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Characterization of particulate organic matters in the water column of the South China Sea using a shotgun proteomic approach

Hong-Po Dong, Da-Zhi Wang,* Minhan Dai, and Hua-Sheng Hong

State Key Laboratory of Marine Environmental Science/Environmental Science Research Center, Xiamen University, Xiamen, China

Abstract

We characterized particulate organic matter (POM) collected from both the surface (41 m and 200 m) and mesopelagic layers (500 m and 1000 m) in the western South China Sea. By using a shotgun proteomic approach, a total of 3035 proteins matching one or more peptides were detected from four POM samples, 505 of which were identified as high-confidence proteins matching two or more peptides. Cyanobacteria was the largest contributor throughout the water column, while crustaceans and dinophytes were the two major groups contributing to the particulate proteins in the POM collected from 200 m. Subcellular locations and biological processes of particulate proteins varied significantly between the 41-m and 200-m layers: photosynthesis-associated proteins were highly abundant in the 41-m layer while tubulins and actins accumulated in the midwaters, especially at the 200-m layer. Porins, adenosine triphosphate synthases, nutrient transporters, molecular chaperones, and ectoenzymes were frequently detected in the POM samples and presented different distribution patterns within the water column, revealing complex biological processes at the different water layers and/or during the sinking of POM. The sources of surface and midwater particulate proteins are different, and the cellular metabolism, generation of energy, and transport processes in POM are attenuated rapidly down ocean water column. Zooplankton fecal pellet packages and membrane encapsulation might play important roles in protecting particulate proteins from degradation.

Particulate organic matter (POM) in seawater plays a key role in oceanic carbon cycling. Through the sinking of POM, carbon is transported into the ocean interior, where most of the POM is recycled. Only a small fraction of POM sinks into the deep ocean and is eventually buried in the sediment, which segregates carbon and influences atmospheric carbon dioxide concentrations and the global carbon cycle (Hedges et al. 2001). Although many efforts have been devoted to the marine POM (reviewed by Volkman and Tanoue 2002; Lee et al. 2004), it is still a poorly understood organic pool in terms of its source, its chemical composition at molecular level, as well as its transformation when transferring from the surface to the deep sea.

POM is composed of both living organisms and nonliving organic matter, with the latter generally being its major component. The relative abundance of living biomass and detritus changes temporally and spatially, and the contribution of living biomass to the bulk POM in surface water is generally an order of magnitude lower than that of detrital organic matter (Volkman and Tanoue 2002). It is known that phytoplankton producers dominate the organic composition of POM in oceanic surface waters, and the plankton community structure generally modulates the recycling and export of organic materials in and out of the euphotic zone. Note that picophytoplankton (< 2 μm), including cyanobacteria such as *Prochlorococcus* and *Synechococcus*, and small eukaryotic algae have been found to contribute substantially to both phytoplankton biomass and production in marine ecosystems (Campbell et al. 1994; Liu et al. 2004). Prokaryote biomass, including photosynthetic prokaryotes, contribute $> 90\%$ of the living biomass of POM at the ocean surface (Wilhelm and Suttle 1999).

Proteins are fundamental components of all living cells and, along with enzymes, hormones, and antibodies, are essential for the proper functioning of an organism. The composition and sequence of amino acids of proteins provide information about their origin and cellular function, including structural organization, stress response, energy production, and conversion. Therefore, characterization of the proteins of POM will improve our understanding of the sources and mechanisms that control the cycling and long-term preservation of organic matter (Nunn and Timperman 2007). Previous studies have demonstrated that proteinaceous materials are a major component of POM (Setchell 1981; Long and Azam 1996). Among others, particulate combined amino acids (PCAAs), a mixture of cellular proteins and detrital combined amino acids, are considered to be major constituents of POM in oceanic surface waters (Tanoue 1996), and are also the largest component (40–50%) of particulate organic carbon (POC) in sinking POM (Hedges et al. 2001). The PCAAs in POM include at least four chemical forms: proteins, acidic materials containing peptides, protein or peptide-specific dye-stainable low-molecular-mass materials, and non-stainable materials, and all are considered to be major components of detrital POM (Saijo and Tanoue 2005).

However, due primarily to the difficulty in protein analysis and identification, the characteristics of POM protein are not well documented to date. A few discrete proteins have been separated and identified from POM using gel-based methods (Saijo and Tanoue 2004, 2005). Our knowledge of the POM dynamics, sources, transfer processes, and its degradation through the water column

* Corresponding author: dzwang@xmu.edu.cn

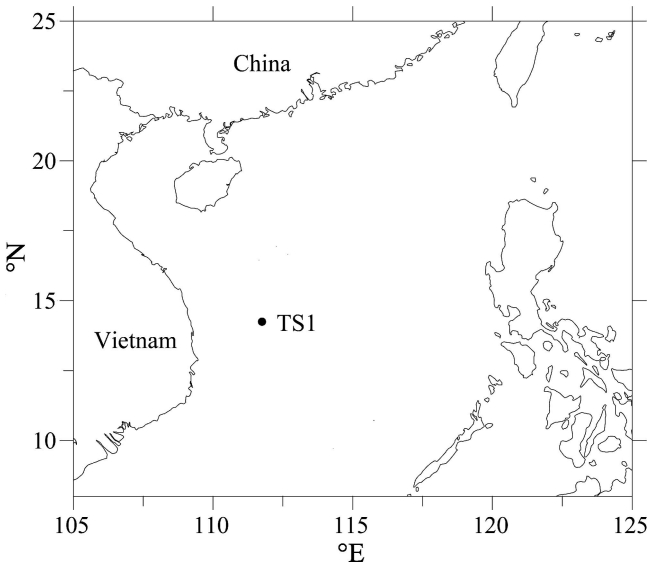

Fig. 1. The sampling location of POM from different water layers in the western South China Sea.

remains largely lacking. Global techniques, such as "shotgun proteomics," provide effective strategies and tools for profiling and identifying proteins in complex protein mixtures, and have been successfully applied to characterize the protein profiles of environmental samples (Ram et al. 2005; Sowell et al. 2009). In contrast to the traditional gel-based method, this approach produces a mixture of peptides through global digestion of the pool of proteins. The mixture is then separated and analyzed with multidimensional liquid chromatography (LC) coupled to a tandem electrospray ionization mass spectrometer, somewhat overcoming the current limitations of two-dimensional electrophoresis (2-DE) technology (Wu and MacCoss 2002). Recently, this method was applied to study marine particulate proteins, showing its powerful ability for analyzing proteins in complex POM samples (Dong et al. 2009).

Here we combined "shotgun" mass spectrometry (MS)–based proteomics with an National Center for Biotechnology Information (NCBI) protein database search to characterize proteins in marine POM. To our knowledge, this study is the first attempt to apply this approach to characterize POM proteins from surface seawater to mesopelagic waters. Using this method, 3035 proteins matching one or more peptides were identified and characterized in POM samples collected from different water layers in the South China Sea (SCS). The pool of particulate proteins consisted of a variety of protein classes from different subcellular locations and biological processes, and the "proteomic fingerprint" of each water layer reflected the presence and activity of organisms in POM as well as the dynamics of POM during the sinking.

Methods

Sample collection—POM samples from different water depths were collected at Sta. TS1 (14°15.495′N, 111°45.563′E; water depth ~ 2390 m) during a summer cruise to the western SCS from 14 August 2007 to 14 September 2007 (Fig. 1). Samples were collected onto 150-mm GF/F glass-fiber filters (Whatman) using an in situ large-volume water-transfer-system sampler (McLane). The depths and filtered seawater volumes were 41 m, 1000 liters; 200 m, 1000 liters; 500 m, 1110 liters; and 1000 m, 1350 liters. Sampling date for these four samples is between 26 August and 27 August 2007. Any visible zooplankton was removed with clean forceps, and all samples were kept frozen at −80°C aboard the ship.

