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Characterization of proteolytic enzyme activities in macroalgae

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Proteases are essential components of cells and participate in processes ranging from photoacclimation and nutrient acquisition to development and stress responses. Virtually nothing is known about this diverse group of enzymes in macroalgae. Methods to measure proteases developed for phytoplankton (caseinolysis, leucine aminopeptidase (LAP) activity and casein zymograms) have been modified to allow use with spectrophotometers as well as spectrofluorometers, so making them accessible to a wider number of users. Applying the methods to a wide range of macroalgae from intertidal zones in south-western Spain and Northern Ireland detected proteases in all species examined. Protease activities differed among species but there was little systematic variation with taxonomic group. Moreover, similar species in Spain and Northern Ireland often showed differences. With the exception of LAP activities in brown algae, protease activities were one to two orders of magnitude greater in macroalgae than those previously measured in phytoplankton, when expressed per unit protein. However, scaling of activities to protein was complicated by significant interferences. Copper-based protein assays gave erroneously high results when applied to the brown algae, and partial purification of proteins by trichloroacetic acid precipitation did not overcome the problem. The higher levels of protease activity suggest that proteolysis plays a more significant role in multicellular than in unicellular algae. Proteases were characterized in terms of activities at different pH and the effect of protease inhibitors. In common with microalgae but in contrast to findings for animals and higher plants, proteases tended to show higher activities in the neutral and alkaline ranges. Many commonly used protease inhibitors (e.g. leupeptin and phenylmethylsulphonyl fluoride) had relatively little effect, which may explain why biochemical work with macroalgal species has traditionally been difficult. Our results suggest that macroalgal proteases are easily measurable but highly variable. A major source of variability that has not been assessed is differing environmental conditions. If this is correct, measurements of proteolytic enzymes may provide a valuable tool for examining biologically relevant changes in environments. Controlled laboratory experiments and seasonal monitoring are the next logical steps towards this goal.

Key words: macroalgae, pH profiles, protease inhibitors–characterization/activity, zymograms

Introduction

Macroalgae are of great importance to coastal processes, including nutrient and oxygen cycling; they dominate primary production in most temperate intertidal zones. The main characteristic of such intertidal zones is the high variability of the environmental factors (i.e. light, temperature and nutrients) affecting growth and survival. Thus, organisms which inhabit these regions must have mechanisms to acclimate to, or otherwise survive, significant environmental stress.

Responses to stress are often mediated at the level of proteins. While stressful environmental conditions can induce synthesis of specific proteins (Fitzgerald *et al.*, 1978), they can also affect protein stability and turnover by increasing the rate of proteolysis of specific proteins (Thiel, 1990). For example, in cyanobacteria, proteases have been implicated in degradation of phycobiliproteins during photoacclimation and nutrient starvation (Foulds & Carr, 1977; Grossman *et al.*, 1993; Collier & Grossman, 1994). Induction of proteases in response to nitrogen or light limitation has also been described in some diatom and chlorophyte species (Berges & Falkowski, 1998).

Beyond stress responses, proteolysis has many other functions, including controlling the development, function and senescence of organelles (Vierstra, 1996), mediating the differentiation of special structures such as heterocysts in nitrogen-fixing cyanobacteria (Thiel, 1990), and also in cell defence (Gottesman, 1998).

In contrast to higher plants and animals, very little is known about proteolytic enzymes in marine photosynthetic organisms. Some information is available for unicellular algae, and some basic biochemical characterization of proteases has been

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carried out in a few species of marine phytoplankton (see Berges & Falkowski, 1996 and references therein), but we can find only three studies of proteases in macroalgae (Kadokami *et al.*, 1990; Matsubara *et al.*, 1998; Wang *et al.*, 1998).

It has been recognized that there is shortage of molecular, biochemical and physiological techniques for quantifying stress in macroalgae and thus for monitoring stress in natural communities and understanding the interacting roles of natural and anthropogenic stresses in intertidal ecosystems (see Davison & Pearson, 1996). In this sense, proteases could be potentially used as biochemical probes if we were able to find some kind of stress-specific response in which proteases were involved.

