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Preservation via macromolecular associations during *Botryococcus braunii* decay: proteins in the Pula Kerogen

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Abstract

The green alga *Botryococcus braunii* was degraded for 201 days under oxic conditions in a flow-through system to test whether the macromolecular algaenan surrounding cells could protect proteins from rapid degradation. Protein loss was ~8× slower for *B. braunii* than for other previously studied phytoplankton, with base extraction consistently removing only a small fraction (<35%) of the total proteinaceous material compared to that seen by acid hydrolysis. Size-exclusion chromatography coupled to evaporative laser-light scattering detection and fluorescence spectroscopy detected proteinaceous materials from 1.7×10^4 to $> 1.5 \times 10^6$ relative molecular mass (M_r) preserved in degraded material. A shift to higher M_r material during *B. braunii* decay and response of protein aggregates to denaturing agents identified hydrophobic and hydrogen-bond interactions as important stabilizing forces for protein preservation. Two-dimensional electrophoresis of proteins extractable from detrital material indicated the predominant presence of modified proteins. Susceptibility of the detrital organic matter to cleavage with proteolytic enzymes indicated the retention of peptide bonds, while incomplete cleavage of aggregates may be due to associations of proteins with algaenans. Using an antibody probe for RuBisCo, the large subunit was not retained in its original form (M_r 55,000), but additional cross-linked or other aggregated products ($> M_r$ 73,000) plus a cleavage product (M_r 24,000) were observed during the decay sequence. In the 201-day-old *B. braunii* detritus, the large contribution of nonpolar > 2-kDa-hydrolyzable amino acids to bulk nitrogen (~50%) suggests that encapsulation and hydrophobic associations protect a fraction of protein from bacterial degradation, and allow for preservation of their products seen as amino acids in the Pula kerogen.

1. Introduction

Proteins are the most abundant nitrogen-containing substances in algae (Lourenço et al., 1998), but generally have been considered relatively labile in the environment with poor preservation potential. Yet evidence now exists for the preservation of high molecular mass proteinaceous material in freshwater, estuarine, and marine environments over relatively short geologic time scales (Nguyen and Harvey, 1997, 1998; Fogel and Tuross, 1999; Pantoja and Lee, 1999). Detailed examination of algal detritus and sediments has recently shown that hydrophobic and hydrogen-bond interactions are important stabilizing forces which lead to the aggregation and preservation of proteinaceous materials (Nguyen and Harvey, 2001). The concurrent observation that such high-molecular-mass aggregated proteins remain susceptible to enzymatic attack after their extraction from the organic matrix (Nguyen and Harvey, 2001) supports the concept of 'encapsulation' (Knicker and Hatcher, 1997), which argues that organic

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materials incorporated within the sedimentary organic matrix are protected from bacterial hydrolysis.

The chlorophyte *Botryococcus braunii* occurs in freshwater and brackish lakes around the world, and is unique in its high concentration of insoluble, non-hydrolyzable, and highly aliphatic cell wall material termed algaenan (Largeau et al., 1980; Berkaloff et al., 1983; Derenne et al., 1989). In some *B. braunii* strains, the polymeric algaenan may comprise more than 30% of the dry biomass (Largeau, 1995). Algaenan is thought to originate from the polymerization of high molecular mass lipids (Metzger and Largeau, 1999), and appears as a trilaminar sheath surrounding the polysaccharide cell wall (Largeau et al., 1980). Scanning electron micrographs of a maar-type Pliocene oil shale (ca. 4 Ma) from Pula (Hungary) have demonstrated the remarkable preservation of algaenan-rich cell walls of *Botryococcus* with the accompanying loss of intracellular contents (Derenne et al., 1997).

The goal of this study was to assess how the refractory algaenan present in the cell walls of *B. braunii* might protect proteins from degradation during the earliest stages of diagenesis. *B. braunii* was subject to oxic degradation to examine the potential for protein preservation and results compared to previous work with other algae under similar conditions. Several recently developed extraction protocols together with multiple analytical approaches were used to characterize the proteins freely released and associated with the refractory cell wall. To test the longevity of preservation, a sample of Pula kerogen rich in *B. braunii* algaenans (Hetényi and Varsányi, 1976) was analyzed to determine if proteinaceous material could be preserved over million year time scales.

2. Materials and methods

2.1. Algal cultures and degradation experiment

The growth of algal cultures, experimental conditions and sampling were performed as previously described (Casadevall et al, 1985; Zang et al., 2001). Briefly, *B. braunii* race A was grown in CHU medium enriched with ^{15}N nitrate until late stationary phase of growth, then degraded for 201 days in a flow-through system with low dissolved organic carbon water from a natural freshwater lake using a similar approach as Harvey et al. (1995). Flow rates of 2 l day^{-1} were maintained thorough the 40 l vessel to provide an average water residence time of 20 days. Larger grazing organisms were excluded by passage of incoming water through a 3 μm filter with degradation mediated by the remaining natural microbial consortia. Sampling was at predetermined time points, with cells and detritus harvested by centrifugation. The pellet was rinsed in distilled water, frozen in LN_2 and lyophilized prior to analysis.

2.2. Determining protein extraction efficiencies and amino acid concentrations

To evaluate the potential for protein preservation in extant algae, four protein extraction methods were performed for lyophilized samples of *B. braunii* collected during late-stationary phase and each compared to the diatom *Thalassiosira weissflogii* extracted in parallel. These methods include determination of: (1) surfactant-soluble protein, (2) NaOH-soluble protein, (3) > 2-kDa-hydrolyzable amino acids, and (4) total-hydrolyzable amino acids.

Surfactant-soluble protein was determined using the following protocol. One to two milligrams dry weight of sample was weighed into a 1.5-ml screw-cap microtube and 500 μl of 0.16% w/v solution of sodium deoxycholate plus 100 μl of 0.1 N NaOH were added. The sample was cooled to 4 °C, ultra-sonicated (5 watts, 1 min) twice with cooling, and 500 μl of the detergent was added. The sample was centrifuged (16,000 × *g*, 10 min.), and the supernatant was transferred to a new microtube. Trichloroacetic acid was added to yield a final concentration of 10% w/v and the sample was incubated at 4 °C for 30 min. The mixture was centrifuged and the pellet washed twice with 1 ml additions of ice-cold ethanol and dried using a Savant speed-vac concentrator. Protein was solubilized in 20 μl of 1 N NaOH and 400 μl of the detergent. After another centrifugation, 100 μl of supernatant was used for total protein estimation using a modified bicinchoninic acid assay (Nguyen and Harvey, 1994). Protein standards were prepared in the same solubilization solution. Concentrations were expressed as protein nitrogen using the protein–nitrogen mass of RuBisCo (0.13 ± 0.01; mean $\pm$ S.E.; $n = 5$) as the protein standard.