The vertical profiles of temperature and salinity were monitored shipboard with a SBE-19-plus conductivity–temperature–depth or pressure unit (Sea-Bird). Phytoplankton biomass (as chlorophyll *a* [Chl *a*]) was analyzed according to standard spectrofluorometric methods (Parsons et al. 1984) using a Hitachi 850 fluorometer. Particulate organic carbon (POC) was analyzed using a carbon–hydrogen–nitrogen analyzer (PE-2400) after carbonate was removed by fuming the filters with concentrated HCl.

Protein extraction and determination in POM—Procedures for extracting the particulate protein were reported previously (Dong et al. 2009) and are briefly described here. The GF/F filter was cut into chips. The chips were suspended in lysis buffer consisting of urea, thiourea, 3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate, Triton X-100, carrier ampholytes, dithiothreitol, and protease inhibitor cocktail, shaken in an ice-cold water bath using an ultrasonic shaker, and then lysed with a sonicator using a microprobe. The solution was incubated at 25°C for 1 h and then centrifuged at 20,000 × *g* for 30 min at 10°C. The supernatant was precipitated with ice-cold 20% trichloroacetic acid (TCA) in acetone. The mixture was centrifuged, and the resultant pellets were rinsed twice with ice-cold acetone and air-dried. Finally, the powder was dissolved in rehydration buffer.

The protein content in the POM concentrate was quantified using the Bradford method. The concentrate was stored in a freezer (−80°C) until further analysis. Unless otherwise noted, all the reagents used in this study were electrophoretic grade.

Shotgun proteomic analysis of POM proteins—The rehydrated POM protein concentrate was loaded onto a 5–12% Bis-Tris gel. Electrophoresis was carried out using the Tris–glycine–sodium dodecyl sulfate (SDS) buffer system (25 mmol L^{-1} Tris, 192 mmol L^{-1} glycine, and 0.1% SDS) on a Hoefer SE 600 apparatus (Amersham). Low-molecular-weight standards were used in the gel, which was fixed and stained with colloidal Coomassie Brilliant Blue G-250 (CBB; Bio-Rad). The gel was cut into four equal pieces to isolate molecular weight (kDa) fractions, followed by reduction, alkylation, and in-gel digestion with trypsin as described previously (Wilm et al. 1996).

The peptides extracted from the gel bands were analyzed by data-dependent tandem mass spectrometry (MS^2) using an on-line liquid chromatography–linear ion trap mass spectrometer (LC-LTQ; Thermo Finnigan). Procedures for

MS analysis were reported previously (Dong et al. 2009). Briefly, the dried peptide extracts were dissolved in 0.1% formic acid and injected onto a C18 peptide trap (Agilent Technologies), and desalted with 0.2% formic acid. Peptides were eluted from the trap and separated on a reversed-phase C18 column with a linear gradient of 0–50% mobile phase B (0.1% formic acid, 84% acetonitrile) in mobile phase A (0.1% formic acid). The LC-MS^2 measurements were made with an LTQ linear ion trap mass spectrometer. To prevent repetitive analysis of the same abundant peptides, dynamic exclusion was enabled with a repeat count of 2 and an exclusion duration of 1.5 min on the LTQ. The LC-MS system was fully automated and under the direct control of an Xcalibur software system (Thermo Finnigan). The 10 most intense ions in every full scan were automatically selected for MS^2 analysis.

Bioinformatic analysis—The protein sequences derived from the MS^2 spectra of tryptic peptides bears taxonomic information about the origin of the proteins. In most cases, the sequences obtained from tryptic peptides are unique to a specific group of organisms, or even one species (Schulze et al. 2005). In this study, a large protein database of potential groups of organisms at Site TS1 was created as previously described (Dong et al. 2009). The proteins identified were grouped on the basis of their taxonomic origin into broader taxonomic levels following the nomenclature of the NCBI taxonomy. Proteins from bacteria were classified into three subgroups: Proteobacteria, Cyanobacteria, and Bacteroidetes, and proteins from eukaryotic algae were classified into seven subgroups: Prasinophyta, Dinophyta, Cryptophyta, Haptophyta, Chrysophyta, Bacillariophyta, and Chlorophyta, based on the classification of phytoplankton functional groups (Reynolds et al. 2002). Other proteins, not belonging to the above groups, were grouped into Archaea, Crustacea, Oomycetes, and Stramenopiles.

The MS^2 spectra from the LTQ data set were searched against the abovementioned database using the SEQUEST algorithm (Thermo Finnigan). All SEQUEST searches were performed using Bioworks 3.2 software (Thermo Finnigan) with the following parameters: fully tryptic peptide, Parent Mass Tolerance, 1.4; and Peptide Mass Tolerance, 1.5. The protein identification criteria were based on Delta CN (≥ 0.1) and Xcorr (one charge ≥ 1.9, two charges ≥ 2.2, three charges ≥ 3.75).

Protein identifications were extracted from the SEQUEST out. file using the in-house software BuildSummary, which combined the peptide sequences into proteins and deleted redundant proteins, as described by Wu and Yates (2003). Because false-positive identifications mainly occur within single peptide-matched proteins, proteins matched by two or more peptides are considered as confident detection (He et al. 2005; Ram et al. 2005). Their biological processes were categorized using the Blast2GO tool on www.geneontology.org (Conesa et al. 2005). The Gene Ontology (GO) project is a major bioinformatics initiative with the aim of standardizing the representation of gene and gene product attributes across species and databases. The project provides structured, controlled vocabularies and classifications that cover several domains of molecular and cellular biology and are freely available for community use in the annotation of genes, gene products, and sequences. The GO database integrates the vocabularies and contributed annotations and provides full access to this information in several formats. Procedures of the Blast2GO analysis were performed as previously described (Dong et al. 2009). Protein subcellular location was predicted using the Proteome Analyst-Subcell Specialization Server 2.5 (http://pasub.cs.ualberta.ca:8080/pa/Subcellular) (Lu et al. 2004).

Results

Vertical variations of particulate protein concentration and other parameters at Sta. TS1—The vertical profiles of particulate protein concentration and other parameters at Sta. TS1 are shown in Fig. 2. Sta. TS1 was located in the center of a cold eddy during the survey period, the thermocline was observed at 30–40-m water depth (Fig. 2A), and the subsurface chlorophyll maximum (DCM) was found at about the 40-m layer (Fig. 2B). Concentrations of particulate protein and POC were high in the euphotic layer (upper 100 m) and reached their maximum at 41 m, with 0.76 $\mu g\ L^{-1}$ particulate protein and 22.25 $\mu g\ L^{-1}$ POC, and then remained relatively constant in the midwater layers (Fig. 2C,D). Chl *a* concentration was high in the upper 50 m and peaked at 41 m (0.65 $\mu g\ L^{-1}$) (Fig. 2B). Vertical profiles of the particulate protein, POC, and Chl *a* concentrations presented a similar variation pattern from surface to midwaters, indicating that the particulate proteins within the water column were regulated by biological productivity.