In a broad-based study such as this, it is important to realize that there are difficult choices to be made. No single set of assays conditions will be optimal for all proteolytic enzymes and conditions that favour one set of enzymes may well inhibit others. For example, the methods used for either assaying certain activities (e.g. addition of reducing agents) or extracting certain membrane-bound proteases (e.g. detergents, high temperatures, rigorous freeze-thaw cycles) would inactivate other proteases. In our work, we have elected to examine those proteases that can be extracted and assayed in Tris buffer, using sonication and freeze-thawing in liquid nitrogen. While there are obvious limitations to this approach, it has been used successfully to examine microalgal proteases (Berges & Falkowski, 1996). Thus, as a first step, the aim of this work was to detect and to describe the basic biochemical features of proteolytic activities in marine macroalgae by adapting the methods developed by Berges & Falkowski (1996) for phytoplankton.

Materials and methods

Sampling sites

A wide variety of the most common macroalgal species (Chlorophyta, Rhodophyta and Phaeophyta) were collected from the intertidal zones near Cádiz (southwestern Spain) and Belfast (Northern Ireland). Collections included species with different morphologies, life cycles and functional forms (*sensu* Littler & Littler, 1980). Samples were carefully selected (only young, apical parts free from visible epiphytes), rinsed with seawater, blotted between two paper sheets to remove adhering water, and frozen and maintained in liquid nitrogen until further analysis.

Homogenization and protein determination

Frozen samples were powdered with a mortar and pestle and resuspended in 50 mM Tris (Tris (hydroxymethyl) aminomethane) buffered to pH 7.5, except in the case of pH-dependence assays (see below). Homogenates were sonicated for three cycles of 10 s at a power setting of 25 W using either a Microson XL2007 Ultrasonic Cell Disruptor (Cádiz) or a VibraCell Sonicator (Belfast). To achieve better cell disruption, sonication was repeated after a further freeze-thaw cycle. Homogenates were centrifuged at 5°C for 5 min at 13 000 *g* (Sigma 2K15 centrifuge in Cádiz or Sanyo Microcentaur centrifuge in Belfast). Supernatants were removed, frozen in liquid nitrogen and stored at −80°C for protein measurements and protease assays.

Protein contents of homogenates were measured using the BCA (bicinchoninic acid) method (Smith *et al.*, 1985, using Sigma Chemical Co. kit BCA-1) with bovine serum albumin (BSA) as a standard. In the case of some brown algae (especially *Fucus* species), homogenates reacted almost instantaneously with the BCA reagent, indicating the presence of interfering compounds. Precipitating protein extracts with trichloroacetic acid (TCA, 10% w/v in acetone) and redissolving did not overcome the problem; in these cases, the Bradford assay (Bradford, 1976) was used. Typically, protein concentrations in homogenates ranged from 0.4 to 8.0 mg ml^{-1}.

Protease assays

Protease assays were carried out largely as described in Sarath *et al.* (1989), measuring either degradation of labelled casein (caseinolysis) or degradation of artificial substrates (L-leucine 7-amido-4-methyl coumarin (Leu-AMC) and L-leucinyl-*p*-nitroanilide (Leu-pNA)) for leucine aminopeptidase (LAP activity). All assays were conducted at 25°C. For both types of assays, spectrophotometric and fluorometric assays were compared, and the methods were inter-calibrated with selected samples. For caseinolysis, fluorometric assays monitoring the degradation of fluorescein isothiocyanate (FITC)-labelled casein (Sigma C0528) were performed in 50 μl total volume (20 μl FITC-casein 0.5% w/v in 50 mM Tris), 10 μl Tris buffer 100 mM and 20 μl homogenate) in microcentrifuge tubes for 1 h (Twinning, 1984). At the end of the incubation, casein was precipitated with 5% w/v TCA, and free FITC liberated by caseinolytic activity was measured using a Perkin Elmer LS50B spectrofluorometer (excitation/emission of 490 nm/525 nm, 10 nm slit widths). The fluorometer was calibrated against standard FITC (Sigma F3651). Equivalent spectrophotometric assays monitoring hydrolysis of azocasein (Sigma A2765) were carried out in 400 μl total volume (250 μl azocasein, 2% w/v in 50 mM Tris buffer, and 150 μl homogenate) in microcentrifuge tubes for 1 h. After TCA precipitation (see above), supernatant was redissolved in NaOH (2 N) and the absorbance of the free chromogenic azo groups produced by cleavage of casein was measured in a spectrophotometer (ATI Unicam) at 440 nm. Activities were calculated using an extinction coefficient of 90 664 M^{-1} cm^{-1}.

