptidase, trypsin, elastase and chymotrypsin were used to examine substrate preference of enzymes in seawater. Mixtures of the sample and substrate were incubated at 25°C for one hour, before and after which the fluorescence of the hydrolytic product, 7-amino-4-methylcoumarin (AMC) was measured with a spectrophotometer and the activity of proteases was assayed as the hydrolysis rate of various MCA substrates.

Isolation of marine bacteria

The seawater samples were spread on 1.5% marine agar plate and incubated at 25°C for 2 days. Colonies with different morphology were isolated and employed for proteolytic activity assay. The classification status of isolates was determined using 16S rDNA sequence. Primers for 16S rDNA were 341F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5' CCG TCA ATT CMT TTG AGT TT 3'). PCR reaction was carried out according to Muyzer *et al.* (1993).

Bacterial protease activity

The colony picked up from the plate was suspended into autoclaved filtered seawater and incubated at 25°C for 2 days with mild shaking. Proteolytic activity in the bacterial suspension was analyzed using the MCA-method as described above.

RESULTS AND DISCUSSION

It is reported that trypsin, chymotrypsin and aminopeptidase showed high activities whereas elastase activity was weak in seawater at the time immediately after sampling (Obayashi and Suzuki, 2005). In this study, all proteolytic activities at day-0 were mostly found in dissolved and cell containing fractions. Trypsin activities were higher and showed a drastic decrease from day-0 to day-

28, whereas aminopeptidase activities increased from day-28 to day-112. Aminopeptidase activities were mainly in the cellular fraction between day-28 to day-84 whereas in day-112, high activity was mainly from the dissolved fraction. Activity of chymotrypsin was low from day-0 to day-84, but was high at day-112, and the activity was mainly attributed from cellular fraction. Relative time course change of these enzyme activities is summarized in Fig. 1. The results of this study in seawater clearly indicate that there were differences in the potential activity levels of the cellular and dissolved fraction. The origin of the free enzymes can be many. Previous studies on the enzymatic activity of heterotrophic bacteria mostly reported that extracellular enzymes may be bound to the cell rather than in the dissolved fraction of seawater. Vives Rego *et al.* (1985) also pointed out that dissolved enzymes account for only a small fraction of the total extracellular enzyme activities in the marine environment, whereas, it is reported that marine bacteria *Alteromonas haloplanktis* release trypsin into the cultures supernatant (Odagami *et al.*, 1993). The changes in the levels of enzyme activities during the 112 days incubation suggested that the microbial activity acting in proteolysis process varies widely in seawater and the bacterial assemblage in the seawater environments is perhaps forced to adapt the changing of the nutrients. Therefore, the bacterial assemblages in the seawater express a higher plasticity in metabolic pathway in order to be able to exploit the changing and limitation of nutrients such as carbon and nitrogen sources that are available for bacterial growth.

Of the 52 bacterial isolates, almost half (42%) were related to *Gammaproteobacteria*, 28% to *Alphaproteobacteria* and 22% to *Cytophaga-Flexibacter-Bacteroides*. A few bacterial species belonged to *Betaproteobacteria*, *Actinobacteria* and *Firmicutes* (Fig. 2). The present study is in accordance with an earlier study that has indicated the dominance of *Gammaproteobacteria* and *Alphaproteobacteria* in the sub-tropic and polar region (Cottrell and Kirchman, 2000). In contrast, the number of *Betaproteobacteria* and *Actinobacteria* were low in the studies of Bouvier and del Giorgio (2007) who has showed that these two classes of bacteria had a potential to grow in an open ocean pelagic environment but were minor in the assemblages due to viral mortality.

In culture, as in nature, the enzyme profiles reflect the bacterium's genotypic identity as well as phenotypic response to environment. In this study, protease activities of all 52 bacterial isolates showed proteolytic activity and the substrate preference varies under identical growth conditions. Each strain specifically hydrolyzed one or several substrates. This confirmed our preliminary observation (Obayashi and Suzuki, 2008). Our study showed that protease activities were always strongest in the cellular fraction ($>0.2 \mu\text{m}$). This finding supports experimental results obtained by Hoppe *et al.* (1998) who observed the high protease activity in $0.2 \mu\text{m}$ to $<3 \mu\text{m}$ bacterial fractions. Significant enzymatic activity in the dissolved fraction ($<0.2 \mu\text{m}$) was detected, indicating that some enzymes were released from the bacterial cells into the surrounding seawater as reported by Odagami *et al.* (1993). Among 52 isolates, *Alphaproteobacteria*, *Cytophaga-Flexibacter-Bacteroides* and *Betaproteobacteria* showed highest

activities in aminopeptidase whereas *Gammaproteobacteria* were active in aminopeptidase and chymotrypsin activities. Trypsin activities were detected from all groups. The activities of elastase were weak in all the bacterial isolates. The results suggest that different bacterial species produce different types of proteases, which should be important in determining the fate of protein in seawater.

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