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Characterization of particulate proteins in Pacific surface waters

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Abstract

We investigated molecular characteristics of particulate proteins in Pacific surface waters using two-dimensional electrophoresis (2DE). Most proteinaceous materials estimated by dye-binding methods were characterized by the 2DE unresolved acidic materials with a broad range of molecular mass and the 2DE unresolved low molecular mass materials with a broad range of isoelectric point. The 2DE unresolved acidic and low molecular mass materials were considered to comprise peptides conjugated with acidic saccharides and degradation products (peptides) of proteins, respectively, which indicates that almost all proteins in living organisms failed to survive in detrital particulate organic matter (POM). Nevertheless, 23 discrete proteins were distinguished by the 2DE. Electrophoretic patterns of the discrete proteins indicated that they were a component of detrital POM. Three discrete proteins were subjected to N terminal amino acid sequence analysis. Two proteins out of three could not be determined because their N termini were blocked, and one protein was determined from the N terminus to the ninth amino acid residue. A homology search revealed that the N terminal amino acid sequence of the protein agreed completely with that of 70 kDa heat shock protein (HSP70) derived from photosynthetic organisms. HSP70 is a major member of the molecular chaperones that protect or repair proteins from damage under conditions of environmental stress. The occurrence of HSP70 in this study demonstrated that phytoplankton were able to induce the molecular chaperone(s). Clarification of factor(s) controlling induction of chaperones will enable us to assess the actual environmental stress on phytoplankton at the biomolecular level.

The particulate organic matter (POM) in surface water is a complex mixture of living biomass and nonliving detritus. The contribution of living biomass to the bulk POM is generally 1 order of magnitude lower than that of detrital organic matter (e.g., Volkman and Tanoue 2002). The particulate combined amino acids (PCAA) are the largest identified fraction in surface POM (e.g., Wakeham et al. 1997), and dye-binding colorimetric methods demonstrated that proteinaceous materials were a major component of POM (Setchell 1981; Long and Azam 1996). Phytoplankton is a primary producer of organic matter in the sea, and most combined amino acids in phytoplankton are in the form of proteins. However, heterotrophic processing may convert cellular proteins of phytoplankton to other forms before its incorporation into detrital pool. At present the chemical nature of the detrital combined amino acids and proteinaceous materials is not well documented, and the processes by which organic matter produced by phytoplankton is transferred to detrital POM are not clear. The characterization of proteinaceous materials in POM at the molecular level is essential to clarifying the dynamics of PCAA, their source, transfer processes, and degradation, in surface water.

Tanoue (1992, 1996) and Tanoue et al. (1996) examined the molecular mass distribution of particulate proteins in oceanic water columns with the use of one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE) and found that particulate proteins were classified into two groups, i.e., background proteins and specific proteins. The background proteins were not resolved by 1D SDS-PAGE, which suggests that they were made up of a large number of proteins of widely ranging molecular mass and that each was present at a relatively low level. The specific proteins that consisted of a small number of individual proteins were distributed over a limited range of molecular mass and were superposed on the background proteins. The specific proteins were recognizable in the POM from oligotrophic surface waters as well as deep waters because of a low level of background proteins, whereas the specific proteins in POM from productive surface waters were barely recognizable because of their high level of background proteins (Tanoue 1996). The inadequate resolution of 1D SDS-PAGE, however, hindered further survey of the variability, universality, and identification of specific proteins, as well as the actual chemical characteristics of background proteins. Apparently, a higher resolution technique for separating the particulate proteins was necessary for their further characterization.

Two-dimensional electrophoresis (2DE) is known as a powerful technique for separating individual proteins from a complex protein mixture (O'Farrell 1975). Proteins are separated according to isoelectric points by isoelectric focusing in the first dimension and according to molecular mass in the second dimension by SDS-PAGE. The 2DE technique has so far been applied only to lake sediment and

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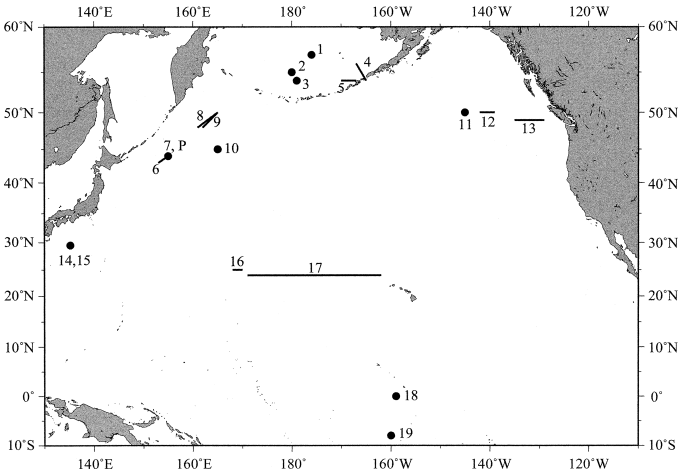

Fig. 1. Locations at which particulate matter in surface waters were sampled during the cruises of KH93-04, KH99-03, KT00-13, and SE01-02. Numbers indicate sample numbers of POM. P indicates a site where plankton sample was collected.

cultured diatoms (Nguyen and Harvey 1998), as well as separating proteins in foraminifera shells from marine sediment (Robbins and Brew 1990) by the geochemical community. We applied 2DE to separate particulate proteins from various oceanic surface waters and report here the source of one specific protein that was found to be widespread in the North Pacific. From the 2DE electrophoretograms of particulate proteins, we also discuss the chemical characteristics of proteinaceous materials in detrital POM.

Materials and methods

Sample collection—Samples of POM in surface waters were collected during four cruises on three vessels: KH93-04, KH99-03 (RV *Hakuho-maru*), KT00-13 (RV *Tansei-maru*), and SE01-02 (TS *Seisui-maru*; Fig. 1; Table 1). The surface waters were obtained by an underway pumping system from the hulls of the ships, and POM was collected onto GF/F glass fiber filters (Whatman, Maidstone) using a continuous filtration system (Tanoue 1996). Plankton samples were collected by vertical towing with a plankton net (Norpac NXX13 bolting cloth with 0.095-mm mesh) from 100 m depth to the surface during the KH99-03 cruise (Table 1) and harvested onto the GF/C glass fiber filter. All samples were kept frozen at −30°C aboard ship and lyophilized ashore. Lyophilized POM, as well as plankton on the surface of the filter, was peeled off carefully together with a small amount of glass fiber from the filter body and ground into powder with a mortar and pestle. The powdered samples were kept frozen at −20°C until analysis.

