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Production of aminopeptidase by marine heterotrophic nanoflagellates

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ABSTRACT: In order to examine the hypothesis that the enzyme levels of heterotrophic nanoflagellates (HNF) are influenced by qualitatively different food bacteria, the production of aminopeptidase by an isolated marine HNF *Jakoba libera*-5(2) fed on natural communities of bacteria and 6 bacterial strains of 5 different taxonomic groups (*Aeromonas*, *Bacillus*, Coryneforms, *Flavobacterium* and *Pseudomonas*) were compared. The aminopeptidase activity (total and free) and abundance of *J. libera*-5(2) significantly differed with the types of food bacteria. The total and free aminopeptidase activities and abundance of *J. libera*-5(2) were at a maximum with *Pseudomonas* spp., followed by *Flavobacterium* sp. and natural communities of bacteria as prey. The values of total aminopeptidase activity of *J. libera*-5(2) with *Pseudomonas* spp., *Flavobacterium* sp. and natural communities of bacteria were 140 ± 6.78 to 285 ± 12.36, 123 ± 11.17 and 38 ± 0.56 µmol h^{-1} l^{-1}, respectively, and those of free aminopeptidase activity were 83 ± 6.15 to 137 ± 5.83, 82 ± 12.18 and 8 ± 0.14 µmol h^{-1} l^{-1}, respectively. *J. libera*-5(2) did not produce any detectable amounts of total and free aminopeptidase while grazing on *Aeromonas*, *Bacillus* and Coryneforms. The chemical characterization of partially purified aminopeptidase of *J. libera*-5(2) produced during grazing on a strain of *Pseudomonas* sp. indicated the enzyme to be metal-chelater-sensitive alkaline serine aminopeptidase with optimal activity at pH 8.0 and 30°C; it was not affected by the major cations of seawater, such as Na^+, Ca^{2+} and Mg^{2+}. The results suggest that at least some marine HNF may significantly contribute to the enzyme pool in marine environments while selectively grazing on bacteria.

KEY WORDS: Aminopeptidase · Grazing · Heterotrophic nanoflagellates · Marine bacteria

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INTRODUCTION

Heterotrophic nanoflagellates (HNF) (2 to 20 µm) are ubiquitous plankton communities in marine environments. Several studies have reported the importance of HNF as the principal grazers of bacteria (reviewed by Sherr & Sherr 2002) and the influence HNF have on both the size distribution of bacterial communities (Andersen et al. 1986, González et al. 1990) and their taxonomical compositions (Hahn & Höfle 1998, Jürgens et al. 1999). A substantial part (10 to 50%) of primary production is channeled through bacteria (Cole et al. 1988), and the bacteria produced are mostly consumed by HNF that differ in their food selectivity and feeding strategies (Fenchel 1986, Nagata 2000). By repackaging bacterial biomass into cells accessible to larger zooplankton (Sherr et al. 1986), and/or fish larvae (Fukami et al. 1999), these organisms function as intermediates in the transfer of energy to higher trophic levels, and they also act as a catalyst for the remineralization and recycling of elements essential for phytoplankton and microbial growth (Goldman et al. 1985, Porter et al. 1985).

Most of the studies on HNF have been focused on seasonal variation (Fukami et al. 1991, Tanaka & Taniguchi 1999), quantification of grazing rates on

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bacteria (reviewed by Landry 1994) and distribution of HNF in different aquatic environments (Nagata 1988, Fukami et al. 1996). There are very few studies available on the metabolism of HNF. Previous studies have stressed that grazing by HNF enhances the metabolic activity of bacteria by releasing dissolved organic carbon (Taylor et al. 1985, Pelegrí et al. 1999), dissolved free and combined amino acids (Andersson et al. 1985, Nagata & Kirchman 1991), dissolved organic phosphorus (Andersen et al. 1986), and colloidal particles (Koike et al. 1990). These released substances are important bacterial substrates and may play a role in the enrichment of the microenvironment (Nagata 2000). However, less attention has been given to the secretion of hydrolytic enzymes. Few studies have reported that, in common with other microorganisms, HNF can also be a novel source of enzymes: acid phosphatase (Nagata & Kirchman 1992b), acid lysozyme (González et al. 1993), acetylglucosaminidase (Vrba et al. 1993, Simek et al. 1994, Zubkov & Sleigh 1998, Sherr & Sherr 1999), α-glucosidase and aminopeptidase (Karner et al. 1994).

HNF have been distinguished from other microorganisms present in the marine planktonic food web by an ability to digest bacterial cell walls almost instantly. A large amount of hydrolytic enzymes must be released to perform this digestion during only a short time (Zubkov & Sleigh 1998). Despite their importance as an enzyme producer, the enzymatic pathways of these organisms are very poorly understood. So far the chemical characterization and ecological implication of these enzymes have not been studied. The enzymatic studies on HNF have been primarily focused on the quantification of either HNF abundance (Simek et al. 1994, Zubkov & Sleigh 1998) or grazing activity (González et al. 1993, Vrba et al. 1993). However, there is no report available on the correlation of enzyme activity with the qualitative aspects of HNF grazing.