Separation and identification of particulate proteins in POM—A typical one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of particulate proteins is shown in Fig. 3. The electrophoresis of all POM samples visualized using CBB stain showed a smeared staining pattern throughout the molecular mass range, and no clear band was recognized due to the heavy background staining. The staining intensity of the POM samples from the 500-m and 1000-m water layers was lower than that of the other two samples (Fig. 3). For each POM sample, four equal slices were cut from the one-dimensional SDS-PAGE gel and each slice was analyzed using the LTQ mass spectrometer. Peak list files obtained from the four fractions were processed separately and the peptide sequences were identified. One or more peptides were assigned to 3035 proteins from four POM samples after removal of contaminants (keratins, trypsin) and redundant proteins, namely 737 in 41-m POM, 915 in 200-m POM, 764 in 500-m POM, and 619 in 1000-m POM (Fig. 4). For confident detection, 505 particulate proteins that matched two or more peptides were identified, accounting for 25%, 13.4%, 13.6%, and 15.2% of the identified proteins in each POM sample from the 41-m, 200-m, 500-m, and 1000-m water layers, respectively. The NCBI accession number, protein name, number of peptides used in the identification, sequence coverage (%), theoret-

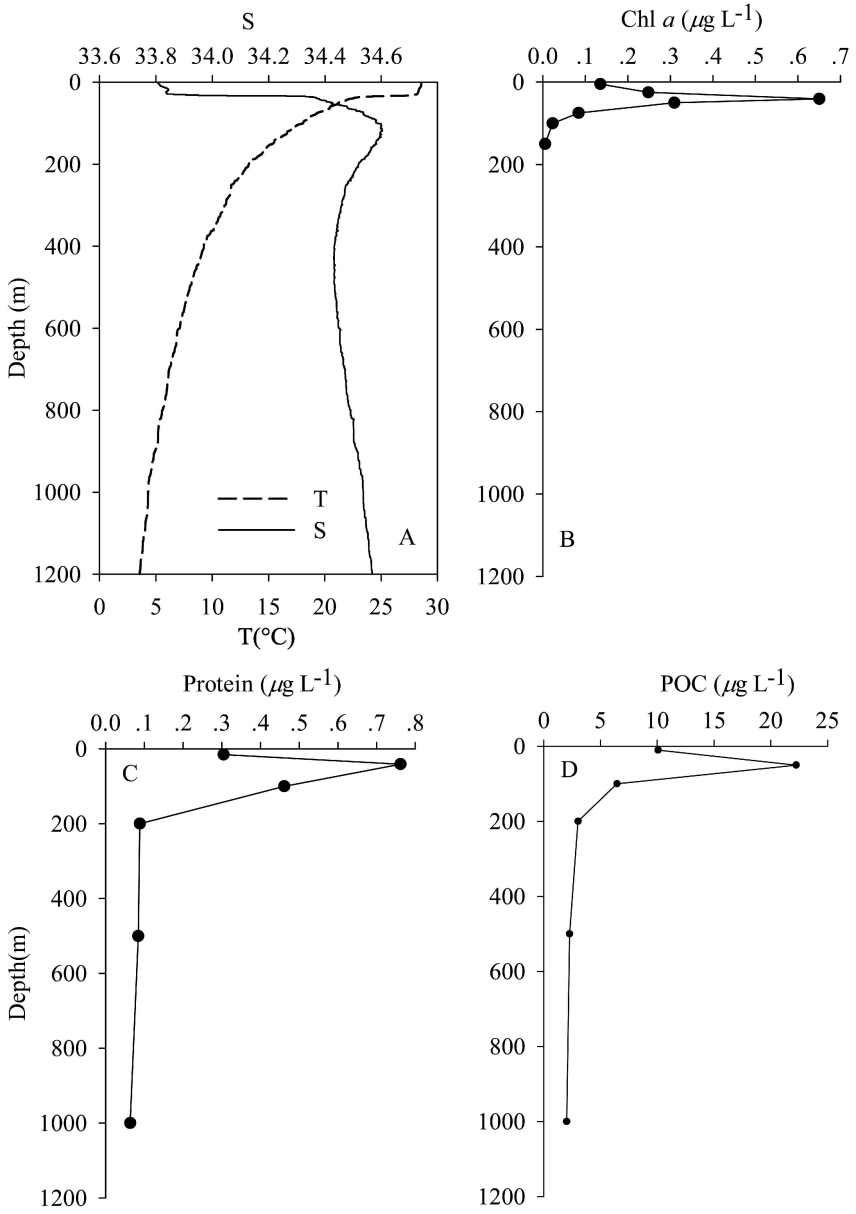

Fig. 2. Vertical profiles of physical and chemical parameters and particulate protein at Sta. TS1 in August 2007 in the western South China Sea. (A) Temperature (T) and salinity (S). (B) Chl *a* concentration. (C) Particulate protein concentration. (D) POC concentration.

ical molecular weight (MW) and isoelectric point (pI), and the subcellular location of all particulate proteins are listed in the Web Appendix (www.aslo.org/lo/toc/vol_55/issue_4/1565a.html).

Overall, about 30% of the proteins identified in the POM samples were novel proteins with no functional assignments based on the BLASTP algorithm. The theoretical pI and MW values of the proteins identified from POM were calculated by DTASelect software. About 60% of the identified proteins had theoretical pI values in the pH range of 4–6.9, and 70% had theoretical MW values between 10 and 60 kDa (Fig. 5). In addition, the MWs and pIs of particulate proteins in each POM sample exhibited similar distribution patterns, indicating that there was a close relationship in particulate proteins among POM within the same water column.

Biological origin of particulate proteins identified in POM—The biological origin of particulate proteins in all POM samples is shown in Fig. 6. At each water layer, cyanobacteria were the largest contributors and accounted for about 32% to 44% of the identified proteins in each POM sample. In addition, although the number of cyanobacteria proteins decreased gradually with depth, a considerable number of proteins derived from cyanobacteria were still detected in POM samples from midwaters, suggesting that there was presumably an abundance of intact cyanobacteria in the POM from midwaters at this

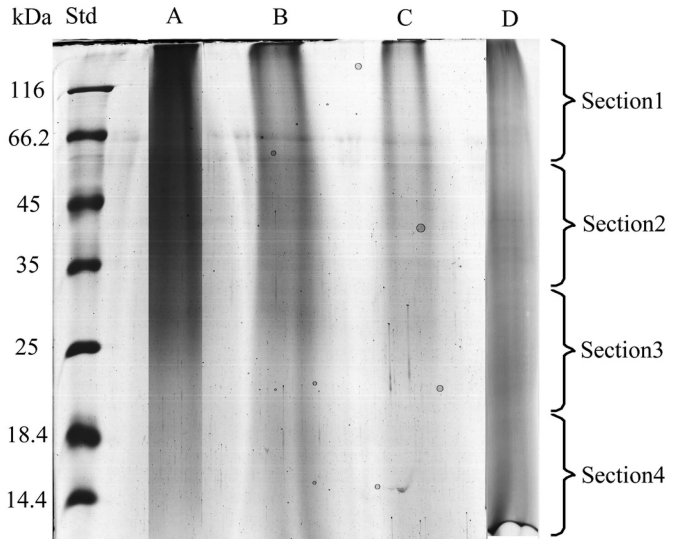

Fig. 3. One-dimensional SDS-PAGE electrophoretogram of particulate proteins precipitated by 20% trichloroacetic acid (TCA) in acetone in POM. The left lane is a molecular weight standard. Lanes A, B, C, and D represent samples from the 200-m, 500-m, 1000-m, and 41-m layers. The gel was stained with CBB and cut into four pieces, i.e., Sections 1, 2, 3, and 4. An ~ 60-μg bovine serum albumin equivalent amount of electrophoretic sample was loaded for each lane.

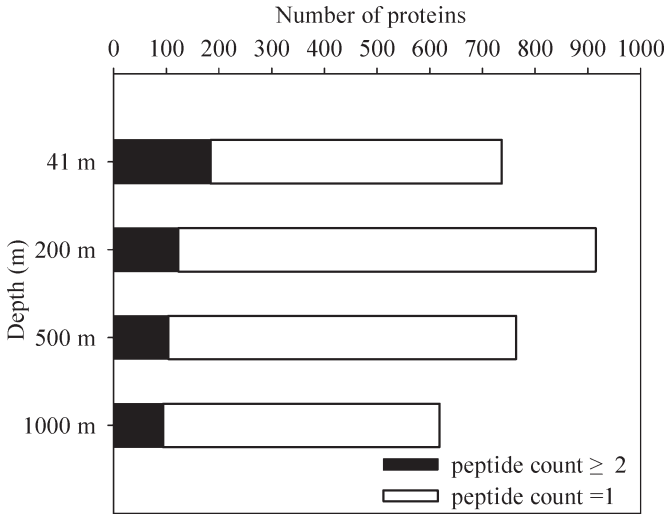

Fig. 4. The number of particulate proteins identified from the POM collected at different water layers at Sta. TS1 in the western South China Sea. In symbol, "peptide count = 1" refers to the number of proteins that matched one peptide.

sampling station. The number of proteins derived from three other bacterioplankton groups, Proteobacteria, Bacteroidetes, and Archaea, were low at the 41-m layer, reached a maximum at the 200-m layer, and subsequently decreased with depth. The variations of protein numbers from Prasinophyta, Dinophyta, and Crustacea were similar to that of bacteria and the highest protein numbers were also found at the 200-m layer. The number of proteins from Haptophyta and Bacillariophyta decreased rapidly with depth. In addition, a high percentage of phytoplanktonic proteins (63%) was detected in the POM sample from the 41-m layer compared to the other three POM samples from midwaters, indicating that phytoplankton dominated the plankton community in surface water and was the major source of surface POM.