The NaOH-soluble protein was obtained as follows: 1–2 mg (dry wt.) of sample was extracted with trichloroacetic acid and acetone using solvents without β-mercaptoethanol. After centrifugation, the pellet was dried and protein was solubilized in 0.1 N NaOH and analyzed in the same way as described for the surfactant-soluble protein method.

Total-hydrolyzable and > 2-kDa-amino acid fractions were analyzed as previously described (Nguyen and Harvey, 1998) with methods for each fraction differing slightly. The > 2-kDa-hydrolyzable amino acid method relied on a solution of cold 10% (w/v) trichloroacetic acid in acetone with sonication to remove extraneous material prior to HCl hydrolysis of the protein pellet and cell debris. Protein estimates thus represent the total quantity of protein. In contrast, quantification of total-hydrolyzable amino acids employed the entire sample, resulting in estimates of the

summed quantity of protein, peptides, and associated amino acids. These samples were hydrolysed for 70 min at 150 °C using 0.5 ml of sequanal grade HCl, quantitatively transferred to microcentrifuge tubes, dried in a centrifuge evaporator, and resuspended in 0.5 ml deionized water. Aliquots of released amino acids were analyzed as trifluoroacyl isopropyl ester derivatives by gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS) according to Silfer et al. (1991). Samples were extracted and analyzed in duplicate or triplicate for each. These estimates were used to constrain protein estimates obtained from all of the other methods.

To examine potential encapsulation during the degradation process, the *B. braunii* detritus was extracted for protein using the NaOH-soluble protein and the > 2-kDa-hydrolyzable amino acid methods. Concentrations and relative abundances of amino acids also were examined in the > 2 kDa and total hydrolyzable amino acid pools. Recovery of arginine in the standard mix was typically low and its estimated concentration among samples should be considered conservative.

2.3. Removal of organic components from algal cells and detritus

Fresh algae and detritus were treated with multiple solvents to remove lipids, pigments, and easily extractable proteins, allowing evaluation of any proteinaceous material protected by the algeanen matrix. Lyophilized material (500 mg) collected at day 0, 60, 120, and 201 were weighed into 50-ml Teflon centrifuge tubes. All extractions were performed with 35-ml solvent volumes with the assistance of ultra-sonication (30 Watts). Centrifugation following each extraction was at 20,000 × *g* for 30 min. Initial extraction used 35 ml additions of ice-cold 10% (w/v) trichloroacetic acid in 90% acetone containing 0.1% β-mercaptoethanol (see Nguyen and Harvey 1998). This extraction was repeated until the supernatant was clear (three times). Any minor amounts of remaining pigments were subsequently extracted with 35 ml additions of 1:1 CH_2Cl_2: methanol. Removal of the 'easily-extractable' protein was achieved with a 1-h incubation in 15 ml of 0.1 N NaOH, with tubes placed on a rotary shaker. The 0.1 N NaOH supernatant containing proteins was concentrated to less than 500 µl with centrifugal ultrafilters (Millipore–Biomax NWWL membrane) having a nominal molecular mass cutoff of 5 kDa. Deionized water (5 ml) was added to the concentrate as a wash step, centrifugation repeated and the final concentrate neutralized with acetic acid prior to storage at −70 °C. The Pula kerogen (a gift from M. Hetényi) was isolated by a sink-float method (Hetényi and Varsányi, 1976) which relies on the use of a series of organic solvent mixtures of varying densities to separate dense minerals from less dense kerogen. Lyophilized subsamples were treated as above.

2.4. Size-exclusion chromatography (SEC) of protein extracts

Prior to analysis, an aliquot of the thawed, NaOH protein extract for each sample was concentrated by trichloroacetic acid precipitation. The pellet was washed with acetone (2×), dried, and then solubilized in 50 mM NH_4HCO_3 or 6 M guanidine·HCl, 50 mM NH_4HCO_3. SEC was performed at room temperature under native conditions using a Bio-Prep SE 1000/17 column (8 × 300 mm 15–20 µm particle size) with 50 mM NH_4HCO_3 as mobile phase at 0.5 ml min^{-1}. Elution was monitored by fluorescence (λ_{ex} = 280 nm; λ_{em} = 340 nm) and by evaporative light scattering detection (ELSD), and under guanidine denaturing conditions by fluorescence and absorbance (280 nm). Protein extracts also were subjected to proteolytic digestion with trypsin and proteinase-K (covalently linked to agarose; Sigma Chem. Co.) for 18 h at 37 °C, with the peptide fragments separated by SEC and detected by ELSD as described in detail elsewhere (Nguyen and Harvey, 2001). Relative molecular masses (M_r) were determined based on SEC calibration with standard proteins (Bio-Rad).

2.5. Two-dimensional gel electrophoresis

The Bio-Rad Mini-PROTEAN II 2-D cell was used for the separation of proteins first by isoelectric focusing (based on charge) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE based on molecular size). Protein extraction and electrophoretic separations were performed as previously described (Nguyen and Harvey, 1998) using 15% mini slab gels containing no SDS. Following electrophoresis, gels were stained using BIO-RAD Silver Stain Plus which can detect a few nanograns of protein.

2.6. Rubisco antibodies and western blotting

The fate of RuBisCo during diagenesis was evaluated using antibodies raised against the large subunit of RuBisCo isolated from *B. braunii* (see Wang and Kolattukudy, 1996). The polyclonal antiserum was a gift from P. Kolattukudy (Ohio State University, USA). Sample preparation required further concentration of the thawed NaOH extracts as described above for SEC, and subsequent solubilization in Laemmli sample buffer. After centrifugation (16,000 × *g*, 10 min), soluble protein was loaded onto a 15% SDS-PAGE gel, and electrophoresis was performed as described by Laemmli (1970). Proteins were transferred from gels onto nitrocellulose membranes using the Bio-Rad mini transblot apparatus, with transfer efficiency assessed by the use of

prestained molecular mass markers (Bio-Rad). The membrane was probed for RuBisCo using the antibodies, and detection was achieved with the Bio-Rad amplified alkaline phosphatase assay. Biotinylated protein standards (Bio-Rad) were used for relative molecular mass determinations. Purified RuBisCo from spinach served as a control.

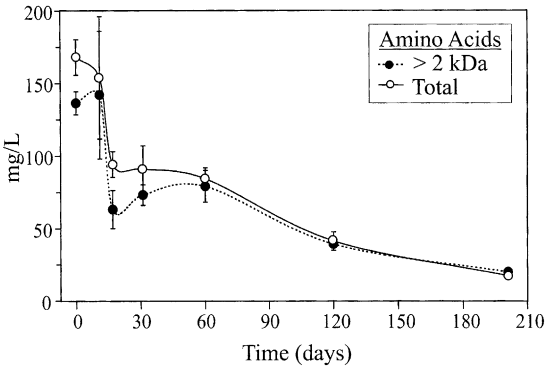

Fig. 1. Time course of (A) > 2 kDa and (B) total hydrolyzable amino acids for the oxic decay of *Botrycoccus braunii*.