A marine bacterium strain, *Vibrio anguillarum*, that had been cultured in the laboratory was harvested by centrifugation (5,500 × g for 20 min), and samples were kept frozen at −80°C until analysis.

Table 1. Locations at which POM in surface waters were sampled.

Sample No.	Sampling location	Sampling date	Sampling volume (L)	Cruise
Bering Sea				
1	57 N, 176 W	5–6 Aug 1999	3201.2	KH99-03
2	55 N, 180 W	4–5 Jul 1999	1633.8	KH99-03
3	54 N, 179 E	9 Aug 1999	2708.6	KH99-03
4	56 N, 167 W–54 N, 165 W	7–8 Jul 1999	2209.8	KH99-03
5	54 N, 167 W–170 W	4–5 Aug 1999	1425.0	KH99-03
Northern north Pacific				
6	43 N, 153 E–44 N, 155 E	27 Jun 1999	5124.0	KH99-03
7	44 N, 155 E	16 Aug 1999	2144.5	KH99-03
8	48 N, 161 E–50 N, 165 E	30 Jun–1 Jul 1999	2826.6	KH99-03
9	50 N, 165 E–48 N, 162 E	15 Aug 1999	2780.8	KH99-03
10	45 N, 165 E	18 Oct 1993	1534.4	KH93-04
11	50 N, 145 W	18–19 Jul 1999	2346.8	KH99-03
12	49 N, 135 W–129 W	20–21 Jul 1999	2548.3	KH99-03
13	50 N, 139 W–142 W	31 Jul–1 Aug 1999	3119.0	KH99-03
Subtropical gyre				
14	29 30 N, 135 15 E	21–22 Apr 2001	4093.1	SE01-02
15	29 30 N, 135 15 E	18–19 Sep 2000	4869.5	KT00-13
16	25 N, 168 E–170 E	24–25 Oct 1993	5623.7	KH93-04
17	24 N, 171 E–22 N, 162 W	27–30 Oct 1993	*	KH93-04
Equatorial Pacific				
18	0 N, 159 W	16–17 Nov 1993	4457.1	KH93–04
19	8 S, 160 W	14–15 Nov 1993	5471.7	KH93–04
Plankton	44 N, 155 E	17 Aug 1999		KH99–03

* The sample volume was unknown.

Protein extraction—Procedures in this study based on the method by Tanoue (1996) were modified for 2D electrophoresis and are briefly described here. By use of a mortar and pestle, each powdered POM sample (about 0.5 g) was homogenized with the sample solution (5 ml) consisting of urea (9.8 mol L^{-1}, Sigma), Nonidet P-40 (2% v/v, Wako), carrier ampholytes (2% v/v Pharmalyte, pH 3–10, Pharmacia), dithiothreitol (DTT, 100 mmol L^{-1}, Wako), bromophenol blue (BPB, 0.01% w/v, Wako), and six protease inhibitors: L-*trans*-expoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) (0.5 μg ml^{-1}, Sigma), phenylmethylsulfonyl fluoride (PMSF, 0.5 mmol L^{-1}, Wako), *N*-tosyl-L-lysylchloromethyl ketone (TLCK, 0.5 mmol L^{-1}, Sigma), Aprotinin (1 μg ml^{-1}, Sigma), Chymostatin (10 μg ml^{-1}, Sigma), and ethylenediaminetetraacetic acid (EDTA, 0.5 mmol L^{-1}, Dojindo). The powdered natural plankton and cultured bacterium samples were also homogenized with the sample solution (1 ml). The solution was incubated at 37°C for 4 h to facilitate solubilization of proteins and then centrifuged at 3,000 × g for 30 min at room temperature. Proteins in the supernatant were desalted and purified by trichloroacetic acid (TCA, Wako) precipitation. TCA (100% w/v) was added to the supernatant to a final concentration of 20% (w/v), and the mixture was allowed to stand in a refrigerator for at least 12 h to precipitate proteins. The solution was centrifuged at 15,000 × g for 1 h at 4°C to remove TCA-soluble materials. The supernatant was discarded, and the TCA-insoluble fraction was washed with TCA solution (5% w/v) once, followed by washing with cold acetone (100%) three times and diethyl ether (100%) once. Finally, the pellet was dried in a vacuum. Unless otherwise noted, all reagents used were electrophoretic grade in this study.

Two-dimensional electrophoresis (2DE)—The dried pellet was redissolved in the above-mentioned sample solution, incubated at 37°C for 4 h, and subjected to 2DE. Before 2DE, the concentration of proteinaceous materials in the sample solution was quantified by the modified Bradford method with a dye reagent containing Coomassie Brilliant Blue-G250 (CBB, Bio-Rad) against bovine serum albumin (BSA, Wako) as a standard (Ramagli and Rodriguez 1985). To equalize sample loading to electrophoresis, about 100 μg or 1 mg of total proteinaceous materials was applied to 2DE, following silver staining or Coomassie Brilliant Blue-R250 (CBB-R, Bio-Rad) staining methods, respectively. A p*I* marker (carbamylated carbonic anhydrase, Carbamylyte calibration kit for 2D electrophoresis, Pharmacia) was added to the electrophoretic sample just before the start of electrophoresis.