In the present paper we aimed to test the hypothesis that the enzyme levels of HNF are influenced by the qualitatively different food bacterial types. Here, we have taken *Jakoba libera*-5(2), natural communities of bacteria and 6 bacteria of 5 different taxonomic groups, and aminopeptidase as the model HNF, qualitatively different prey organisms and the hydrolytic enzyme, respectively. Additionally, the partial biochemical characterization of aminopeptidase released by *J. libera*-5(2) was also investigated.

MATERIALS AND METHODS

Isolation of HNF and prey bacteria. A strain of HNF was isolated from natural seawater from the Pacific Ocean, Kochi-ken, Japan, by using seawater medium enriched with rice-grain and serial dilution techniques. It measured 4 to 5 µm in length and had a cell volume of 24 μm^3. This strain was tentatively identified as *Jakoba libera* strain 5(2) (Ruinen) Patterson (Patterson 1990). The clonal culture of *J. libera*-5(2) was routinely maintained in rice-grain culture medium. Bacteria present in the rice-grain enrichment culture were considered to be the natural communities of marine bacteria (NCB). Six bacterial strains (Ae-B1, Fl-B1, GPR-2, GPR-3, Ps-B2 and Ps-B6) were also isolated from Pacific Ocean, Kochi-ken, surface seawater samples and were subsequently identified with an array of biochemical tests according to Bergey's *Manual of Determinative Bacteriology* (Holt et al. 1994). These bacterial strains were tentatively identified as *Aeromonas* sp. (Ae-B1), *Bacillus* sp. (GPR-2), Coryneforms (GPR-3), *Flavobacterium* sp. (Fl-B1) and *Pseudomonas* sp. (Ps-B2 and Ps-B6).

Bacterial sizes. Bacterial cell size was determined by a computer-assisted image analysis of 4,6-diamidino-2-phenylindole (DAPI) stained bacterial cells (Fukami et al. 1991) with an epifluorescence microscope (Olympus BX60) equipped with a charge-coupled device camera (SenSys 400, Photometrics). Length (L) and width (W) were measured from the photographs by using IP Lab Scientific Imaging Software (Scanalytica). Cell volume (V) was calculated as $V = 4/3[\pi(W/2)^3] + [\pi(W/2)^2 \times (L - W)]$ (González et al. 1990).

Grazing experiment. The isolated pure culture of bacterial strains were grown in a FeTY medium, consisting of 0.5 g l^{-1} trypticase peptone, 0.05 g l^{-1} yeast extract, 0.01 g l^{-1} ferric citrate and 80% (v/v) aged seawater at pH 7.5 (Fukami et al. 1992) for 72 h. The bacteria cells were harvested by centrifugation (5500 × g, 30 min) at 4°C. The pellets were washed repeatedly with 0.22 µm filtered and autoclaved aged seawater to remove the cultivation medium, and resuspended in 0.22 µm filtered and autoclaved aged seawater. Before the grazing experiments were performed, the resuspension of each bacteria was filtered through 0.8 µm membrane to remove bacterial aggregates. The natural bacteria present in the culture of HNF were eliminated by stepwise dilution techniques (Pelegrí et al. 1999). The HNF culture with natural prey bacteria was transferred serially by adding 0.1 ml (in the case of prey bacteria Ps-B2, Ps-B6 and Fl-B1) or 1 ml (in the case of prey bacteria Ae-B1, GPR-2 and GPR-3) of HNF culture to 100 ml of the high-density resuspension of axenic culture of each prey bacterium (10^7 cells ml^{-1}). HNF in the inoculated cultures were allowed to grow to stationary phase, which usually occurred 4 to 6 d after HNF inoculation. The above reinoculation method was repeated several times to obtain an HNF-bacterium predator–prey system by successively di-

luting natural bacteria. In addition, cultures were monitored regularly to assure purity of the prey bacteria by observing the typical rod shape and size (Table 1) with an epifluorescence microscope. No contamination was recorded during the experiment.

Prior to the grazing experiments, HNF were cultured for several generations with monoxenic cultures of 6 individual isolated bacteria as prey. The grazing experiments were conducted in 250 ml screw-cap bottles containing 100 ml of 0.22 µm filtered and autoclaved aged seawater (pH 8.0). HNF used as inocula were previously cultured on the same bacterial isolate as that used in the grazing experiment. The initial concentrations of HNF set in the growth experiments were 2.0×10^3 cells ml^{-1}. The initial concentrations of prey bacteria ranged from 9.4–10.1 $\times$ 10^6 cells ml^{-1}. The natural communities of bacteria were obtained from the flagellate rice-grain culture by filtering through a 0.8 µm membrane. More than 98% of the HNF were removed by this filtration method. Bacteria were harvested from the filtrate by centrifugation (5500 $\times$ *g*, 30 min) at 4°C. After repeated washing with 0.22 µm filtered and autoclaved aged seawater, bacterial cells were resuspended in 0.22 µm filtered and autoclaved aged seawater with a concentration of 9.4–10.1 $\times$ 10^6 cells ml^{-1}. Bacteria-only controls were also prepared for each bacterium and natural communities of bacteria by adding the initial concentration of individual prey bacterium to the 100 ml of 0.22 µm filtered and autoclaved aged seawater (pH 8.0) in a 250 ml screw-cap bottle. The 6 bacterial strains and natural communities of bacteria did not grow in the bacteria-only control culture; instead, their cell abundance decreased slightly with time.