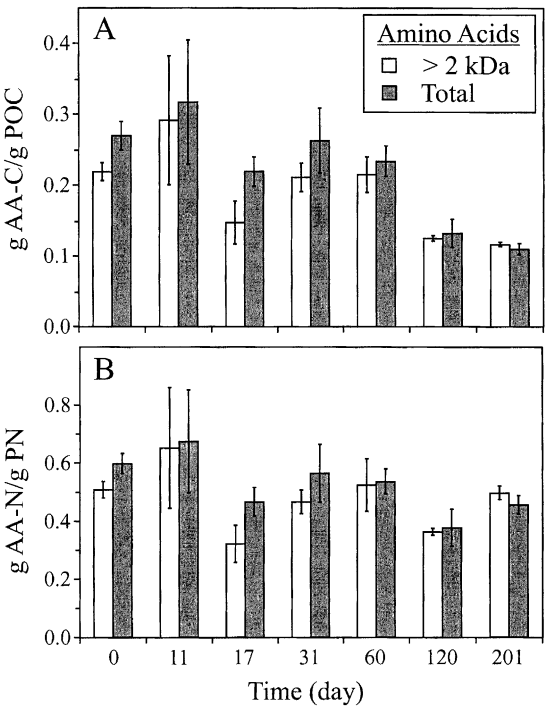

Fig. 2. The contribution of amino acids to (A) particulate organic carbon and (B) particulate nitrogen during *Botrycoccus braunii* decay under oxic conditions.

3. Results

3.1. Kinetics of degradation

Similar to bulk organic material (Nguyen and Harvey, 1997), total hydrolyzable amino acids were lost rapidly over the first 17–30 days of the incubation with a slower decline thereafter (Fig. 1). A concentration of 168 mg/l amino acids was present at the start of the decay experiment, with 17 mg/l remaining after the 201-day incubation. Amino acids associated with the protein (> 2 kDa) fraction comprised 81% of all hydrolyzable amino acids in late-stationary phase cells, and 94–100% of the total amino acid pool from 60 through 201 days of decay (Fig. 2). Due to the large contribution of proteins to the total hydrolyzable amino acid pool, loss rates for the >2 kDa and total hydrolyzable amino acid fractions were identical (Figs. 1 and 2), with a first-order decay constant of 3.2 ± 0.9 year^{-1} ($r^2=0.84$) for the entire 201 days. This rate of protein decay for *B. braunii* is substantially slower (~8 fold) than protein loss seen for previously studied phytoplankton under similar conditions (see Fig. 3; Harvey et al., 1995; Nguyen and Harvey, 1997). Based on bacterial cell densities during the experimental period (avg. $6.4\pm1.1\times10^6$ cells ml^{-1}) the contribution of bacterial protein to the total protein pool was estimated to be <1%.

3.2. Protein extraction efficiencies for extant algae and B. braunii *detritus*

Extraction of protein from extant *B. braunii* was inefficient using surfactant and alkaline solutions, with 18% and 53% of the total protein content (based on total hydrolyzable amino acids) released, respectively

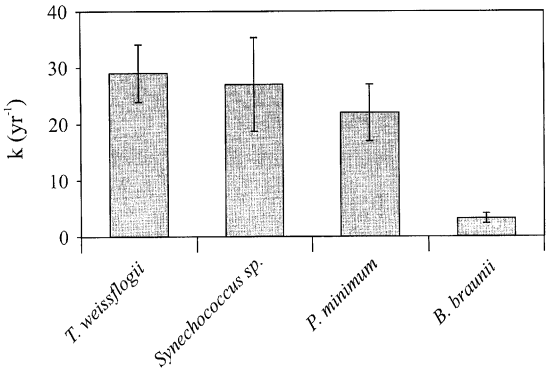

Fig. 3. Comparison of first-order decay constants (±95% CL) for > 2 kDa acid-hydrolyzable amino acids during the oxic degradation of four phytoplankton species: *Thalassiosira weissflogii* (diatom), *Synechococcus* sp. (cyanobacterium), *Prorocentrum minimum* (dinoflagellate), and *Botrycoccus braunii* (green alga).

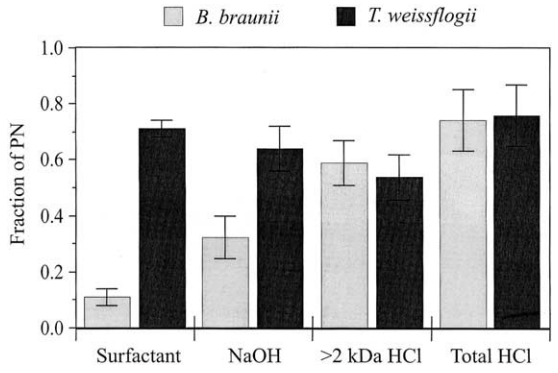

Fig. 4. Comparison of protein extraction efficiencies for late-stationary cultures of *Botrycoccus braunii* and *Thalassiosira weissflogii*. Increasing rigor of extraction is shown from left to right with total hydrolyzable amino acids used to estimate total protein. Mean ± 1 S.D. of replicate analysis.

(Fig. 4). In contrast, the diatom *T. weissflogii* was amenable to protein extraction with all methods employed (Fig. 4). The total or 'true' protein content in *B. braunii* cells or detritus was thus based on the summation of > 2-kDa amino acids released by rigorous acid hydrolysis. Alkaline protein extraction remained problematic for detrital material throughout *B. braunii* decay, with acid-hydrolyzable protein (> 2 kDa) accounting for 36–52% of particulate nitrogen while NaOH-extractable protein comprised only 12–17% of PN. As a result, the fraction of proteins protected or resistant to NaOH extraction varies from 24–35% of PN.

3.3. Amino acids during diagenesis: composition and contributions to POC and PN

Total and > 2-kDa-hydrolyzable amino acids comprised 27% and 22% of POC, respectively, in late-stationary phase cells (Fig. 2 A; day 0). Contributions of these two amino acid pools to bulk carbon showed a gradual decline during algal decay, with both pools comprising 11% of POC after 201 days. Hydrolyzable amino acids were a more important contributor to PN in stationary phase cells, with 51–60% of cellular nitrogen derived from amino acids during the decay (Fig. 2B). Half of the nitrogen could still be attributed to proteinaceous material in the 201-day detritus.

