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Detection and partial characterization of dissolved glycoproteins in oceanic waters

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Abstract

The widespread occurrence of dissolved proteins as well as porin proteins was confirmed in water columns at five stations located in the North Pacific, the northern North Pacific, and the Bering Sea, where no investigation has heretofore been made with regard to dissolved protein. The major dissolved proteins detected in this study were determined to be glycosylated, based on the detection of an aldehyde group formed by the periodate oxidation of the electrophoretically separated dissolved proteins. The cross reactivity against lectins to the glycoproteins indicated that there were two types of linkage between sugar chain and polypeptide; one was an N-linked sugar chain and the other was an O-linked sugar chain. In this study, a previously reported dissolved protein with an apparent molecular weight of 40 kilodaltons was identified as an OmpA-like protein, not a porin protein but a major outer membrane protein of most pathogenic gram-negative bacteria. The survival of porin proteins in dissolved organic matter is thought to give rise to the resistant structure of these proteins; consequently, unidentified dissolved proteins could also be porin proteins. However, the occurrences of glycoproteins and an OmpA-like protein demonstrated that proteins other than porin proteins account for the majority of dissolved proteins, since no glycosylated porin protein is known. There must therefore be an additional mechanism by which dissolved proteins are protected from biological attack. The possibility that glycoprotein sugar chains can help preserve other dissolved proteins in seawater, as well as the glycoproteins themselves, is discussed herein.

Dissolved organic matter (DOM) in seawater plays an important role in marine ecosystems through the microbial loop (e.g., Azam 1998). Our knowledge of DOM has been rapidly increasing over the last decade (e.g., review by Ogawa and Tanoue [2003]); however, DOM is still the least understood organic reservoir in terms of its actual source, chemical nature, and formation processes.

The ultimate source of DOM in the sea is the organic matter produced by phytoplankton (e.g., Nagata 2000). However, microbial derived D-enantiomers of amino acids were detected from DOM greater than 1 kilodalton (kDa) (McCarthy et al. 1998) and from bulk DOM (Dittmar et al. 2001). Detection of methylated and *N*-acetyl aminosugars from DOM greater than 1 kDa indicated a bacterial contribution to dissolved carbohydrate in seawater (Boon et al. 1998). Laboratory experiments also demonstrated that microbial activity played a role in the formation and determination of the chemical composition of DOM (Meon and Kirchman 2001; Ogawa et al. 2001). Growing evidence strongly indicates that bacterial organic constituents form a part of DOM.

The occurrence of fewer than 30 dissolved proteins in oceanic seawater and the identification of porin proteins, which are trans–outer membrane channel proteins of gram-negative bacteria, provide direct evidence that bacterial macromolecules are a source of DOM greater than 10 kDa (Tanoue et al. 1995; Suzuki et al. 1997; Yamada et al. 2000). Many proteins in organisms are known to contain covalently bound sugars, termed glycoproteins, and their sugar chains have been shown to play a key function in complex cellular events (e.g., Winterburn and Phelps 1972). Glycoproteins are produced in marine environments; however, apart from a small number of porin homologue proteins, the chemical features, including glycosylation, of these dissolved proteins remain unknown. Plainly, further investigations are required to clarify the chemical nature and sources of dissolved proteins in seawater.

In this study, we examined the distributions of dissolved proteins in water columns in the east and west sides of the northern North Pacific, the Bering Sea, and the North Pacific, where no investigations have been made with regard to dissolved proteins, and we reconfirmed the widespread occurrence of protein molecules in DOM. We detected glycoproteins among the dissolved proteins examined in this study, and we report the results of a partial characterization of sugar chains and discuss the possible role of sugar chains in glycoproteins in DOM.

Sampling

Samples of seawater were collected at five stations: two stations, B (25°00′N, 165°00′E; water depth, 5,983 m) and M2 (35°00′N, 165°00′E; water depth, 5,886 m) on the KH95-3 cruise of the R/V *Hakuho-maru* from 19 October to 24 November 1995 and three stations, K (44°00′N, 155°00′E; water depth, 5,325 m), BE (55°00′N, 180°00′E; water depth, 3,672 m), and P (50°00′N, 145°00′W; water depth, 4,252 m) on the KH99-3 cruise from 25 June to 25 August 1999 (Fig. 1). Seawater samples were collected with

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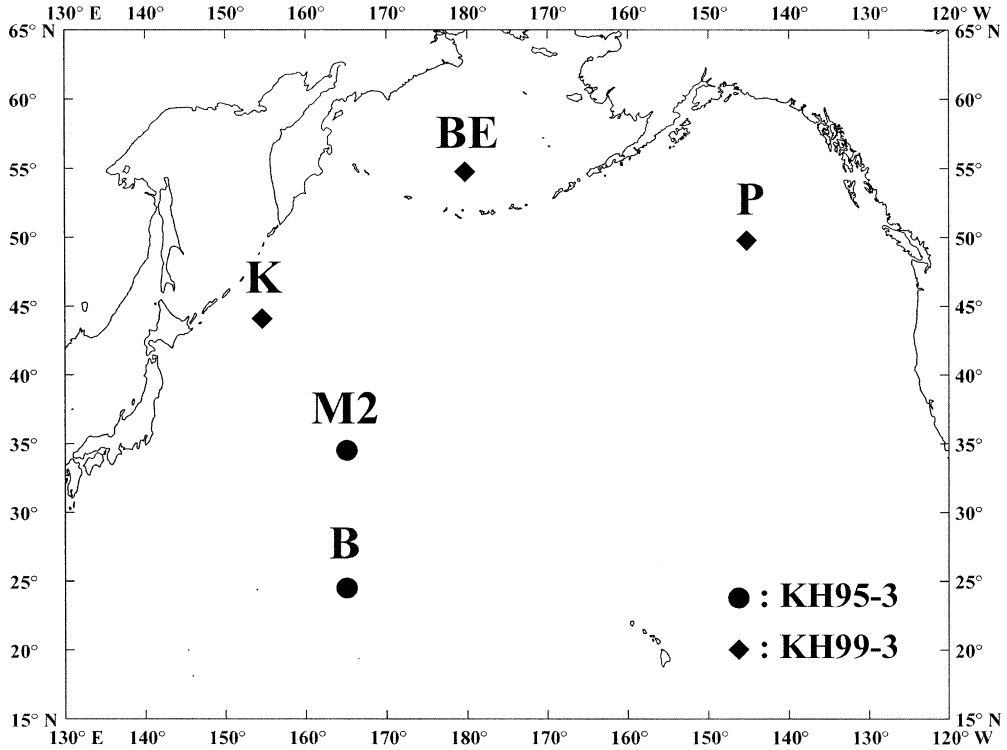

Fig. 1. Sampling stations of the KH-95-3 and the KH-99-3 cruises.

a Niskin bottle mounted on a CTD-Rosette multiple sampler. A plastic bucket was used for samples at a depth of 0 m.

Methods

Sample preparation of dissolved protein analyses—The procedures for sample preparation (filtration, concentration, desalting, and purification) of dissolved proteins are illustrated in Fig. 2. The procedures for concentration and desalting were previously reported (Tanoue 1995) and are described here briefly.

Samples of seawater were filtered through a glass fiber filter (GF/F; Whatman) immediately after sampling. With the large-volume samples (more than 10 liters; Sta. M2 and B from KH95-3 cruise and Sta. P from KH99-3 cruise), sodium azide (NaN_3: 5 mmol final concentration) and sodium dodecyl sulfate (SDS: 0.01% [w/v] final concentration) were added to the filtrates. The filtrates were stored in a cold room (approximately 4°C) on the KH95-3 cruise and in the ship laboratory on the KH99-3 cruise until the preconcentration and desalting were performed aboard ship. The small-volume samples (less than 10 liters, Sta. K and BE from the KH99-3 cruise) were kept frozen (−20°C) until concentration and desalting were performed ashore.

All filtrates were concentrated by a tangential ultrafiltration system with a filter having a nominal molecular mass cut-off of 10 kDa (10,000 NMMCO filter, low protein-binding regenerated cellulose; Millipore) and desalted with desalting solution (NH_4HCO_3: 35 mmol, SDS: 0.01% [w/v]). For the large-volume samples, the Pelicon™ ultrafiltration system (Millipore; 4,650 cm^2 of filter area) was used for preconcentration aboard ship at room temperature. The Pelicon™-preconcentrated samples (approximately 300 ml) were further concentrated (see below) aboard ship on the KH95-3 cruise. The preconcentrated samples from KH99-3 were kept frozen (−20°C) during the cruise until further concentration ashore. For small-volume samples, after additions of NaN_3 (5 mmol final concentration) and SDS (0.01% [w/v] final concentration), preconcentration and desalting with the Pelicon™ ultrafiltration system were carried out at constant temperature (35°C). The preconcentrated samples (approximately 300 ml) were kept frozen (−20°C) until further concentration.