Bottles were incubated in the dark at 20°C without shaking, and at predetermined intervals the abundances of HNF and bacteria as well as aminopeptidase production rates were determined. Abundances of HNF and bacteria were determined by staining with DAPI (Fukami et al. 1991). A schematic of the grazing experiment is shown in Fig. 1. All the measurements were conducted in triplicate, and data are expressed as means ± standard deviation.

The net cellular production (10^4 cells ml^{-1}) of HNF during the grazing experiment was determined by the difference between the highest and initial HNF abundance (Pérez-uz 1996).

Aminopeptidase assay. The aminopeptidase activities in the cell-free filtrate (0.22 µm filtrate) and unfiltered culture of HNF *Jakoba libera*-5(2) grazing on qualitatively different bacteria (culture of HNF + bacteria) and in the bacteria-only control were measured fluorometrically using 7-l-leucyl-4-methyl-coumarinyl-amide (Leu-MCA) as substrate (Hoppe 1983). Leu-MCA has already been established as a model substrate for aminopeptidase activity in the marine environment (see Patel et al. 2000, and references

Table 1. Characterization of food bacterial strains. Cell size was determined at an early stationary phase

Name of bacteria	Motility	Mean ± SD (µm) Length	Width	Volume
Ps-B2 (*Pseudomonas* sp.)	+	1.50 ± 0.36	0.32 ± 0.04	0.112 ± 0.011
Ps-B6 (*Pseudomonas* sp.)	+	1.40 ± 0.19	0.43 ± 0.06	0.183 ± 0.032
Fl-B1 (*Flavobacterium* sp.)	+	1.33 ± 0.13	0.30 ± 0.03	0.087 ± 0.026
Ae-B1 (*Aeromonas* sp.)	+	1.05 ± 0.34	0.28 ± 0.02	0.058 ± 0.021
GPR-2 (*Bacillus* sp.)	+	1.66 ± 0.64	0.40 ± 0.05	0.191 ± 0.046
GPR-3 (Coryneforms)	+	1.60 ± 0.17	0.45 ± 0.08	0.231 ± 0.062

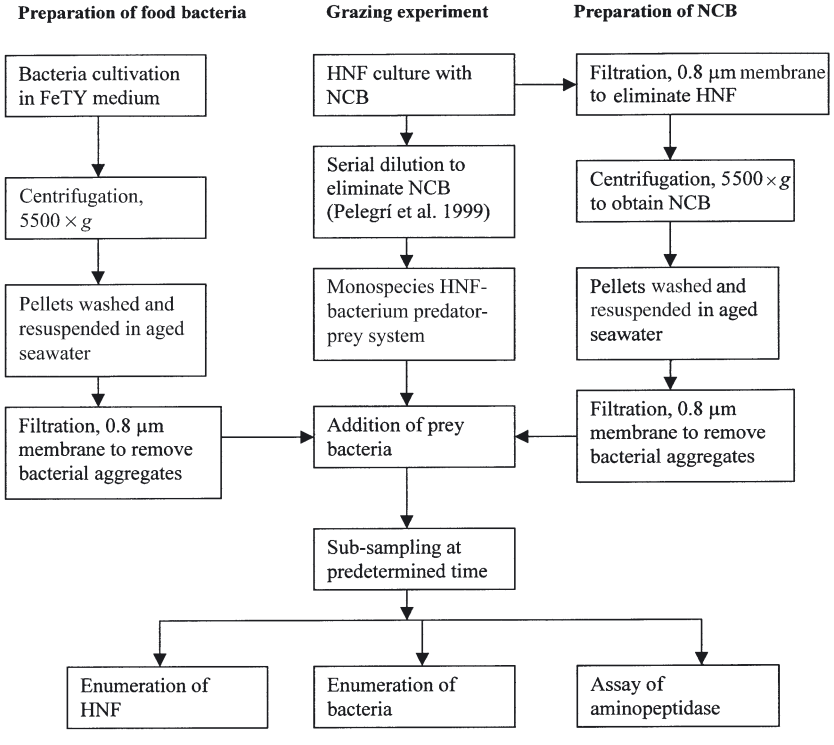

Fig. 1. Schematic of the experiment: HNF grazing on different food bacteria

cited therein). The activity in the cell-free 0.22 µm filtrate was referred to as the free enzyme activity, and that in the unfiltered sample as the total enzyme activity. The cell-free filtrate was prepared by gently filtering the sample through a 0.22 µm Millipore filter (Millipore) with a syringe. The assay mixture was composed of a 0.25 ml subsample of culture filtrate or the unfiltered culture of HNF + bacteria, 4.25 ml of 10 mmol l^{-1} Tris-HCl buffer (pH 8.0) and 0.5 ml of 0.5 mmol l^{-1} Leu-MCA (dissolved in dimethylsulphoxide). The final concentration of Leu-MCA in the assay mixture was 50 µmol l^{-1}. This concentration was found to produce the highest rate of hydrolysis in the preliminary kinetic experiment. The assay mixture was incubated at 30°C for 1 h. Fluorescence intensity was measured before and after a 1 h incubation. In the case of total enzyme activity, the assay mixture was filtered using the syringe filtration method as above, and then the fluorescence intensity of the filtrate was measured. The fluorescence intensity was determined at wavelengths of 365 nm for excitation and 440 nm for emission (Shimadzu RF-500 spectrofluorophotometer). Blanks with 0.22 µm filtered and autoclaved aged seawater samples were processed in parallel. After correction of the fluorescence through the blanks, the catalytic activity was estimated from fluorescence of standard 7-amino-4-methylcoumarin (AMC). One unit of enzyme activity was expressed as µmol of AMC produced from the hydrolysis of Leu-MCA per hour per liter of sample under the experimental conditions.