Subcellular location of particulate proteins—In this study, the identified proteins were divided into two groups according to their biological origin: prokaryotic proteins and eukaryotic proteins, and subsequently classified into subcellular compartments using Proteome Analyst—Subcell Specialization Server 2.5 (University of Alberta Bioinformatics Research Group).

Of the prokaryotic particulate proteins, those with unknown cellular localization accounted for 41% to 45% of the prokaryotic proteins in POM samples (Fig. 7A). Except for these unknown proteins, cytoplasmic proteins were the largest component of prokaryotic particulate proteins (~ 24–29%) in POM samples, and the highest protein number was observed in the 200-m layer and then decreased gradually with the depth. In the 41-m layer, 149 proteins were classified as inner membrane proteins, representing 26% of the particulate proteins, including adenosine triphosphate (ATP) synthases, light-harvesting proteins, photosystem II(I) proteins, cytochromes, transporters, porins, Na^+ or H^+ antiporters, channels, and other cellular localization proteins. The maximum number of membrane proteins was detected in the POM sample from the 41-m layer and the number decreased with depth, suggesting that membrane proteins were subject to degradation during the sinking of POM from the surface to midwaters. A total of 101 proteins from prokaryotes were classified as extracellular proteins in all of the POM samples, including various ectoenzymes, such as galactosidase, betaglucanase, aminopeptidase, esterase, peptidase, protease, amylase, polygalacturonase, and lipase, and the ectoenzymes varied with the water depth (*see* Web Appendix). For example, beta-galactosidase, metalloendopeptidases, and metalloproteinase were only detected in the 41-m sample, chitinase in the 200-m sample, amylases in the 500-m and 1000-m samples, and thioesterases detected in 200-m, 500-m, and 1000-m samples. These results demonstrated that the components of organic matter in POM samples from different water depths were distinct from each other. In addition, the type and the number of ectoenzymes in the 200-m and 500-m samples were higher than that in 41-m and 1000-m samples, suggesting that organic matter from these two water layers was more complicated and diverse.

For the eukaryotic particulate proteins, the cytoplasmic, nuclear, and chloroplastic proteins were the main components in all of the POM samples. The maximum number of cytoplasmic proteins from eukaryotes was observed in the 200-m water layer and then decreased rapidly with water depth. The number of nuclear proteins varied a little from the surface to the midwaters. Chloroplastic proteins comprised about 41% of the eukaryotic particulate proteins in the POM sample from the 41-m layer (Fig. 7B), and declined to about 6% to 7% in the other three POM samples from midwaters. This result implied that highly efficient photosynthesis occurred in the 41-m water layer and the majority of chloroplastic proteins were degraded

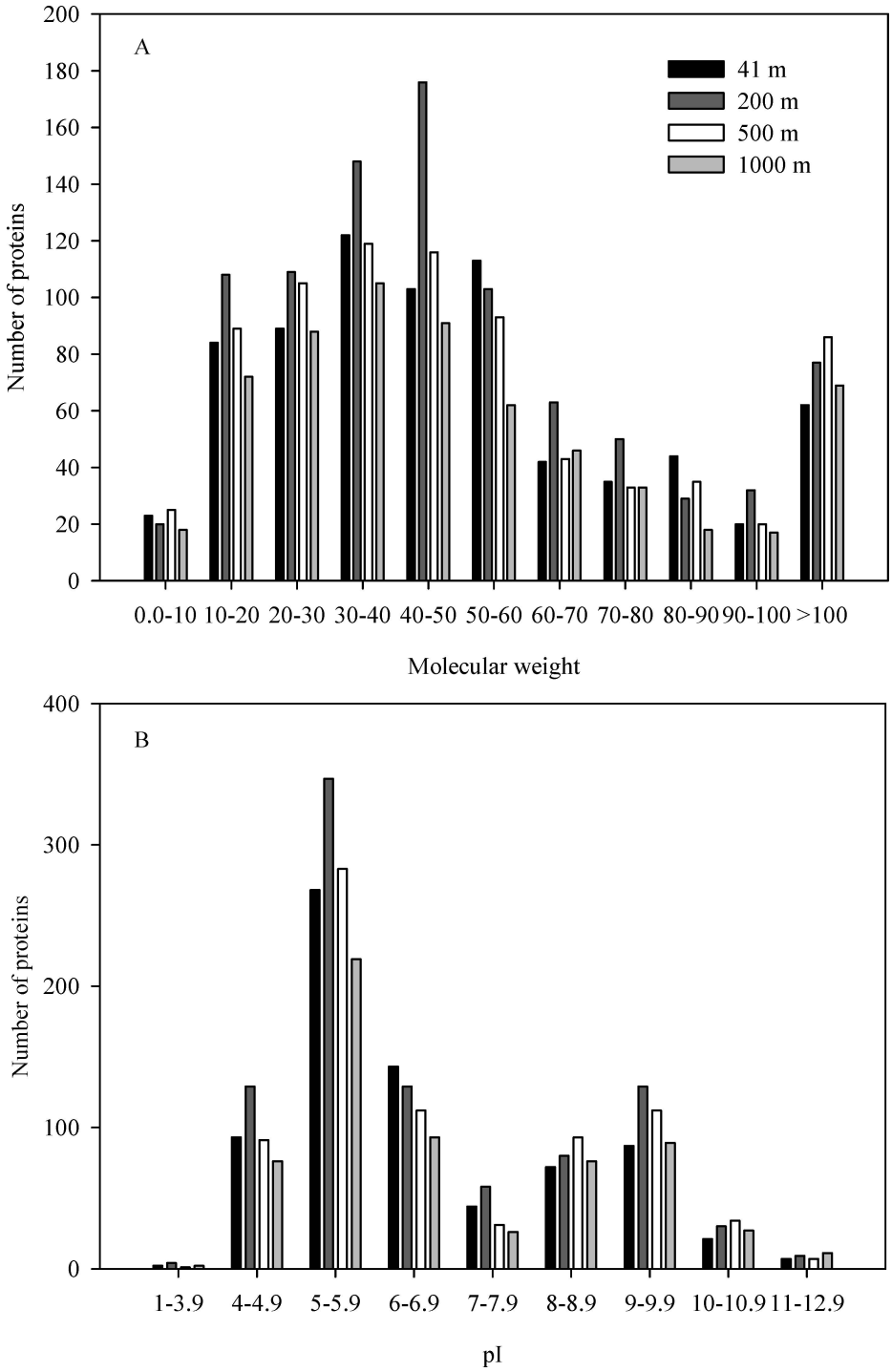

Fig. 5. Distribution of molecular weights and isoelectric points of all the proteins identified in the POM from the four water depths at Sta. TS1 in the western South China Sea. (A) Molecular weight distribution. (B) pI distribution.

during the sinking of POM from the surface to the midwaters.

Biological processes of particulate proteins—The identified particulate proteins were functionally categorized based on universal GO annotation terms using the Blast2GO tool against the non-redundant protein database. Totally, about 47% to 56% of the particulate proteins in POM samples were linked to at least one annotation term within the GO biological process category, while the remaining 44% to 53% of particulate proteins were with unknown functions or unannotated.

