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Characterization of the particulate protein in Pacific surface waters

by Eiichiro Tanoue[1]

ABSTRACT

The molecular characteristics of particulate protein in the surface waters along transects from 45N to 25S in the central Pacific are reported. The majority of particulate-combined amino acids (PCAA) was in the form of protein molecules in the samples from the northern North Pacific and Equatorial regions, namely, productive areas, while PCAA was mainly present as nonproteinaceous amino acid in the subtropical regions, namely, oligotrophic areas. Thus, it appears that the chemical form of PCAA, one of the major constituents of particulate organic matter (POM), varies meridionally.

Two characteristic groups of particulate protein were identified from meridional differences in their molecular distribution. The first group, derived directly from cellular proteins of living organisms, was made up of a large number of proteins, each present at a relatively low level, which gave smeared electrophoretograms and were considered to be "background" proteins. The background proteins contributed greatly to the total protein, as well as to PCAA, and they appeared to be readily remineralized. The second group included a small number of specific proteins with a limited range of molecular masses. This group was prevalent in oligotrophic areas, an indication that proteins from specific sources survive and accumulate as a consequence of their resistance to degradation.

A protein with an apparent molecular mass of 45 kilodaltons (kDa), a member of the second group, was commonly found at low latitudes and the partial N-terminal amino acid sequence indicated that the 45-kDa protein was a single protein species that has not previously been reported. Thus, a single and identifiable protein molecule appears to be very widespread at low latitudes.

1. Introduction

The particulate organic matter (POM) in surface water that is retained on a given filter is a mixture of the organic constituents of the living biomass and of nonliving detritus. The relative levels of living biomass and detritus change temporally and spatially and the estimations of living biomass in POM have received considerable attention (e.g., Holm-Hansen, 1969; Eppley *et al.,* 1977; Dortch and Packard, 1989; Cho and Azam, 1990; Kirchman *et al.,* 1993; Yanada and Maita, 1995).

Particulate combined amino acids (PCAA), which are liberated by the hydrolysis of macromolecules, represent the largest identified fractions of POM in oceanic surface waters (e.g., Tanoue *et al.,* 1982; Sharp, 1983; Tanoue, 1985). Protein is the major cellular constituent of phytoplankton and 85% of phytoplankton nitrogen is in the form of protein

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(e.g., Billen, 1984). Cellular proteins in living organisms might be transformed into the detrital "combined amino acids" or "proteinaceous compounds" through biogeochemical processes. The PCAA in surface waters represent a mixture of cellular proteins of organisms and of detrital combined amino acids. The dynamics of the two types of PCAA are expected to be quite different. However, they have not been distinguished from each other in previous studies of PCAA.

Direct estimations of particulate protein have been taken by fluorometric and colorimetric methods, such as Lowry's method (Parsons and Strickland, 1962; Clayton *et al.*, 1988), the biuret reaction (Danilenko, 1977), the ninhydrin method (Handa *et al.*, 1972), the fluorescamine assay (Packard and Dortch, 1975; Garfield *et al.*, 1979), the Coomassie blue assay (Setchell, 1981) and the bicinchoninic acid assay (Nguyen and Harvey, 1994). Such methods are convenient, rapid and suitable for analysis of multiple samples. However, they provide limited information about the nature and molecular characteristics of proteins and do not give any clues that allow us to trace the processes of transformation of cellular proteins to detrital-combined amino acids in POM. Thus, to date, the chemical form of PCAA has not been well documented.

It has been reported that about 97% of the cellular material of living organisms consists of macromolecules and three groups can be distinguished: lipids; periodic molecules, such as peptidoglycans and polysaccharides; and informational macromolecules, such as nucleic acids and proteins (Gottschalk, 1986; Chróst, 1991). During the transformation of organic constituents of the living biomass to those of the detritus, such macromolecules are subjected to the activities of autolytic enzymes of the organisms themselves, with successive degradation through higher and lower trophic levels. Since the first stage of the degradation of macromolecules is dependent on enzyme-substrate relationships, such alterations must be strongly controlled by the nature of the macromolecules (Chróst, 1991; Billen, 1991). It is, therefore, essential to obtain information at the macromolecular level about marine organic matter for a better understanding of the dynamics of the organic matter and its fate in the sea.

Tanoue (1991, 1992) used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to analyze particulate protein in the water columns of the northern North Pacific and demonstrated that large numbers of proteins with widely ranging molecular masses are present at relatively low levels in POM from the surface waters, while specific proteins with a limited range of molecular masses make up the major protein species in POM from intermediate and deep waters. The very different electrophoretic patterns suggested that the chemical form of PCAA differs through the water column. Tanoue *et al.* (1995) and Tanoue (1995) revealed that a relatively limited number of protein species can be detected as major proteins in the dissolved phase in the water columns in the Pacific. Despite the fact that the marine biological community produces a vast number of different proteins, there is an indication that particular proteins survive selectively and accumulate as a consequence of their specific nature, in POM as well as in DOM, in the water column.

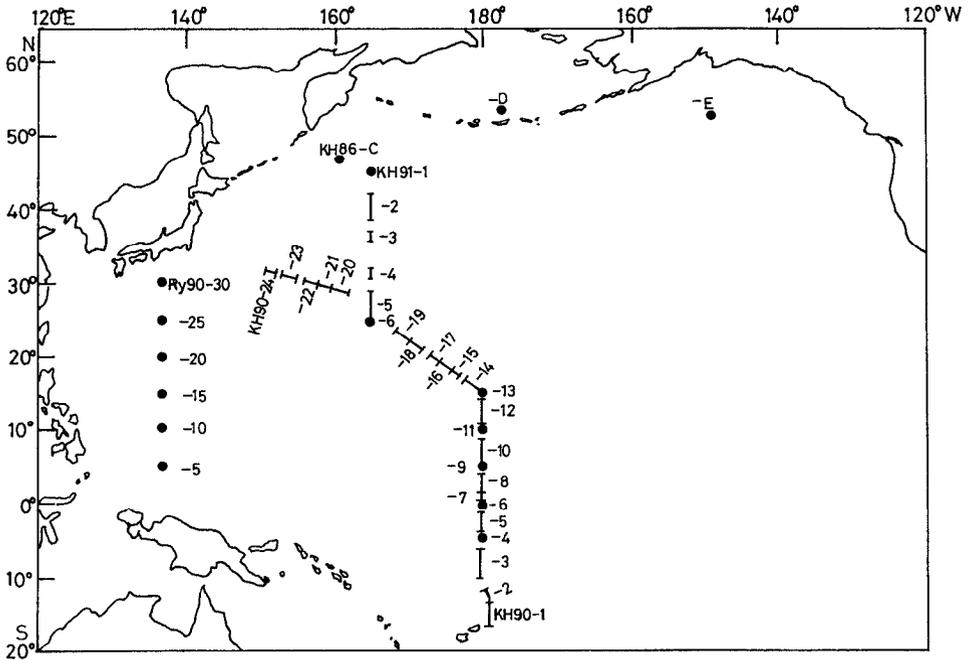In the present report, patterns of particulate proteins after PAGE, as well as concentra-

Figure 1. Locations at which particulate matter in surface waters was sampled during the cruises Ry90-01 (sample nos. Ry90-5 to Ry90-30), KH90-2 (KH90-1 to KH90-24) and KH91-3 (KH91-1 to KH91-6). Stations (KH86-C, -D and -E) in the northern North Pacific and in the Bering Sea for which SDS-PAGE patterns of particulate proteins were previously reported (KH86-D and -E; Tanoue, 1992) are also shown. Details are given in Table 1.

tions of particulate organic carbon (POC), particulate nitrogen (PN), PCAA and particulate protein, are provided for surface waters in a variety of oceanic regions from the northern North Pacific to the Equatorial Pacific. On the basis of the molecular characteristics of the particulate proteins, the chemical form of PCAA is discussed.