In the case of the bacteria-only culture, both the free and total enzyme activities were determined as mentioned above.

Preparation of aminopeptidase for biochemical characterization. Because of the highest enzymatic activity and net cellular production of HNF *Jakoba libera*-5(2) with the food bacteria *Pseudomonas* sp. (Ps-B2), the aminopeptidase of the culture filtrate (0.22 µm filtrate) of HNF grazing on *Pseudomonas* sp. (Ps-B2) was partially purified and characterized. The cell-free filtrate (0.22 µm filtrate) of the 5 d old culture of HNF grazing on bacteria (culture of HNF + Ps-B2) was treated as a crude enzyme preparation. The enzyme in the crude preparation was precipitated with 80% (w/v) ammonium sulphate. The precipitate was collected by centrifugation (5500 × *g*, 30 min) at 4°C, then dissolved in 10 mmol l^{-1} Tris-HCl buffer (pH 7.2) followed by dialysis (molecular weight cut off 15 kd) for 24 h at 4°C against the same buffer. The dialyzates were pooled and used for further studies.

Effect of pH and temperature. The pH-dependent activity of partially purified aminopeptidase was examined using the wide range of buffer systems containing 10 mmol l^{-1} citrate (pH 3.0 to 6.0), phosphate (pH 7.0), Tris-HCl (pH 8.0), borax-NaOH (pH 9.0 to 10.0) and glycine-NaOH (pH 11.0 to 12.0). The assay conditions were the same as described above, except buffers of different pH (3.0 to 12.0) were used for the assay mixture. The effect of temperature on enzyme activity was evaluated by incubating the assay mixture at different temperature (5 to 60°C at 5°C increments) for 1 h. To study the heat stability of the enzyme, substrate-free enzyme solutions were held at various temperature (5 to 60°C at 5°C increments) for 30 min, followed by cooling to 0°C before addition of the assay mixture.

Effect of metal ions and inhibitors. The enzyme was pre-incubated with individual metal ions and inhibitors at 20°C for 5 min. Separate blanks with individual metal ions and inhibitors were prepared. The residual activity was measured.

RESULTS

The isolated HNF *Jakoba libera*-5(2) was subjected to batch culture grazing experiments with NCB, and 6 bacterial strains of 5 different taxonomic groups — *Pseudomonas* spp. (Ps-B2 and Ps-B6), *Flavobacterium* sp. (Fl-B1), *Aeromonas* sp. (Ae-B1), *Bacillus* sp. (GPR-2) and Coryneforms (GPR-3) — as prey. The abundance of *J. libera*-5(2) varied with the types of prey bacteria. *J. libera*-5(2) had its highest abundance of 9.83 (± 0.58) × 10^4 cells ml^{-1} with the prey bacteria Ps-B2 (Fig. 2A), followed by 6.61 (± 1.26) × 10^4 cells ml^{-1} with Ps-B6 (Fig. 3A), 4.20 (± 0.97) × 10^4 cells ml^{-1} with Fl-B1 (Fig. 4A) and 2.63 (± 0.81) × 10^4 cells ml^{-1} with NCB (Fig. 5A). *J. libera*-5(2) did not grow (HNF abundance ca. 0.22 × 10^4 cells ml^{-1}) with the prey bacteria Ae-B1, GPR-2 and GPR-3. Generally with the appearance of HNF in the cultures of *Pseudomonas* spp. (Ps-B2 and Ps-B6), *Flavobacterium* sp. (Fl-B1) and natural communities of bacteria (NCB) resulted in a sharp decline in bacterial numbers (58-72, 46 and 33% of the control, respectively) parallel to an increase in both free and total aminopeptidase activity (Figs. 2B, 3B, 4B & 5C).

In the bacteria-only control of NCB, the aminopeptidase activity (free and total) was stable and roughly followed the bacterial density (Fig. 5B). After the inoculation of HNF, the free and total aminopeptidase activities increased 12- and 15-fold, respectively (Fig. 5C).

In Ps-B2, Ps-B6 and Fl-B1, the total aminopeptidase of HNF followed the trend in HNF abundance and reached its highest value at Day 5 (285 ± 12.36 µmol h^{-1} l^{-1}, Fig 2B), Day 6 (140 ± 6.78 µmol h^{-1} l^{-1}, Fig. 3B) and Day 6 (123 ± 11.17 µmol h^{-1} l^{-1}, Fig. 4B), respectively. The free enzymes of HNF with Ps-B2, Ps-B6 and Fl-B1 as prey reached a stationary phase after 3 to 5 d of HNF addition, and yielded the highest value 137 ±