Overall, the number of proteins involved in cellular metabolic processes decreased rapidly with depth (from 271

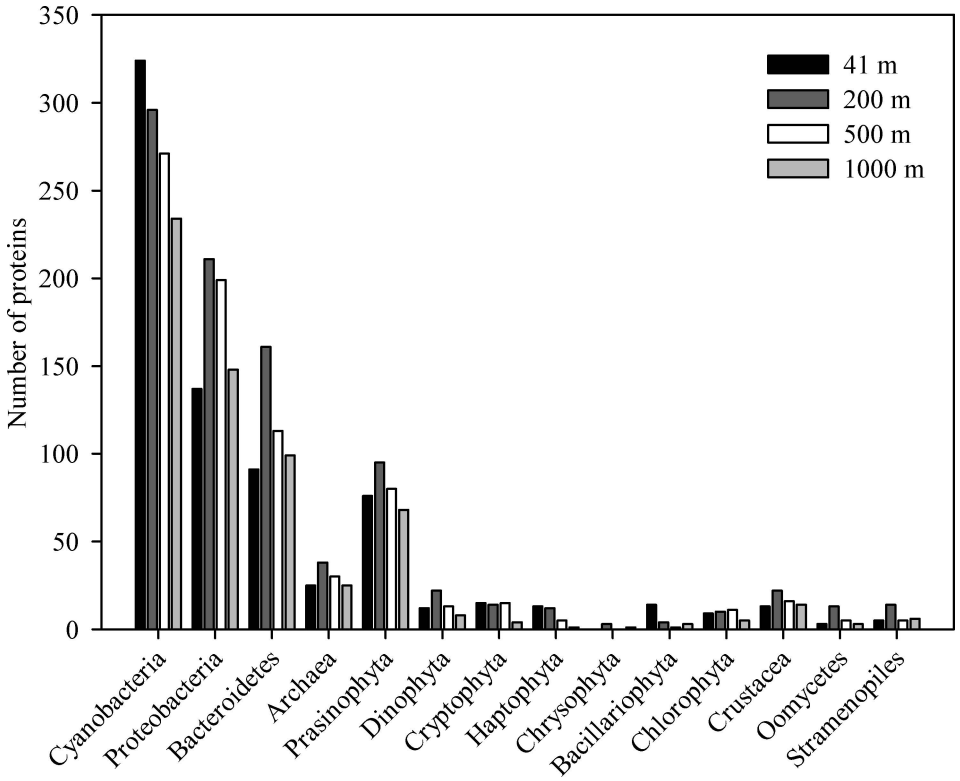

Fig. 6. Distribution of phylogenetic groups contributing to all identified proteins in the POM from the four water layers in the western South China Sea.

at 41 m to 118 at 1000 m), while the maximum number of proteins involved in primary metabolic processes and macromolecule metabolic processes was observed in the 200-m layer and decreased with depth, especially from 500 m to 1000 m (Fig. 8), suggesting that biological activities in POM were attenuated rapidly down ocean water columns. The highest proportion of peptides matching photosynthesis proteins was found in the POM from the 41-m layer, accounting for 44.4% of the total peptides for POM, including many protein complexes involved in photosystem I and photosystem II, but peptides that matched this group of proteins accounted for only 0.6% to 1.5% of the total peptides in the other POM samples (Table 1). In addition, the number of proteins involved in photosynthesis and the generation of precursor metabolites and energy in the POM sample from the 41-m water layer was significantly greater than that of proteins from the other POM samples. In the POM sample from the 41-m water layer, 99 proteins were identified to be involved in photosynthesis (Fig. 8; Web Appendix), including ribulose 1,5-bisphosphate carboxylase or oxygenase, light-harvesting complex protein, cytochrome, and phycoerythrin. Among them, 49 proteins were located in the chloroplasts of eukaryotic algae and 50 were located on the inner membrane (photosynthetic membrane) of cyanobacteria. In other POM samples from midwaters, especially the 200-m water layer, the most frequently detected proteins were involved in cellular component organization and biogenesis or cellular localization (Web Appendix; Fig. 8). Cytoskeleton organization and biogenesis, including tubulins and actins, were the predominant processes in cellular component organization and biogenesis in this study. Across three samples from midwaters, peptides that matched proteins involved in these functions comprised 10% to 63.8% of the total peptides, whereas they accounted for only 2% of the total peptides in the POM sample from the 41-m layer (Table 1). In addition, tubulin and actin proteins were among the most prevalent proteins detected from the POM samples from the 200-m, 500-m, and 1000-m water layers (Web Appendix). In the POM sample from 200 m, a total of 71 tubulin and actin proteins were identified, accounting for 7.8% of the total proteins, and most of them were high-abundance proteins (Table 1). Obviously, the particulate protein profiles between the surface and midwaters were significantly different, possibly since the particulate protein composition of the surface waters might be determined by the structure and activities of the biological community, while the particulate protein composition of midwaters was a result of the accumulation of organism-derived detritus.

Transport function is defined as the directed movement of substances (such as macromolecules, small molecules, and ions) into, out of, within or between cells, or within a multicellular organism (http://www.geneontology.org/). Figure 8 shows that the number of proteins involved in transport functions decreased with depth (from 120 at 41 m to 26 at 1000 m), suggesting that the cell's capacity to import nutrients for metabolism in POM declines with increasing depth. Peptides that matched transporters involved in nutrient uptake in the 41-m POM sample

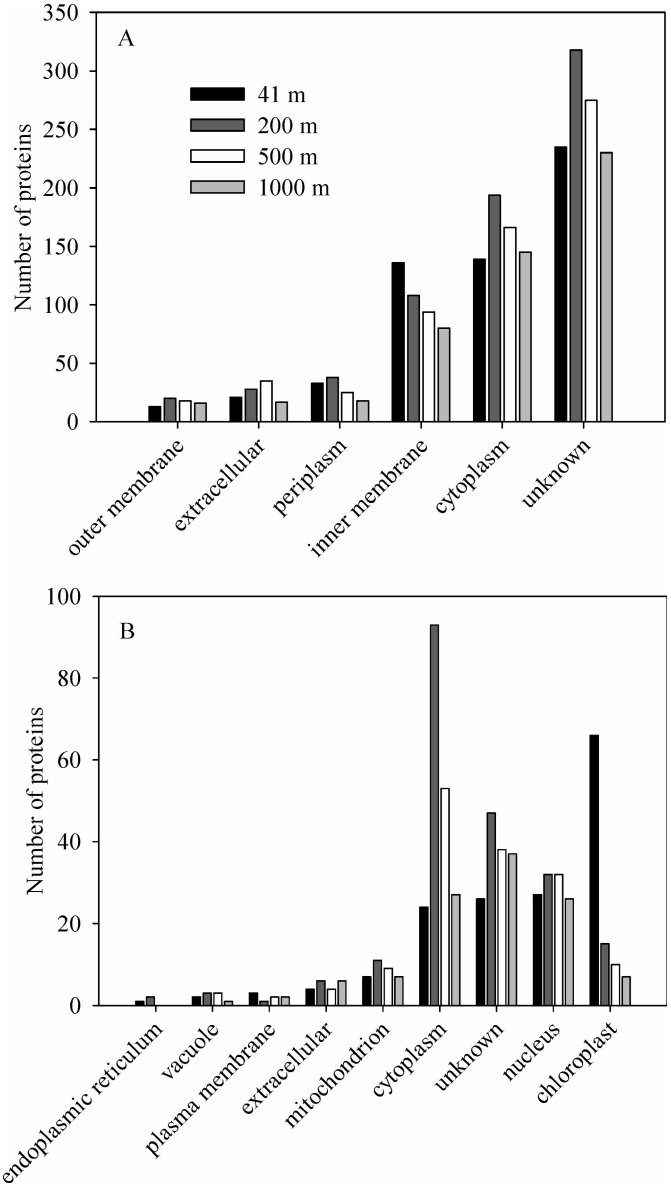

Fig. 7. Classification of all identified proteins by subcellular localization. (A) Subcellular localization for particulate proteins from prokaryotes. (B) Subcellular localization for particulate proteins from eukaryotes.

comprised 6.8% of the total peptides, which was higher than that in the POM samples from the midwaters (2.5% to 4.9%) (Table 1). In all of the POM samples, two of the most abundant transporters detected were involved in urea and ammonium uptake (*see* Web Appendix).