2. Materials and methods

a. Materials. POM was collected by the filtration of surface waters on GF/F glass fiber filters during the three cruises, namely, Ry90-01, KH90-2 and KH91-3 (Fig. 1, Table 1). Surface seawater was collected by a submersible pump at six stations along 137E and POM was collected on GF/F glass fiber filters (150 mm in diameter; Whatman, Maidstone, U.K.) under a hydraulic pressure of ca. 150 mm Hg during the cruise of M. S. *Ryofu-maru* (Ry90-01) of the Japan Meterological Agency, from January 22, 1990 to January 31, 1990. During the two cruises of R. V. *Hakuho-maru* (Ocean Research Institute, University of Tokyo) from September 3, 1990, to October 25, 1990 (KH90-2), and from May 9, 1991, to June 8, 1991 (KH91-3), seawater was pumped up from the bottom of the ship (about 5 m below the surface) and introduced into a tank (capacity, 20 liters) with a continuous

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

Sample no.	Range of locations	Sampling dates	Sample volume (l)
KH91-1	44°58'N,164°53'E–45°02'N,165°11'E	12–13 May 1991	281
KH91-2	42°40'N,165°07'E–38°39'N,165°06'E	16–17 May 1991	345
KH91-3	37°09'N,165°05'E–35°35'N,165°04'E	17–18 May 1991	415
KH91-4	32°22'N,165°02'E–29°26'N,165°08'E	18 May 1991	471
KH91-5	29°24'N,165°02'E–25°25'N,165°00'E	19 May 1991	506
KH91-6	24°59'N,164°59'E–24°59'N,165°00'E	19–20 May 1991	616
Ry90-30	30°00'N,137°00'E	22 January 1990	160
Ry90-25	25°00'N,137°00'E	23 January 1990	160
Ry90-20	20°06'N,137°00'E	25 January 1990	160
Ry90-15	15°01'N,137°00'E	26 January 1990	160
Ry90-10	10°03'N,137°00'E	30 January 1990	160
Ry90-5	05°04'N,137°00'E	31 January 1990	160
KH90-24	32°30'N,150°22'E–31°57'N,152°14'E	5 September 1990	550
KH90-23	31°46'N,152°51'E–30°48'N,155°56'E	5–6 September 1990	315
KH90-22	30°46'N,156°05'E–30°10'N,157°49'E	6 September 1990	1439
KH90-21	30°09'N,157°52'E–29°09'N,159°39'E	6 September 1990	491
KH90-20	29°32'N,159°42'E–28°34'N,162°10'E	6–7 September 1990	747
KH90-19	23°57'N,168°27'E–22°31'N,170°21'E	8–9 September 1990	570
KH90-18	22°29'N,170°23'E–21°14'N,172°01'E	9 September	510
KH90-17	20°35'N,172°51'E–19°13'N,174°38'E	9–10 September 1990	590
KH90-16	19°11'N,174°40'E–18°12'N,175°56'E	10 September 1990	534
KH90-15	18°11'N,176°40'E–16°52'N,177°39'E	10 September 1990	685
KH90-14	16°49'N,177°42'E–15°00'N,179°59'E	10–11 September 1990	749
KH90-13	15°00'N,179°59'E	11 September 1990	1385
KH90-12	14°48'N,180°00'E–10°40'N,180°00'E	12 September 1990	1420
KH90-11	10°37'N,180°00'E–09°34'N,179°54'E	12–13 September 1990	1476
KH90-10	09°31'N,179°54'E–05°02'N,180°00'E	13–14 September 1990	1804
KH90-9	05°06'N,180°00'E	14 September 1990	1782
KH90-8	04°44'N,179°59'E–01°46'N,180°00'E	14–15 September 1990	1603
KH90-7	01°44'N,180°00'E–00°00'N,179°59'E	15 September 1990	1133
KH90-6	00°01'N,179°54'E	15 September 1990	1340
KH90-5	00°29'S,179°59'E–05°01'S,179°59'E	16 September 1990	1759
KH90-4	05°00'S,179°58'E	16 September 1990	1343
KH90-3	05°51'S,179°58'E–10°01'S,179°59'E	17–18 September 1990	1127
KH90-2	10°01'S,179°59'W–13°04'S,179°00'W	18–19 September 1990	1512
KH90-1	13°48'S,179°01'W–17°46'S,178°49'W	19–20 September 1990	1083

overflow. Seawater from the tank was filtered through a GF/F glass fiber filter via a specially designed filtration apparatus with a filtration area of 285 × 420 mm^2 under a hydraulic pressure of ca. 40 mm Hg and POM was collected continuously from time to time, usually twice a day, during the cruises.

The particulate matter collected on GF/F filters was kept frozen at $-30°C$ on board ship after visible plankton had been removed by hand with forceps and it was lyophilized on

land as soon as possible. Lyophilized samples were stored in a deep freezer ($-60°C$) until analysis. Special attention was paid to ensure samples remained frozen at all times prior to analysis.

The surface seawater temperature (SST) and salinity were monitored during KH90-2 and SST, and salinity and *in situ* fluorescence were monitored during KH91-3 for the same surface water.

b. Methods. Procedures for analyzing particulate protein were reported previously (Tanoue, 1991, 1992) and are described here only briefly. Particulate protein was extracted with a sample buffer solution of Tris-HCl (pH 6.8, 62.5 mM), sodium dodecylsulfate (SDS; 2%, w/v), 2-mercaptoethanol (5%, v/v) and heated (100°C) for 3 min. After cooling, urea (8 M, final conc.) was added to facilitate solubilization of particulate protein. After centrifugation at 2,000 $\times$ g for 5 min at room temperature, the supernatant was adjusted to pH 6.8 and analyzed by SDS-PAGE. In the present study, the simplest possible procedures were employed, unless otherwise noted. Thus, no preconcentration, desalting or purification of particulate proteins in the electrophoretic samples was included to avoid a biased molecular distribution of particulate protein due to any pretreatment. A GF/F filter was treated in the same manner as the POM sample to serve as a control at the start and the end of the preparation of samples from each cruise and no protein was detected in the control samples.

SDS-PAGE and procedures for staining and destaining (Coomassie brilliant blue-R250, CBB-R staining method) were performed as described by Laemmli (1970), using ready-made continuous-gradient gels (5–20%; PAGEL℗, NPG-520 type; Atto, Tokyo) or hand-made discontinuous gels (Tanoue, 1992). The following standard proteins (Sigma, St. Louis, MO), with their molecular masses in kilodaltons (kDa), were used: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).

Prior to the analyses of POC and PN, the filter with POM was allowed to stand in a desiccator filled with HCl vapor for 6 hr to remove carbonate. Then, the filter was dried in an oven for 4 hr at 80°C. POC and PN were determined with a CHN-analyzer (model MTS-2; Yanaco, Kyoto, Japan).

The fluorometric method (fluorescamine method; Udenfriend *et al.,* 1972) was used for determination of PCAA after hydrolysis of combined amino acid with hydrochloric acid (6 M) at 105°C for 24 hr. After the hydrolysate was dried *in vacuo,* the residue was redissolved in borate buffer (pH 9.0) and the solution was centrifuged at 1,500 $\times$ g for 15 min. Fluorescamine reagent (0.01%, w/w, in dried acetone; Roche Diagnostics, Tokyo) was added to the supernatant and the reaction mixture was stirred efficiently on a Vortex. The intensity of fluorescence (excitation, 390 nm; emission, 480 nm) was calibrated with a standard solution of glycine.