Synthetic substrates were used to measure LAP activity. For fluorometric assays, degradation of Leu-AMC (Sigma L2145) was monitored continuously with a Perkin Elmer LS50B spectrofluorometer (excitation/emission of 380 nm/460 nm, 10 nm slit widths). The reaction was performed in a 1 ml cuvette containing 500 μl substrate

(2 mM in distilled water), 500 μl Tris buffer (100 mM) and 20–40 μl homogenate. The fluorometer was calibrated against standard free AMC (Sigma A9891). For spectrophotometric assays, degradation of Leu-pNA (Sigma L2158) was monitored continuously with an ATI UNICAM spectrophotometer at 400 nm, in a 1-ml cuvette with 700 μl total volume (350 μl Leu-pNA 2 mM, 150 μl homogenate, 200 μl Tris buffer 50 mM). LAP activity was defined as the activity leading to the cleavage of Leu-AMC or Leu-pNA, regardless of the nature of the enzymes involved (Berges & Falkowski, 1996). Activities were calculated using an extinction coefficient of 10500 M^{-1} cm^{-1} for nitroanilide (Sarath *et al.*, 1989).

Electrophoretic measurements

Zymograms of protease activities were prepared largely as described in Berges & Falkowski (1996). Briefly, samples prepared in Tris buffer (see above) were spun at 13 000 *g* to remove cell fragments, loaded on an equal protein basis and separated on 10% polyacrylamide gels that did not contain SDS or reducing agents. Immediately after electrophoresis, gels were immersed in a 2% (w/v) casein solution in 50 mM Tris buffer (pH 7.5), incubated at 25°C for 1–1.5 h, and then rinsed with distilled water and stained with Coomassie Brilliant Blue R-250 (Sarath *et al.*, 1989). Protease activity appeared as clear bands against a blue background. In species where poorly resolved high-molecular-mass bands were observed, we attempted to improve resolution using gradient gels (ProSieve 50 gel solution) or deoxycholic acid (0.25% w/v) and Triton X-100 (0.5% v/v) polymerized in the gels (see Hames, 1990). Zymograms were scanned (Agfa Studio Star Desktop Colour Scanner), and the area and mean intensity of the bands were quantified by an image analysis program (NIH Image version 1.55).

Species survey

To test assays and select species for further study, a survey of caseinolytic and LAP activity and of zymograms was carried out in nearly 40 species (listed in Table 1). Because the survey was intended to be semi-quantitative, and mindful of potential difficulties with protein assays in some species, homogenates were prepared using approximately equal blotted wet masses of different species.

Characterization of protease activities

Assays to characterize protease activity were carried out for selected species in Belfast using spectrofluorometric methods, and in Cádiz using spectrophotometric methods. For selected species (*Ulva rigida, Codium decorticatum, Stypocaulon scoparium, Dictyota dichotoma, Pterocladiella capillacea* and *Gracilaria* sp. in Cádiz), substrate dependence of reactions was quantified for substrate concentrations ranging from 0 to 1.2 mM for azocasein and 0 to 3 mM for Leu-pNA. Data were fitted to a Michaelis–Menten equation using an iterative non-linear curve-fitting procedure (Kaleidagraph for Macintosh version 3.0).

For selected species (*Ulva rigida, Stypocaulon scoparium* and *Pterocladiella capillacea* in Cádiz), the pH dependence of protease activities was determined using a 50 mM MES (2-[*N*-morpholino]ethanesulphonic acid) buffer (Sigma M5287) for pH 6.0–6.5 and 50 mM bis-Tris propane buffer (Sigma B 6755) for pH 7.0–10.0. Extracts were prepared at the same pH as for the assays. Activities were scaled to that found at pH 7.5.