Further concentration and desalting were carried out with a Minitan™ ultrafiltration system (Millipore; 240 cm^2 of filter area). On the KH95-3 cruise (samples from Sta. M2 and B), concentration and desalting were performed at room temperature aboard ship, and the Minitan™-concentrated samples (approximately 50 ml) were kept frozen (−20°C) until the next treatment. On the KH99-3 cruise (Sta. K, BE, and P), after samples were thawed ashore, they were concentrated and desalted at constant temperature (35°C), and the Minitan™-concentrated samples (approximately 50 ml) were kept frozen (−20°C) until the next treatment.

The concentrated and desalted samples were dried in a Speed Vac© Plus concentrator (model SC210A, Savant), redissolved in a small volume of desalting solution (2–10 ml) to make crude samples (Fig. 2), and stored at −20°C until use.

The concentration and desalting were carried out in different conditions (i.e., immediate preconcentration of large-volume samples aboard ship or freezing and storage of

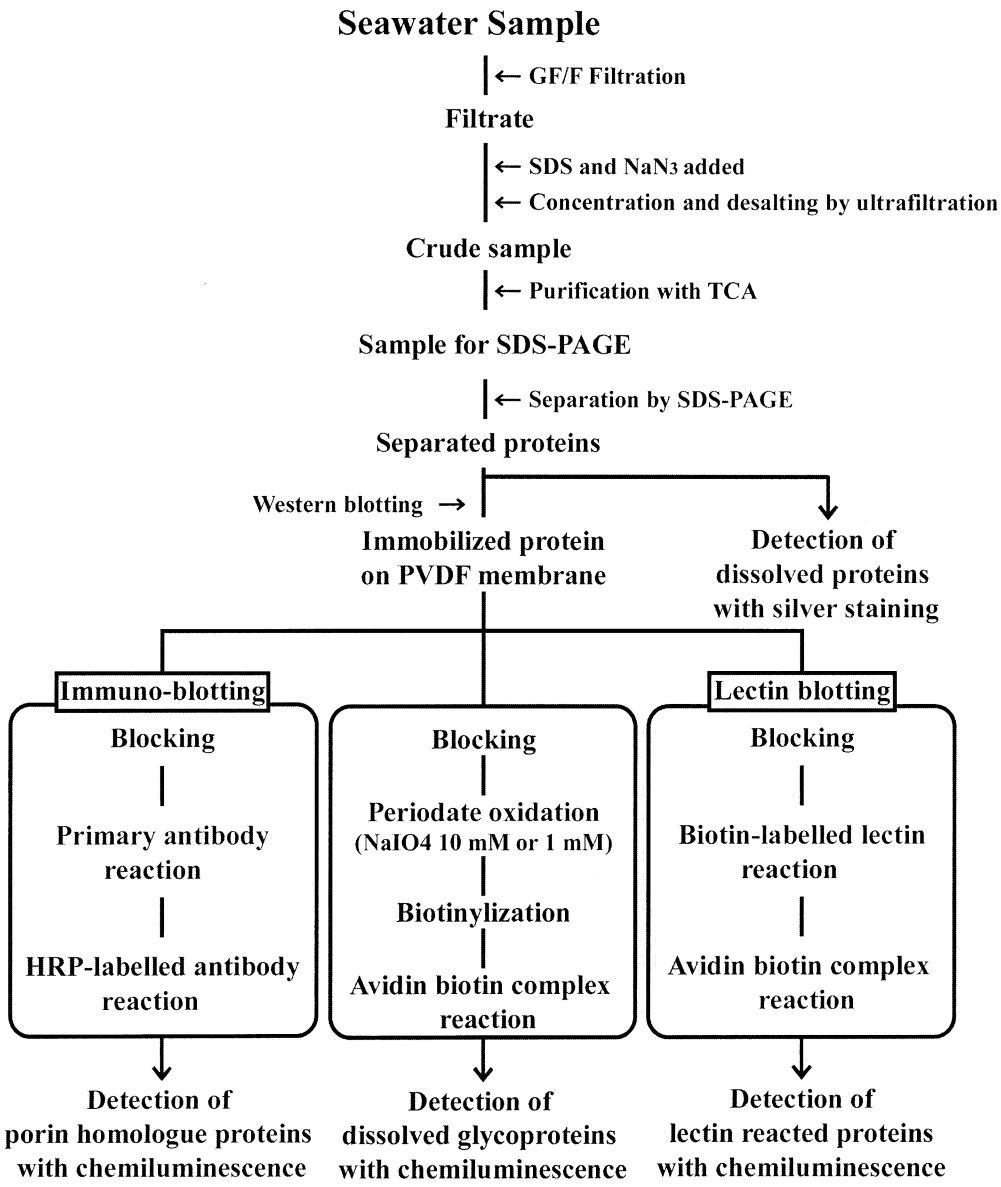

Fig. 2. Flow chart for each analysis in this study.

small-volume samples, and room temperature or 35°C temperature during concentration and desalting). The different treatments did not lead to obvious differences in the final electrophoretic patterns of dissolved proteins, although systematic comparisons were not made between the different treatments.

During the freezing and storage, the conformation of some dissolved proteins could have been destroyed and denaturated. Since complete denaturation and reduction of any disulfide bonds in proteins were accomplished in this study by the use of SDS and 2-mercaptoethanol with heating (Fig. 2), denaturation did not affect the final results. Thus, proteins and/or their subunits are defined as proteins in this study.

During the concentration of dissolved proteins at room temperature, SDS was sometimes precipitated as a solid at low room temperature. Because the critical micellar temperature of SDS in NaCl/sodium phosphate buffer (NaCl; 0.1 mol, sodium phosphate; 0.05 mol, pH 7.4) is 23°C (Helenius and Simons 1975). Once SDS was precipitated, repeated rounds of the purification step were required for removal of excess SDS from the crude samples (see below and Fig. 2). It was desirable to prevent SDS precipitate formation. SDS possesses strong bactericidal activity and is used for protein extraction from organisms at room temperature or 37°C (Andrews 1986). Since no bacterial activity needed to be considered, concentrations of the small-volume samples and further concentration of the preconcentrated large-volume samples were carried out at 35°C.

Proteins in the crude samples (Fig. 2) were purified by precipitation with trichloroacetic acid (TCA). TCA-insoluble low-density material, in which lipids and proteins were associated, was sometimes observed during the TCA precipitation treatment (Tanoue 1995), and isopropyl alcohol was effective for removing lipids from this material. A previ-

ously reported method of TCA precipitation (Tanoue 1995) was modified in this study as follows. The TCA solution (100%; w/v) was added to the crude sample (10%; w/v, final concentration) on ice and left at 4°C overnight. Then, TCA/isopropyl alcohol solution (5%; w/v) was added to the final TCA concentration of 7% (w/v) on ice and again left at 4°C overnight. TCA concentrations in a range of 5–20% (w/v) did not influence the final electrophoretograms of dissolved proteins (Tanoue 1995). The solution was centrifuged to remove TCA/isopropyl alcohol–soluble materials at 15,000 × g at 4°C for 30 min. After centrifugation, the supernatant was discarded, and the precipitate was resuspended by vigorous homogenization with a sonication system (Ultrasonic stirrer, Youkai-kun™, model USS-1, Nihonseiki) in TCA/water solution (5%; w/v). The homogenate was centrifuged again and the supernatant was discarded. This step was repeated twice, after which the precipitate was washed twice with 99% ethyl alcohol and then with diethyl ether. The precipitate was then air-dried.

Separation and detection of dissolved proteins—The separation and detection of dissolved proteins were performed by SDS–polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). Samples were solubilized in SDS-PAGE sample buffer (Tris/HCl: 0.0625 mol [pH 6.8], 2-mercaptoethanol: 5% [v/v], SDS: 2% [w/v], glycerol: 3% [v/v], bromophenol blue: 0.002% [w/v]) and then boiled at 100°C for 3 min. A dissolved protein sample (5 μl), equivalent to that in 200 ml of original seawater, was applied to each lane of a 12.5% handmade gel. Electrophoresis was performed at the constant current of 20.5 mA for 30 min. Molecular weight markers (Molecular Weight Marker ''Daiichi'' · III: Daiichi Pure Chemicals) were used for reference: phosphorylase b (97 kDa), albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa). After electrophoresis, the proteins on the gel were visualized by silver staining using the 2D-Silver Stain · II ''Daiichi'' kit (Daiichi Pure Chemicals).