Two-dimensional electrophoresis was carried out according to the modified method of O'Farrell (1975). The first-dimensional electrophoresis (isoelectric focusing), which used an immobilized pH gradient (IPG) strip (11 cm, a linear gradient between pH 3 and 10.5, Pharmacia), was carried out by the horizontal electrophoresis system, Multiphor II (Pharmacia) as described by Bjellqvist et al. (1982). Running conditions for the first dimensional separation were as follows. The IPG strip was allowed to run at 300 V for the initial 3 h. The electric voltage was linearly increased from 300 to 2,000 V during an additional 5 h, followed by running at 2,000 V for a further 8 h. All steps were run at 20°C. After the first dimension that separated proteins based on charge differences, the IPG strip was equilibrated with an equilibration buffer solution consisting of Tris-HCl (50 mmol L^{-1}, pH 6.8), urea (6.0 mol L^{-1}), SDS (3% w/v), DTT (50 mmol L^{-1}), and BPB (0.01% w/v) for 20 min at room temperature.

The second-dimensional SDS-PAGE was performed to separate proteins based on their molecular mass difference (Laemmli 1970). A handmade polyacrylamide slab gel (1 × 130 × 138 mm^3) containing 12.5% acrylamide, 0.33% *N*,*N*′-methylene-bisacrylamide, and 0.1% SDS in Tris-HCl (0.38 mol L^{-1}) buffer, pH 8.8, was used. Gel solutions were polymerized by the addition of 0.05% ammonium persulfate and 0.05% *N*,*N*,*N*′,*N*′-tetramethylenediamine. The electrode buffer solution was composed of Tris (25 mmol L^{-1})–glycine (129 mmol L^{-1}), pH 8.3, containing 0.1% SDS. SDS-PAGE without stacking gel was performed at a constant current of 10 mA until the dye (BPB) in the IPG strip was transferred to the polyacrylamide gel and thereafter at a constant current of 30 mA until the dye reached the bottom of the gel. Molecular mass markers (SDS-PAGE molecular weight standards, low range, Bio-Rad) were also separated simultaneously for reference: phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

After electrophoresis, proteins on the gel were stained by the silver staining or CBB-R staining methods. After the method of Oakley et al. (1980), silver staining was performed using a silver stain kit according to the manufacturer's instructions (2D-silver stain-II, Daiichi). CBB-R staining and destaining was also performed by the method of Laemmli (1970). No protein was detected in the control sample in which GF/F filters were used in the same manner as in the sample POM (data not shown). In this study, complete proteins as well as their subunits were defined as proteins.

N terminal sequence analysis—After 2DE, the proteins were transferred from polyacrylamide gel to polyvinylidene difluoride (PVDF) membranes (Fluortrans, Pall Ultrafine Filtration) by the electroblotting method according to Matsudaira (1987). The electroblotting was performed using a semidry blotting system (Nihon-Eido) at 0.5 mA cm^{-2} for 3 h at room temperature. The blotted membrane was stained with CBB-R (0.1% w/v) in methanol (50% v/v) for 2 min, destained with methanol (50% v/v) and acetic acid (10% v/v) for 5 min, washed with methanol (70% v/v) for 2 min, and then dried in a vacuum. The visualized spots of proteins on the PVDF membrane were cut out and transferred to a protein sequencer (Model 494, Perkin Elmer, Applied Biosystems Division). The sequencing was carried out according to the manufacturer's instruction manual. The phenylthiohydantoin (PTH)-amino acid that was generated by automated Edman degradation was identified using an online high-performance liquid chromatography system (model 785A, 140D, Perkin Elmer).

Results

Typical 2D electrophoretograms of particulate proteins, as well as a cultured bacterium and natural plankton samples, are shown in Fig. 2. The 2DE enabled us to separate more than a thousand proteins from a single strain of bacterium (e.g., O'Farrell 1975). Many protein spots were clearly distinguished in this study on the electrophoretograms of living specimens visualized by the silver staining method, i.e., over 500 proteins from a cultured bacterium (Fig. 2A) and about 100 proteins from the natural net plankton (Fig. 2B). An electrophoretogram of POM (Fig. 2C) visualized by the silver staining method showed a smeared staining pattern on an acidic area, but no spot was recognized because of the smeared and heavy background staining. Although the same amount (100 μg BSA equivalent by the dye-binding method) of proteinaceous materials was loaded in the respective electrophoreses, the unresolved background staining was significant in POM (Fig. 2C) but not in living specimens (Fig. 2A,B). The electrophoretogram of the POM sample (Fig. 2D), which was obtained by the identical electrophoretic sample seen in Fig. 2C, was visualized by the CBB-R staining method after a tenfold amount (1 mg BSA equivalent) of proteinaceous materials was loaded. Although 10 times as much sample was loaded, the background staining was at a relatively low level, and six discrete protein spots were visualized in Fig. 2D.

Using the CBB-R staining method, a limited number of discrete protein spots were visible and distinguished in all POM samples examined (Fig. 2D–H). Besides the discrete protein spots, two characteristic electrophoretic patterns were commonly observed on the POM electrophoretograms. One was the above-mentioned unresolved smeared background staining on the acidic region (p*I* range 4–7) with molecular mass ranging from more than 97 kDa to less than 14 kDa, which indicates that there were electrophoretically unresolved acidic materials in POM. This unresolved smeared staining was particularly significant on silver staining gel (Fig. 2C). Another was that the bottom of the gel was heavily stained with a broad p*I* range by the CBB-R staining method in POM samples, which indicates that low molecular mass (less than 10 kDa) materials with a broad p*I* range (3–10.5), particularly the 5–7 p*I* range, were enriched in oceanic surface POM.

Molecular masses and p*I*s of individual discrete protein spots in oceanic surface POM are listed in Table 2. The molecular mass and p*I* of a protein are its distinctive features. Given spots on different 2DE gels that have the same molecular masses and p*I*s are thought to be the identical protein species, provided that the electrophoretic patterns on the 2DE gels are similar to each other (Bjellqvist et al. 1994; Corbett et al. 1994; Wang et al. 2000). Since the electrophoretic patterns of particulate proteinaceous materials were very similar (Fig. 2D–H), it was concluded that protein spots showing the same molecular mass and p*I* in POM from different areas were the same protein species. In Table 2, protein spots in individual POM samples (vertical columns) that were identified to be of the same molecular mass and p*I* among the different POM samples were tentatively identified as the same protein species. The protein species identified in this study were wrapped up in the horizontal columns (1–23).