To provide information about the classes of proteases present in the extracts, the effects of different protease inhibitors on caseinolysis and LAP activity were studied in selected species (*Ulva* sp., *Enteromorpha* sp., *Porphyra* sp., *Fucus vesiculosus, F. serratus* and *Pelvetia canaliculata* in Belfast and *Stypocaulon scoparium, Pterocladiella capillacea* and *U. rigida* in Cádiz). The inhibitors used were phenylmethylsulphonyl fluoride (PMSF, Sigma P7626; final concentration 3.3 mM), 4-[2-aminoethyl]-benzenesulphonyl fluoride (AEBSF, Sigma A8456; final concentration 1 mM), leupeptin (Sigma, L9783; final concentration 1.6 μg ml^{-1}), pepstatin A (Sigma P4265; final concentration 160 μg ml^{-1}) and EDTA (Sigma E5134; final concentration 10 mM) following Berges & Falkowski (1996). For inhibitors that were added dissolved in organic solvents (pepstatin A and PMSF), control reactions were run with equivalent quantities of the solvents and results were corrected for solvent effects.

Another means of characterizing proteases is to determine the breakdown products they generate from a particular substrate. For degradation assays with selected species (*Ulva rigida, Pterocladiella capillacea* and *Stypocaulon scoparium* from Cádiz), 300 μl of the protein BSA (1% w/v in Tris 50 mM, pH 7.5) (Sigma A7906) was added to 200 μl of crude homogenates. Replicate samples were either precipitated immediately with TCA (10% w/v in acetone), or incubated for 1 h at 25°C followed by precipitation. Homogenates were prepared for electrophoresis and separated as previously described (Berges & Falkowski, 1996). Gels were stained and analysed as for zymograms (see above), comparing patterns of proteins in samples before and after incubations.

Protease stability

To assess the stability of protease activities in homogenates of tissue, extracts of selected species (*Ulva* sp., *Enteromorpha* sp., *Porphyra* sp., *Fucus vesiculosus, F. serratus* and *Pelvetia canaliculata* in Belfast and *Stypocaulon scoparium, Pterocladiella capillacea* and *U. rigida* in Cádiz) were measured for caseinolytic and LAP activities immediately after homogenization, and then repeatedly at intervals up to 24 h. Homogenates were kept at 24°C during this period.

Results

Assay method inter-comparisons

For 18 different homogenates of five different species, LAP measurements were made using both fluorometric (Leu-AMC substrate) and spectrophotometric (Leu-pNa substrate) methods. A regression of hydrolysis rates of Leu-AMC versus

Table 1. Caseinolytic activity, leucine-aminopeptidase (LAP) activity, and proteolytic bands in zymograms in a variety of macroalgae from Belfast and Cádiz