Proteins on gels were quantified by the dye-elution method (Ball, 1986; Tanoue, 1991; 1992). After SDS-PAGE, staining and destaining, 5-mm sections of the gel were cut out.

Dye (CBB-R) was extracted with organic solvent from the dye-protein complex in the matrix of each piece of gel and absorbance of the dye was measured at 595 nm, with the extraction solution as the blank. Blank values obtained as mean values from 6–8 pieces of gels of similar size, which had been cut out from lanes loaded with sample buffer only on the same gel, were subtracted from experimental values. The total amount of protein on the gel was quantified by summing the amounts of protein in the individual pieces of gel, with bovine serum albumin (BSA) as the standard.

The reaction with fluorescamine results in products with different fluorescence yields with different amino acids and the reagent reacts with primary amines but not with proline or hydroxyproline, which are not primary amines (Udenfriend *et al.,* 1972). There are significant protein-to-protein variations in the efficiency of the formation of the dye-protein complexes (Pollard *et al.,* 1978; Ball, 1986; Tanoue, 1992). Therefore, it should be emphasized that amounts of PCAA and particulate protein reported herein are relative values.

The concentration and purity of the proteins of interest in the samples from gels after electrophoresis, prepared as mentioned above, were not sufficient for the analysis of N-terminal amino acid sequence because of the deliberate omission of preconcentration and purification steps. For preconcentration of samples for electrophoresis, we examined the utility of small-scale dialysis, a water-absorbing gel, a pressure-driven disposable ultrafiltration unit (Molcut-II®; Nihon Millipore, Tokyo) and precipitation with trichloro-acetic acid. However, such methods did not give satisfactory results because all the procedures resulted in concentration not only of the protein of interest but also of many other proteins, as well as of non-proteinaceous organic compounds, in POM.

For sequencing of N-terminal amino acids, the band of a protein of interest was extracted by passive diffusion from the gel after SDS-PAGE of a sample that had not been subjected to pretreatment. The bands of protein with the same electrophoretic mobility on multiple gels that had been loaded with multiple samples, collected from various areas, were cut out with a razor blade from the gels. The resulting pieces of gel that contained the band of the protein of interest were combined. For extraction of the protein from the gel matrix, the combined pieces of gel in a diluted solution (1/10) of sample buffer were allowed to stand first for three months in a refrigerator (4°C) and then at room temperature for three months, with occasional agitation. The protein in the diluted sample buffer was concentrated with a pressure-driven disposable ultrafiltration unit (Molcut-II®) with filter with a nominal molecular mass cut-off of 10 kDa (filter material, low protein-binding regenerated cellulose). The concentrated sample was subjected to electrophoresis and the protein in the gel was electroblotted onto a polyvinylidene difluoride membrane (PVDF membrane; Immobilon®, 0.45 µm pore size; Millipore, Bedford, MA) as described by Matsudaira (1987). The protein band on the PVDF membrane was sequenced with a sequencer (model 477A; Applied Biosystems, Foster City, CA, USA) that was equipped with a system for on-line reverse-phase HPLC of the phenylthiohydantoin (PTH) derivatives of amino acids that

were generated by automated Edman degradation. Detailed procedures for determination of sequences of N-terminal amino acids were reported by Tanoue *et al.* (1995).

3. Results

a. Distributions of POC, PN, PCAA and particulate protein. The meridional distributions of organic compounds in POM from surface waters collected during the two cruises are summarized in Figure 2. Meridional differences were evident in concentrations of POC, PN, PCAA and particulate protein along the transects. The concentrations of these materials were high in subarctic waters (north of 41N; Anma *et al.,* 1990), which are characterized by low SST and low salinity (Fig. 3). Concentrations of POC, PN, PCAA and particulate protein decreased rapidly toward the south, while both SST and salinity increased toward the south. The steep gradients in such concentrations in the area north of 34N correspond to the region of the Kuroshio extension (North Pacific Current; Kawai, 1972). The region is thought to be a transitional area between the Subarctic and the Subtropical regions (Masuzawa, 1972). A steep gradient of the intensity of *in situ* fluorescence was also recorded in this region (Fig. 3). The meridional gradients observed in the present study correspond to previous observations, in the North Pacific, of steep meridional gradients in the composition and biomass of phytoplankton and zooplankton species (Johnson and Brinton, 1963; McGowan and Williams, 1973; Ishizaka *et al.,* 1994), the *in situ* fluorescence of chlorophyll *a* (Chl *a;* Pak *et al.,* 1988) and the surface concentration of Chl *a* (Dickson and Wheeler, 1993). The correspondence between meridional changes in the concentrations of particulate protein, POC, PN, PCAA and the *in situ* fluorescence indicate that the amount of particulate protein in surface waters is closely associated with biological productivity.

In areas south of 34N, which correspond to the subtropical gyre, the concentrations of the various materials varied but did not show significant meridional variation, with the exception of values of C/N. C/N values were low, being less than 6, in the areas north of 34N and they increased to around 7 between approximately 30N and 18N. They appeared to decrease again to around 6 toward the south. In areas associated with high values of C/N, it appeared that particulate protein made a lower contribution to PCAA (protein/PCAA). Lower values of protein/PCAA were particularly significant in areas around 20N. Concentrations of POC, PN and particulate protein, as well as C/N values along the transect of 137E, were approximately the same to those along the transects of 165E and 180° (Fig. 4). The contributions of particulate protein to PCAA were a little higher than their counterparts in the subtropical gyre.