From 19 POM samples from the Bering Sea to the equatorial Pacific (Fig. 1), 23 discrete proteins were detected in the molecular mass range from 16 to 144 kDa and in the p*I* range from 4.1 to 8.6 (Table 2). Four discrete proteins (protein species 4, 6, 8, and 15) were universally detected from different oceanic regions. One protein (12) was detected in the northern North Pacific and Bering Sea, and two others (9 and 13) were found in the North Pacific subtropical gyre and equatorial Pacific. The numbers of protein spots detected in POM from the subtropical gyre and the Gulf of Alaska tended to be larger than those from the Bering Sea and the northern North Pacific, implying that more protein spots were observed in POM from the oligotrophic areas than from the productive areas.

In Fig. 2D–H, same amount (1 mg BSA equivalent) of electrophoretic sample was applied. In terms of the original filtered seawater equivalent, the amounts of sample loaded were larger in POM samples from the oligotrophic areas than those of the productive areas. For example, 140 and 102 liters of the original filtered seawater equivalent amounts of electrophoretic samples were loaded for POM samples from the productive areas (10 and 18 in Fig. 2E,F, respectively), while 235, 236, and 458 liters equivalent amounts of samples were used for samples from the oligotrophic areas (17, 16, and 15 in Fig. 2D, G, and H, respectively). The smeared background staining was stronger in samples from the productive areas than the oligotrophic areas, which indicates that the background staining materials were relatively rich in POM from the productive areas. There was a possibility that some protein spots were not distinguished in POM from the productive areas because of the high background intensity. However, loading of a small amount of sample resulted in weak staining intensity of the entire electrophoretograms and the numbers of protein spots did not increase but decreased in POM from the productive areas (data not shown). Since the interference of the background staining was not eliminated, the regional tendencies in the distributions of protein species and their numbers were not clear at present.

To characterize the discrete proteins, we tried to determine the amino acid sequence of protein spots. Although we encountered technical difficulties, i.e., differences in blotting efficiencies among the protein spots, interference of background staining materials, and sample limitation, three spots (protein species 4 and 9 in sample 16 and protein species 8 in sample 10 in Table 2) could be subjected to N terminal amino acid sequence analysis. Two proteins (species 4 and 8 in samples 16 and 10, respectively) were observed from various oceanic regions, and one (9 in sample 16) was detected from the subtropical and equatorial Pacific (Fig. 1, Table 2). Two (8–10 and 9–16) out of three spots could not be determined due to blocking of their N termini, and one spot (4–16) was successfully determined from the N terminus to the ninth amino acid residue (GKVVGIDLG).

The amino acid sequence was further subjected to a homology search with the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1997) through the DNA Data Bank of Japan (DDNJ) homology search (http://