Species	Order	Site	Caseinolytic activity	LAP activity	Zymogram
CHLOROPHYTA					
Chaetomorpha sp.	Cladophorales	Be	+	n.d.	L
Cladophora rupestris (Linnaeus) Kützing	Cladophorales	Be	+ +	+ +	L
Codium fragile (Suringar) Hariot	Bryopsidales	Be	+	n.d.	H, L
Codium decorticatum (Woodward) M.A. Howe	Bryopsidales	Ca	+	+ + +	H, L
Derbesia sp.	Bryopsidales	Ca	+ +	n.d.	0
Udotea petiolata (Turra) Børgesen	Bryopsidales	Ca	n.d.	+ +	H
Enteromorpha intestinalis (Linnaeus) Link	Ulvales	Be	+ +	+	H, L
Ulva lactuca Linnaeus	Ulvales	Be	+ + +	+	H, L
Ulva rigida C. Agardh	Ulvales	Ca	+ +	+ + +	0
RHODOPHYTA					
Porphyra umbilicalis (Linnaeus) Agardh	Bangiales	Be	+ +	+	H
Porphyra sp.	Bangiales	Ca	n.d.	+ +	H
Delesseria sanguinea (Hudson) Lamouroux	Ceramiales	Be	+	n.d.	H, L
Halopitys pinastroides (Gomel) Kützing	Ceramiales	Ca	+ +	+	0
Heterosiphonia sp.	Ceramiales	Ca	n.d.	0	0
Phycodrys rubens (Linnaeus) Batters	Ceramiales	Be	+	n.d.	H
Polysiphonia sp.	Ceramiales	Be	+	n.d.	H
Corallina officinalis Linnaeus	Corallinales	Be	+ + +	+ + +	0
Corallina elongata J. Ellis & Solander	Corallinales	Ca	+	+ + +	0
Jania rubens (Linnaeus) J.V. Lamouroux	Corallinales	Ca	0	+ + +	0
Peyssonnelia sp.	Cryptonemiales	Ca	+ + +	n.d.	n.d.
Gelidium pusillium (Stackhouse) Le Jolis	Gelidiales	Be	+ +	n.d.	H, L
Pterocladiella capillacea (S.G. Gmelin) Santelices & Hommersand	Gelidiales	Ca	+ +	+ + +	L
Callophyllis laciniata (Hudson) Kützing	Gigartinales	Be	+	n.d.	H, L
Chondrus crispus Stackhouse	Gigartinales	Be	+ +	+	H, L
Gigartina stellata (Stackhouse) Batters	Gigartinales	Be	0	n.d.	H, L
Schottera nicaëensis (J.V. Lamouroux *ex* Duby) Guiry & Hollenberg	Gigartinales	Ca	n.d.	+	0
Gracilaria sp.	Gracilariales	Ca	+ +	+ +	0
Palmaria palmata (Linnaeus) Kuntze	Palmariales	Be	+	n.d.	0
Plocamium cartilagineum (Linnaeus) Dixon	Plocamiales	Be	+	n.d.	H
Plocamium cartilagineum (Linnaeus) Dixon	Plocamiales	Ca	n.d.	+	H
Lomentaria articulata (Hudson) Lyngbye	Rhodymemiales	Be	+ +	n.d.	H, L
PHAEOPHYTA					
Desmarestia aculeata (Linnaeus) Lamouroux	Desmarestiales	Be	+	n.d.	H
Dictyota dichotoma (Hudson) Lamouroux	Dictyotales	Be	+	n.d.	L
Dictyota dichotoma (Hudson) Lamouroux	Dictyotales	Ca	+ +	+	L
Ectocarpus siliculosus (Dillwyn) Lyngbye	Ectocarpales	Be	+ +	+ + +	H
Ascophyllum nodosum (Linnaeus) Le Jolis	Fucales	Be	+	n.d.	H
Fucus serratus Linnaeus	Fucales	Be	+	0	H, L
Fucus spiralis Linnaeus	Fucales	Be	n.d.	0	H
Fucus vesiculosus Linnaeus	Fucales	Be	+ + +	0	H, L
Himanthalia elongata (Linnaeus) Gray	Fucales	Be	n.d.	0	n.d.
Pelvetia canaliculata (Linnaeus) Decasne & Thuret	Fucales	Be	+ + +	0	H, L
Halidrys siliquosa (Linnaeus) Lyngbye	Fucales	Be	n.d.	+	n.d.
Sargassum muticum (Yendo) Fendsholt	Fucales	Be	n.d.	+	n.d.
Alaria esculenta (Linnaeus) Greville	Laminariales	Be	+	n.d.	H, L
Laminaria hyperborea (Gunnerus) Foslie	Laminariales	Be	+	0	H
Laminaria saccharina (Linnaeus) Lamouroux	Laminariales	Be	n.d.	+ + +	n.d.
Stypocaulon scoparium (Linnaeus) Kützing	Sphacelariales	Ca	+ + +	+	L

Samples from Belfast (Be) were measured by spectrofluorometric assays; samples from Cádiz (Ca) by means of spectrophotometric assays. Proteolytic activity is expressed on a qualitative scale: + + + , high activity; + +, medium activity; +, low activity; 0, no activity. H, High molecular weight (about 200 kDa) proteases; L, low molecular weight (about 20 kDa) proteases; 0, activity not detected in zymograms. n.d., samples not determined.

Leu-pNA gave a strong linear relationship ($R^2 = 0.97$) with a slope of 1.06 (± 0.04; not significantly different from 1 at $p < 0.05$). The relationship between caseinolytic activities measured using FITC-casein and azocasein was more variable; the limit of detection for the spectrophotometric method was considerably higher than for the spectrofluorometric method. The two assays were significantly related ($R^2 = 0.54$) with a slope of 0.22 (± 0.051); i.e. activities calculated using azocasein tended to give results about 5 times lower than those using FITC-casein.