In the equatorial region, contributions of particulate protein to PCAA varied significantly even though concentrations of POC, PN, PCAA and protein did not show clear meridional differences in this region. The Equatorial Pacific, encompassed by a relatively oligotrophic and tropical environment, is the site of upwelling and, as a consequence, of highly productive areas (Betzer *et al.,* 1984; Chavez and Barber, 1987; Bender and McPhaden, 1990). The productivity of this area is meridional and zonal (e.g., Thomas,
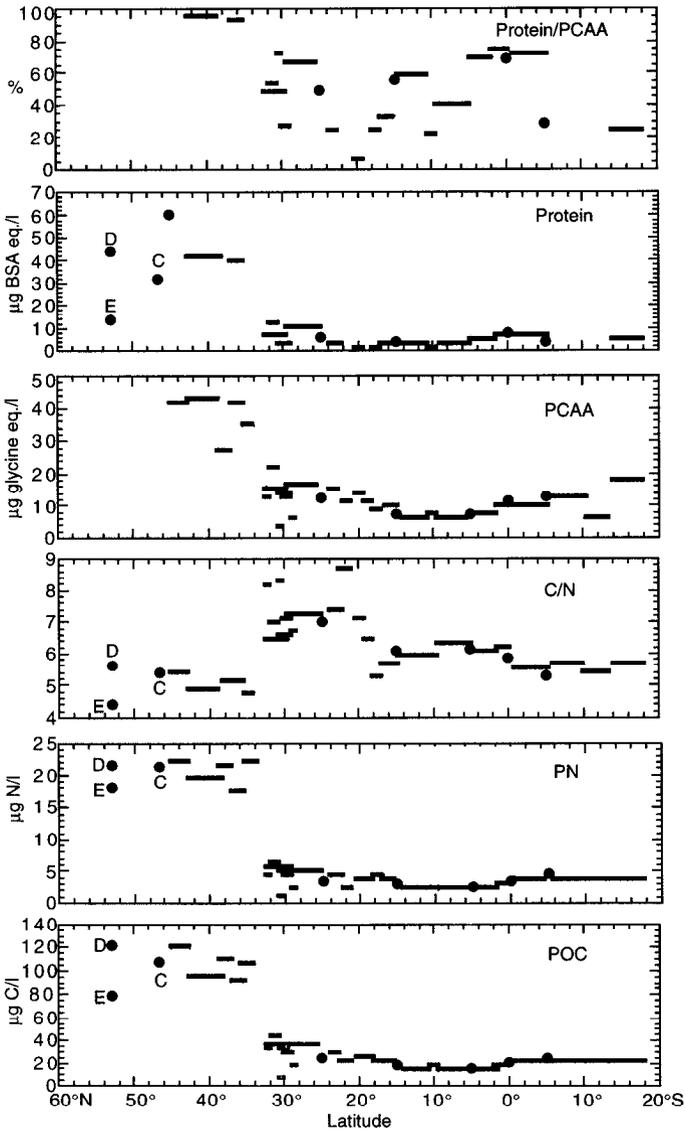
Figure 2. Meridional distributions of POC (µg C/l), PN (µg N/l), C/N (w/w), PCAA (µg relative to a glycine standard/l), protein (µg relative to a BSA standard/l) and protein/PCAA (%) along the cruise tracks of KH90-2 and KH91-3. Closed circles represent samples collected from fixed location (stations) and the bars represent the range of the locations of collected sample (see Fig. 1 and Table 1). Previously reported data (C, D and E) are included.
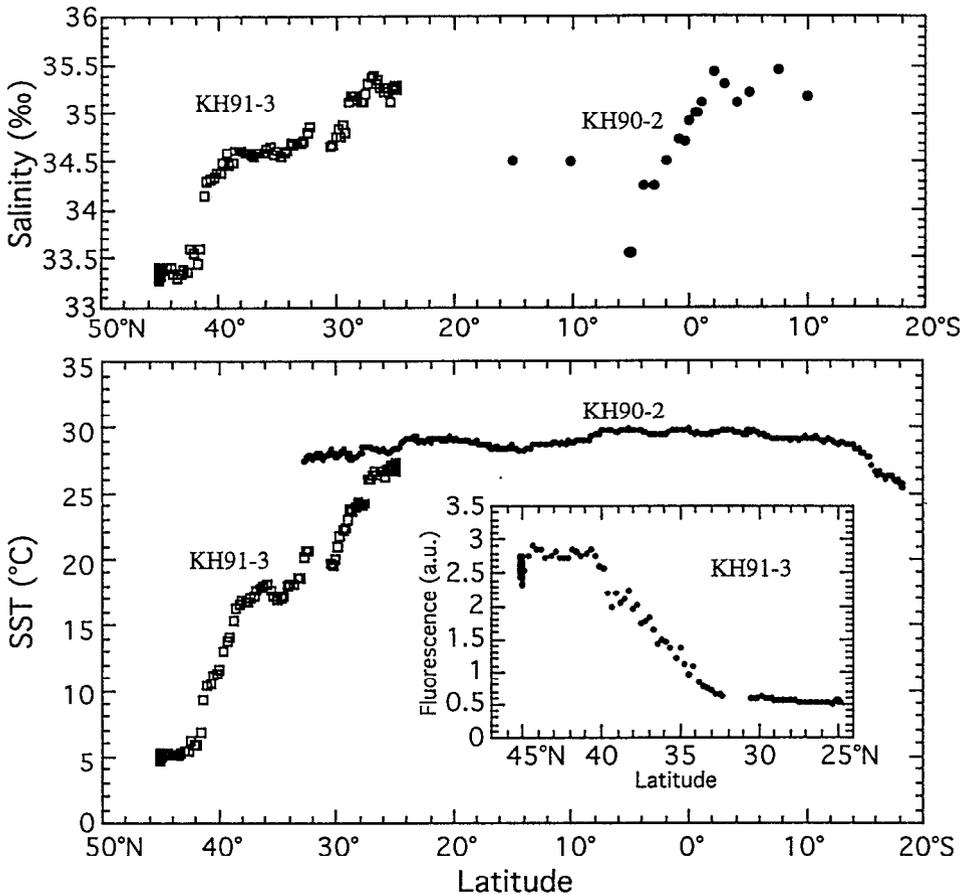
Figure 3. Salinity (%) and SST (°C) along 165E (KH91-3) and 180° (KH90-2) and *in situ* fluorescence intensity (arbitrary units, a.u.) along 165E.

1979; Wyrtki and Kilonsky, 1984; Bender and McPhaden, 1990). The area of the North Equatorial Counter Current (NECC), characterized by low salinity (Fig. 3) and extending from about 4N to about 10N (Philander, 1990), appeared to coincide with relatively low values of protein/PCAA (Fig. 2). The protein/PCAA values appeared to be high in areas of the North Equatorial Current (NEC) and the South Equatorial Current (SEC) that are located to the north and south of the NECC, respectively, at sites along the path of the westward-flowing surface current which is due to the upwelling in the east. The values of protein/PCAA decreased in the area south of the SEC, namely, the subtropical region of the South Pacific. Similar patterns of levels of dissolved organic carbon (DOC) and pCO_2 were observed on the same cruise (Tanoue, 1993; Ishii and Inoue, 1995), with both parameters being lower in the area of the NECC than those in the NEC and in the SEC, indicating relatively low biological productivity in areas of the NECC. Contributions of particulate
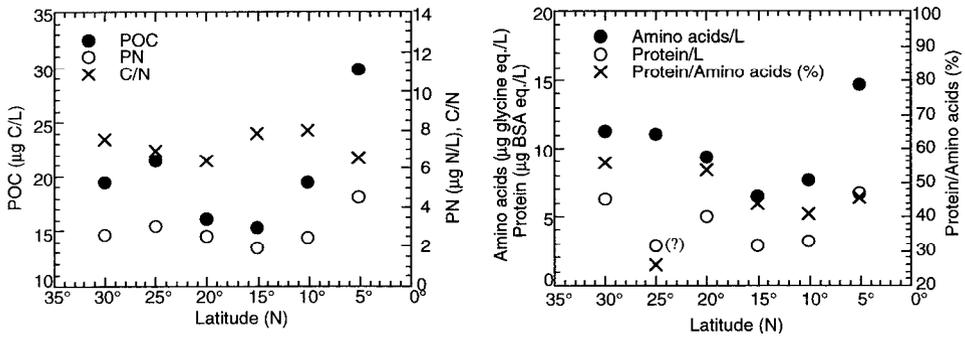
Figure 4. Concentrations of POC (μg C/l), PN (μg N/l), PCAA (μg relative to a glycine standard/l) and particulate proteins (μg relative to a BSA standard/l), and C/N and Protein/PCAA (%) along 137E (Ry90-01).

protein to PCAA were positively correlated with the primary productivity of the respective water masses.

b. Molecular masses of particulate proteins. The electrophoretograms of the particulate protein examined in the present study are shown in Figure 5. The particulate proteins in surface waters had a wide range of molecular masses. Proteins from 14 kDa to 66 kDa were separated by electrophoresis, and proteins were also present with molecular masses greater than 66 kDa and less than 14.3 kDa. In the case of the three northern samples along 165E (KH91-1, -2 and -3 in Fig. 1), the gel was stained heavily and uniformly in the region between 14 kDa and 66 kDa (Fig. 5a; KH91-1, -2 and -3). Significant amounts of low-molecular-mass proteins were concentrated at the migration front (bottom of the gel), but no clear bands of protein were visible. The electrophoretograms were similar to those obtained from samples of surface waters and of plankton (Fig. 5c, d and e) collected from more northern areas (C, D and E in Fig. 1).