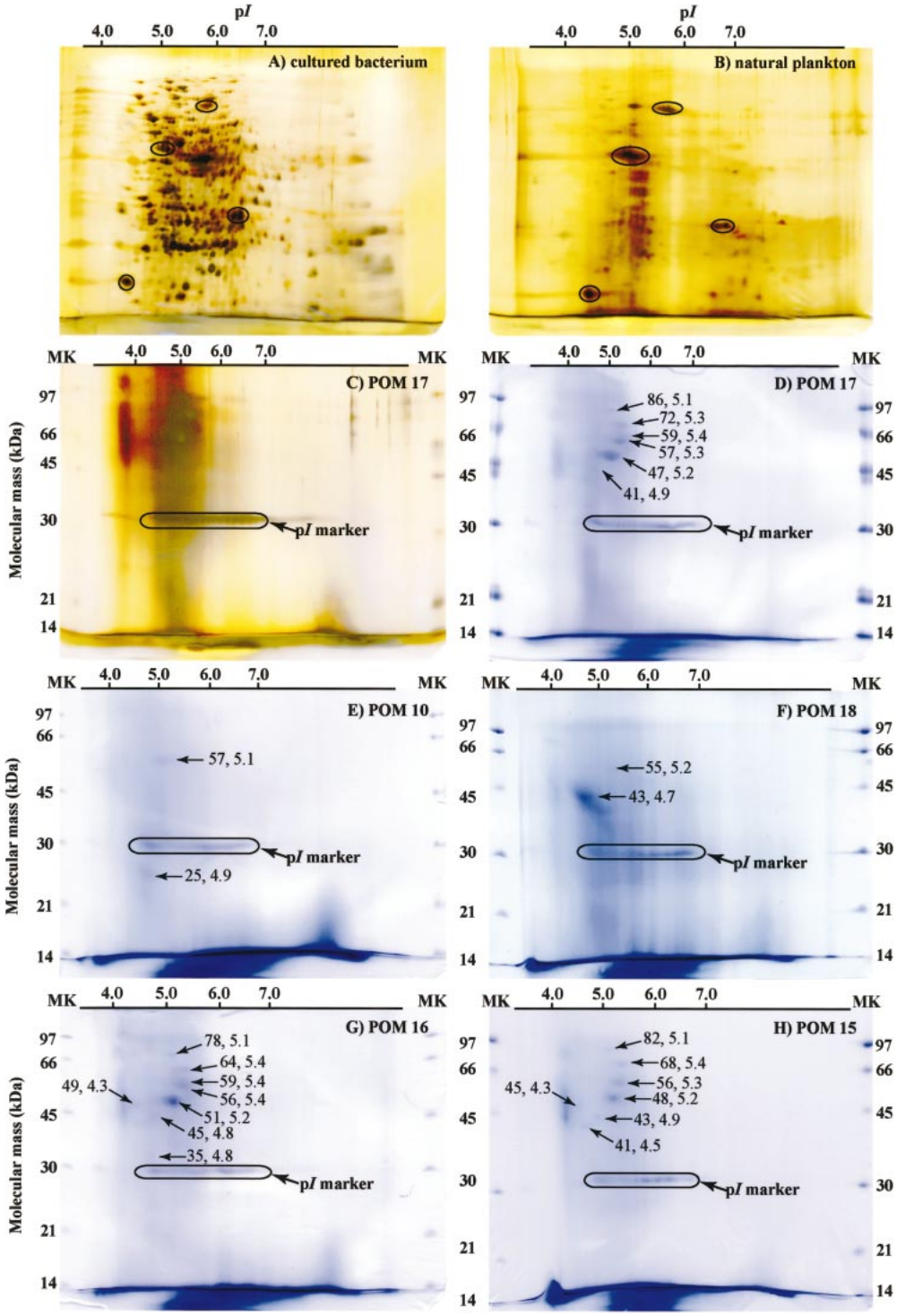

Fig. 2. Two-dimensional electrophoretograms of (A) a cultured bacterium, (B) natural plankton, and (C, D, E, F, G, and H) particulate proteins. Proteins on the gels were stained by silver staining (A, B, and C) and CBB-R staining (D, E, F, G, and H) methods. Individual protein spots are indicated by molecular mass/p*I* in electrophoretograms of particulate proteins (C, D, E, F, G, and H). Amount of samples in C, D, E, F, G, and H were equivalent to 12, 235, 140, 102, 236, and 458 liters of the original seawater equivalent, respectively. Numbers in parentheses represent sample numbers of POM. In two electrophoretograms (A and B), the internal molecular mass, p*I* markers (circled) constituted by serum albumin (66.2 kDa, 5.44), ovalbumin (45 kDa, 4.96), carbonic anhydrase (31 kDa, 6.46), and trypsin inhibitor (21.5 kDa, 4.42) were used. In six electrophoretograms (C, D, E, F, G, and H), carbamylated carbonic anhydrase (p*I* range 4.8–6.7) was used as the internal p*I* marker. The molecular mass markers (MK) on the left- and right-hand lanes were separated simultaneously at the second dimensional SDS-PAGE.

Table 2. Molecular masses and p*I*s (MM in kDa, P*I*) of discrete particulate proteins visualized by CBB-R staining method. Protein species in different POMs were tentatively identified as the same species from similarities of molecular masses, P*I*s, and 2-DE electrophoretic patterns. Figures in bold were subjected to N-terminal amino acid sequence determination. Horizontal broken lines are arbitrarily drawn for the reader's convenience.

P.S.*	Bering Sea					Northern north Pacific	
(S.N.)†	1	2	3	4	5	6	7
1 (144, 4.3)		144, 4.3					
2 (103, 5.8)					103, 5.8		
3 (97, 4.6)							
4 (78–88, 4.9–5.2)				83, 5.0		80, 5.1	
5 (65, 5.1)							
6 (63–72, 5.2–5.4)	66, 5.2		63, 5.3	64, 5.2		64, 5.2	66, 5.2
7 (56–59, 4.6–5.4)		56, 5.6					
8 (53–57, 5.1–5.5)	56, 5.2	53, 5.5	54, 5.2	55, 5.2		54, 5.2	55, 5.2
9 (47–52, 5.1–5.2)							
10 (45, 5.1)							
11 (43–49, 4.3–4.7)			48, 4.4				
12 (43–47, 5.3–5.8)	45, 5.4	43, 5.8	45, 5.4			45, 5.5	46, 5.4
13 (39–45, 4.8–5.1)							
14 (39, 4.8)							
15 (36–41, 4.5–5.3)		36, 5.1	39, 5.1	38, 5.1	39, 5.2	40, 5.2	38, 5.2
16 (37–38, 5.1–5.3)			37, 5.1		38, 5.3		
17 (32–35, 4.8–4.9)							
18 (34, 5.2)							
19 (25–28, 4.6–5.1)		26, 5.2	26, 4.8	26, 4.7	27, 4.7		27, 4.7
20 (24, 4.2–4.8)		24, 4.8			24, 4.2		
21 (20, 8.6)							
22 (16, 6.8)							
23 (16, 7.1)							
N.P.‡	3	7	7	5	5	5	5

* Protein species: numbers in parentheses are ranges of molecular masses and p*I*s.
† Sample number.
‡ Number of protein spots visualized by CBB-R staining method in each POM sample.

www.ddbj.nig.ac.jp/), which covered five amino acid sequence databases, i.e., Protein International Resource (PIR), SWISS-PROT, DDBJ Amino acid Sequence Database (DAD), the Proteins Data Bank sequence taken from the header (PDBSH), and Protein Research Foundation (PRF). The result is shown in Table 3. The N terminal amino acid sequence (GKVVGIDLG) of protein species 4 in sample 16 agreed (100% homology) with proteins collectively known as the 70 kDa heat shock protein (HSP70) reported from four freshwater cyanobacteria (*Anabaena* sp., *Anabaena variabilis*, *Gloeobacter violaceus*, *Nostoc linckia*, *Synechocystis* sp.), two marine cyanobacteria (*Prochlorococcus marinus*, *Synechococcus* sp.), chloroplasts of one marine cryptomonas (*Guillardia theta*), one marine diatom (*Odontella sinensis*), and two marine red algae (*Porphyra umbilicalis* and *Porphyra purpurea*).

Discussion

Two-dimensional electrophoresis unresolved acidic materials in surface POM—The amino acid sequence, as well as electrophoretograms of living bacterium (Fig. 2A) and natural mixed plankton (Fig. 2B), verified that the proteins in samples were separated at high resolution under the present 2DE conditions. Nevertheless, electrophoretograms of POM were characterized by unresolved acidic materials with a broad range of molecular mass (Fig. 2C), which suggests that the acidic materials might not be proteins able to be separated by 2DE (acidic materials, hereafter).

The results of 1D SDS-PAGE suggested that the specific proteins were superposed on the unresolved and smeared background proteins, which were thought to represent numerous cellular proteins directly derived from living organisms (Tanoue 1992, 1996; Tanoue et al. 1996). The acidic materials detected by 2DE were thought to correspond to the anticipated background proteins detected by 1D SDS-PAGE. The cellular proteins in living organisms had to distribute a broad range of p*I* region and be focused as spots; however, the background staining was only evident on the acidic region and showed smeared electrophoretic pattern on the 2DE gels. The 2DE in this study demonstrated that the anticipated background proteins were undetectable. Levels of individual proteins in living organisms might be far below the detection limit, in case a protein/peptide-specific but low-sensitivity CBB-R staining method was used (detection limit, 0.1 μg; Gygi et al. 2000). The individual proteins in living organisms were also not detected by the highly sensitive silver staining method (detection limit, 1 ng; Gygi et al. 2000)

Table 2. Extended.