Species surveys

Using the assays adapted from work on phytoplankton, caseinolytic and LAP activities were easily detected in almost every species, and the majority of species also showed clear proteolytic bands on zymograms (Table 1). Few other generalizations can be made. In terms of caseinolytic activities, the Ulvales displayed the highest activities among the chlorophytes. It was also noted that samples of bleached *Enteromorpha* sp. had increased activity compared with healthy green specimens (data not shown). *Corallina officinalis* and *Peyssonnelia* sp. showed the highest activities among the rhodophytes, and members of the Fucales and *Stypocaulon scoparium* among the phaeophytes. For LAP activities, the highest values were recorded for *Ulva rigida* and *Codium decorticatum,* and the coralline rhodophyte species. In contrast, generally lower LAP activity was found in brown algae, especially in members of the Fucales and Laminariales, where no activity was found for some species (Table 1). For zymograms, there appeared to be no taxonomic patterns. Some species showed both low ($\leqslant$20 kDa) and high ($\geqslant$200 kDa) molecular mass proteases (e.g. *Ulva* sp., *Codium* spp., *Callophyllis laciniata, Pelvetia canaliculata* and *Fucus vesiculosus*), while others showed either only low (e.g. *Dictyota dichotoma, Stypocaulon scoparium* or *Pterocladiella capillacea*) or only high (e.g. *Udotea petiolata, Porphyra* sp., *Plocamium cartilagineum, Laminaria hyperborea*) molecular mass bands (Table 1, Fig. 1). In general, the high-molecular-mass bands detected were poorly resolved. Using gradient gels or detergents such as deoxycholic acid and Triton X-100 polymerized in the gels did not improve resolution (data not shown).

Enzyme characterization

In terms of substrate dependence, Michaelis–Menten kinetics fitted the data from all the species examined, except for *Gracilaria* sp. where no evidence of saturation was observed for LAP assays (Table 2). LAP activities and caseinolytic activities were not always related. For example, relatively high activities for both methods were observed in *Codium decorticatum,* but *Dictyota dichotoma* had the highest activity for caseinolysis but one of the lower LAP activities measured. K_s values for LAP activities ranged from 0.24 to 2.57 mM, and from 0.03 to 2.34 mM for caseinolytic activities. *Stypocaulon scoparium* showed the lowest affinity for both assays, but K_s values in other species were not well related between assays.

Protease activities were affected by pH ($p<0.05$) in all the species tested (Fig. 2). The pH profiles for caseinolytic and LAP activities were quite similar, especially in the acidic ranges. Some interspecific

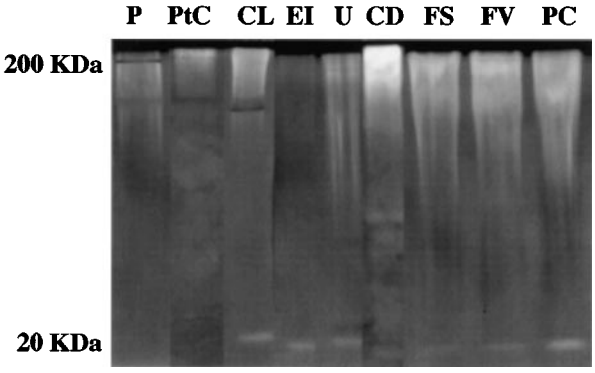

Fig. 1. Zymogram of caseinolytic activity in 10% polyacrylamide gel from homogenates of *Porphyra* sp. (P), *Pterocladiella capillacea* (PtC), *Callophyllis laciniata* (CL), *Enteromorpha intestinalis* (EI), *Ulva* sp. (U), *Codium decorticatum* (CD), *Fucus spiralis* (FS), *F. vesiculosus* (FV) and *Pelvetia canaliculata* (PC). Gels were placed in a 2% (w/v) casein solution in 50 mM Tris buffer (pH 7.5), incubated at 25°C for 1 h. Gels were stained with Coomassie Brilliant Blue R-250 and protease activity appeared as clear bands. Apparent molecular weights are marked according to protein standards denatured in SDS.