On the electrophoretograms of the three southern samples along 165E (KH91-4, -5 and -6 in Fig. 5a), by contrast, distinct bands of individual proteins were observed over a limited range of molecular masses, superimposed on the smeared "background" proteins (Tanoue, 1992). The boundary between the waters that yield the two types of electropho-retogram was between the 30 and 35N along 165E (Figs. 1 and 5a). Several clear bands of protein were also visible with a limited range of molecular masses between 36 kDa and 66 kDa in samples collected from 30N to 5N along 137E (Fig. 5b). The POM collected over wide areas from about 18S to 32N along 180° in the central Pacific (Fig. 1) yielded electrophoretic patterns (Fig. 5f, g and h) that were essentially the same as those obtained along 137E and those of the three southern samples along 165E. Distinctive bands of individual proteins were found over a limited range of molecular masses between 36 kDa and 66 kDa, and they were superimposed on the smeared background proteins. Relative intensities of staining of individual bands of protein, as well as of background proteins, differed among the samples examined. For example, staining intensities were low and
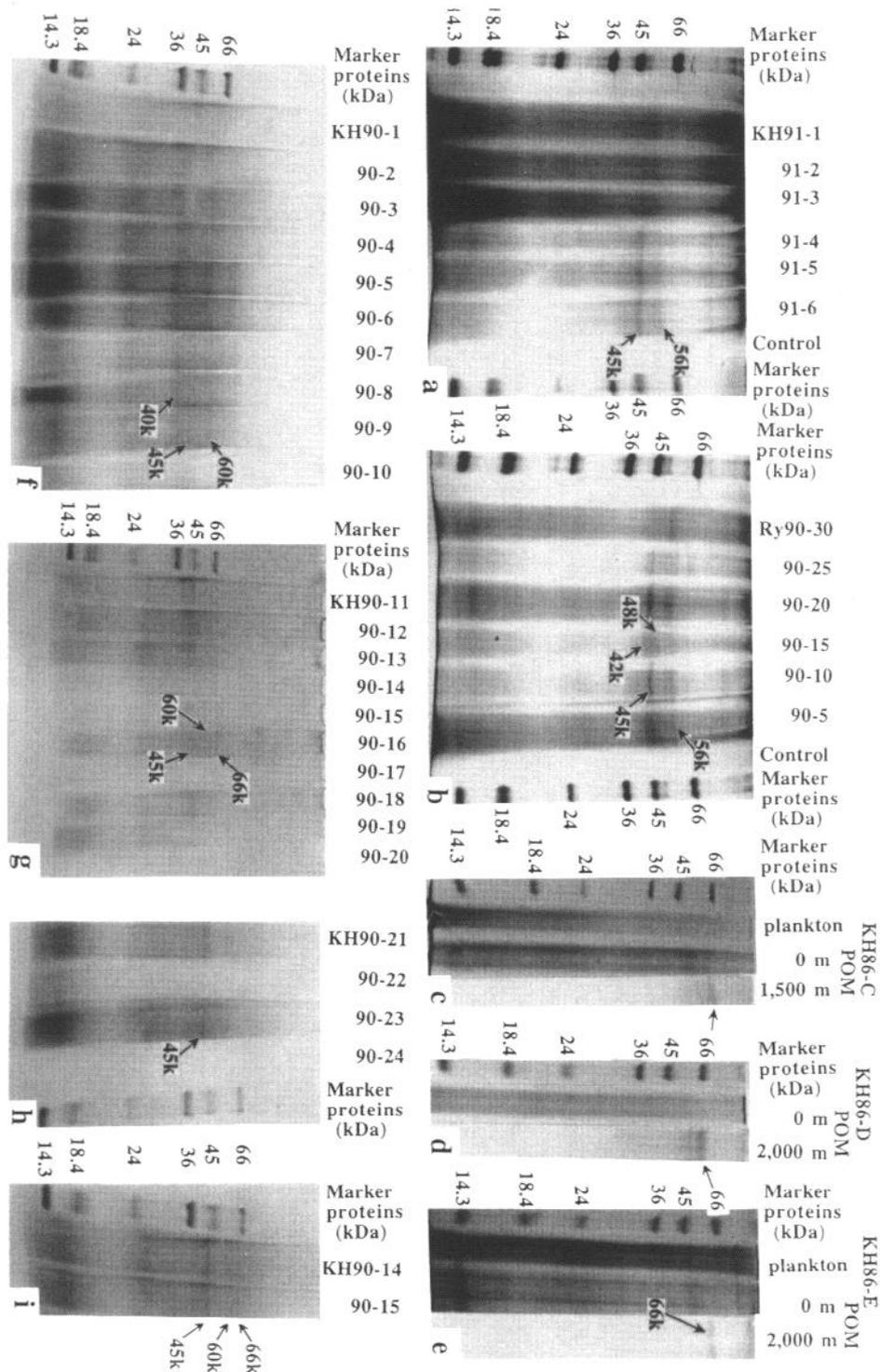
Figure 5. Electrophoretograms of particulate protein along 165E (a) 137E (b) and 180° (f, g, h and i). Electrophoretograms of mixed plankton and of POM from surface and deep waters at stations KH86-D and -E (Tanoue, 1992) and KH86-C in the northern North Pacific and Bering Sea are also shown in c, d and e. SDS-PAGE was performed with a hand-made discontinuous gel system, with 12.5% acrylamide for the separation gel and 2.5% acrylamide for the spacer gel for a, b, c, d and e. A ready-made continuous gel was used for f, g, h and i. Electrophoretograms obtained from samples that had been concentrated with a disposable ultrafiltration unit (Molcut-II™) are shown in i. Plankton samples were collected at each station with a Norpac net (Tanoue, 1992).
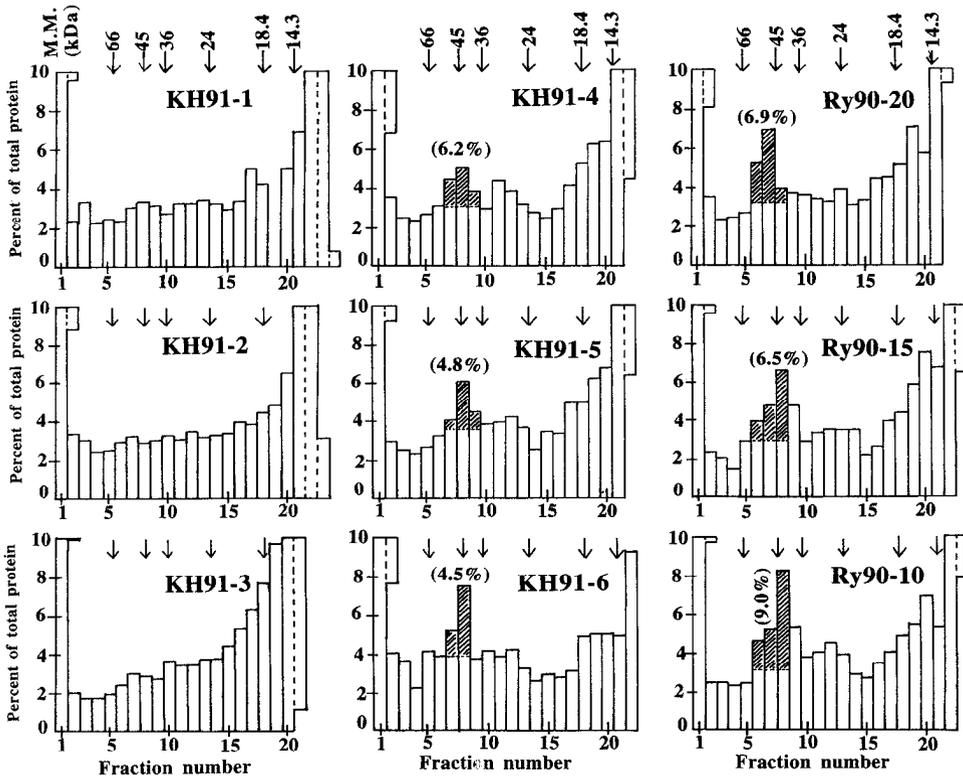
Figure 6. Relative abundance of the proteins in 5-mm pieces of gel for samples collected along 165E and 137E. Shaded fractions represent the contribution (%) of the proteins in the distinct bands to the total protein.

individual protein bands were unclear on electrophoretograms from KH90-14, -15, -22 and -24 (Fig. 5g and h), collected from the subtropical region. However, distinct bands were detectable in such samples after concentration of electrophoretic samples (KH90-14 and -15) with a pressure-driven disposable ultrafiltration unit (Molcut-II™; Fig. 5i).