Northern north Pacific						Subtropical gyre				Equatorial Pacific	
8	9	10	11	12	13	14	15	16	17	18	19
											97, 4.6
	80, 5.2		85, 4.9	88, 5.1		83, 5.1	82, 5.0	**78, 5.1**	86, 5.1		83, 5.1
											65, 5.1
66, 5.4	64, 5.4		64, 5.2	65, 5.3	65, 5.3	66, 5.4	68, 5.4	64, 5.4	72, 5.3		64, 5.4
								59, 5.4	59, 5.4		58, 4.6
56, 5.3	54, 5.3	**57, 5.1**	54, 5.2	55, 5.3	55, 5.3	55, 5.3	56, 5.3	56, 5.4	57, 5.3	55, 5.2	55, 5.4
						52, 5.1	48, 5.2	**51, 5.2**	47, 5.2		
						45, 5.1					
							45, 4.3	49, 4.3		43, 4.7	44, 4.6
				46, 5.3	47, 5.4						
						41, 4.9	43, 4.9	45, 4.8	41, 4.9		39, 5.1
				39, 4.8							
40, 5.2	37, 5.3		37, 5.0	38, 5.2	37, 5.3		41, 4.5				
						32, 4.9		35, 4.8			
				34, 5.2							
26, 4.9	25, 5.0	25, 4.9	26, 4.6			28, 5.1					
					20, 8.6						
				16, 6.8							
				16, 7.1							
4	5	2	5	9	5	8	7	8	6	2	8

because of the interference due to the unresolved acidic materials. In conclusion, there may not be a significant amount of previously defined background proteins directly derived from living organisms.

The two-dimensional electrophoretograms of lake sediments and decayed diatoms in the degradation experiment were also characterized by unresolved acidic materials with a broad range of molecular mass, and the staining intensities of unresolved acidic materials relatively increased with the progresses of degradation (Nguyen and Harvey 1998). The acidic materials were not recognized on electrophoretograms of living specimens (Fig. 2A,B), which suggests that the acidic materials occurred as detritus in POM. Long and Azam (1996) found that CBB-stained particles were similar in shape and size range to polysaccharide-containing transparent exopolymers. Their result implied that peptides contained in the transparent exopolymers were stained by CBB. In this study, lipid materials and saccharides, which did not conjugate to peptide chain, were eliminated during the TCA precipitation and during electrophoresis (Pitt-Rivers and Impiombato 1968), respectively. Although occurrences of carbohydrate moieties were not confirmed, indirect evidence implied that the acidic materials in this study were peptides conjugating with acidic saccharides.

Two-dimensional electrophoresis unresolved low molecular mass materials—Except for the disulfide bond, the sample preparation procedure for 2DE did not cleave any covalent bonds but instead destroyed noncovalent interactions among proteins (peptides) or between proteins (peptides) and other organic matter (Andrews 1986; Tanoue 1991). Since proteins in living organisms were resolved by the present 2DE condition (Fig. 2A,B), the low molecular mass materials were not directly derived from living organisms but were degradation products of proteins (peptides, hereafter).

The peptides did not comprise free amino acids and oligopeptides in POM sample because the CBB-R did not stain peptides of less than seven amino acid residues (Mayer et al. 1986). In the present SDS-PAGE condition, no molecular-sieve effect was exerted on the separation of peptides with molecular masses of less than approximately 10 kDa. Therefore, the peptides defined in this study might have peptide chains longer than seven amino acid residues, but their molecular masses were smaller than approximately 10 kDa. Exogenously added glycine was located on the front (bottom) of the gel when electrophoresis was terminated. Interestingly, the electrophoretic mobility of the peptides was faster than that of glycine (Fig. 2). Electrophoretic mobilities of such peptides did not depend on their molecular masses but on how much they were negatively charged. The individual charges of peptides are influenced by the binding of the SDS anion, which gives all molecules a negative charge (e.g., Pitt-Rivers and Impiombato 1968). The rapid electrophoretic mobility suggested the peptides had a high affinity for SDS.

Table 3. Proteins in databases that agreed (100% homology) with the amino acid sequence from N-terminus to ninth amino acid residue (GKVVGIDLG) of discrete protein (4–16) in detrital POM. Results of homology search carried out on 21 November 2003 are shown.

Protein	MM* (kDa)	p*I**	Organism	Taxon	Environment	Organelle	Sequence from
HSP70	67	4.7	*Odontella sinensis*	Diatom	marine	chloroplast	plastid
HSP70	68	4.8	*Porphyra umbthcalis* (strain Avonport)	Red alga	marine	chloroplast	plastid
HSP70	68	4.8	*Porphyra purpurea* (strain Avonport)	Red alga	marine	chloroplast	plastid
HSP70	68	5.0	*Gullardia theta* (*Cryptomonas* Phi)	Cryptomonas	marine	chloroplast	plastid
HSP70	68	5.0	*Gullardia theta* (*Cryptomonas* Phi)	Cryptomonas	marine	chloroplast	plastid
HSP70	77	5.0	*Anabaena* sp. (strain PCC 7120)	Cyanobacteria	freshwater		nucleotide
HSP70	77	5.0	*Anabaena* sp. (strain PCC 7120)	Cyanobacteria	freshwater		nucleotide
HSP70	77	5.0	*Anabaena variabilis* (strain PCC 7937)	Cyanobacteria	freshwater		nucleotide
HSP70	68	5.0	*Gloeobacter violaceus* (strain PCC7421)	Cyanobacteria	freshwater		nucleotide
HSP70	§	§	*Nostoc linckia*	Cyanobacteria	freshwater		—
HSP70	68	4.7	*Synechococcus* sp. (strain WH 8102)	Cyanobacteria	marine		nucleotide
HSP70	67	4.7	*Synechocystis* sp. (strain PCC 6803)	Cyanobacteria	freshwater		nucleotide
HSP70	67	4.7	*Synechocystis* sp. (strain PCC 6803)	Cyanobacteria	freshwater		nucleotide
HSP70	68	4.6	*Synechocystis* sp. (strain PCC 6803)	Cyanobacteria	freshwater		nucleotide
HSP70	68	4.8	*Prochlorococcus marinus* (strain SS120)	Cyanobacteria	marine		nucleotide
HSP70	68	4.8	*Prochlorococcus marinus* (strain MIT9313)	Cyanobacteria	marine		nucleotide
HSP70	68	4.8	*Prochlorococcus marinus* (strain MED4)	Cyanobacteria	marine		nucleotide

* Theoretical p*I* and MM was computed by the compute p*I*/Mw tool of ExPASy (expert protein analysis system: http://us.expasy.org/).