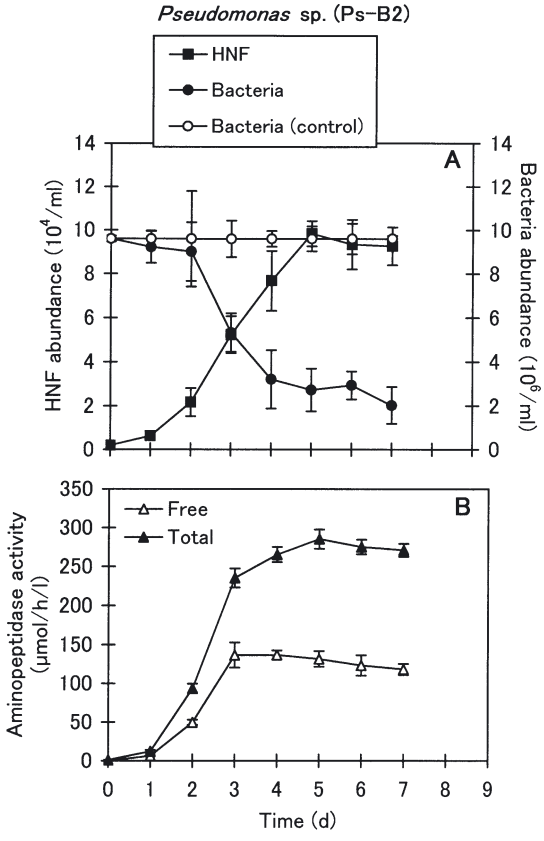

Fig. 2. Batch culture of heterotrophic nanoflagellates *Jakoba libera*-5(2) with *Pseudomonas* species (Ps-B2) as prey. (A) HNF and bacterial abundance, and (B) free and total aminopeptidase activity produced by HNF *J. libera*-5(2). Error bars are SD of triplicate measurements. SD are given when they exceed dimension of the symbols

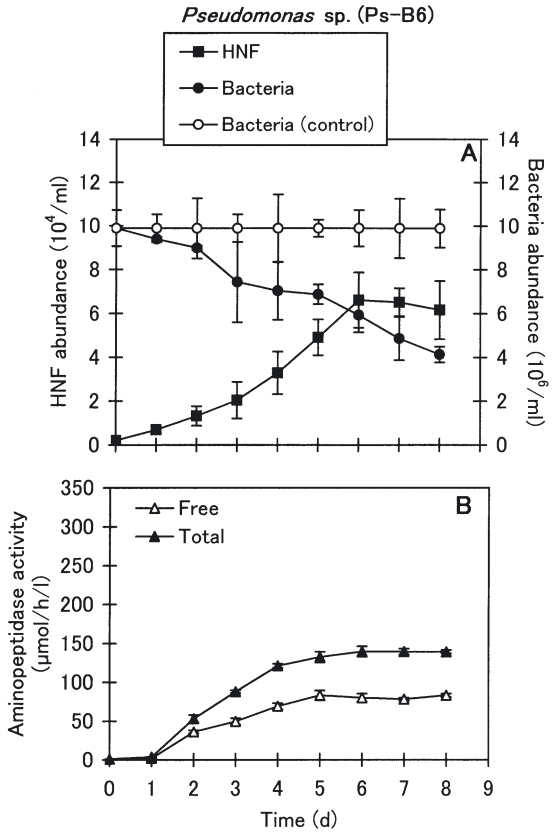

Fig. 3. Batch culture of heterotrophic nanoflagellates *Jakoba libera*-5(2) with *Pseudomonas* species (Ps-B6) as prey. (A) HNF and bacterial abundance, and (B) free and total aminopeptidase activity produced by HNF *J. libera*-5(2). Error bars are SD of triplicate measurements. SD are given when they exceed dimension of the symbols

5.83 μmol h^{-1} l^{-1} at Day 4, 83 ± 6.15 μmol h^{-1} l^{-1} at Day 5 and 82 ± 12.18 μmol h^{-1} l^{-1} at Day 6, respectively. The HNF *Jakoba libera*-5(2) did not produce any measurable amounts of free and total aminopeptidase with Ae-B1, GPR-2 and GPR-3 as prey bacteria, but some total aminopeptidase always appeared in all predator–prey systems after HNF inoculation. In contrast, aminopetidase activity was not detected in the axenic culture of 6 food bacterial (Ps-B2, Ps-B6, Fl-B1, Ae-B1, GPR-2 and GPR-3) strains when cultivated with aged seawater, FeTY medium (data not shown) and 0.8 μm culture filtrate of HNF (previously grown on the same bacterial strain) (data not shown). As aminopeptidase was absent in both nutrient-depleted aged seawater, and nutrient-rich (FeTY and 0.8 μm culture filtrate of HNF) medium, nutrient-depleted aged seawater medium was used in the grazing response of HNF on different food bacteria because of its advantage in minimizing the changes in food bacterial concentration by not supporting bacterial growth (Eccleston-Parry & Leadbeater 1994, Zubkov & Sleigh 2000).