Immunoblotting—To detect dissolved porin homologue protein, immunoblotting (Towbin et al. 1979) was carried out (Fig. 2) with the use of a primary antiserum (α-48DP N-14) raised from a 48-kDa dissolved protein, which was identified as a homologue of the porin P of *Pseudomonas aeruginosa* (Tanoue et al. 1996). The immunoreactivity of this antiserum (α-48DP N-14) was reported by Suzuki et al. (1997).

The proteins on the gel after SDS-PAGE were transferred onto a PVDF membrane (Immobilon-P™ Transfer membrane, Millipore) using the Milli Blot-SDE transfer system (Millipore) at 2.5 mA cm^{-2} for 30 min, following the method of Kyhse-Andersen (1984; Western blotting). After Western blotting, the membrane was blocked with a solution of phosphate buffer saline (PBS; NaCl: 137 mmol, KCl: 2.7 mmol, Na_2HPO_4: 8 mmol, KH_2PO_4: 1.5 mmol) containing skimmed milk (1%; w/v). After blocking, the porin homologue on the PVDF membrane was reacted with the primary antiserum (α-48DP N-14). It was then reacted with the secondary antibody, which was a horseradish peroxidase (HRP)–labeled anti-rabbit IgG (goat) (Cosmo Bio). The HRP-labeled α-48DP N-14–reacted proteins on the PVDF membrane were detected by enhanced chemiluminescence (Whitehead et al. 1979) using a commercially available kit (ECL™; Amersham Pharmacia Biotech). The proteins reacted on PVDF membrane were exposed to autoradiography film (Hyperfilm™ ECL™ high-performance chemiluminescence films, Amersham). Film was developed using an X-ray film developer (GBX developer and replenisher, Kodak). Development was stopped using a solution of acetic acid (3%; v/v), and the film was fixed using X-ray film fixer (GBX fixer and replenisher; Kodak), washed with water, and dried.

Detection of dissolved glycoproteins—As shown in Fig. 2, dissolved glycoproteins were detected by the combination of the following methods: SDS-PAGE (Laemmli 1970), Western blotting (Kyhse-Andersen 1984), periodate oxidation (Kondo et al. 1991), and enhanced chemiluminescence (Whitehead et al. 1979). The detection of dissolved glycoproteins was carried out with the use of a commercially available kit, G. P. Sensor™ (Honen), based on the method of Kondo et al. (1991), and the above-mentioned enhanced chemiluminescence detection kit (ECL™). After SDS-PAGE followed by Western blotting (Fig. 2), glycoproteins immobilized on the PVDF membrane were blocked with Tris-Tween solution I (Tris-HCl: 10 mmol [pH 7.4], NaCl: 0.5 mol, Tween 20: 0.05% [w/v]). The sugar constituents of the glycoproteins were then subjected to periodate oxidation. It is known that hexose, pentose, hexosamine, and sialic acid are the major constituents of sugar chains of glycoproteins and that sialic acid is easily degradable by periodate oxidation in comparison with other sugar moieties. Two conditions for periodate oxidation were employed in this study. In the ''weak'' condition, oxidation on ice with 1 mmol $NaIO_4$ solution was employed for detection of sialic acid. The sugar constituent detected under this condition contained sialic acid, termed sialic oligosaccharide in this study. The ''strong'' oxidation at room temperature with 10 mmol $NaIO_4$ solution was used for detection of other sugar moieties, termed oligosaccharides in this study. Both oxidations were performed in the dark. The aldehyde group formed by the periodate oxidations was coupled with biotin hydrazide. Biotin hydrazide–sugar chains were then coupled with HRP-Avidin in a solution containing Tris-Tween solution II (Tris-HCl: 10 mmol [pH 7.4], NaCl: 0.15 mol, Tween 20: 0.05% [w/v]) and HRP-Avidin (0.1%; w/v). The HRP-labeled glycoproteins were detected by enhanced chemiluminescence.

Lectin blotting—Lectin blotting (e.g., Taka et al. 1996) was employed to estimate the partial structure of sugar chains in the glycoproteins. The linkage between sugar chain and amino acid residue of the polypeptide chain has been classified into two types; one is an N-linked sugar chain in which the sugar chain is attached to an asparagine residue, and the other is an O-linked sugar chain in which the sugar chain is attached to a serine or threonine residue (Marshall and Neuberger 1970). Two lectins, concanavalin A (ConA, from *Canavalia ensiformis*), which recognizes the N-linked sugar chain, and peanut lectin (PNA, from *Arachis hypogaea*), which recognizes the O-linked sugar chain, were used to distinguish the linkage type of the glycoproteins in this

study. After SDS-PAGE followed by Western blotting (Fig. 2), the immobilized glycoproteins on the PVDF membrane were blocked with Tris-Tween solution II, and the membrane was soaked in the solution of the lectin conjugated with biotin (0.0005% biotin-lectin in Tris-Tween solution II). Lectin reacted with glycoproteins according to its selectivity in this step. Glycoproteins that reacted with lectin on the PVDF membrane were transferred into HRP-Avidin solution to form the avidin–biotin complex. The HRP-labeled lectin that reacted with glycoproteins was detected by enhanced chemiluminescence.

Results

Occurrences of dissolved proteins and porin homologue protein—Electrophoretograms of dissolved proteins from surface through deep waters at five stations (Fig. 1) in the North Pacific (Sta. B and M2) and the northern North Pacific (Sta. K, BE, and P) are shown in Fig. 3. Dissolved proteins were detected throughout the water columns at all stations. There are a small number of proteins that are clearly visible as bands within a relatively limited molecular weight range and a lack of depth-related trends in terms of number and species of proteins detected in the water columns. The characteristic electrophoretic pattern of dissolved proteins in this study was similar to those obtained from various other oceanic areas (see review by Tanoue 2000). Among the dissolved proteins detected in this study, proteins with apparent molecular weights of approximately 39 kDa and 48 kDa proteins were the most prominent. A 34-kDa protein was also prominent, except in samples from the intermediate and deep waters at Sta. B (Fig. 3). Two notched bands with molecular weights ranging from 51 to 61 kDa were found and were particularly conspicuous at Sta. M2 (Fig. 3). These bands are well-known artifactual bands from SDS-PAGE sample buffer (e.g., Ochs 1983). They were distinguishable in their shapes from dissolved protein bands in seawater, and they were ignored in this study.

The characteristic electrophoretograms of dissolved proteins were not produced by the living organisms, including bacteria that were passed through the GF/F filter. Analysis of even a single bacterium on SDS-PAGE yields a large number of proteins observed over a wide range of molecular weights. So, the gel was stained relatively uniformly and showed a smeared electrophoretic pattern (Tanoue 2000). The number of bacteria decreased two orders of magnitude from the surface to deep water at stations examined in this study (data not shown). Nevertheless, the fact that a small number of dissolved proteins were detected as bands without the depth-related trend in electrophoretograms (Fig. 3), as mentioned above, indicated that the effects of proteins in living organisms were insignificant and did not need to be considered with regard to the major and common proteins discussed in this study.

A 48-kDa protein, one of the common dissolved proteins in the study, was detected in samples from water columns of the central and equatorial Pacific and from the surface waters of the Antarctic and Indian Oceans (Tanoue 2000). It was identified as a homologue of porin P of the gram-negative bacterium, *P. aeruginosa* (Tanoue et al. 1995; Suzuki et al. 1997). In order to verify that the 48-kDa protein detected in this study was also a homologue of porin P, the cross reactivity of the antibody against the porin P homologue (α-48DP N-14) was examined for samples from three stations in the east and west sides of the northern North Pacific and the Bering Sea (Fig. 4), where the occurrence of porin homologue dissolved proteins has not been reported. The molecular weight of the porin P protein was 48 kDa on SDS-PAGE (Worobec et al. 1988). The antibody reacted reliably with a dissolved protein band at the anticipated molecular weight. Thus, it was concluded that the porin P homologue was also present as one of the major dissolved proteins in the water columns of sites in the northern North Pacific and the Bering Sea, indicating that the porin P homologue was widespread in oceanic waters.

The antibody did not react with the 34-kDa protein, one of the major proteins, or other proteins, but it reacted with the 39-kDa protein, another major dissolved protein, from the three stations (Figs. 3, 4). The 39-kDa protein might have an epitope similar to that of porin P because the antibody used in this study was polyclonal. However, it was unlikely that the 39-kDa protein was a porin homologue because it was glycosylated (see below). The above-mentioned artifactual bands are known to appear when polyclonal antibodies are used as the immunoblotting probe (Bérubé et al. 1994), and these bands also reacted with the antibody in this study.