† All amino acid sequences in four databases were deduced from nucleotide (plastid) sequences. Methionine (initial codon) was N-terminus from the nucleotide sequencing (Reith and Munholland 1991) and the N-terminus of the mature HSP70 started at glycine (Tsugeki and Nishimura 1993).

‡ N-terminal amino acid sequence in this study agreed with 29 sequence data with 100% homology and agreed with 53 data with 88% homology (agreed with eight out of nine) in four databases. Among the data with 100% homology, identical protein was reported in different databases. Sequence data overlaps were combined.

§ Amino acid sequence was submitted to another database Q93N29(TrEMBL) and was deduced from the fragment of Dnak gene that encoded HSP70. MM and p*I* of the protein could not be computed.

‖ Amino acid sequence of discrete protein (4–16) agreed with HSP70 from N-terminus in two databases (DAD and SWISS-PROT), but identical sequence was also found from 19th amino acid residue in two databases (DAD and PIR).

The staining intensity of the CBB-R staining method was directly proportional to the amount of proteins on the gel (Tanoue 1992). The staining characteristics of POM (Fig. 2D–H) indicated that a majority of POM visualized by the CBB-R staining method comprised not proteins but peptides (less than 10 kDa) and above-mentioned acidic materials. It has been reported from dye-binding colorimetric estimations or histochemical observations that there were discrete proteinaceous particles (e.g., Setchell 1981; Mayer et al. 1986) and that proteinaceous materials were the major fraction in detrital POM (Long and Azam 1996). It was considered that almost all proteins in organisms did not survive but were degraded to peptides or acidic materials during the transfer process from organisms to detrital POM, and peptides and acidic materials may represent previously defined proteinaceous materials in detrital POM. The occurrences of the peptides and acidic materials in POM suggested that noncovalent associations among detrital organic materials might have played an important role for survival of the peptides and the acid materials in detrital POM in seawater. However, the actual mechanism by which acid materials and small-sized peptides did not transfer to DOM but remained as a constituent of detrital POM remains unclear.

Occurrence of discrete proteins and HSP70 in surface POM—The homology search is not restricted to the sequence from N terminus but looks for identical sequence in any parts of entire peptide chain of protein in the database. Nevertheless, the N terminal amino acid sequence in this study only agreed (100% homology) with eight HSP70s from N terminus to ninth amino acid residue (Table 3). HSPs are major members of the molecular chaperones that assist in the assembly, folding, and translocation of other proteins in biochemical processes inside the cells of organisms (Ellis 1987; Gething and Sambrook 1992). The sequence of the protein spot (4–16) only agreed with HSP70s from cyanobacteria and the chloroplasts of eukaryotic marine algae but did not agree with HSP70s of other organisms, which indicates that this protein was derived from the HSP70 of marine photosynthetic organisms widespread in the surface waters (Table 2).

A question was raised as to whether the HSP70 was derived from living phytoplankton or detritus in POM. The number of cellular proteins in living organisms is numerous, for example, about 200 protein spots were distinguished on 2DE gel on the thylakoid membrane fraction isolated from *Synechocystis* sp. (Wang et al. 2000). In the heat shock response experiments of two psychrotrophic yeasts isolated from Arctic environments, several HSPs including HSP70 were expressed but their relative abundances were not significant (Berg et al. 1987). The individual proteins of living organisms in POM presented far below the detection limit as mentioned above. Therefore, it was unlikely that only HSP70 was exceptionally induced at a detectable level, whereas other cellular proteins were not in living organisms, even if we assumed that a level of HSP70 significantly increased when plankton was subjected to environmental stress. The fact that the discrete proteins were detected by

Table 3. Extended.

Agree from†	Reference	Accession (database)‡
N-terminus	Kowallik et al. (1995)	S78277 (PIR); P49463 (SWISS-PROT); Z67753-40 (DAD)
N-terminus	Reith and Munholland (1991)	S19660 (PIR); X62240-1 (DAD); 1802278A (PRF)
N-terminus	Reith and Munholland (1995)	S73236 (PIR); P30723 (SWISS-PROT); U38804-129 (DAD)
N-terminus	Douglas and Penny (1999)	AF041468-109 (DAD); 2509453DX (PRF)
N-terminus	Wang and Lin (1991)	A41609 (PIR); P29215 (SWISS-PROT); 182418A (PRF)
N-terminus	Kaneko et al. (2001)	AG2179 (PIR); Q9ZEJ6 (SWISS-PROT); AP003591-178 (DAD)
N-terminus	Pohl direct submission	AJ132707-1 (DAD)
N-terminus	Pohl et al (1997)	O05714 (SWISS-PROT); Y13044-1 (DAD)
N-terminus	Nakamura et al. (2003)	AP006582-266 (DAD)
N-terminus	Dvornyk et al. direct submission	AF388880-1 (DAD)
N-terminus	Palenik et al. (2003)	BX569695-317 (DAD)
N-terminus	Chitnis and Nelson (1991)	C39025 (PIR); P22358 (SWISS-PROT); M57518-1 (DAD); 1703313C (PRF)
N-terminus	Kaneko et al. (1995)	D63999-42 (DAD); D90903-72 (DAD)‖
N-terminus	Kaneko et al. (1996)	S75209 (PIR)‖; P73098 (SWISS-PROT)
N-terminus	Dufresne et al. (2003)	AE017166-220 (DAD)
N-terminus	Rocap et al. (2003)	BX572101-275 (DAD)
N-terminus	Rocap et al. (2003)	BX572094-243 (DAD)

the 2DE and their number in the individual POM samples was six in average and nine in the maximum (Table 2) indicates that the discrete proteins occurred at a high level in terms of single proteins. These results suggested that the discrete proteins including HSP70 did not derive directly from living organisms but accumulated in POM as detrital proteins.