We partially purified and characterized the free aminopeptidase of *Jakoba libera*-5(2) + Ps-B2 as prey bacteria because of the highest enzymatic activity and cell abundance of the HNF. The optimum pH of the partially purified enzyme was 8.0 (Fig. 6). At pH 9.0 and 10.0, 72 and 26% of the maximum enzyme activity retention was observed. The effect of temperature on enzyme activity and stability is shown in Fig. 7. The highest activity was observed at 30ºC. The enzyme activity sharply decreased above 30ºC. The relative activity at 40ºC was 41%. The aminopeptidase was inactivated by exposure to a temperature of 45ºC for 30 min. Taking the results in these experiment, together, it is concluded that this enzyme is relatively heat-labile. Addition of calcium did not stabilize the enzyme activity under high temperature. The partially purified aminopeptidase was unaffected by the major cations of seawater, such as Na^+, Ca^{2+} and Mg^{2+}, even at a higher concentration (10 mmol l^{-1}) (Table 2). However, the activity was suppressed by Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+} at 1 mmol l^{-1}. Metal protease inhibitors (EDTA and or-

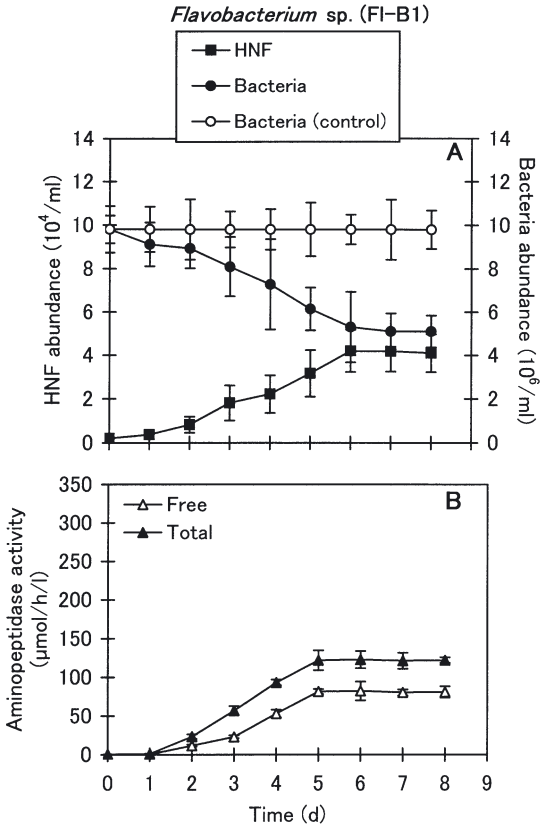

Fig. 4. Batch culture of heterotrophic nanoflagellates *Jakoba libera*-5(2) with *Flavobacterium* species (Fl-B1) as prey. (A) HNF and bacterial abundance, and (B) free and total aminopeptidase activity produced by HNF *J. libera*-5(2). Error bars are SD of triplicate measurements. SD are given when they exceed dimension of the symbols

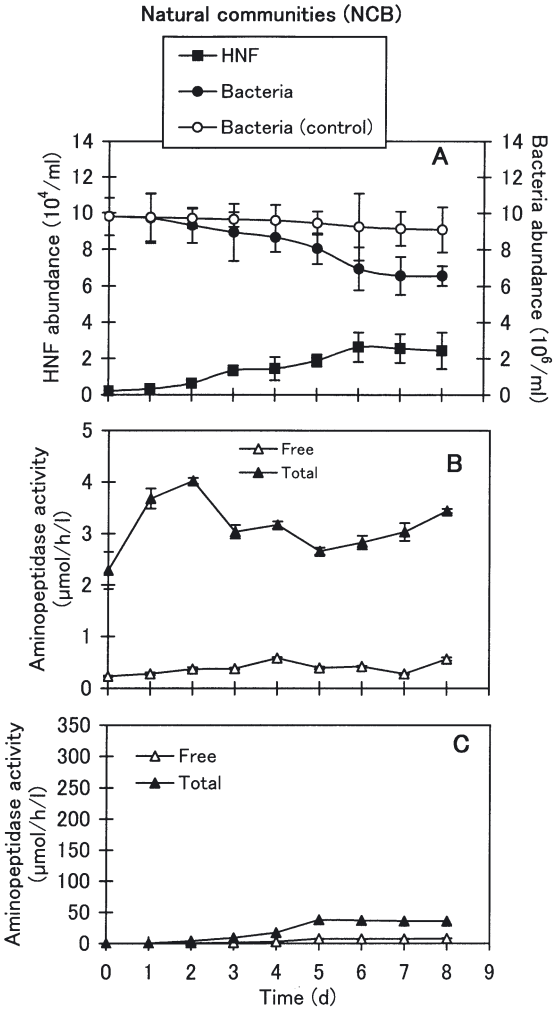

Fig. 5. Batch culture of heterotrophic nanoflagellates *Jakoba libera*-5(2) with natural communities of bacteria (NCB) as prey. (A) HNF and bacterial abundance, (B) free and total aminopeptidase activity of NCB, and (C) free and total aminopeptidase activity produced by HNF *J. libera*-5(2). Error bars are SD of triplicate measurements. SD are given when they exceed dimension of the symbols. Note that the enzyme activity has different scales in (B) and (C)

thophenanthroline) strongly inhibited activity, suggesting that aminopeptidase requires metal ions for the catalysis. This enzyme was also inhibited by thiol protease inhibitor (parachloromercuribenzoic acid, iodoacetic acid and iodoacetamide), indicating the involvement of a sulphhydryl group at the active site of the enzyme. Moreover, the pH profile of the enzyme and its inhibition by serine protease inhibitors (leupeptin and phenylmethylsulphonyl fluoride) indicate this enzyme to be an alkaline serine aminopeptidase.