Reinvestigation of N-terminal amino acid sequences of the dissolved proteins—Tanoue et al. (1995) carried out an homology search of 11 amino acid sequence data of dissolved proteins in the Protein Identification Resource International database (PIR), through the Japan International Protein database. Currently, the data bank of amino acid sequences is rapidly growing in protein chemistry. We repeated the homology analysis with the BLAST program (Altschul et al. 1997) through a Data Bank of Japan (DDBJ) homology search (http://www.ddbj.nig.ac.jp/), which covered five amino acid sequence databases: i.e., PIR, SWISS-PROT, DDBJ Amino Acid Sequence Database (DAD), the Protein Data Bank sequence taken from the header (PDBSH), and Protein Research Foundation (PRF).

The amino acid sequence from the N-terminus (GTVTTDGADIVIKT) of the 48-kDa dissolved protein from the North Pacific station (45°10.3′N, 165°34.4′E) agreed with those of the porin P and porin O proteins of *P. aeruginosa*, with 100% homology. The porin O protein of *P. aeruginosa*, the newly 'hit' protein, is an anion-specific, phosphate-selective porin, the same as the porin P protein, and immunochemical reactivity is also similar to that of the porin P protein (Hancock et al. 1992). The porin O protein was just three amino acids longer than the porin P protein and has a lower affinity for both chloride and phosphate than does the porin P protein (Siehnel et al. 1992). Although the porin O protein genetically differs from the porin P protein (Hancock et al. 1992; Siehnel et al. 1992), the porin P and porin O proteins of *P. aeruginosa* are indistinguishable in terms of the molecular weights and immunochemical reactivity in this study. The porin O protein is apparently a porin

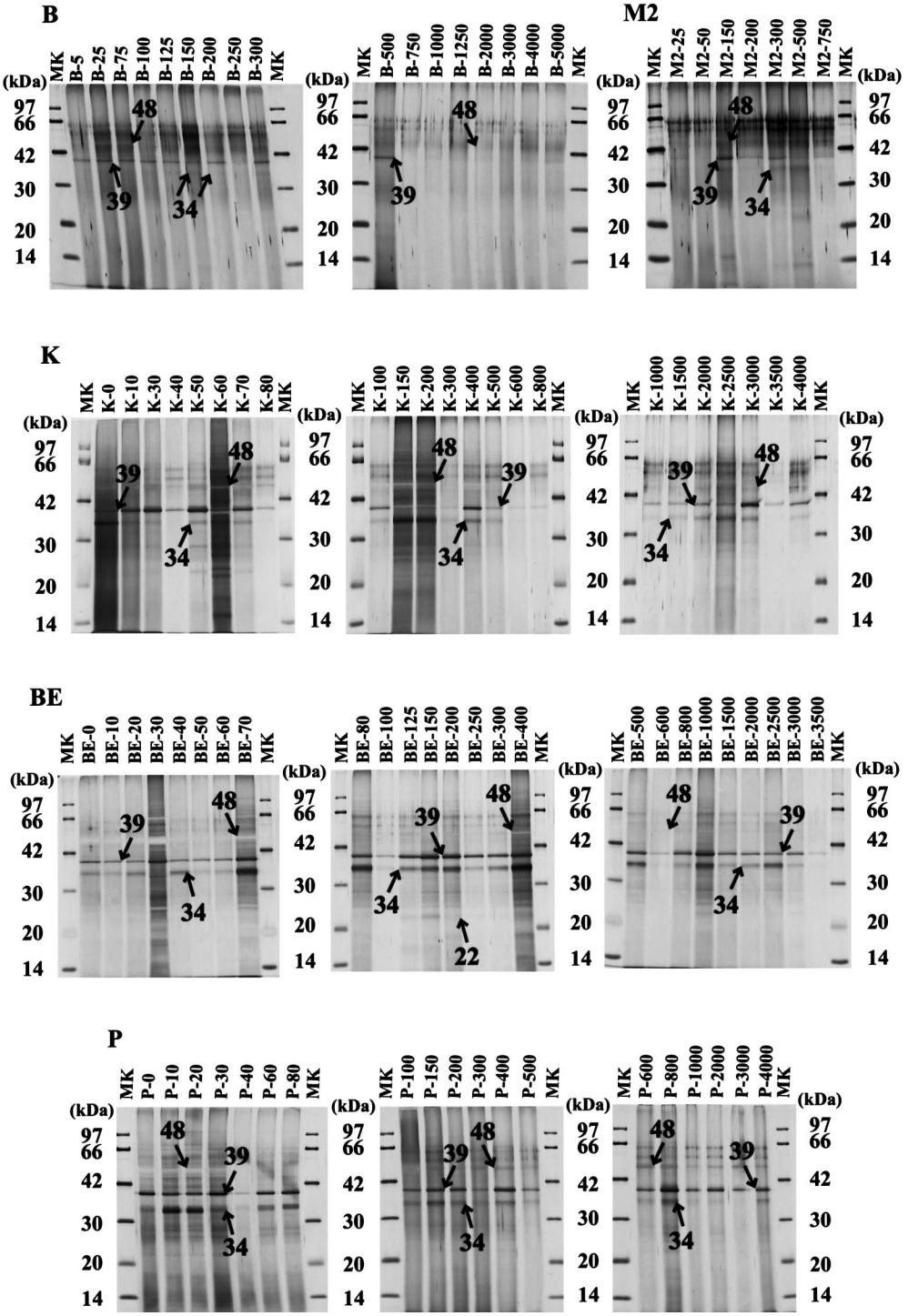

Fig. 3. Depth profiles of dissolved proteins at Sta. B, M2, K, BE, and P. Sample for each lane represents a station and sampling depth. MK on the left- and right-hand lanes denotes size marker protein. Molecular weight is indicated as kDa for each dissolved protein band, as well as for the marker protein. Amounts of samples loaded on the gel were equivalent to that in 200 ml of the original seawater in each case.

P homologue protein, and the 48-kDa proteins were collectively called porin P homologues in this study.

Reinvestigation of the N-terminal amino acid sequence in this study demonstrated that the amino acid sequence (TVTVTPLMLGYTFQL) of the 40-kDa dissolved protein from the same station (Tanoue et al. 1995) agreed (100% homology) with a major outer membrane protein (OmpA-like protein) of *Acinetobacter* spp., one of the gram-negative

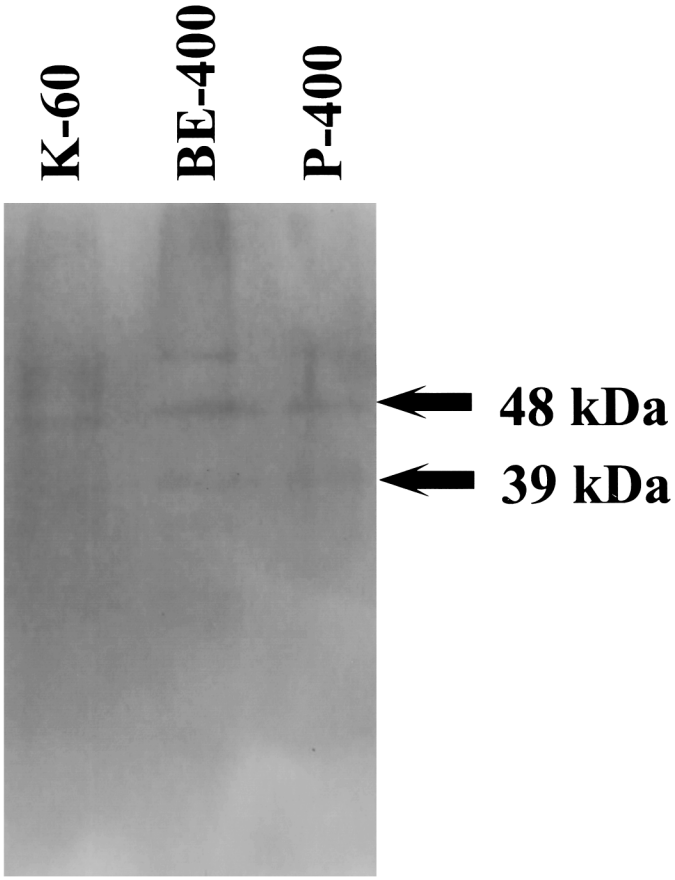

Fig. 4. The results of immunoblotting with α-48DP N14 antiserum. Sample for each lane represents a station and sampling depth. Amounts of samples loaded on the gel were equivalent to that in 2 liters of the original seawater in each case.

bacterium of the *Pseudomonas* group, reported by Ofori-Darko et al. (2000). OmpA was identified to be a major outer membrane protein of *Escherichia coli*, and an OmpA-like protein was found to be the major outer membrane protein in most gram-negative pathogenic bacteria (Lugtenberg and Alphen 1983). We performed a further FASTA program (Pearson and Lipman 1988) investigation through a DDBJ homology search, which covered DNA sequence as well as amino acid sequence databases from PIR, SWISS-PROT, DAD, PDBSH, and PRF. The best score, a high homology of more than 90%, was obtained with only the 40-kDa dissolved protein, indicating that the 40-kDa protein was similar to a member of the OmpA family of *Acinetobacter baumannii* (92.308%; Gribun et al. unpubl. data) and the OmpA-like protein of *Acinetobacter radioresistans* (90.909%; Toren et al. 2002). The 37-kDa and 34-kDa dissolved proteins from the subtropical Pacific station (24°35.0′N, 170°0.1′E) and the 39-kDa dissolved protein from another subtropical Pacific station (22°47.1′N, 158°04.6′W), did not exhibit a high similarity score to other proteins of more than 90%. No score was obtained for the 30-kDa dissolved protein from the North Pacific station (45°10.3′N, 165°34.4′E), indicating that no such protein was reported in amino acid sequence and DNA sequence databases.