The number of proteins involved in the stress response in the POM sample from the surface was greater than that in the POM samples from midwaters (Fig. 8). There are two main kinds of important proteins in this group: chaperone proteins and ubiquitin proteins. The number of chaperone proteins, including heat-shock protein 70, 60-kDa chaperonin 2, heat-shock protein 90, and molecular chaperone DnaK, decreased with depth (from 10 at 41 m to four at 1000 m), and peptides that matched these proteins accounted for 1.5% of the total peptides in the 41-m POM sample, which was significantly higher than that in the other POM samples (0.7%) (Table 1). Polyubiquitin and ubiquitin proteins, a group of highly conserved regulatory proteins ubiquitously expressed in eukaryotes were among the most prevalent proteins detected in the 41-m POM sample (Web Appendix). More ubiquitin proteins were observed in the POM sample from surface water compared to the POM samples from midwaters.

In the POM sample from 41 m, ATP synthases, which are involved in the generation of precursor metabolites and energy, were among the most abundant proteins (Web Appendix). A total of 25 ATP synthases were detected and the peptides that matched the proteins accounted for 10.4% of the total peptides (Table 1). In addition, ATP synthases were frequently detected in the POM samples from midwaters but the abundance was significantly lower than that in the surface POM sample. Peptides that matched the ATP synthases comprised 1.1% to 5.8% of the total peptides in the POM samples from midwaters.

Discussion

Source of proteins in POM—It is well known that the POM collected onto the GF/F filters using in situ filtration methods includes the suspended POM (SPOM) as well as part of the sinking POM (reviewed by Volkman and Tanoue 2002). Using principal components analysis, Sheridan et al. (2002) reported that the suspended particles in surface waters (0–200 m) from the equatorial Pacific Ocean are composed primarily of labile phytoplankton materials and that they present a similar pigment, lipid, and amino acid composition as that of the surface phytoplankton. Also, they are less degraded than particles sinking out of the euphotic zone, whereas midwater suspended particles (200–1000 m) contain phytodetritus derived from particles exiting the euphotic zone (105 m) as well as being diluted with zooplankton and microbial source materials. In this study, the phytoplankton communities at the sampling station were comprised of cyanobacteria, bacillariophytes, haptophytes, chlorophytes, cryptophytes, prochlorophytes, prasinophytes, and dinophytes. Of these, the cyanobacteria in the upper 150 m were the most abundant autotrophic picoplankton, with the maximum abundance if *Prochlorococcus* is classified within the cyanobacteria (Huang et al. pers. comm.). Recently, Liu et al. (2007) reported that the integrated maximum abundance of *Prochlorococcus* occurred in the upper 150 m in the summer, which contributed up to 80% of the total autotrophic biomass at the Southeast Asia Time Series Station in the SCS. By tracing the source organisms of proteins identified using MS^2 spectra, proteins from almost all of the above-mentioned phytoplankton classes were detected in a POM sample at the 41-m layer, and the percentage of cyanobacterial proteins was the highest, up to 44.0% in all functional groups (Fig. 6), which reflects the high abundance and activity of cyanobacteria in this area. However, care needs to be taken with such an explanation, since cyanobacterial proteins were the most abundant compared

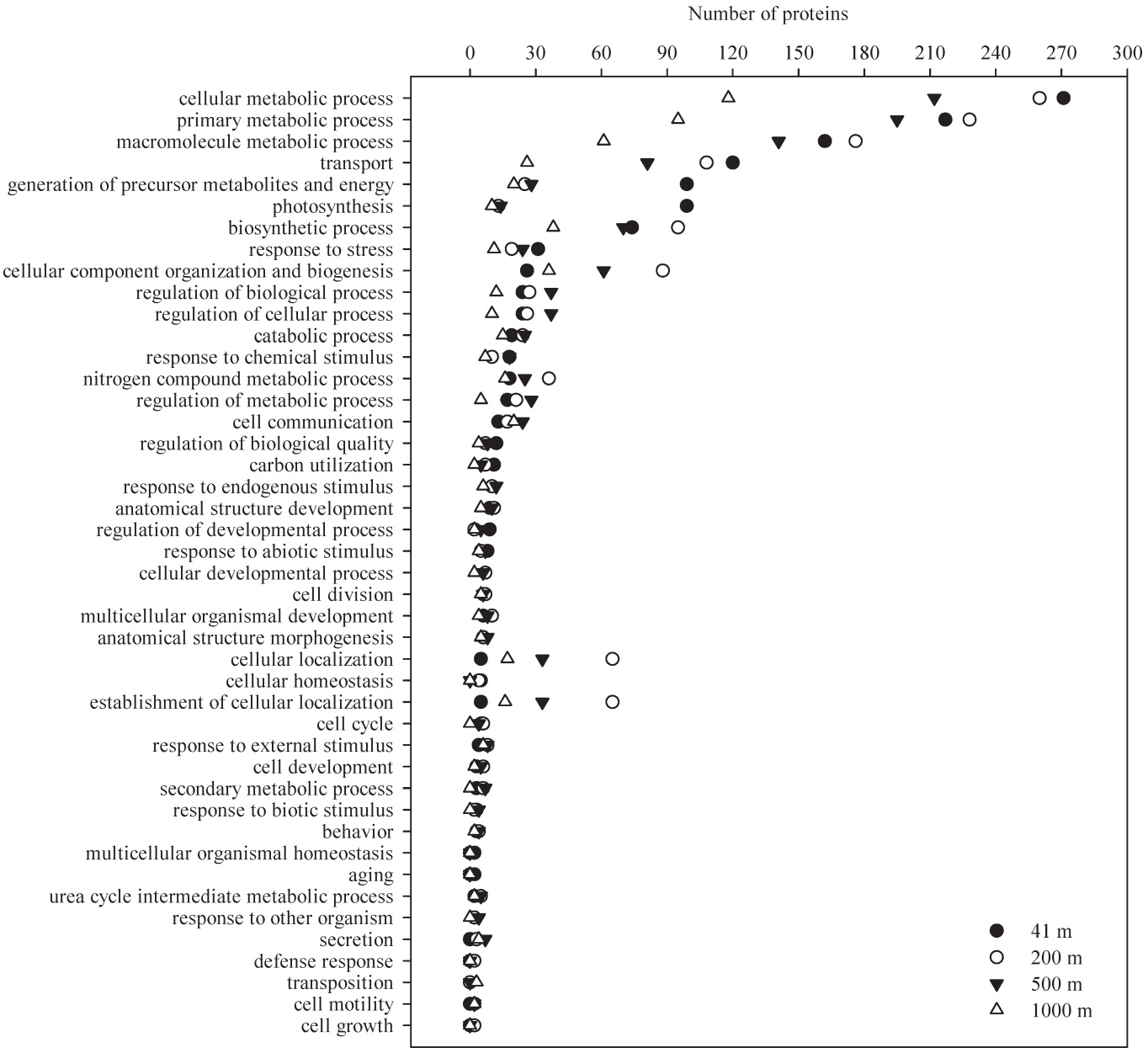

Fig. 8. Gene Ontology (GO) biological process terms for all identified proteins in the POM from 41-m, 200-m, 500-m, and 1000-m water depths. GO is a structured, controlled vocabulary that describes gene products in terms of their associated biological processes, cellular components, and molecular functions in a species-independent manner.

to those from other marine organisms in the present protein database.