Figure 6 shows the relative levels of proteins determined at 5-mm intervals on gels from samples along 165E and 137E. Proteins concentrated at the migration front in each case ranged from 9.3 to 22.7% of the total, but the relative abundance of proteins at the migration front decreased from north to south. In the separate pieces of gels that represented proteins of between 14.3 kDa and 66 kDa, each 5-mm section was relatively uniformly stained or was more heavily stained toward regions of higher mobility. No clear peaks were obtained from the three northern samples (KH91-1, -2 and -3), which yielded smeared electrophoretograms with unrecognizable distinct bands (Fig. 5a). In the three southern samples (KH91-4, -5 and -6), which yielded distinct bands, the relative abundances of proteins between 45 kDa and 66 kDa were a little higher than those of the higher-

and lower-mobility proteins. The peak identified from the separate gel fractions corresponded to the areas on gels where distinct bands had been visible. The relative abundance of proteins in each 5-mm gel fragment from the samples along 137E (Fig. 6) also revealed clear peaks in separate fractions between 45 kDa and 66 kDa (Fig. 5b).

To estimate the contributions of the proteins in these distinct bands to the total protein, a correction for background proteins was made because each fraction included background proteins. We assumed that the background proteins were uniformly distributed and that the amounts of the background proteins were equivalent to those elsewhere on the gel. The contributions of the proteins in the distinct bands to the total protein (shaded fractions in Fig. 6) were calculated to be in the range of 4.5–6.2% for the three southern samples along 165E and in the range of 6.5–9.0% along 137E, respectively. Since we analyzed the samples along 137E that gave the most distinct bands, the values are maximal. Thus, the amounts of specific proteins might not exceed 10% of the total particulate protein in surface water, even when distinct bands are clearly visible.

The resolution of electrophoretograms of POM was inadequate because we did not purify the particulate protein during the preparation of samples for electrophoresis. Inadequate purification of electrophoretic samples leads to errors in estimations of molecular masses (e.g., Tanoue, 1995). Thus, the molecular masses of the specific proteins were tentatively determined from simple comparisons of their electrophoretic mobilities with those of marker proteins on the same gel. Proteins with apparent molecular masses of approximately 56 kDa and 45 kDa were found to be the major specific proteins in the samples along the transects of 137E and 165E (Fig. 5a and b). Proteins with apparent molecular masses of 42 kDa and 48 kDa were also found in samples along 137E (Fig. 5b). Among the series of 24 samples along 180° (Fig. 5f, g and h), the 45-kDa protein was commonly found as one of the major specific proteins. A protein with an apparent molecular mass of approximately 60 kDa was also found in the majority of samples and a 66-kDa protein was found in the sample designated KH90-16.

The 45-kDa protein was found in all samples collected from the areas south of 34N during the three cruises along the transects of 165E, 137E and 180°. Fifteen electrophoretic samples, designated KH90-1, -2, -6, -7, -8, -9, -10, -11, -12, -13, -14, -16, -18, -20 and -22 (Fig. 5f, g and h), collected from 15S to 30N in the central Pacific, were subjected to electrophoresis in triplicate. Each band that represented the 45-kDa protein was cut out from the gel and the resultant 45 pieces of gel were pooled as one sample and the 45-kDa protein was extracted. Since the efficiency of extraction was extremely low in the preliminary experiments (data not shown), the pooled sample was prepared for repeated electrophoresis. Re-electrophoresis of the combined extract after a 6-month extraction period yielded a clear, sharp, single and reproducible band at the anticipated position after the second SDS-PAGE. Thus, we verified that the 45-kDa protein was a single protein species, as judged by SDS-PAGE. In this case, the background proteins were insignificant, and this protein was subjected to analysis of its N-terminal amino acid sequence analysis (Table 2). The first 12 residues from the N-terminus of the protein were identified. A single

Table 2. N-terminal amino acid sequence of the 45-kDa protein obtained by automated Edman degradation.

Cycle	1	2	3	4	5	6	7	8	9	10	11	12
45-kDa protein	Gly	Thr	Gln	Pro	Asn	Pro	Ser	Pro	Ala	Ser	Pro	Val

amino acid was detected at every step of Edman degradation, a result that indicated that the 45-kDa protein from samples collected over a wide area of the central Pacific was a single species. However, no similar N-terminal amino acid sequence was retrieved during a homology search of the Protein Identification Resource International database (PIR-International, version 39.0), made through the Japan International Protein Information Database (JIPID).

4. Discussion

a. Particulate protein and PCAA. The majority of the PCAA was in the form of protein molecules in the surface waters in the northern North Pacific and Equatorial regions, namely, in productive areas, while a small fraction the PCAA was in the form of protein molecules and the PCAA was mainly present as nonproteinaceous amino acids in the subtropical gyre, namely, in an oligotrophic area (Fig. 2). Amounts of nonproteinaceous amino acids, obtained by subtracting the amount of protein on a gel from that of PCAA on a filter, were relatively high in the subtropical region (data not shown).

Since the amounts of particulate protein or PCAA were relative values, as mentioned above, the contributions of particulate protein to PCAA were also relative values. The amino acid spectra of POM closely resemble those of the plankton, and the amino acid compositions of marine organisms are also very similar (e.g., Romankevich, 1984; Cowie and Hedges, 1992). Meridional differences in concentrations of PCAA might not be the result of differences in fluorescence yield due to variations in amino acid composition. Since more than 90% of the particulate protein was in a form of background protein, which consisted of a large number of proteins, each protein was apparently present at a low level (Figs. 5 and 6), and variations in protein composition might not give rise to meridional differences in concentrations of particulate protein as a consequence of differences in formation of dye-protein complexes. Thus, the relative changes in the contributions of particulate protein to PCAA with latitude seem unlikely to have been due to analytical uncertainties. However, the values determined in the present study are first-order approximations. At the present time, it is difficult to quantify proteins on gels after SDS-PAGE (Tanoue, 1991). A new approach that allows us to estimate particulate protein is required if we are to verify meridional difference in the contributions of particulate protein to PCAA (Fig. 2). Such an approach should provide important information about the chemical form of PCAA.

SDS-PAGE resolves proteins with large molecular masses. Small peptides are lost to some extent and amino acids are lost completely because these small compounds diffuse from the gel matrix during staining and destaining. Nonproteinaceous PCAA did not,

therefore, consist of large proteins but might have consisted of peptides and/or amino acids. Since oligopeptides and amino acids are minor components of living organisms, as mentioned above, and since the levels of nonproteinaceous PCAA were not correlated with those of POC, PN, *in situ* fluorescence and particulate protein, it seems unlikely that nonproteinaceous PCAA was derived directly from living organisms. Such small com-pounds were retained on the GF/F filter probably via their association with macromol-ecules that were large enough to be retained on the filter. Thus, it seems likely that the majority of the PCAA in oligotrophic areas might exist as oligopeptides and/or amino acids that have been incorporated into the polymeric detritus.