To simplify comparisons of amino acid distributions, individual protein amino acids were grouped based on side chain chemical property. For the total hydrolyzable pool, nonprotein amino acids constituted another group. Since most or all of the total hydrolyzable amino acid pool consisted of higher molecular mass (> 2 kDa) material, these two pools showed similar compositions and trends. Amino acids with *nonpolar* side chains comprised the bulk (59%) of all amino acids in late-stationary phase *B. braunii*. This was followed by amino acids with *polar-charged* (26%) and *polar-uncharged* (15%) side chains. By day 201 of the decay period, amino acids in these three groups were 65, 19, and 16%, respectively (Table 1). Overall, there was an increase in nonpolar amino acids, while a decrease was observed for amino acids with a polar-charged side chain, both of which were attributed to shifts in several individual amino acids. For the nonpolar amino acids, glycine and alanine increased significantly during diagenesis from their high initial mole%'s; valine, isoleucine, and phenylalanine showed no significant changes; and leucine and proline decreased. The decrease in amino acids with a polar-charged side chain was largely due to glutamic acid, and lysine and arginine in the > 2-kDa pool, which decreased significantly during the degradation sequence. Among all amino acids with a polar-uncharged side chain (hydroxyproline, serine, threonine, and tyrosine), only serine showed a small, albeit significant, increase. Mass spectrometry of unlabeled amino acid standards and of the hydrolyzate of the *B. braunii* detritus confirmed that the amino acids were isotopically enriched in ^{15}N and thus algal in origin.

Hydrolyzable amino acids liberated from the kerogen comprised 0.034 ± 0.010% of OC and 1.02 ± 0.32% (mean ± 1 S.D.; $n = 2$) of N. Amino acids were characterized by a composition consistent with the diagenetic alteration of this pool during *B. braunii* decay (Fig. 5). Contributions of glycine and alanine to the total were high, with mole%'s at 24 and 21, respectively. The high values are consistent with the relative enrichment seen for these two nonpolar amino acids during early diagenesis. Proline was the only amino acid with a nonpolar side chain with a decrease during the diagenetic sequence. Tyrosine, with a polar-uncharged side chain, decreased in mole% from 2.2% in extant algae to 0.5% in the kerogen. Amino acids with a polar-charged side chain were depleted in the kerogen by 44–100% relative to the extant algae. The nonprotein amino acids β-alanine and γ-aminobutyric acid were absent from cells and barely above detection limits in the 201-day detritus of *B. braunii* (Table 1), but together they comprised 1.8% of all amino acids in the 4 Ma Pula kerogen (Fig. 5).

3.4. M_r distributions and chemical nature of intact proteinaceous materials

SEC coupled to an ELSD detector observed proteinaceous materials of a broad molecular weight range (M_r from 2000 to 1,500,000) in late-stationary phase cells (Fig. 6, native, day 0). Proteinaceous 'aggregates,' material eluting in the void volume, also were found. Low to very high M_r (5000–1,500,000) proteins were observed in the detritus from days 60 to 201, with an increased contribution from aggregates (Fig. 6, native).

The NaOH-extractable material could not be hydrolyzed completely to lower M_r material by trypsin treat-

Table 1
Average mole% composition and precision (±2 S.D.; $n=2$) of total hydrolyzable amino acids during the 201-day decay of *Botrycoccus braunii* and for Pula kerogen[a]

Time (days)	Nonpolar side chain						Uncharged-polar side chain				Charged-polar side chain				Nonprotein		
	Gly	Ala	Val	Leu	Ile	Pro	Phe	Hpro	Ser	Thr	Tyr	Asp	Glu	Lys	Arg	BALA	GABA
0	13.6	15.2	6.9	9.1	4.0	7.1	3.9	0.79	7.1	5.2	2.2	9.5	10.2	4.4	0.95	0	0
11	13.7	15.5	7.0	9.0	4.4	6.4	4.2	0.51	6.5	5.1	2.2	10.4	10.4	4.1	0.63	0	0
17	13.9	16.0	7.2	9.1	4.2	6.3	4.1	0.51	6.67	5.1	2.05	10.3	10.0	4.0	0.62	0	0
31	14.3	17.0	7.2	8.9	4.2	6.2	4.1	0.48	6.8	5.01	2.2	10.0	9.3	3.8	0.43	0	0
60	14.2	16.4	7.2	8.9	4.30	6.17	4.5	0.48	7.27	5.2	2.4	10.5	8.6	3.6	0.29	0	0
120	15.3	17.2	7.1	8.0	4.1	6.1	4.1	0.50	7.8	5.3	2.2	10.4	7.9	3.7	0.27	0	0
201	17.9	20.6	6.9	6.52	3.91	5.5	3.33	0.64	8.5	5.22	1.7	9.7	6.7	2.4	0.12	0	0.29
Pula	24.0	21.4	7.9	7.3	4.7	3.1	3.0	1.4	5.6	9.2	0.46	5.3	3.8	0.99	0	1.25	0.56
±2 S.D.																	
0	1.1	0.34	0.18	0.32	0.17	0.22	0.41	0.05	0.28	0.17	0.12	0.23	0.49	1.8	0.84	0	0
11	1.2	1.5	0.43	0.64	0.20	0.10	0.12	0.02	0.60	0.59	0.15	0.80	1.29	0.38	0.32	0	0
17	1.1	1.4	0.15	0.66	0.22	0.33	0.23	0.01	0.02	0.67	0.09	0.19	0.80	1.9	0.75	0	0
31	1.5	3.5	0.36	0.14	0.19	0.36	0.35	0.08	0.23	0.04	0.48	0.87	0.99	1.5	0.52	0	0
60	1.8	1.0	0.17	0.35	0.03	0.04	0.51	0.01	0.04	0.20	0.31	0.67	0.94	0.11	0.15	0	0
120	3.3	3.2	0.16	0.84	0.25	0.10	0.63	0.06	0.66	0.68	0.43	1.6	1.4	0.22	0.03	0	0
201	0.63	0.94	0.24	0.09	0.04	0.11	0.03	0.02	0.47	0.04	0.10	0.23	0.23	0.39	0.09	0	0.43
Pula	0.43	3.0	1.8	1.7	0.60	0.99	1.8	0.72	3.8	2.9	0.13	0.56	0.87	0.39	0	0.82	0.37

[a] Protein amino acids are ordered into three groups based on side chain chemical property. Nonprotein amino acids are a fourth group.