Table 2. Kinetic parameters for proteolytic enzyme activities measured from several species of seaweeds

	Caseinolytic activity			LAP activity		
Species	V_{max} (U (g protein)$^{-1}$)	K_s (mM)	r^2	V_{max} (U (g protein)$^{-1}$)	K_s (mM)	r^2
Ulva rigida	0.039 ± 0.009	0.03 ± 0.06	0.60	0.75 ± 0.24	0.70 ± 0.66	0.75
Codium decorticatum	0.192 ± 0.066	0.78 ± 0.52	0.92	3.75 ± 0.08	0.33 ± 0.03	0.99
Stypocaulon scoparium	0.132 ± 0.132	2.34 ± 3.23	0.88	0.59 ± 0.54	2.57 ± 4.08	0.72
Dictyota dichotoma	0.317 ± 0.115	0.10 ± 0.17	0.50	0.66 ± 0.20	2.15 ± 1.24	0.92
Pterocladiella capillacea	0.037 ± 0.005	0.26 ± 0.21	0.92	3.59 ± 0.60	0.24 ± 0.17	0.88
Gracilaria sp.	0.057 ± 0.005	0.05 ± 0.06	0.67	$Y = -60 + 0.60$ (Leu-pNA)		0.88

Units of activity (U) represent micromoles of product released per minute (azopeptide fragments from azocasein for caseinolytic activity or *p*-nitroanilide from leucine-*p*-nitroanilide for LAP activity). Enzyme kinetics were fitted to a Michaelis–Menten model (or linear model for LAP activity in *Gracilaria* sp.) using iterative methods. Each value represents the mean of three determinations made on the same homogenate (±SE of the mean).

differences were found, but again these did not seem to relate to taxonomic affiliations. Both activities were lower at acidic and alkaline than at neutral pH, especially for *Stypocaulon scoparium* where no LAP activity was detected at pH 6. Peaks were observed at pH 8 in *S. scoparium* for LAP activity and in *Ulva rigida* for caseinolysis. In contrast, activities in the rhodophyte *Pterocladiella capillacea* showed less variation with pH, and highest values in the alkaline range.

There were also wide differences in the effect of protease inhibitors on activities in different species. The protease inhibitors were categorized as ineffective (no difference from controls without inhibitors), moderately effective (<30% inhibition relative to controls, $p<0.05$) or highly effective (>30% inhibition relative to controls, $p<0.05$). On this basis, leupeptin, and pepstatin A were only highly effective against caseinolytic activity for *Stypocaulon scoparium* (Table 3), while EDTA was highly effective in *S. scoparium, Ulva* sp. and *Pelvetia canaliculata*. PMSF was highly effective against caseinolysis in *S. scoparium,* but only moderately effective in *Pterocladiella capillacea*. In contrast, AEBSF was highly effective against caseinolysis in *U. rigida, Ulva* sp., *Enteromorpha intestinalis, Fucus vesiculosus* and *F. serratus*. With respect to LAP activity, leupeptin and pepstatin A were ineffective for the three species tested in Cádiz, AEBSF was only effective for *P. capillacea,* and EDTA and PMSF caused a strong inhibition in *S. scoparium, P. capillacea* and *U. rigida*.

Assays for BSA degradation did not reveal any bands that corresponded to degradation products (data not shown). Based on changes in the density of the BSA bands on the gels, *Pterocladiella capillacea* showed higher BSA degradation rate (0.023 mg BSA (mg protein)$^{-1}$ h^{-1}) than *Ulva rigida* (0.013 mg BSA (mg protein)$^{-1}$ h^{-1} and *Stypocaulon scoparium* (0.003 mg BSA (mg protein)$^{-1}$ h^{-1}).

Protease stability

Caseinolytic activities were quite stable in *Stypocaulon scoparium* and *Pterocladiella capillacea* at