Occurrence of dissolved glycoproteins—We attempted to detect dissolved glycoproteins by detection of their sugar chain constituents. Two types of sugar chains (i.e., oligosaccharides and sialic oligosaccharides) detected from five stations are compared in Fig. 5.

Both oligosaccharide and sialic oligosaccharide were evident throughout the water columns at Sta. B and M2 (Fig. 5). Oligosaccharide was clearly detected on the 39-kDa protein, the most prominent protein, at these two stations (Fig. 3). Sialic oligosaccharide was also detected on this protein, although the staining intensity was low, indicating that the 39-kDa protein had both types of sugar constituents, but the contribution of sialic oligosaccharide might be small. The 34-kDa protein was faintly visible by silver staining of samples from Sta. B and M2 (Fig. 3), and oligosaccharides and sialic oligosaccharides were also detected faintly on this protein. The staining patterns of this glycoprotein were different from those of the 39-kDa glycoprotein (Fig. 5). For example, the 34-kDa protein band was clearly visible by silver staining of samples from B-150, B-200, and M2-300 (Fig. 3). Sialic oligosaccharide was visible but no oligosaccharide band was recognized in samples of B-150 and M2-300 (Fig. 5). On the other hand, oligosaccharide was also visible in the sample from B-200. The different staining intensities of the 34-kDa glycoprotein in terms of the oligosaccharide and sialic oligosaccharide might imply a heterogeneous nature of the sugar constituents, even if the electrophoretic mobility was the same. The sugar chains were not detected clearly on the 48-kDa protein band; however, oligosaccharides and sialic oligosaccharides were faintly observed in a few samples.

In samples from the northern North Pacific (Sta. K and P; KH99-3 cruise) and the Bering Sea (Sta. BE; KH99-3 cruise), the typical electrophoretic patterns (Fig. 3) were selected, and the sugar chain staining patterns of these samples were compared (Fig. 5). The 34-kDa and 39-kDa proteins were the most prominent throughout the water columns at three stations (Fig. 3). Both sugar constituents were clearly detected in the 34-kDa protein. The oligosaccharides and the sialic oligosaccharides were clearly detected on the 39-kDa proteins, except in the samples from K-0 and K-200, in which the amounts of the 39-kDa protein were low (Fig. 3). Other than in the samples of K-0 and K-200, the oligosaccharide and sialic oligosaccharide were equally stained on the 39-kDa protein (Fig. 5), indicating that the 39-kDa glycoprotein had both sugar constituents. The 48-kDa protein was also observed in the entire water column at three stations, as mentioned above (Fig. 3). Clear oligosaccharide and faint sialic oligosaccharide signals were observed in this protein from samples from BE-10, BE-200, BE-2500, and P-150. These oligosaccharides were not detected clearly in this protein in samples from K-2000 and P-4000.

Linkage between sugar chain and polypeptide—The results of lectin blotting are shown in Fig. 6. The sugar chain of the 34-kDa glycoprotein reacted with ConA but not with PNA, indicating this glycoprotein had the N-linked sugar

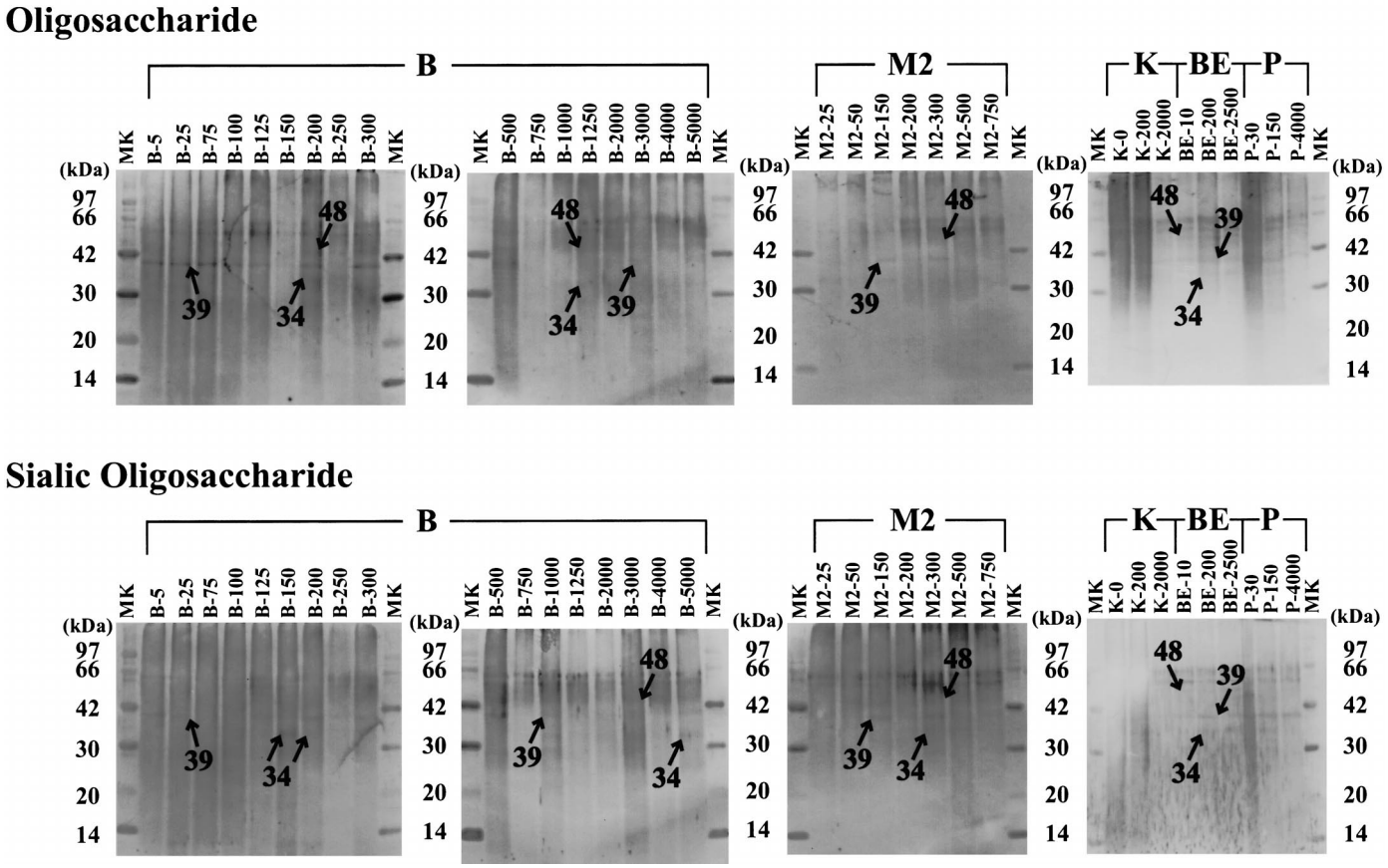

Fig. 5. The detection of oligosaccharide and sialic oligosaccharide constituents of dissolved proteins. Sample for each lane represents a station and sampling depth. MK on the left- and right-hand lanes denotes size marker protein. Molecular weight is indicated as kDa for each dissolved protein band as well as for the marker protein. Amounts of samples loaded on the gel were equivalent to that in 1 liter of the original seawater in each case.

chain. The 39-kDa glycoprotein reacted with both lectins, indicating this glycoprotein has at least two types of sugar chains, one was the N-linked sugar chain and the other was the O-linked sugar chain. The ConA faintly reacted with the 48-kDa protein band, indicating that the N-linked sugar chain was in this protein band.