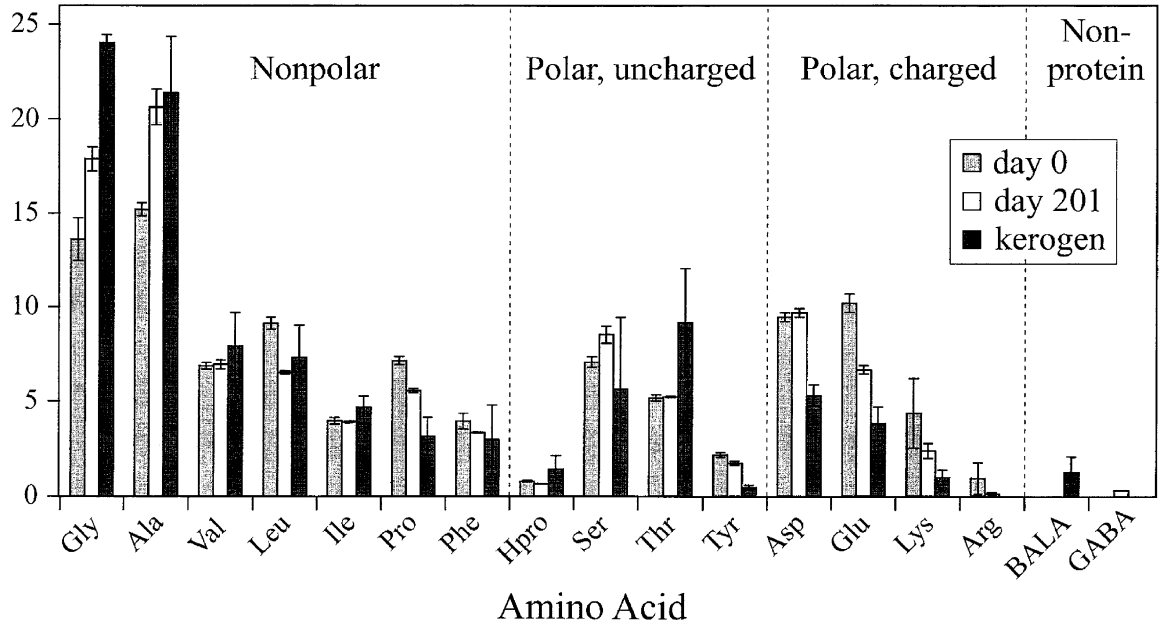

Fig. 5. Comparison of total hydrolyzable amino acid composition present in initial and 201-day degraded *Botrycoccus braunii* cells and 4-Ma Pula kerogen. Error bars represent mean±2 S.D. of replicate subsamples obtained from the degradation vessel.

ment within 3 h, and little or no further hydrolysis was observed even after 18 h (Fig. 6). Most cleavage products had M_r 1000–44,000. Minimal cleavage was also observed for the aggregates from stationary phase cells (day 0). A fraction of aggregated proteins were observed for detritus from days 60 to 201 of the degradation sequence, after the protease incubation. Also, some proteins with M_r 44,000–1,000,000 remained after the trypsin digestions (note the flat ELSD response just above the baseline). Unlike the ELSD responses, fluo-

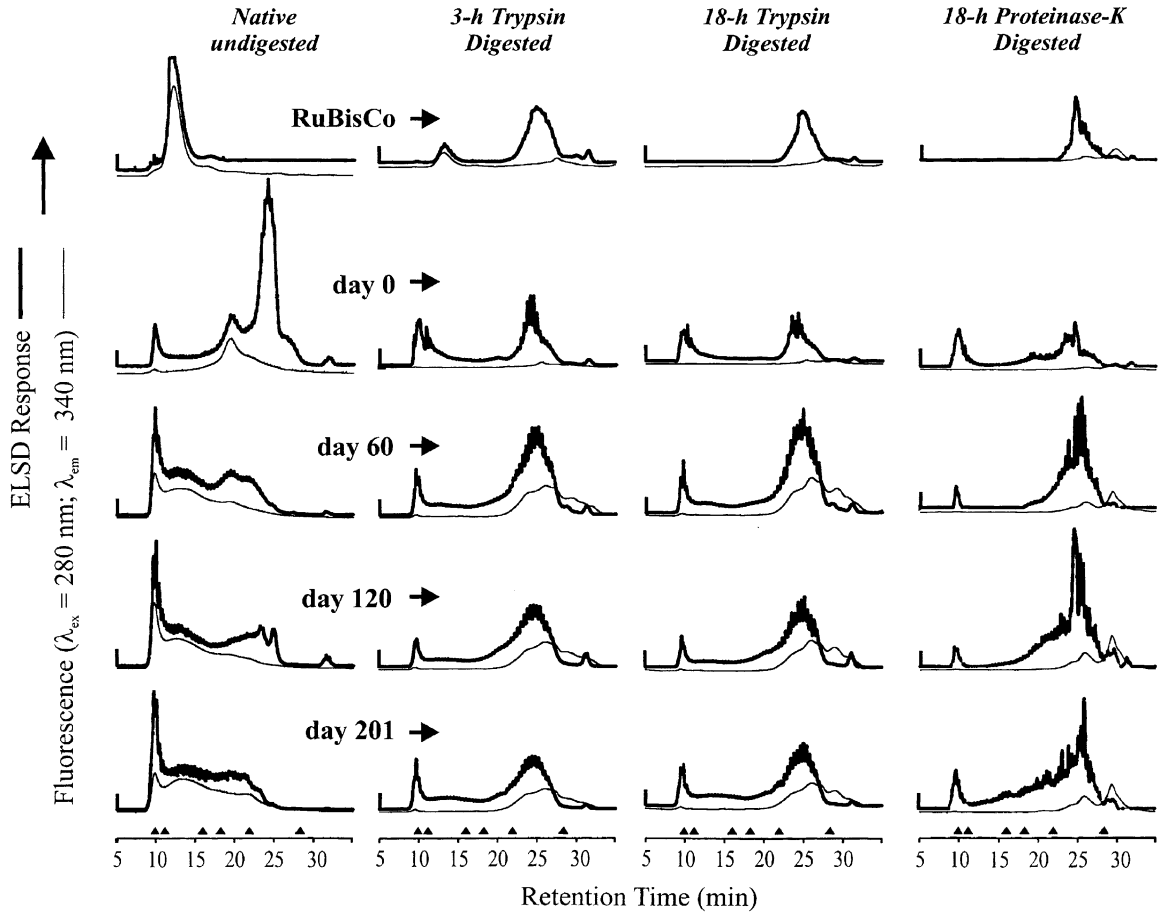

Fig. 6. SEC chromatograms of native and enzyme-digested, NaOH-extractable proteinaceous material from different stages of *Botrycoccus braunii* decay. Relative molecular mass ($\times 10^3$) markers are shown and decrease from left to right: 1500, 670, 158, 44, 17, 1.35.

rescence associated with the aggregates and > M_r 17,000 proteins was minimal or completely lost to low M_r (<17,000) products following trypsin digestion. 18-h digestions with proteinase-K yielded similar elution profiles as observed for the trypsin digestions (Fig. 6). On closer inspection, however, some additional cleavages were observed with the nonspecific protease, in particular, for aggregates and M_r 44,000–1,000,000 proteins from days 60 and 120. Based on comparisons with the negative control (buffer plus protease), the contribution of low M_r material from protease auto-digestion or other products was <0.3%.

Absorbance measurements indicated that the low M_r (2000–30,000) polypeptides observed by ELSD stationary-phase *B. braunii* (day 0) were characterized by a weak tryptophan fluorescence (Fig. 7, native). Tryptophan is a relatively minor constituent of proteins (Voet and Voet, 1990), and its likelihood of being found in small polypeptides is low. In general, both absorbance and ELSD measurements revealed similar M_r distributions and a shift towards higher M_r substances during diagenesis (compare Figs. 6 and 7, native).