The HSP70 identified in this study survived from the N terminus to the ninth amino acid residue, and its molecular mass (78–88 kDa, Table 2) was similar or rather larger than those of HSP70s from the databases (Table 3), which indicates that the HSP70 in detrital POM might survive from N terminus to, probably, C terminus without much modification. The amino acid sequences of other two proteins could not be determined due to their blocked N termini. It has been well documented that many proteins have blocked N termini; consequently, Edman degradation does not proceed (e.g., Brown 1979; Tsugita et al. 1994; Kamo et al. 1995). The present results are not unusual. Occurrences of proteins blocking their N termini also indicated that the N termini of these proteins survived during the transfer and accumulation from in vivo proteins in source organism(s) to detrital POM.

The present results stand in sharp contrast to the results of dissolved proteins. The sequence analysis of 11 trials succeeded without exception on seven dissolved protein species with different molecular masses (Tanoue et al. 1995), including samples from the same sites (samples 10 and 16) used in this study (Fig. 1). Some of the dissolved proteins were derived from bacterial membrane proteins (Tanoue 1995; Suzuki et al. 1997; Yamada and Tanoue 2003), while HSP70 in surface POM was derived from photosynthetic organisms. It has been known that the blocked N terminus occurs with high frequency in proteins from eukaryotes but does not appear to be extensive in proteins from prokaryotic cells (Brown and Roberts 1976; Brown 1979; Bjellqvist et al. 1994; Cash 1998). The fact that the two proteins had blocked N termini suggested that they were a different type of protein from the dissolved proteins and that their source(s) might be different. This discussion, however, did not exclude the possibility that the discrete proteins in POM were derived from heterotrophic organisms, since some proteins from a virus (Narita 1958) to mammals reportedly had blocked N termini.

Factors controlling occurrence of discrete proteins including HSP70 in surface POM—Only 23 protein species were distinguished as discrete proteins in detrital POM throughout the samples (Table 2), and there were about six discrete proteins on average in the individual samples. The question now arises as to why a limited number of discrete proteins survive in oceanic surface POM. At present, the source and chemical nature of the discrete proteins other than HSP70 are unknown. The nature of HSP70 might be useful as a clue to discuss mechanism(s) by which discrete proteins survived in detrital POM. A spatial variability in the occurrence of discrete proteins was evident, even if we assume that 23 protein species were correctly identified by 2DE (Table 2). No HSP70-like protein band was observed in the 1D SDS-PAGE electrophoretograms of POM from intermediate and deep waters (Tanoue 1992), although no data are available for the occurrence of HSP70 in POM below the euphotic zone. The distribution of discrete proteins implied that they were not necessarily refractory, while they were components of detrital POM. There must be some mechanism for survival and accumulation of particular proteins in detrital POM.

Bacterial membrane or its remnants were an important source of proteins and amino acids in dissolved organic matter (DOM; e.g., Tanoue et al. 1995; McCarthy et al. 1998; Dittmar et al. 2001). It was hypothesized that bacterial membrane materials might be protected against microbial attack (Nagata et al. 1998). HSP70 locates in the stroma of chloroplasts or cyanobacteria but is not a membrane protein (Tsugeki and Nishimura 1993). Occurrences of N termini

blocked proteins also suggested that source or sources of discrete proteins in POM might differ from that of dissolved proteins. The bacterial membrane hypothesis emerged from the studies of DOM may not be applicable to the mechanism of survival and accumulation of discrete proteins in detrital POM.

Yamada and Tanoue (2003) reported occurrences of dissolved glycoproteins and proposed a mechanism that dissolved glycoproteins formed macromolecular fabrications due to the glutinous nature of sugar chains in glycoproteins, and these structures facilitated the survival of dissolved biomolecules. HSP70 is apt to interact with other proteins/peptides and can also interact directly with fatty acids because of its nature as a molecular chaperone (Kiang and Tsokos 1998). HSP70s might acquire resistance to degradation by association with other materials in detrital POM. An association with other detrital materials in POM might play an important role in the survival of discrete proteins. As compared with the 2DE patterns of living specimens, discrete protein spots of POM tended to locate in high molecular mass and acidic regions, which indicates that discrete proteins were negatively charged in seawater environments. Chemical characteristics of individual discrete proteins including those ionic in nature might support such association. Apparently, chemical characterization is needed to clarify the mechanism of survival and accumulation of discrete proteins.

HSP levels are increased when a cell is subjected to stress, including heat shock or a change in ambient temperature, exposure to free radicals, infection by pathogens, and tissue injuries (Kiang and Tsokos 1998). HSP70 (protein species 4) was widely detected in samples from subtropical areas (Fig. 1; Table 2). Phytoplankton in the subtropical area might be exposed to unfavorable environmental conditions (e.g., high temperature, strong irradiance, nutrient limitation) and be forced to adapt to acquire tolerance by inducing high levels of HSPs. Occurrences of HSP70 in the productive areas were spatially variable, which might reflect the temporal variability of environmental conditions in these areas. The spatial variability of HSP70 implies that HSP70 may be semilabile material, and the occurrence of HSP70 seems to be related to source intensity, although the mechanism of survival and accumulation is a key factor for occurrence of HSP70. At present it is not clear what factor(s) control the source intensity of HSP70.

The identification of HSP70 in this study demonstrated that phytoplankton induces a molecular chaperone. Further investigations are apparently needed to clarify what kind of phytoplankton induces HSP70, what kind of environmental factors actually give rise to stresses on phytoplankton physiology, and what kind of molecular chaperones other than HSP70 are induced by marine phytoplankton. Answering such questions will give us a tool to assess actual environmental stresses on phytoplankton and may reveal one of the controlling mechanisms of phytoplankton community dynamics at the biomolecular level.

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