It has been demonstrated that monomeric organic compounds, including amino acids, bind abiologically to dissolved macromolecules or are transformed biologically into polymeric DOM (Carlson *et al.*, 1985; Brophy and Carlson, 1989). Authentic proteins have also been demonstrated to be transformed abiotically to refractory DOM in seawater (Keil and Kirchman, 1994). Laboratory experiments have focused on the processes of formation of refractory DOM from the labile amino acids and protein. Although no direct evidence that such processes occur in the POM has yet been reported, it is likely that such reactions could occur to transform cellular proteins, peptides and amino acids from living organisms into detrital combined amino acids. Peptides and amino acids that have been incorporated into macromolecules are known to be quite refractory to degradation (Carlson *et al.*, 1985; Brophy and Carlson, 1989; Keil and Kirchman, 1994), while the majority of cellular proteins in organisms is readily remineralized within hours or days (Hollibaugh and Azam, 1983; Coffin, 1989; Tupas and Koike, 1990; Keil and Kirchman, 1993). Thus, the dynamics of PCAA in surface water might vary meridionally. Namely, PCAA in subarctic waters might be readily remineralized because PCAA is mainly in the form of protein, while PCAA in subtropical waters might be inactive because the majority of PCAA is in the form of inanimate nonproteinaceous compounds.

b. Background proteins and specific proteins. As a consequence of the horizontal changes in the distribution of proteins, two different types of electrophoretogram were generated by samples from the areas north and south of 34N (Figs. 1 and 5). The geographic boundary between the two electrophoretic patterns coincided with the steep gradients in concentra-tions of particulate protein, POC, PN, PCAA and *in situ* fluorescence intensity (Fig. 2). In one case, only background proteins were seen on the electrophoretograms. The levels of background proteins were closely correlated with those of POC, PN, PCAA, *in situ* fluorescence intensity and particulate protein. The background proteins were distributed over a wide range of molecular masses and were evident in POM from the subarctic waters; they yielded smeared electrophoretograms. In the second case, specific proteins were distributed over a limited range of molecular masses between approximately 40 kDa and 66 kDa and were visible as bands on the gel, superimposed on relatively low levels of background proteins in the samples from the low-latitude areas where low levels of POC, PN, PCAA, *in situ* fluorescence intensity and particulate protein were recorded. Only six

specific proteins were recognizable as major specific proteins, as shown in Figure 5. Even in samples from the subtropical gyre, where background proteins were less heavily stained and no clear bands were found because of the very low levels of particulate proteins, specific proteins with molecular masses of 66 kDa, 60 kDa and 45 kDa were visualized upon concentration of the samples (Fig. 5i). We can conclude that the number of specific proteins is small but some of the specific proteins do occur in the POM from low-latitude areas.

The specific proteins did occur in the surface POM from subarctic waters, as indicated by the distinguishable bands of protein generated by such samples. For example, bands of protein with apparent molecular masses of 66 kDa and of around 45 kDa were visible but unclear due to the high levels of background proteins in surface samples from KH86-C and KH86-E, respectively (Fig. 5c and e). However, the background proteins represented quantitatively almost 100% of the particulate protein in the subarctic waters. Even in the samples from the subtropical gyre, in which the specific proteins were clearly detectable, background proteins accounted for more than 90% of the particulate protein (Fig. 6). The level of particulate protein in surface waters actually represents that of background proteins, regardless of the oceanic region.

The background proteins in POM gave smeared electrophoretograms and SDS-PAGE of entire organisms also yielded smeared electrophoretograms (Fig. 5c and e; O'Farrell, 1975). Moreover, levels of the background proteins decreased rapidly with depth, generally reaching an unrecognizable level in deep waters (Fig. 5c, d and e; Tanoue, 1992). Thus, the background proteins represent cellular proteins of living organisms and they are susceptible to biological degradation. Organisms retained on the GF/F filter were mainly a mixture of autotrophic phytoplankton and heterotrophic bacteria. Numbers of bacteria in surface waters at the same locations as those at which the POM samples designated KH91-1 and -6 had been collected were about $1.2–1.4 \times 10^6$ cells/ml (depths of 0–11 m) and about $0.2–0.7 \times 10^6$ cells/ml (depths of 0–10 m), respectively (Hara, personal comm.). The amounts of protein from the bacterial population were calculated to be 12–14 µg/l and 2–7 µg/l for samples from KH91-1 and -6, respectively, on the assumption that the amount of organic matter in a bacterial cell is twice that of carbon (20 fg C/cell; Lee and Fuhrman, 1987), that 50% of the organic matter is protein (Romankevich, 1984), and that 50% of bacteria were retained on the GF/F filter (Lee and Fuhrman, 1987). Concentrations of Chl. *a* were 0.7 µg/l (depths of 0–10 m) and 0.05 µg/l (depths of 0–10 m) at the sites of samples KH91-1 and -6, respectively. The amounts of protein from the phytoplankton population were calculated to be 21 µg/l and 1.5 µg/l for samples designated KH91-1 and -6, respectively, on the assumption that the amount of organic matter in phytoplankton is twice that of carbon while that of carbon is 50 times of that of Chl. *a* (Cho and Azam, 1990), that 30% of organic matter is protein (Mayzard and Martin, 1975), and that 100% of phytoplankton is retained on the GF/F filter regardless of the latitude (Chavez *et al.*, 1995). Although the electrophoretograms could not differentiate the sources of background proteins, the contribution of proteins from the phytoplankton population to the background

proteins appeared to be equal to or larger than that from the bacterial population in the sample designated KH91-1, from the subarctic region. In the sample KH91-6, from the subtropical region, bacterial proteins made a greater contribution than those from phytoplankton.

c. *Characteristics of specific proteins.* Among the clearly visualized proteins, a protein with an apparent molecular mass of approximately 45 kDa was commonly visualized as one of the major proteins in POM on the basin scale. The sample that was subjected to analysis of the N-terminal amino acid sequence was composed of multiple samples collected from 31N to 14S. Nevertheless, a single N-terminal amino acid sequence was obtained (Table 2). The fact that multiple samples gave a single N-terminal sequence indicates that the 45-kDa protein was 1) a true protein and 2) a single species. The results also indicate that the 45-kDa protein in multiple samples was in an identical chemical form, from the N-terminus to, probably, the C-terminus and that the protein survives without modification in surface POM. The present results are, moreover, consistent with results from other samples of surface seawater collected from the equatorial area (Java Sea), through the Indian Ocean, to the Antarctic Ocean (Tanoue *et al.,* 1996). It appears that the 45-kDa protein, a single species of protein, is distributed in low-latitude areas on a basin scale.

The specific proteins were clearly evident in oligotrophic areas and appeared not to be correlated directly with primary production. These specific proteins were minor components of the particulate protein. It is noteworthy that the PCAA was of three main types, namely, background proteins, specific proteins and nonproteinaceous compounds, in terms of its dynamics.