Denaturing conditions had a negligible effect on the M_r distribution of proteins/polypeptides from late-stationary phase cells based on absorbance (Fig. 7), indicating the monomeric nature of these polypeptides. Fluorescence did decrease for the proteins with M_r 17,000–44,000, probably due to solvent quenching of tryptophan upon unfolding. Unlike the proteins from day 0, denaturing conditions resulted in a shift towards lower M_r products for proteins from days 60 to 201, as indicated by absorbance and fluorescence measurements (Fig. 7). Aggregates in the 201-day-old detritus appeared to be more recalcitrant to the guanidine·HCl conditions. Proteinaceous material from extracts of the Pula kerogen was below the detection limit of the ELSD and so protease digests were not conducted.

Two-dimensional gel electrophoresis revealed that late-stationary phase cells (day 0) were characterized by proteins/polypeptides of M_r 2000–60,000 with acidic

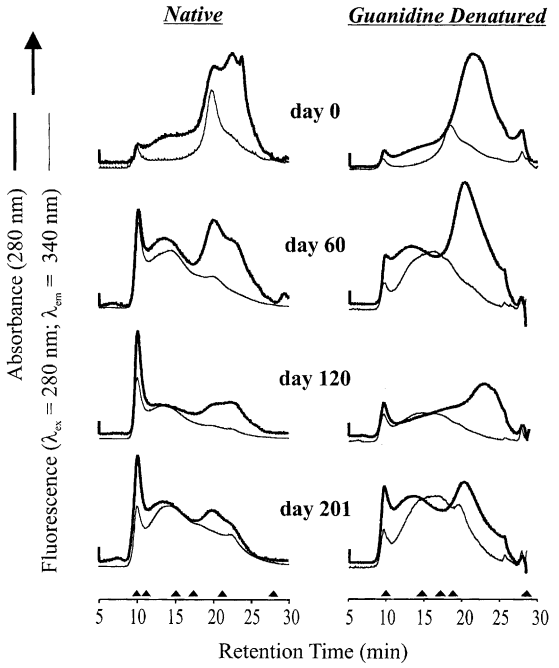

Fig. 7. SEC chromatograms of NaOH-extractable proteinaceous material from different stages of *Botrycoccus braunii* decay, under native and guanidine·HCl denaturing conditions. Relative molecular mass ($\times 10^3$) markers from left to right for native conditions: ~1500, 670, 158, 44, 17, 1.35; for denaturing conditions: ~1500–335, 158, 44, 17, 1.35.

pI's (Fig. 8). Protein hydrolysis and modification were rapid, with only nine of the ca. 90 original protein spots scored in stationary-phase cells also observed at day 11. However, other new spots were observed by this time. Protein composition from days 11, 60, 120 and 201 showed little change, with the most intense staining observed in an unresolved, acidic (average pI ~5) and M_r 25,000–200,000 region (Fig. 8). With biological samples, some proteins are difficult to solubilize or may remain associated with other proteins even in the presence of high concentrations of detergents and denaturing agents (Nguyen and Harvey, 1998). This may explain some of the streaking associated with the protein separations. Interestingly, negatively-stained isoforms, or charge variants, of a protein designated by the letter 'a' persisted throughout the incubation period. Silver typically binds to various chemical groups such as sulfhydryl and carboxyl moieties in macromolecules, although the detection method we applied (i.e., Bio-Rad Silver Stain Plus) does not stain highly glycosylated proteins or metalloproteins; these proteins would appear as white spots or bands on a yellowish background. In general, the M_r distribution determined from electrophoresis compared well to that observed by SEC under denaturing conditions. Both methods revealed the high abundance of the M_r 2000–30,000 proteins/polypeptides in late-stationary phase *B. braunii* (compare Figs. 7 and 8 at day 0).

3.5. Fate of RuBisCo

When proteins were separated on the basis of molecular size by electrophoresis and then probed with the antibodies raised against the large subunit of RuBisCo, multiple cross-reactions were observed (Fig. 9). The large subunit as well as two other proteins were visible in late-stationary phase *B. braunii* (day 0). The large subunit per se (M_r 55,000) does not appear to be well preserved, but traces were present throughout the 201-day incubation (Fig. 9). The smaller protein (M_r 24,000) may be an autolytic degradation product in the late-stationary phase cells and was detected for up to 120 days. The larger algal protein (M_r 73,000), which was also observed in the pure, spinach RuBisCo, may be a covalently modified product. Cross-reactivity of the antiserum with higher M_r protein products was also observed during the degradation sequence.

4. Discussion

Although hydrolyzable amino acids were efficiently removed during the microbially mediated diagenesis of *B. braunii*, amino acids associated with higher molecular weight material (– 2 kDa) still accounted for half of the residual nitrogen after 201 days of decay. Based on native SEC, much of the proteinaceous material retained appeared to be relatively high M_r (1.7×10^4 to 1.5×10^6). Using two-dimensional gel electrophoresis to take 'snapshots' of proteins in time, it was evident that many of the original proteins were lost, and the residual material consisted of proteins or their modified products with M_r between 2000 to > 200,000. Most of this proteinaceous material was acidic in nature (average pI ~5) and poorly resolved. What is striking about these observations is that similar findings have been seen for proteinaceous materials in phytodetritus derived from other algae and in sedimentary material from a marine sapropel (Nguyen and Harvey, 1998, 2001). These results challenge the long-held notion that high molecular mass proteinaceous material is unlikely to survive except in the presence of sufficient mineral surfaces or other inorganic matrices.

Hydrophobic and hydrogen-bond interactions between proteinaceous materials and with nonprotein components have recently been proposed as major forces for the stabilization of nitrogen in sediments (Nguyen and Harvey, 2001). The results with *B. braunii* whose cell walls comprise a "classical" polysaccharide inner wall and algaenan-rich outer wall (Largeau et al., 1980), support the importance of noncovalent forces for protein preservation. One possibility is that the