The PNA strongly reacted with two bands having apparent molecular weights of 20 kDa and 22 kDa in samples from K-0, K-200, and BE-200; however, the bands were not visible by the ConA treatment. The 20- and 22-kDa proteins were not clearly distinguishable by silver staining because of the high background at Sta. K, but a band with a molecular weight of 22 kDa was faintly visible on the sample from BE-200 (Fig. 3). Neither of the 20- and 22-kDa proteins was detected by the two periodate oxidations (Fig. 5), but they were heavily stained by lectin blotting, indicating that the sugar chains of these low–molecular weight glycoproteins are short, but rich in O-bonds. The staining patterns of sugar constituents and the linkage types of the 20- and 22-kDa proteins were different from those of 34-kDa, 39-kDa, and 48-kDa glycoproteins. Thus, another type of glycoproteins (20 kDa and 22 kDa) were present in which the sugar chains conjugated with peptides through the O-linked sugar chain, and the O-linked sugar chains were short but rich in number.

Discussion

The occurrence of dissolved proteins has been reported from the surface to deep oceanic waters at five stations located in the subarctic, subtropical, and equatorial Pacific and in the surface waters of the Indian and the Southern Oceans (Tanoue et al. 1996; Tanoue 2000). The results observed in the present study at two stations located on the east and west sides of the northern North Pacific (Sta. K and P) and the Bering Sea (Sta. BE) and at two stations located in the North Pacific (Sta. B and M2) reconfirmed the widespread occurrence of dissolved proteins in oceanic water columns. The molecular distribution of dissolved proteins differed among the five stations (Fig. 3); however, some proteins detected in this study were similar to those reported previously. In terms of SDS-PAGE patterns, the proteins with molecular weights of approximately 34, 39, and 48 kDa commonly detected in this study (Fig. 3) were also found in subtropical and equatorial stations in a previous study (Tanoue et al. 1996).

Particularly, Tanoue et al. (1995) reported that the 48-kDa protein in seawater was a homologue of porin P of the gram-negative bacterium *P. aeruginosa.* The reanalysis of the N-terminal amino acid sequence (24 steps) showed that the first-terminal 24 residues of the 48-kDa dissolved protein

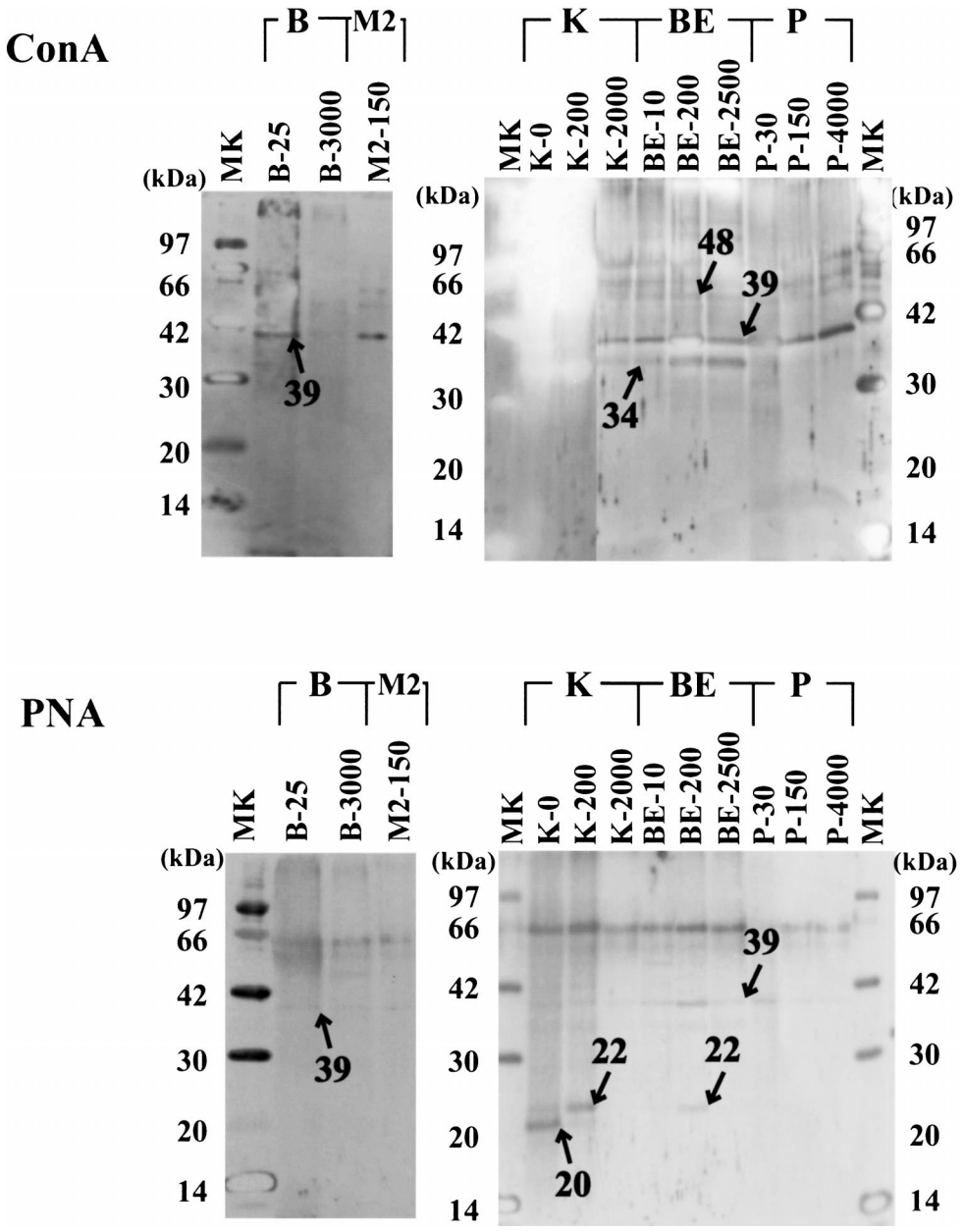

Fig. 6. The results of lectin blotting with ConA and PNA. Sample for each lane represents a station and sampling depth. MK on the left- and right-hand lanes denotes size marker protein. Molecular weight is indicated as kDa for each dissolved protein band as well as for the marker protein. Amounts of samples loaded on the gel were equivalent to that in 1 liter of the original seawater in each case.

again agreed (100%) with those of the porin P protein (data not shown). In this study, the 48-kDa dissolved protein was commonly observed through the water columns in the study areas (Fig. 3), and since an antibody raised from a 48-kDa protein (Suzuki et al. 1997) reacted with the 48-kDa dissolved protein in this study (Fig. 4), the widespread occurrence of the porin P homologues in the oceanic water columns was reconfirmed.

The porin P protein is one of the trans–outer membrane channel proteins. These proteins serve as a molecular sieve of the outer membrane of gram-negative bacteria and are collectivity known as porins (e.g., Nikaido 1994). It has been thought that porins are resistant against enzymatic and bacterial attack because of their trimer form structure (Worobec et al. 1988; Schirmer 1998). Every gram-negative bacterium has its own porin, whose physical properties are quite similar to those of other porins, but each porin has its own molecular weight (e.g., Lugtenberg and Alphen 1983; Worobec et al. 1988). Therefore, it is possible to speculate that there are many kinds of porins with different molecular weights present in DOM.

It was reported that the antibody raised from the porin

protein (Omp35La) of a pathogenic marine bacteria, *Vibrio anguillarum,* reacted with the 34-kDa dissolved protein in samples from the North Pacific and the Antarctic Oceans (Suzuki et al. 1997) and also with the 18-, 32-, and 37-kDa dissolved proteins in samples from coastal areas (Yamada et al. 2000). These observations support the idea that porin proteins other than porin P homologue are present in DOM. The occurrence of glycoproteins (Figs. 5, 6) strongly demonstrated that proteins other than porin proteins accounted for the majority of dissolved proteins, because no glycosylated porin is known (Schirmer 1998) and the 34- and 39-kDa proteins, the major dissolved proteins, were glycosylated.

Oligosaccharide and sialic oligosaccharide were both detected on the 48-kDa protein in some samples (Fig. 5). It was observed from the amino acid sequence that the 48-kDa protein band was a mixture of two proteins (Tanoue et al. 1995). Thus, it is presumed that the gel electrophoresis band of porin P homologues in some samples in this study was also a mixture of other protein(s). The protein concomitant with the 48-kDa protein was a glycoprotein, in which the sugar chain was attached to a polypeptide with N-linkage (Fig. 6).

The partial characterization of the sugar chains of the glycoproteins in this study indicated the heterogeneous nature of these sugar chains in terms of their sugar constituents and linkage types (Figs. 3, 5, 6). The heterogeneity of sugar chains in glycoproteins has been well understood because the population of sugars attached to an individual protein is controlled by posttranslational modifications; namely, the sugar constituents of an individual glycoprotein depend on the physiological conditions of cell in which the glycoproteins are synthesized (e.g., Dell and Morris 2001). Apparently, the one dimensional PAGE (SDS-PAGE) in this study was a first step, and the high-resolution technique of two-dimensional electrophoresis (e.g., Nguyen and Harvey 1998) will be required for further characterization of individual proteins in DOM.