DISCUSSION

Batch culture grazing experiments are the convenient way of examining HNF behavior in the presence of different prey organisms. They are relatively quick and simple, and they generate useful growth kinetic data to measure bacterivory (Fenchel 1982, Eccleston-Parry & Leadbeater 1994). The drawback of batch culture is that the prey bacteria are initially abundant and then their density can be rapidly decreased due to predation by HNF. However, these pulsed prey grazing experiments may be better mimics of the natural prey environment encountered by HNF than continuous culture experiments. Pelagic HNF encounter locally enriched patches of prey in the sea, more often in the form of marine snow (Azam & Long 2001). The ability of HNF to locate prey by chemosensing (Sibbald et al. 1987) and to rapidly synthesize food vacuoles and increase cell volume when exposed to abundant prey appears to be an adaptation used to exploit temporary

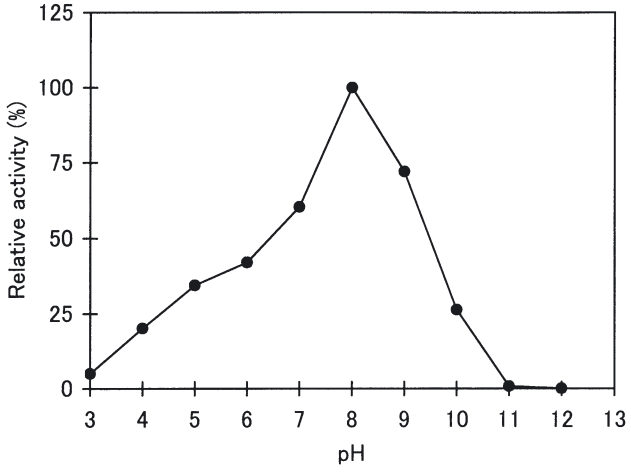

Fig. 6. Effect of pH on partially purified free aminopeptidase activity of *Jakoba libera*-5(2) with *Pseudomonas* sp. (Ps-B2) as prey bacteria. The buffers used were 10 mmol l^{-1} citrate (pH 3.0–6.0), phosphate (pH 7.0), Tris-HCl (8.0), borax-NaOH (pH 9.0–10.0) and glycine-NaOH (pH 11.0–12.0)

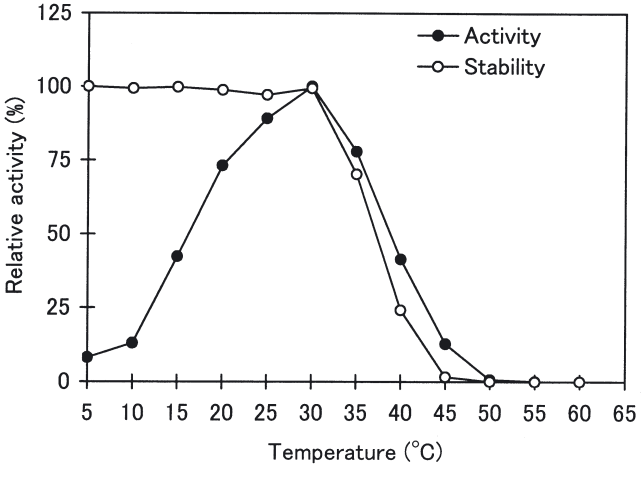

Fig. 7. Effect of temperature on the partially purified free aminopeptidase activity of *Jakoba libera*-5(2) with *Pseudomonas* sp. (Ps-B2) as prey bacteria

enriched microenvironments rather than a continuous steady-state prey supply.

Previous reports have suggested that the aminopeptidase activity, which was ubiquitous in marine environments and responsible for catalyzing the peptide bonds, was mostly bound to the bacterial cell membrane (Rosso & Azam 1987), and heterotrophic bacteria are usually the sole producers of it (Chróst 1990). As to the optimal activity (pH 8.0 to 9.0) (Fig. 6) and thermostability (below 30°C) (Fig. 7), the aminopeptidase of *Jakoba libera*-5(2) is able to function in the enzyme catalyzed organic matter cycling in marine environment. Furthermore, they might be responsible for the 'hyperproduction of enzyme' in organic aggregates,

Table 2. Effect of metal ions and inhibitors on aminopeptidase activity. The activity is expressed as a percentage of the activity in the absence of metal ion and inhibitor. The partially purified enzyme solution was pre-incubated with the individual metal ion and inhibitor at 20°C for 5 min. Separate blanks with individual metal ions and inhibitors were prepared. PCMB: para-chloromercuribenzoic acid. PMSF: phenylmethylsulphonyl fluoride

Metal ions and inhibitors	Concentration (mmol l^{-1}) 0.1	1	10
Ca^{2+}	100	100	100
Co^{2+}	43	22	13
Cu^{2+}	0	0	0
Hg^{2+}	0	0	0
Mg^{2+}	100	100	100
Mn^{2+}	83	47	22
Na^{+}	100	100	100
Zn^{2+}	56	43	30
EDTA	3	1	0
Iodoacetic acid	13	9	0
Iodoacetamide	21	11	6
Leupeptin	0	0	0
Orthophenanthroline	10	4	0
PCMB	1	0	0
PMSF	6	3	0

where they densely colonize, along with the attached bacteria (Cho & Azam 1988).