Diatoms and coccolithophores are the main components of the $>$ 3-μm phytoplankton in the ocean. Coccolithophores are the best-known haptophytes covered by calcium carbonate platelets, while diatoms have silica shells and often produce biological glues, which may promote aggregation and hence faster sinking rates (Boyd and Newton 1999). This study demonstrated that proteins from these two phylogenetic groups generally decreased rapidly with depth, suggesting that these large algae can sink rapidly after death, and thereby contribute significantly to the downward flux of organic carbon (Fig. 6). However, for smaller Prasinophyte and Dinophyte, sinking is very slow, so zooplankton fecal pellets may contribute to the accumulation of proteins from these two phylogenetic groups at 200 m.

Saijo and Tanoue (2004) distinguished 23 discrete proteins from 19 surface POM samples from the Bering Sea to the equatorial Pacific using the 2-DE method, and concluded that the proteins resolved on 2-DE electrophoretograms were not directly derived from living organisms but from detrital POM. In this study, we detected 737 proteins in a POM sample from the 41-m layer using one-dimensional electrophoresis gel-based LC-MS^2. These proteins were involved in various biological processes,

Table 1. Proportion of protein groups with distinctive features detected in the POM samples from different water layers in the South China Sea.

Protein group	Depth (m)	Number of proteins	Percentage of total proteins (%)	Number of peptides	Percentage of total peptides (%)
Proteins involved in photosynthesis	41	99	13.4	891	44.4
	200	13	1.4	17	0.6
	500	14	1.8	19	1.5
	1000	10	1.6	11	1.3
Proteins involved in cytoskeleton organization and biogenesis	41	6	0.8	41	2.0
	200	71	7.8	1681	63.8
	500	34	4.5	312	25.0
	1000	14	2.3	85	10.0
Transporters	41	53	7.2	136	6.8
	200	51	5.6	65	2.5
	500	32	4.2	62	4.9
	1000	24	3.9	33	3.9
Chaperone proteins	41	10	1.4	31	1.5
	200	7	0.8	19	0.7
	500	5	0.7	9	0.7
	1000	4	0.6	6	0.7
ATP synthases	41	25	3.4	208	10.4
	200	10	1.1	30	1.1
	500	13	1.7	72	5.8
	1000	9	1.5	40	4.7

including photosynthesis, nitrogen compound metabolic processes, carbon utilization, cellular component organization and biogenesis, stress responses, and cell communication, indicating that a large number of the identified particulate proteins might be derived directly from living organisms, for it is improbable that so many proteins involved in diverse metabolic processes in cells could be protected from biodegradation as detrital proteins. During the sampling period, the 41-m layer had the maximum Chl *a* concentration and an abundance of phytoplankton. On the basis of the number of peptides identified and sequence coverage of MS, POM from the 41-m layer was enriched with photosynthesis proteins and ATP synthases, especially the light-harvesting complex of cyanobacteria and eukaryotic algae. Peptides that matched proteins associated with photosynthesis functions accounted for 44.4% of the total peptides for POM (Table 1). The prevalence of proteins involved in photosynthesis, carbon fixation, and energy generation in POM might reflect in situ growth of phytoplankton and accumulation of phytoplankton biomass in the subsurface chlorophyll maximum layer in the oligotrophic SCS. These results demonstrated that the particulate proteins in the POM samples from the upper 100 m in the SCS were primarily comprised of cellular proteins from living organisms.

Midwater SPOM is a very complicated mixture of small heterotrophic organisms, diatom cysts, copepod eggs, ''minipellets'' from microzooplankton, fragments of metazoan carcasses, fecal pellets, and phytodetritus. Previous studies have shown that zooplankton can alter the suspended particle composition by consuming suspended particles and thus influence the midwater suspended particle pools through disaggregation of fecal pellets (Wakeham and Lee 1989; Sheridan et al. 2002). During the survey period, crustacean-originated proteins increased from the surface to midwaters (Fig. 6), indicating that zooplankton might be the most important source of midwater POM. Microbes also directly degrade suspended particles or transform organic materials derived from sinking particles, and thus contribute biomass to the midwater suspended particle pools (Sheridan et al. 2002). This study also found that proteins derived from Proteobacteria, Bacteroidetes, and Archaea increased from the surface to midwaters (Fig. 6). In addition, an abundance of living bacteria and cyanobacteria is also found in the sinking particles and suspended particles within the whole water column and even in the deep-sea sediment at 4500 m (Lochte and Turley 1988). Thus, it is likely that a part of the proteins identified from the midwater particles originated from in situ living bacteria.

Degradation and preservation of particulate proteins—Previous studies reported that the integral thylakoid membrane protein is stable under high-light conditions, but degrades rapidly upon transition to low light or darkness (Adam 1996). In addition, when cyanobacteria are deprived of an essential nutrient, the phycobilisomes are specifically and rapidly degraded (Collier and Grossman 1994). The present study identified 99 photosynthesis proteins from the POM in the 41-m layer, with the majority being integral-membrane protein complexes from the photosystem I and photosystem II reaction centers. However, only a few photosynthetic membrane proteins were detected in the midwater POM compared to the surface POM. A total of 37 were annotated as proteins involved in photosynthesis in the midwater POM. Moreover, peptides that matched proteins involved in photosynthesis accounted for 44.4% of the total peptides in the

POM from the 41-m layer, while it decreased to 0.6% to 1.5% of the total peptides in the POM from midwaters (Table 1). These results indicated that the photosynthesis proteins were prone to degradation and the majority of them were degraded during the sinking of the POM from the euphotic layer.

Tanoue (1996) demonstrated that membrane proteins of marine organisms in surface water are one source of the specific proteins identified in the POM. Kinetic studies of cytoplasmic vs. membrane proteins during degradation showed that proteins protected by a membrane layer are lost at a slow rate (Borch and Kirchman 1999). In this study, a few photosynthetic membrane proteins were detected as high-confidence proteins in midwater POM, such as photosystem II D2 protein, C-phycoerythrin, and photosystem II PsbC protein. In addition, ATP synthases localized in membranes were also frequently detected in the POM samples from midwaters. This suggested that such membrane proteins might be more likely to be protected than other proteins, therefore accumulating in detrital POM from midwaters. In cyanobacteria or eukaryotic algae, the thylakoid or photosynthetic membranes may occasionally form a liposome-like ball and then become incorporated into the detrital macromolecules after cell breakage due to various reasons, and thus become resistant to degradation. Liposome-like particles form spontaneously from membrane fragments produced during digestion, egestion, and viral lysis (Shibata et al. 1997; Borch and Kirchman 1999).

Interestingly, many high-abundance tubulins, which are annotated as related to cellular component organization and biogenesis, were detected in midwater particles, in particular from the 200-m water layer, and the number of these proteins decreased with depth (*see* Web Appendix; Table 1). Tubulin is the main component of the microtubule, one of the cytoskeletons formed by the self-assembly of α- and β-tubulin heterodimers. Both α- and β-tubulin are the same in overall structure, and each contains a core β-sheet surrounded by helices (Downing and Nogales 1998*b*). This protein is unable to interact with other proteins or peptides or fatty acids, such as heat-shock protein 70 as a molecular chaperone, though it can interact with other proteins in implementing microtubule functions (Downing and Nogales 1998*a*). Thus, tubulins have no special structural resistance to proteolysis. In fact, tubulin is unstable and easily degraded (Ishizaki et al. 1988). Moreover, tubulin is not a membrane protein and, therefore, there must be special mechanisms for survival and accumulation of tubulin in midwater POM.