In a homology reinvestigation of previous sequence results (Tanoue et al. 1995) of the dissolved proteins, OmpA-like protein was newly found as a source protein. The OmpA protein forms a small nonspecific diffusion channel and is not a porin protein (Nikaido 1983). This protein has a nonphysiological function but serves as a phage receptor (Schweizer and Henning 1977) and also contributes to the pathogenicity of bacteria (Weiser and Gotschlich 1991). OmpA is closely associated with lipopolysaccharide, one of the important components of the gram-negative bacteria outer membrane (Karch et al. 1983). The occurrence of OmpA-like protein, as well as of glycoproteins, in DOM was counter to the speculation that the unidentified dissolved proteins were also porin proteins.

Both the OmpA and porin P protein have a large proportion of β-sheet structure (Worobec et al. 1988; Koebnik 1999). The β-sheet has a very rigid structure held together by hydrogen bonds that connect the outside bonds in neighboring chains (Alberts et al. 1994). The large amount of β-sheet structure in bacterial outer membrane proteins sets them apart from most other proteins (Siehnel et al. 1990). Wells et al. (2001) commented that complex N- and O-linked glycoproteins occurred in membrane-bound or secreted proteins that were synthesized in the endoplasmic reticulum and Golgi apparatus. The 39-kDa dissolved protein, a dominant constituent of the dissolved proteins, had N- and O-linked sugar chains (Fig. 6). Therefore, it was inferred that dissolved proteins other than porin P homologues and OmpA-like protein were also membrane-bound proteins. Because porin and OmpA protein are channel proteins in the outer membrane, these membrane proteins might have interacted with other membrane components. One possible explanation for the results of this study is that the bacterial membrane or its remnants encapsulate and protect dissolved proteins in seawater (Tanoue 1995; Hedges et al. 2000; Ogawa and Tanoue 2003 [and references therein]). This idea is consistent with the detection of D-enantiomers of amino acids of peptidoglycans in bacterial outer membranes in DOM (McCarthy et al. 1998; Dittmar et al. 2001).

Unfortunately, this explanation is not satisfactory, because all the dissolved proteins we have so far identified have pathogenic sources. Specifically, the dissolved proteins identified in seawater are not derived from the bulk bacterial population but from certain species of bacteria (Suzuki et al. 1997, 2000). At present, it is not clear whether the pathogenic nature of the source bacteria ultimately causes the survival of the dissolved proteins or rather that observations are accidental results stemming from the fact that pathogenic bacteria have been well investigated. Even if the membrane or its remnants of specific bacteria groups selectively survived, there must be more dissolved proteins present in DOM, because there were a large number of proteins in a single marine bacterial outer membrane (e.g., Suzuki et al. 1997). Consequently, there must be an additional mechanism by which proteins transferred to DOM are protected.

It is known that the sugar chain in glycoproteins is located outside of the protein molecule (Tomiya et al. 1988). Glycoproteins are also apt to associate with other molecules inside a cell as a result of their glutinous nature, giving rise to the sugar chain locations on the protein molecules. As a result of their diverse characteristics, glycoproteins play many roles in living organisms. For example, they serve as a resistance agent for some proteases (Kelly and Alpers 1973) and as an agglutination factor (Yen and Ballou 1974). It is reported that the sugar chain plays an important role for supramolecular fabrication, (i.e., large complex formation from small molecules via noncovalent associations); for example, hemoglobin forms supramolecules that are dependent on these sugar chains, even though carbohydrates occupy only 0.5% (wt) of hemoglobin (Ebina et al. 1995).

If dissolved glycoproteins have such glutinous nature, they might be adhering to various DOM components in seawater. In marine environments, it has been reported from laboratory experiments that organic–organic interactions produced the refractory amino acids and/or protein (Carlson et al. 1985; Keil and Kirchman 1993, 1994). Particularly, using artificially synthesized glucosylated RuBPcase, Keil and Kirchman (1993) demonstrated that glycosylated protein was degraded more slowly than protein or sugars alone. They hypothesized that glycosylation or other protein–sugar complexes produced less labile protein and dissolved combined amino acids in seawater. Since there were no data on the occurrence of glycoproteins (Keil and Kirchman 1993),

it was unclear whether glycosylated proteins were formed abiotically in situ and/or biotically in seawater.

An abiotic glycosylation (Maillard reaction) has been proposed for the formation mechanism of biologically refractory humic substance in soil and sediment (e.g., Hedges 1988; Yamamoto and Ishiwatari 1989). Current molecular level and functional group analyses have indicated that abiotic glycosylation is seldom evident in DOM (e.g., Hedges et al. 2000; Ogawa and Tanoue 2003). In this study, oligosaccharides and sialic oligosaccharides were detected (Fig. 5), and these oligosaccharides were attached to polypeptides through O- or N-linkages (Fig. 6). All these structures are well known in glycobiology but are difficult to form abiotically. Therefore, the glycoproteins observed in this study were thought to be derived from components of living organisms. Here, we hypothesized that the glycoproteins from organisms form macromolecular fabrications with other DOM components in seawater through the glutinous nature of sugar chains.

Glycoproteins are found in a wide range of organisms as well as in membrane and cytoplasm proteins of individual cells. The partial characterization of sugar chains in this study did not provide direct information on the source or function of glycoproteins seen in the DOM pool. However, the above-mentioned glycobiological findings, the glutinous nature of sugar chains in glycoproteins, as well as previous experimental results (Keil and Kirchman 1993) allow us to speculate that the sugars of glycoproteins play an important role for the survival of biomolecules in DOM. The sugars of glycoproteins serve as a linker in the formation of molecular–molecular associations, which facilitate the survival of dissolved proteins and other biomolecules in seawater.

References

ALBERTS, B., D. BRAY, J. LEWIS, M. RAFF, K. ROBERTS, AND J. D. WATSON. 1994. Molecular biology of the cell, 3rd ed. Garland Publishing.

ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG, W. MILLER, AND D. J. LIPMAN. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucl. Acids Res. **25:** 3389–3402.

ANDREWS, A. T. 1986. Preparative gel electrophoresis, p. 178–204. *In* A. R. Peacocke and W. F. Harrington. [eds.], Electrophoresis. Theory, techniques, and biochemical and clinical applications, 2nd ed. Oxford Univ. Press.

AZAM, F. 1998. Microbial control of oceanic carbon flux: The plot thickens. Science **280:** 694–696.

BÉRUBÉ, B., L. COUTU, L. LEFIÉVRE, S. BÉGIN, H. DUPONT, AND R. SULLIVAN. 1994. The elimination of keratin artifacts in immunoblots probed with polyclonal antibodies. Anal. Biochem. **217:** 331–333.

BOON, J. J., V. A. KLAP, AND T. I. EGLINTON. 1998. Molecular characterization of microgram amounts of oceanic colloidal organic matter by direct temperature-resolved ammonia chemical ionization mass spectrometry. Org. Geochem. **29:** 1051–1061.

CARLSON, D. J., L. M. MAYER, M. L. BRANN, AND T. H. MAGUE. 1985. Binding of monomeric organic compounds to macromolecular dissolved organic matter in seawater. Mar. Chem. **16:** 141–153.

DELL, A., AND H. R. MORRIS. 2001. Glycoprotein structure determination by mass spectrometry. Science **291:** 2351–2356.

DITTMAR, T., H. P. FITZNAR, AND G. KATTER. 2001. Origin and biogeochemical cycling of organic nitrogen in the eastern Arctic Ocean as evident from D- and L-amino acids. Geochim. Cosmochim. Acta **65:** 4103–4114.

EBINA, S., K. MATSUBARA, K. NAGAYAMA, M. YAMAKI, AND T. GOTOH. 1995. Carbohydrate gluing, an architectural mechanism in the supramolecular structure of an annelid giant hemoglobin (lectin/linkers). Proc. Natl. Acad. Sci. USA **92:** 7367–7371.

GRIBUN, A., Y. NITZAN, I. PECHATNIKOV, G. HERSHKOVITS, AND D. J. KATCOFF. The pore forming protein of *Acinetobacter baumannii*—a member of the OmpA family. Available at: http://ftp.ddbj.nig.ac.jp:8000/cgi-bin/get_entry2.pl?database=dad&query=AF390104-1.

HANCOCK, R. E. W., C. EGLI, R. BENZ, AND R. J. SIEHNEL. 1992. Overexpression in *Escherichia coli* and functional analysis of a novel PP_i-selective porin, OprO, from *Pseudomonas aeruginosa.* J. Bacteriol. **174:** 471–476.