It was reported previously that the level of background proteins decreased generally to an unrecognizable level in POM from the intermediate and deep waters and that specific proteins with a limited range of molecular masses, between 45 kDa and 66 kDa, were major protein constituents of the POM (Fig. 5c, d and e; Tanoue, 1992). Tanoue (1995) extracted dissolved proteins from seawater and found out that a limited number of protein species accounted for the majority of dissolved protein components. Such observations led to the proposal of a mechanism whereby selected protein species from marine organisms are transferred to DOM and accumulate by virtue of the nature of the proteins (Tanoue, 1995; Tanoue *et al.,* 1995). The present observations indicate that such a proposal is consistent with and applicable to the dynamics of particulate protein in surface waters on a basin scale. In terms of the details, however, more complex processes must be considered. For example, the 45-kDa protein that was identified in the POM on a basin scale from subtropical and tropical surface waters was not found in POM from deep waters at Stations C, D and E (Figs. 1 and 5c, d and e) where proteins with molecular masses of approximately 66 kDa were predominant (Tanoue, 1992). A 66-kDa protein was also found in POM from surface waters (Fig. 5g and i). However, it is not known at the present time whether the two proteins were identical.

d. Sources of specific proteins. The 45-kDa protein had a characteristic N-terminal amino acid sequence (Table 2). Proline was found at the 4th, 6th, 8th and 11th positions among 12 amino acid residues from the N-terminus. Several proline-rich proteins that are associated with the actin cytoskeleton of eukaryotic cells have been reported (Donnelly *et al.,* 1993). However, no homologies were found between proteins of this type and the 45-kDa protein isolated in the present study. Since no similar sequence of N-terminal amino acids was retrieved from the database, the N-terminal amino acid sequence failed to provide any direct source-related information.

The 45-kDa protein found in the surface POM in the present study was not found as one of the major protein components in the dissolved phase. Fewer than thirty individual proteins accounted for the major dissolved protein components in the surface water (Tanoue, 1995) at a station where the POM sample designated KH91-1 was collected (Fig. 1). At the station where the POM sample designated KH91-6 was collected and where the 45-kDa protein was clearly visible on gels (Fig. 5a), two proteins with apparent molecular masses of 48 kDa and 37 kDa were the major dissolved protein components in surface water (Tanoue, 1995). The 48-kDa protein, one of the most common protein species in the dissolved phase, had an electrophoretic mobility close to that of the 45-kDa protein in the particulate phase in the present study. However, the N-terminal amino acid sequence of the 45-kDa protein in the present study (Table 2) differs from that of the 48-kDa protein in the dissolved phase (Tanoue *et al.,* 1995). It should be noted that a homology search is effective for any part of a polypeptide chain. Thus, no amino acid sequence similar to the N-terminal amino acid sequence of the 45-kDa protein was found in any part of the peptide chain of the 48-kDa protein in the dissolved phase and vice versa. Therefore, the 45-kDa protein in the surface POM and the 48-kDa protein in the dissolved phase appear to be different proteins. Six major dissolved protein species, other than the 48-kDa protein, having molecular masses of 40 kDa, 39 kDa, 37 kDa, 34 kDa and 30 kDa, were examined (Tanoue *et al.,* 1995) and each was different from the 45-kDa protein in the particulate phase.

The question now arises as to why the specific protein species differ between the particulate and dissolved phases. The levels of specific proteins in the particulate phase might be at least one order of magnitude lower than those of the major dissolved proteins. Amounts of samples KH91-4, -5 and -6 used for the detection of specific proteins were equivalent to 8.7–12 liters of original seawater, while the specific dissolved proteins in surface waters were detected from the equivalent of 1 liter of original seawater (Tanoue, 1995) at the same locations as those at which POM samples designated KH91-1 and -6 were collected. Since specific proteins in the particulate phase are minor components in the water column, it is possible that the 45-kDa protein was present in the dissolved phase but that it was present below the limit of detection when we examined the dissolved proteins.

The question also arises as to why the major dissolved protein species were not detected in the particulate phase. Differences in sources and in the physical states *in situ* of specific proteins between the dissolved and particulate phases provide possible explanations for the

differences in the molecular profiles of particulate and dissolved proteins. In the present study, complete denaturation and reduction of any disulfide bonds in the particulate proteins were accomplished by heating with SDS and 2-mercaptoethanol. Therefore, covalently linked polypeptide subunits were effectively separated and any disulfide bonds or other noncovalent bonds, such as ionic and hydrogen bonds and apolar interactions, were disrupted. Although we have no information about the possible oligomeric form of the 45-kDa protein in the particulate phase, the size (diameter) of the native form of the 45-kDa protein cannot exceed more than several dozen nanometers and must be less than 0.1 μm since the length of a fibrous protein, for example, myosin (molecular mass, 500 kDa) is less than 140 nm (Haschemeyer and Haschemeyer, 1973). Since the 45-kDa protein was retained on the GF/F filter (nominal pore diameter, 0.7 μm), the "native" 45-kDa protein *in situ* might interact noncovalently with other macromolecules that are large enough to be retained on a GF/F filter. By contrast, the major dissolved proteins might not interact with such macromolecules *in situ* and, thus, these proteins would pass through the GF/F filter during filtration.

The 48-kDa protein in the dissolved phase was identified as porin, a pore-forming channel protein in membranes of Gram-negative bacteria (Tanoue *et al.*, 1995). If the specific proteins, including the 45-kDa protein, in the particulate phase are similar proteins derived from bacteria, their behavior in the water column, as well as during the process of filtration, might be expected to be similar. Since the 45-kDa protein was only observed in the surface POM and not in POM from deep waters and, moreover, since the physical state of the 45-kDa protein might be different *in situ* from that of dissolved proteins, the 45-kDa protein in POM appears not to be similar to dissolved proteins but might be derived from a common organism that lives in surface waters in oligotrophic oceanic regions.

Largeau and co-workers demonstrated, through comparative chemical and morphological studies of fossil remains, that biomacromolecules of extant organisms are preserved without or with only minor transformation during the processes of sedimentation and diagenesis (e.g., Largeau *et al.*, 1986, 1991; Tegelaar *et al.*, 1989). Such biomacromolecules are selectively preserved because of their nonhydrolyzable and refractory nature, which is based on a network of long, saturated hydrocarbon chains, in the cell walls or protective envelopes of organisms (Largeau, 1995). Proteins are a different type of biomacromolecule altogether. The 45-kDa protein was denatured by SDS. Moreover, the 45-kDa protein, once denatured and transferred onto the blotting membrane after SDS-PAGE, released N-terminal amino acids upon Edman degradation. Thus, the persistence of the 45-kDa protein might not be explained simply by the "selective preservation" hypothesis. Laursen *et al.* (1996) demonstrated, from a biomimetic proteolytic enzymatic approach, that detrital POM in estuarine waters might be selectively enriched in membrane fragments of cells with associated proteins since lower rate constants for proteolysis were obtained for the membrane fraction than for the cytoplasmic fraction after the physical separation of subcellular fractions of phytoplankton cells. There are also indications that proteins in membranes or cell walls are assimilated insufficiently by protozoa, zooplankton

or deposit feeders (Nagata and Kirchman, 1992; Mayer *et al.,* 1995) because such proteins are protected from access by proteolytic enzymes (Walker and Fearnley, 1986).

Membrane proteins of organisms in surface water seem to be the probable source of the specific protein identified in the present study. This suggestion does not, however, exclude other possibilities. For example, a process whereby cytoplasmic proteins that have been incorporated into the detrital macromolecules become refractory to degradation might occur. Such processes have been proposed by Keil and Kirchman (1994) for the formation of refractory DOM, as mentioned above, and by Mayer and co-workers for the sedimentary pool of large peptides (>7–15 amino acid residues) that are protected by some kind of matrix (Mayer *et al.,* 1986, 1995).

It is quite clear that we have only just begun to understand the occurrence of discrete protein molecules in the sea. If we can trace the source of the 45-kDa protein we should gain information not only about the dynamics of particulate protein but also about the processes involved in the transfer of proteins from cellular components of organisms to inanimate pools of particulate and dissolved phases in the sea. Further information on the specific proteins observed in the present study is required to trace the processes of their production, transportation and decomposition and to characterize the relevant dynamics of biopolymers in the sea.

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