The functionally active hydrolytic enzymes are synthesized by HNF and secreted into pinocytosis vesicles or food vacuoles of HNF (Chróst 1990); release of extracellular enzymes from living HNF cells has also been reported (Nagata & Kirchman 1992b, Vrba et al. 1993, Simek et al. 1994). Although little is known about the enzyme production mechanisms of HNF, Nagata & Kirchman (1992b) hypothesized that HNF released digestive enzymes, which are embedded inside the partially digested bacterial cell walls, probably as a function of egestion. The ecological implication of these enzymes is still unknown. They have pH optima clearly different from bacterial prey mediated enzymes. Karner et al. (1994) observed the accumulation of aminopeptidase activity (pH optimum at pH of natural seawater) in a culture medium of natural communities of HNF fed on either live or heat-killed bacteria of natural assemblages of marine bacteria. However, our results showed that the release of aminopeptidase (both free and total) by *Jakoba libera*-5(2) as measured at pH 8.0 with a fluorogenic substrate can be greatly influenced by the prey bacteria. *J. libera*-5(2) produced aminopeptidase while grazing on *Pseudomonas* spp. (Ps-B2 and Ps-B6) (Figs. 2B & 3B), *Flavobacterium* sp. (Fl-B1) (Fig. 4B) and NCB (Fig. 5C). There was no enzyme activity detected with *Aeromonas* (Ae-B1), *Bacillus* (GPR-2) and Coryneforms (GPR-3).

Table 3. Net cellular production and ranges of total and free aminopeptidase activity of HNF *Jakoba libera*-5(2) fed with qualitatively different food bacteria. The net cellular production of HNF during the grazing experiment was determined by the difference between the highest and the initial HNF abundances. Each range represents the minimum and maximum of the enzyme activity that occurred during the batch culture grazing experiment

Bacteria	Net cellular production (10^4 HNF cells ml^{-1})	Range of total aminopeptidase activity (μmol h^{-1} l^{-1})	Range of free aminopeptidase activity (μmol h^{-1} l^{-1})
Ps-B2	9.63 ± 1.30	1.2–285	0.1–137
Ps-B6	6.41 ± 1.87	1.1–140	0.3–83
Fl-B1	4.0 ± 0.29	0.6–123	0.1–82
NCB	2.43 ± 0.083	0.1–38	0.04–8.6
Ae-B1	0.019 ± 0.005	0–0.4	0–0.2
GPR-2	0.017 ± 0.006	0–0.2	0–0.1
GPR-3	0.015 ± 0.0.005	0–0.3	0–0.1

The 6 qualitatively different prey bacteria used in this study did not produce aminopeptidase when axenically cultured in nutrient-depleted aged seawater and a nutrient-rich (FeTY and 0.8 μm culture filtrate of HNF) medium. Overall, the production mechanisms of aminopeptidase by aquatic bacteria are poorly understood because of the complexity of proteolytic enzymatic systems (Chróst 1991, Mohapatra et al. 2003). Chróst (1990) mentioned that some aquatic bacteria control the regulation of enzyme synthesis that largely depends on the physico-chemical conditions of the surrounding habitat. Non-detection of aminopeptidase activity in these 6 qualitatively different prey bacterial strains might be due to the repression of enzyme synthesis by chemical constituents of the cultivation medium, and/or the growth conditions used in this study.

The production rate of aminopeptidase of *Jakoba libera*-5(2) was directly proportional to the net cellular production of HNF (Table 3). These results indicate that among the different qualitative food bacterial strains tested, were the most palatable food for *J. liberia*-5(2) *Pseudomonas* spp. (Ps-B2 and Ps-B6), followed by *Flavobacterium* sp. (Fl-B1) and natural communities of bacteria (NCB). They did not like *Aeromonas* (Ae-B1), *Bacillus* (GPR-2) and Coryneforms (GPR-3) as food. When our results are compared with those of Karner et al. (1994), the variation in results might be attributed to the phenotypic traits of bacteria and HNF, and/or the environmental parameters. Earlier reports mostly presented data on the quantitative relationship of abundance, and/or bacterivory of HNF with extracellular enzyme activity of HNF (Vrba et al. 1993, Simek et al. 1994, Zubkov & Sleigh 1998). To our knowledge, this is the first report to correlate HNF enzyme activity with qualitative aspects of HNF grazing.

The role of alkaline serine aminopeptidase released by *Jakoba libera*-5(2) in feeding processes is still unknown, we speculate that because some HNF graze on attached bacteria (Caron 1987, Zubkov & Sleigh 2000) the secretion of aminopeptidase by HNF could be important in the production of dissolved free amino acids by hydrolysis of proteins from organic aggregates and detritus that may nurture the preferred bacterial growth (Nagata & Kirchman 1992a). In organic nutrient-depleted oceanic waters, perhaps certain individual amino acids, especially serine, or a combination of amino acids, which have been known as a potential attractant for bacteria (Adler 1966, 1975), produced during the hydrolysis of proteins in the water column may function as a chemical signal by the flagellate to attract and/or nurture the prey bacteria (Andersson et al. 1985). To understand fully the nature and properties of this enzyme in ecological processes, it is essential to isolate, purify and characterize it to a greater extent.

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