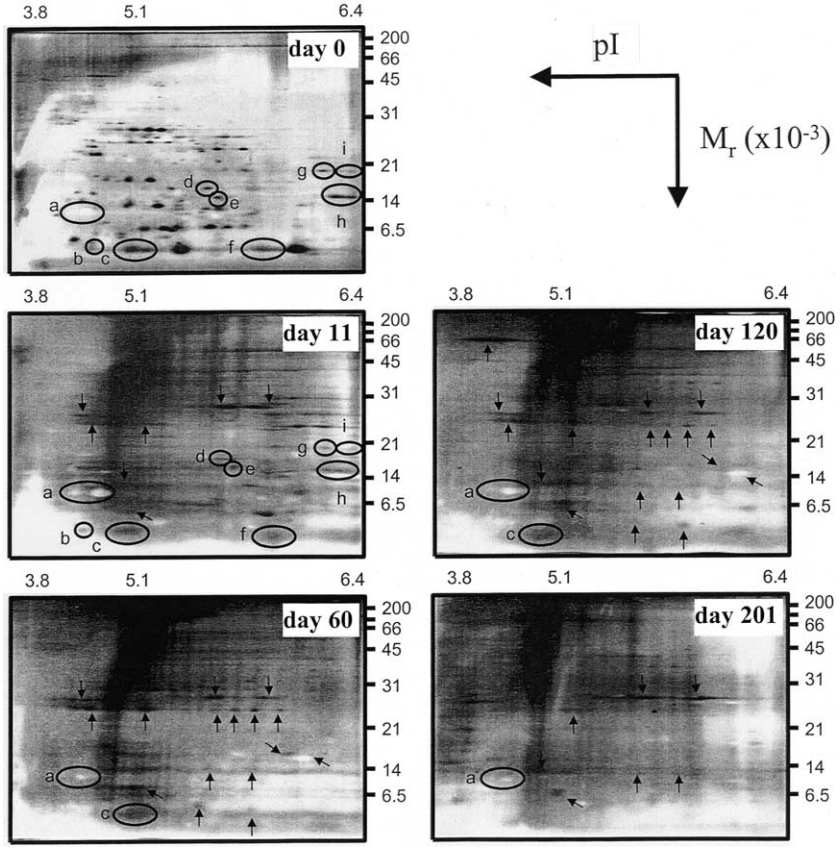

Fig. 8. Two-dimensional electrophoretograms of detergent-extractable proteins from different stages of *Botrycoccus braunii* decay. Circled and labeled spots indicate proteins or polypeptides common to all gels. Arrows point to some common spots observed during the degradation; note the positions and directions of the arrows when comparing gels. For day 0, the white spots designated by the letter 'a' were highlighted due to their poor contrast against the gel background.

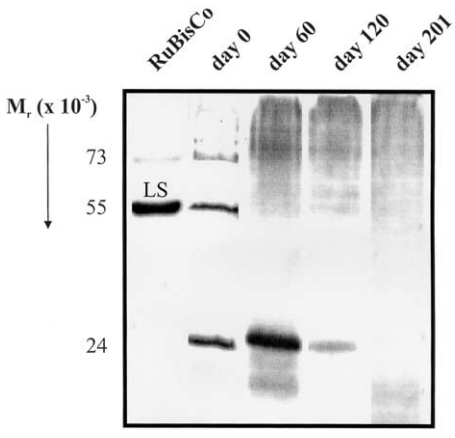

Fig. 9. Western blot of proteins probed with antibodies raised against the large subunit (LS) of RuBisCo from *Botrycoccus braunii*. Lanes from left to right: purified RuBisCo from spinach; NaOH protein extracts from days 0 to 201 of *B. braunii* decay. For each algal sample, protein had been extracted from an equivalent dry weight of material.

preserved aggregates are simply proteins which have unfolded after cellular disruption, with the exposure of hydrophobic polypeptide regions leading to various inter- and intra molecular associations (Nguyen and Harvey, 2001). A second possibility is that the surviving aggregates originated as proteins associated with cell walls (e.g., membrane proteins) which remain encapsulated by lipids or fragments of algaenan-composed outer walls. Kinetic studies of cytoplasmic versus membrane proteins during degradation have shown that proteins protected by a membrane layer are lost at a slower rate (Laursen et al., 1996; Nagata et al., 1998; Borch and Kirchman, 1999); algaenans could also provide a similar physical barrier and one which is more effective as evident by the very low degradation rates observed. Compared to detritus derived from diatoms (Nguyen and Harvey, 2001), *B. braunii* algaenans also appear to play a more effective role, allowing low M_r proteinaceous material to be preserved in addition to high M_r aggregates. Since these substances are susceptible to enzymatic attack *after* its extraction from the organic matrix,

it suggests that an encapsulation pathway for preservation is operative. In the case of late-stationary phase *B. braunii*, the inability of trypsin and the nonspecific proteinase-K to hydrolyze the extracted aggregates suggests that those aggregates might include: (1) tightly encapsulated proteins, (2) cross-linked proteins, and/or (3) nonproteinaceous substances such as the macromolecular algaenans. As diagenesis proceeds, the relative contribution from other types of unprotected proteins appears to increase, since these aggregates are more susceptible to attack. The detrital aggregates which are not digested may represent the original, refractory macromolecular material found in stationary phase *B. braunii*.

An important observation is that even the 'labile' protein fraction of *B. braunii* was lost at half to one third the rate observed for previously studied phytoplankton (Nguyen and Harvey, 1997). Although the cell walls of the different marine phytoplankton (including dinoflagellate, diatom and cyanobacterium) might be expected to possess varying degrees of resistance to degradation, proteins within *B. braunii* cells were substantially more resistant to loss. Indeed, Corre et al., (1996) has previously observed that Chlorophytes with an algaenan-composed trilaminar outer wall were substantially more resistant to detergents than those devoid of algaenans. The extremely low extraction of protein from *B. braunii* with detergent or alkaline organic solvents reflects the chemically-resistant nature of the algaenan matrix (Largeau et al., 1980; Metzger and Largeau, 1999) and can explain the reduced rates of protein decay for *B. braunii* as compared to the other algae which do not contain these polymeric structures.

The rapid loss of the RuBisCo large subunit as seen by decreased antibody binding could be from multiple fates, including autolytic and/or bacterial hydrolysis, or extensive abiotic modifications which altered the antigenic sites and prevented recognition. The use of polyclonal antibodies should allow for the detection of various modified forms of RuBisCo, since there will be a suite of antibodies, each detecting a particular conformation or short amino acid sequence. Cross-linking of proteins not only occurs during diagenesis (Collins et al., 1992; Nguyen and Harvey, 1998), but in some cases even before cellular death (Wilson et al., 1995; Wang and Kolattukudy, 1996). The oxidative cross-linking of RuBisCo large and small subunits might be a contributing factor to the formation of the 73-kDa protein observed in late-stationary phase cells. Cross-reaction of the antibodies to high M_r proteins in the detritus might be due to RuBisCo's incorporation into the refractory macromolecular pool. Abiotic modifications to RuBisCo also appears a possible route during diagenesis, based on stable isotopic analysis of the isolated protein as well as on the use of anti-melanoidin antibodies (Fogel and Tuross, 1999).

The analysis of protein amino acid compositions proved useful for the evaluation of diagenetic alterations in the organic matter. The significant enrichment of alanine and glycine, together with the relatively stable contributions of most other nonpolar amino acids, would increase the hydrophobic character of the detritus as the diagenesis progressed. Their increase would further stabilize the remaining protein aggregates. In the Pula kerogen, alanine and glycine comprised 46% of all amino acids, supporting the contention that such increased hydrophobic interactions are an important mechanism for preservation. An amino acid not typically measured in environmental samples is hydroxyproline (for a review, see Keil et al., 2000), which can be found in glycosylated cell wall proteins of certain algae including chlorophytes (McConville et al., 1982). The hydroxyproline content of late-stationary phase *B. braunii* was 0.8 mole% (Table 1), comparable to the 0.5% seen in the green algae *Dunaliella tertiolecta* (McConville et al., 1982). Although one might predict the enrichment of hydroxyproline during diagenesis due to its association with the cell wall, no increase in the relative amounts of this polar-uncharged amino acid was observed (Fig. 5). This apparent paradox can be explained by the association of hydroxyproline with the non-resistant polysaccharide inner wall of *B. braunii* rather than the resistant algaenan outer wall (Largeau et al 1980; Metzger and Largeau, 1999). As a consequence, hydroxyproline is degraded at similar rates as other cellular contents with no enrichment.