HEDGES, J. I. 1988. Polymerization of humic substances in natural environments, p. 45–58. *In* F. H. Frimmel and R. F. Christman [eds.], Humic substances and their role in the environment. Wiley.

———, AND OTHERS. 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Org. Geochem. **31:** 945–958.

HELENIUS, A., AND K. SIMONS. 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta. **415:** 29–79.

KARCH, H., J. GMEINER, AND K. NIXDORFF. 1983. Alteration of the immunoglobulin G subclass responses in mice to lipopolysaccharide: Effects of nonbacterial proteins and bacterial membrane phospholipids or outer membrane proteins of *Proteus mirabilis.* Infect. Immun. **40:** 157–165.

KEIL, R. G., AND D. L. KIRCHMAN. 1993. Dissolved combined amino acids: Chemical form and utilization by marine bacteria. Limnol. Oceanogr. **38:** 1256–1270.

———, AND ———. 1994. Abiotic transformation of labile protein to refractory protein in sea water. Mar. Chem. **45:** 187–196.

KELLY, J. J., AND D. H. ALPERS. 1973. Blood group antigenicity of purified human intestinal disaccharidases. J. Biol. Chem. **248:** 8216–8221.

KOEBNIK, R. 1999. Structural and functional roles of the surface-exposed loops of the β-barrel membrane protein OmpA from *Escherichia coli.* J. Bacteriol. **181:** 3688–3694.

KONDO, M., H. HARADA, S. SUNADA, AND T. YAMAGUCHI. 1991. Increased selectivity in the detection of glycoproteins on nitrocellulose membranes by washing with sodium hydroxide solution. Electrophoresis **12:** 685–686.

KYHSE-ANDERSEN, J. 1984. Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10:** 203–209.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

LUGTENBERG, B., AND L. V. ALPHEN. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737:** 51–115.

MARSHALL, R. D., AND A. NEUBERGER. 1970. Aspects of the structure and metabolism of glycoproteins. Adv. Carb. Chem. Biochem. **25:** 407–478.

MCCARTHY, M. D., J. I. HEDGES, AND R. BENNER. 1998. Major bacterial contribution to marine dissolved organic nitrogen. Science **281:** 231–234.

MEON, B., AND D. L. KIRCHMAN. 2001. Dynamics and molecular

composition of dissolved organic material during experimental phytoplankton bloom. Mar. Chem. **75:** 185–199.

NAGATA, T. 2000. Production mechanisms of dissolved organic matter, p. 121–152. *In* D. L. Kirchman [ed.], Microbial ecology of the oceans. Wiley.

NGUYEN, R. T., AND H. R. HARVEY. 1998. Protein preservation during early diagenesis in marine waters and sediments, p. 88–112. *In* B. A. Stankiewicz and P. F. Van-Bergen [eds.], Nitrogen-containing macromolecules in the bio- and geo-sphere. American Chemical Society.

NIKAIDO, H. 1983. Proteins forming large channels from bacterial and mitochondrial outer membranes: Porins and phage lambda receptor protein. Methods Enzymol. **97:** 85–100.

———. 1994. Porins and specific diffusion channels in bacterial outer membranes. J. Biol. Chem. **269:** 3905–3908.

OCHS, D. 1983. Protein contaminants of sodium dodecyl sulfate–polyacrylamide gels. Anal. Biochem. **135:** 470–474.

OFORI-DARKO, E., Y. ZAVROS, G. RIEDER, S. A. TARLÉ, M. V. ANTWERP, AND J. L. MERCHANT. 2000. An OmpA-like protein from *Acinetobacter* spp. stimulates gastrin and interleukin-8 promoters. Infect. Immun. **68:** 3657–3666.

OGAWA, H., Y. AMAGAI, I. KOIKE, K. KAISER, AND R. BENNER. 2001. Production of refractory dissolved organic matter by bacteria. Science **292:** 917–920.

———, AND E. TANOUE. 2003. Dissolved organic matter in oceanic waters. J. Oceanogr. **59:** 129–147.

PEARSON, W. R., AND D. J. LIPMAN. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:** 2444–2448.

SCHIRMER, T. 1998. General and specific porins from bacterial outer membranes. J. Struct. Biol. **121:** 101–109.

SCHWEIZER, M., AND U. HENNING. 1977. Action of major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. J. Bacteriol. **129:** 1651–1652.

SIEHNEL, R. J., C. EGLI, AND R. E. W. HANCOCK. 1992. Polyphosphate-selective porin OprO of *Pseudomonas aeruginosa*: Expression, purification and sequence. Molec. Microb. **6:** 2319–2326.

———, N. L. MARTIN, AND R. E. W. HANCOCK. 1990. Function and structure of the porin proteins OprF and OprP of *Pseudomonas aeruginosa*, p. 328–342. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan [eds.], *Pseudomonas*: Biotransformations pathogenesis and evolving biotechnology. American Society for Microbiology.

SUZUKI, S., T. FUJITA, N. KIMATA, K. KOGURE, AND E. TANOUE. 2000. Isolation of bacteria with membrane proteins homologous to *Vibrio anguillarum* porin Omp35La. Microb. Environ. **15:** 189–195.

———, K. KOGURE, AND E. TANOUE. 1997. Immunochemical detection of dissolved proteins and their source bacteria in marine environments. Mar. Ecol. Prog. Ser. **158:** 1–9.

TAKA, J., T. SATO, T. SAKIYAMA, H. FUJISAWA, AND K. FURUKAWA. 1996. Bovine pituitary membrane glycoproteins contain β-*N*-acetylgalactosaminylated N-linked sugar chains. J. Neurochem. **66:** 852–859.

TANOUE, E. 1995. Detection of dissolved protein molecules in oceanic waters. Mar. Chem. **51:** 239–252.

———. 2000. Proteins in the sea—synthesis, p. 383–463. *In* N. Handa, E. Tanoue, and T. Hama [eds.], Dynamics and characterization of marine organic matter. Terrapub/Kluwer.

———, M. ISHII, AND T. MIDORIKAWA. 1996. Discrete dissolved and particulate proteins in oceanic waters. Limnol. Oceanogr. **41:** 1334–1343.

———, S. NISHIYAMA, M. KAMO, AND A. TSUGITA. 1995. Bacterial membranes: Possible source of a major dissolved protein in seawater. Geochim. Cosmochim. Acta **59:** 2643–2648.

TOMIYA, N., T. YAMAGUCHI, J. AWAYA, AND M. KURONO. 1988. Structural analysis of asparagine-linked oligosaccharides of porcine pancreatic kallikrein. Biochemistry **27:** 7146–7154.

TOREN, A., E. ORR, Y. PAITAN, E. Z. RON, AND E. ROSENBERG. 2002. The active component of the bioemulsifier alasan from *Acinetobacter radioresistens* KA53 is an OmpA-like protein. J. Bacteriol. **184:**165–170.

TOWBIN, H., T. STAEHELIN, AND J. GORDON. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350–4354.

WEISER, J. N., AND E. C. GOTSCHLICH. 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59:** 2252–2258.

WELLS, L., K. VOSSELLER, AND G. W. HART. 2001. Glycosylation of nucleocytoplasmic proteins: Signal transduction and O-GlcNAc. Science **291:** 2376–2378.

WHITEHEAD, T. P., L. J. KRICKA, T. J. N. CARTER, AND G. H. G. THORPE. 1979. Analytical luminescence: Its potential in the clinical laboratory. Clin. Chem. **25:** 1531–1546.

WINTERBURN, P. J., AND C. F. PHELPS. 1972. The significance of glycosylated proteins. Nature **236:** 147–151.

WOROBEC, E. A., N. L. MARTIN, W. D. MCCUBBIN, C. M. KAY, G. D. BRAYER, AND R. E. W. HANCOCK. 1988. Large-scale purification and biochemical characterization of crystallization-grade porin protein P from *Pseudomonas aeruginosa.* Biochim. Biophys. Acta **939:** 366–374.

YAMADA, N., S. SUZUKI, AND E. TANOUE. 2000. Detection of *Vibrio (Listonella) anguillarum* porin homologue proteins and their source bacteria from coastal seawater. J. Oceanogr. **56:** 583–590.

YAMAMOTO, S., AND R. ISHIWATARI. 1989. A study of the formation mechanism of sedimentary humic substance-II. Protein-based melanoidin model. Org. Geochem. **14:** 479–489.

YEN, P. H., AND BALLOU, C. E. 1974. Partial characterization of the sexual agglutination factor from *Hansenula wingei* Y-2340 type 5 cells. Biochemistry **13:** 2428–2437.

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