Microtubule is the main component of the flagella or cilia of eukaryotes. Typical flagella or cilia consist of a cylinder of nine doublet microtubules and two additional single microtubules known as the central pair (Mitchell 2000). In addition, ciliary or flagellar microtubules are more stable than cytoplasmic microtubules (Behnke and Forer 1967). Thus, it is likely that cilia or flagella covered by a specialized plasma membrane may be resistant to biodegradation. Since many zooplankton (such as flagellates and ciliates) in the ocean have flagella or cilia (Carty and Wujek 2003; Gong et al. 2006), such zooplankton source material could contribute to suspended particle pools in midwaters through disaggregation of fecal pellets derived from herbivorous, carnivorous, or coprophagous feeding (Sheridan et al. 2002). So tubulin in the form of flagella or cilia, or even microtubules packed in zooplankton fecal pellets, might be protected from degradation and then combined with other detrital material in the POM. Moreover, these slender, fiber-like flagella or cilia might play an important role in starting the aggregation process and the formation of particles as well as stabilizing the structure of the aggregation by directly interacting with organic or inorganic matrices.

In POM from midwaters, actins were also highly abundant in addition to tubulins. Actin is the most abundant protein in many eukaryotic cells and can form an actin filament (double helical polymers of actin globular subunits all arranged head-to-tail), which is the main structural component of the filopodia and microvilli (Mogilner and Rubinstein 2005). In addition, actin microfilaments have been found in higher protists (Mitchell and Zimmerman 1985). In the *Rhizopoda* and *Myxomyceta*, cell motility is regulated by actin-myosin contraction, which sometimes acts in parallel with the microtubules (Métivier and Soyer-Gobillard 1988). As a consequence, it is postulated that actin in particles in the form of actin filaments or organelles, like tubulins, may also be protected from degradation.

Specific proteins in POM—In the ocean, the ambient CO_2 concentrations for photosynthetic organisms can vary across orders of magnitude and often become the limiting factor for carbon acquisition. Many photosynthetic microorganisms use a CO_2-concentrating mechanism to maximize photosynthesis under limiting CO_2 conditions. In this study, carboxysome proteins induced by inorganic carbon limitation in *Prochlorococcus* as suggested by Woodger et al. (2003) were detected in the 41-m POM sample and ribulose 1,5 bisphosphate carboxylase or oxygenase (RuBisCo) was also found. The carboxysome is thought to concentrate carbon dioxide to overcome the inefficiency of RuBisCo (Yeates et al. 2008). These results suggest that *Prochlorococcus* in the POM sample from the subsurface chlorophyll maximum layer might be stressed by inorganic carbon.

Nitrogen is an essential macronutrient in marine environment, the non-availability of which in suitable form or concentration often limits biological production. In this study, the most frequently detected transport proteins in the four POM samples are involved in urea uptake (*see* Web Appendix), and these transport proteins originated from *Prochlorococcus*. These results demonstrated that urea, the most common form of dissolved organic nitrogen in the open ocean (Anita et al. 1991), is a crucial nitrogen source for *Prochlorococcus* within the water column. Ammonia monooxygenases from Crenarchaeota were detected as high-confidence proteins in 200-m, 500-m, and 1000-m POM samples. Ammonium transporters in Crenarchaeota were also detected. Ammonia monooxygenase is the key enzyme responsible for the conversion of ammonia to nitrite. Marine Crenarchaeota are relatively

abundant in deep neritic waters and in the meso- and bathypelagic zones of the ocean (Herndl et al. 2005). Recently, metagenomic studies have revealed that Crenarchaeota express genes related to bacterial ammonia monooxygenase (Nicol and Schleper 2006). Furthermore, a marine chemolithoautotrophic Crenarchaeota strain was isolated that can use ammonia as a sole energy source (Konneke et al. 2005). These results indicated the potential role of Crenarchaeota in nitrification processes in the ocean. The detection of ammonia monooxygenase in this study further confirmed the presence and expression of genes related to ammonia monooxygenase in Crenarchaeota, and suggested that the Crenarchaeota present in the mesopelagic layer of the SCS might be involved in nitrification. Archaeal nitrification may be an important process in the biogeochemical cycling of nitrogen in the SCS.

Iron has also been shown to be one of the limiting elements in the ocean and plays important roles in regulating the growth of phytoplankton and bacteria (Guan et al. 2001). In our study, 12 receptors or transporters of siderophore-iron complexes were identified with one peptide in the POM samples from the four water layers, and TonB-dependent receptors, a family of beta-barrel proteins involved in iron uptake in gram-negative bacteria were also detected, probably implying that planktonic bacteria experienced iron stress during the sampling period and that iron plays an important role in marine plankton in this area.

Porins in bacteria were frequently detected in the 41-m POM sample and also in other POM samples. By conferring permeability properties to the outer membrane, porins play a crucial role in the bacterium's antibiotic susceptibility and survival to various environmental conditions (Delcour 1997). Therefore, the detection of these porins within the water column suggests that porins play a significant role in resistance to nutrient limitation in the surface or low temperature and high pressure in midwaters. Furthermore, 26 molecular chaperones, such as heat-shock protein 90, heat-shock ClpB protein, Hsp 100, molecular chaperone DnaK, and 60-kDa chaperonin 2, were identified in the POM samples from the four water layers (Table 1), and 11 of these were high-confidence proteins. Expression of molecular chaperones levels increase when a cell is subjected to various environmental stresses (Hartl and Hayer-Hartl 2002). Therefore, additional molecular chaperones were found in the upper 200-m layer in this study, indicating that plankton in the upper ocean were subjected more to environmental stresses.

Some specific ectoenzymes were also detected with one peptide from our POM samples. Aminopeptidase was found in all of the POM samples, while chitinase was only found in the POM sample from the 200-m water layer and glucosidase was detected in the POM samples just from the upper 200 m. Such distinct distributions of these ectoenzymes are probably related to the stability of the enzymes themselves as well as the trophic status in the particles at different depths. Poremba (1995) reported that aminopeptidase is critical for the degradation of POM in the whole water column. Rath et al. (1993) showed that leucine aminopeptidase activity is inversely correlated with leucine concentration, indicating the high expression of this enzyme at the low free amino acid concentrations typical of oligotrophic situations. In the oligotrophic SCS, it is likely that low free amino acid concentration within the whole water column might lead to the ubiquitous distribution of aminopeptidase in particles. Furthermore, high concentrations of oligo- and polymeric glycosidic compounds might result in the induction of glucosidase systems in cells (Rath et al. 1993). The detection of glucosidases in the upper 200 m likely indicates the abundance of glycosidic compounds in these particles. Chitinase is considered as a representative of the various extracellular bacterial enzymes involved in chitin degradation in detrital POM in marine environment (Smucker and Kim 1991).The finding of chitinase in POM of the 200-m water layer indicated that this layer might contain significant amount of chitinous matter, which is probably from zooplankton, such as copepods and detritus. The high abundance of crustacean- and dinophyte-originated particulate proteins in POM from the 200-m layer supports this hypothesis.

It is known that the composition of POM is still largely uncharacterized at the molecular level, and as organic matter produced in the euphotic zone of the ocean sinks through the mesopelagic zone, its composition changes significantly (Lee et al. 2004). The present study has revealed the change in protein composition of marine POM through the water column, reflective of the sources, transformation, and degradation of marine POM. The characteristics of the particulate proteins from different water depths indicated that the sources of surface and midwater POM are different, with the former being mainly derived from living organisms and cyanobacteria as the major contributor, while the latter is composed of detrital organisms. During the sinking of the POM out of the euphotic zone, the majority of photosynthesis-associated proteins were degraded, but various important proteins involved in organic and inorganic nutrient transport or utilization, stress responses or adaptations, and energy generation were detected, indicating that active biological processes occur in the POM at each water layer. Accumulation of tublins and actins in midwater POMs, especially in the 200-m layer suggested that fecal pellet packages of zooplankton might protect these proteins from degradation.

It is important to point out that there exists a large set of unidentified proteins, which could have provided insights of the metabolic and biogeochemical processes of the POM in the ocean. With a more complete metagenomic database of marine organisms and the development of MS techniques, we will likely gain more information about the processes involved in POM remineralization and the biological mechanisms occurring in POMs from a proteomic scale, which will certainly improve our understanding of the sources, transfer processes, and dynamics of particulate proteins in the POM.

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