Although nonprotein amino acids β-alanine and γ-aminobutyric acid have been suggested as indicators of organic matter degradation state (Cowie and Hedges, 1994), they were absent or detectable only at trace levels in the detritus of *B. braunii*, even after 201 days of degradation under oxic conditions. While the relative abundances of these two amino acids in the Pula kerogen were higher than in the 201-day detritus, they remained minor components. A previous study of organic-rich systems has shown that decay constants of these nonprotein amino acids are similar to those of the protein amino acids under oxic and anoxic conditions (Nguyen and Harvey, 1997) and their concentrations are not enhanced downcore in a 4000-year-old marine sapropel (Nguyen and Harvey, 1998). These results and the present study suggests that biological processing is not responsible for the predominance of β-alanine and γ-aminobutyric acid in ancient pelagic clay sediments that was observed by Whelan (1977). Rather, abiotic processes such as adsorption may be more important controls for the elevated contributions of these amino acids in sediments.

Recently, Dauwe et al. (1999) presented a statistical argument suggesting that amino acids are robust indicators of diagenetic status. Results of laboratory incubations here and Pula kerogen analysis indicate that

nonprotein amino acids per se cannot easily infer the degradation state for all types of samples or environments. Certain protein amino acids in *B. braunii* detritus and the kerogen, however, do appear to reflect the highly degraded state of the organic matter. While such an approach is certainly useful, the present results suggest that amino acids alone cannot provide a quantitative measure for assessing diagenetic status.

An important question to consider is the nature of organic nitrogen in detritus and sediments. If proteinaceous material represents about half of the nitrogen in *B. braunii* detritus, then what comprises the remaining fraction? At least a portion of the nitrogen in an organic-rich sapropel of Mangrove Lake, Bermuda was composed of proteinaceous material not susceptible to hydrolysis using the traditional HCl extraction (Knicker and Hatcher, 1997). Although minerals may play an important role in organic matter preservation (Mayer, 1994; Keil et al., 1994), the low abundance of minerals together with the predominance of N in amide linkages in the sapropel led Knicker and Hatcher (1997) to propose encapsulation as a mechanism for the protection of proteins. For *B. braunii* detritus, it appears likely that algaenans act as a barrier, preventing the efficient hydrolysis of proteins, and resulting in the reduced amino acids yields. This scenario seems likely when one considers that hydrolyzable amino acids typically comprise 70–80% of the nitrogen in stationary-phase phytoplankton (Lourenço et al., 1998), yet only 60% of the nitrogen in stationary-phase *B. braunii* could be attributed to amino acids. The ancient Pula kerogen is an extreme example, and only 1% of the nitrogen present could be attributed to HCl-hydrolyzable amino acids even though Nguyen et al. (2003) have observed that the amide linkage remains the dominant nitrogen signal present. Some fraction of the remaining nitrogen also could represent non-HCl-hydrolyzable amino acid, but the low amounts of amino acids present precluded alternative methods in this study.

It is important to note that Knicker and Hatcher (1997) defined proteinaceous encapsulation operationally based on ^{15}N NMR and pyrolysis GC/MS analysis of material which could not be released by HCl hydrolysis. Zang et al. (2000) has subsequently shown that peptides incorporated into the structure of humic acids can also be protected from HCl hydrolysis. This definition differs from the more rigorous definition of encapsulation used here, i.e. which includes proteinaceous material that *cannot be extracted by NaOH* as well as that not amenable to HCl hydrolysis. Nevertheless, it illustrates the ability of macromolecular organic material to act as a barrier to protein hydrolysis.

The role of algaenans in *Botryococcus* to act as a barrier and to retard the loss of proteins is also supported by recent NMR evidence. ^{15}N and 2D ^{15}N ^{13}C NMR analysis by Zang et al. (2001) using subsamples of material generated in this study observed a dominant amide signal in the algal and detrital residues of *B. braunii* even after solvent and NaOH extraction. This signal remained prominent in highly degraded material (201 days), with no significant appearance of heterocyclic nitrogen or melanoiding product observed.

In addition to proteins, other important biological structures containing nitrogen are nucleic acids and pigments. However, these other molecules are relatively minor constituents of cells and would not be expected to account for a large fraction of the remaining, uncharacterized detrital nitrogen. Nucleic acids typically only comprise ~5% of the nitrogen in phytoplankton (Lourenço et al., 1998), and deoxyribonucleic acid is readily decomposed (Poinar, 1998). Chlorophyll or its degradation products are not expected to be major components of the uncharacterized nitrogen, since the pigment comprises only ~1% of the nitrogen in phytoplankton (Lourenço et al., 1998), and porphyrins are minor contributors to sedimentary organic nitrogen (Stankiewicz and van Bergen, 1998). Although pigment concentrations were not determined, the debris still remained green beyond 120 days of decay in the absence of light. The encapsulation of pigments in addition to proteins by the refractory cell wall matrix is evident by the fact that trichloroacetic acid and acetone extractions could not effectively remove chlorophyll from extant *B. braunii*. Algaenans may play an important role in the preservation of multiple 'labile' substances via encapsulation or related mechanisms, providing additional time for more resistant hydrophobic aggregations to form.

5. Conclusions

The large fraction of proteinaceous material in the detritus of *B. braunii* which cannot be extracted after rigorous extraction compared to other algal detritus support the hypothesis that algaenans act as physical barriers for the encapsulation and preservation of labile organic matter. Molecular weight shifts in proteinaceous material to substantially higher molecular masses (M_r ~10^6) suggest that noncovalent aggregations also occur during diagenesis and are important for preservation. The increased contribution of protein amino acids with nonpolar side chains (glycine and alanine) during the decay, together with the observed susceptibility of aggregates to dissociation with various denaturants argues that hydrophobic interactions play an essential role in preservation of *B. braunii* proteins as seen in other algae (Nguyen and Harvey, 2001). In the Pula kerogen, the presence of hydrolyzable amino acids with a highly nonpolar composition appears to reflect the importance of both algaenans and hydrophobic inter-

actions as preservative agents for proteinaceous material during even extensive cellular degradation. Recent observations using TMAH thermochemolysis have also observed amino acids in polar fractions of ancient (140 Ma) kerogens from the Kashpir Oil Shales (Mongenot et al., 2001), including the identification of alanine and glycine (Riboulleau et al., 2002), which suggests that this process may be widespread. The combination of algaenan encapsulation and hydrophobic aggregations appears to allow small amounts of proteinaceous material to be retained in *B. braunii* detritus and survive over geologic time scales.

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