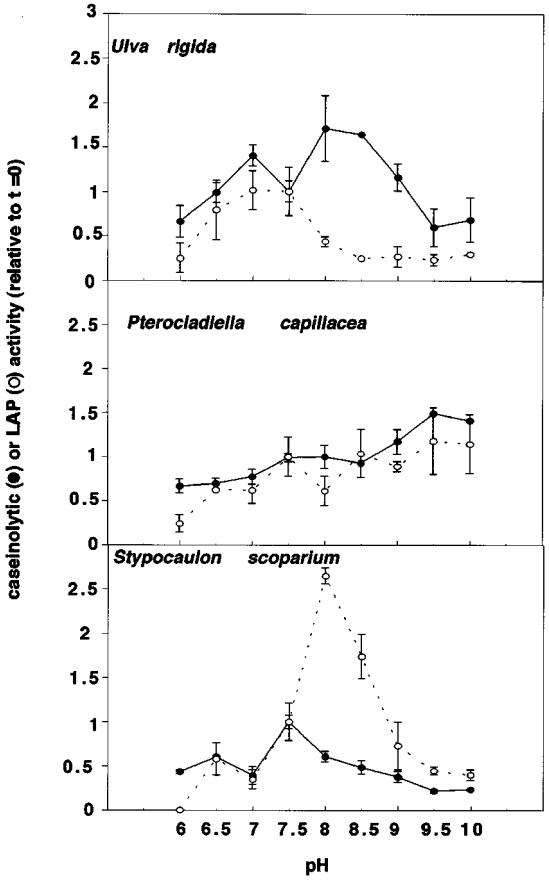

Fig. 2. pH profiles of caseinolytic activity (continuous lines) or LAP activity (broken lines) for three species of marine macroalgae. LAP activity was defined as the activity leading to the cleavage of Leu-pNA, regardless of the nature of the enzymes involved (Berges & Falkowski, 1996). Activities were measured in 50 mM MES buffer (pH 6.0–6.5) and 50 mM Tris buffer (pH 7.0–10.0). Activities were scaled to that found at pH 7.5. Each point represents the mean of three separate homogenates; error bars represent ± 1 SE.

Table 3. Effects of protease inhibitors on caseinolytic activity or LAP activity for eight species of marine macroalgae

	Relative caseinolytic activity					Relative LAP activity				
Species	AEBSF	EDTA	PMSF	Leupeptin	Pepstatin A	AEBSF	EDTA	PMSF	Leupeptin	Pepstatin A
Pterocladiella capillacea	103 (28.1)	99.0 (16.5)	56.5 (6.6)	96.1 (28.9)	94.2 (4.8)	34.3 (8.6)	28.6 (12.5)	22.2 (13.5)	88.6 (20.0)	80.0 (15.4)
Stypocaulon scoparium	123 (7.5)	0.0 (0.0)	0.0 (0.0)	56.5 (2.0)	20.0 (20.0)	75.1 (15.0)	15.2 (15.2)	0.0 (0.0)	82.6 (37.5)	79.2 (15.0)
Fucus vesiculosus	56.7 (6.5)	134 (10.0)	n.d.	90.9 (11.3)	28.1 (3.8)	n.d.	n.d.	n.d.	n.d.	n.d.
Fucus serratus	24.8 (12.0)	150 (28.3)	n.d.	86.5 (15.2)	81.3 (36.5)	n.d.	n.d.	n.d.	n.d.	n.d.
Pelvetia canaliculata	203 (10.2)	24.7 (1.3)	n.d.	92.5 (4.8)	82.4 (2.1)	n.d.	n.d.	n.d.	n.d.	n.d.
Enteromorpha intestinalis	50.4 (14.0)	75.5 (9.0)	n.d.	112.0 (8.0)	114.0 (6.0)	n.d.	n.d.	n.d.	n.d.	n.d.
Ulva rigida	31.0 (3.2)	120.7 (26)	68.2 (37.0)	73.8 (25.0)	127 (27.4)	86.4 (25.3)	0.0 (0.0)	0.0 (0.0)	77.3 (9.1)	66.7 (11.1)
Ulva sp.	77.8 (1.0)	16.1 (5.0)	n.d.	90.8 (2.0)	67.5 (2.0)	n.d.	n.d.	n.d.	n.d.	n.d.

Final concentrations of inhibitors were 1.6 μg ml^{-1} leupeptin, 160 μg ml^{-1} pepstatin A, 10 mM EDTA, 1 mM 4-[2-aminoethyl]benzenesulphonyl fluoride (AEBSF) and 3.3 mM phenylmethylsulphonyl fluoride (PMSF). Activities are expressed as percentages of activity in control assays. Each value represents the mean of three assays (±SE of the mean). n.d., not determined.

room temperature (Fig. 3). For other species, activities were well replicated, but varied considerably over time. For example, caseinolytic activities in *Ulva* spp. and *Porphyra* sp. declined by about 50% within 2 h, but while they remained low in *Porphyra,* they increased to original levels in *Ulva* spp. by 6 h. In *Fucus vesiculosus* and *Pelvetia canaliculata* there were substantial increases in caseinolytic activities over time. In species where LAP activities were also measured (*S. scoparium, P. capillacea* and *U. rigida*), the two assays tended to show similar patterns.

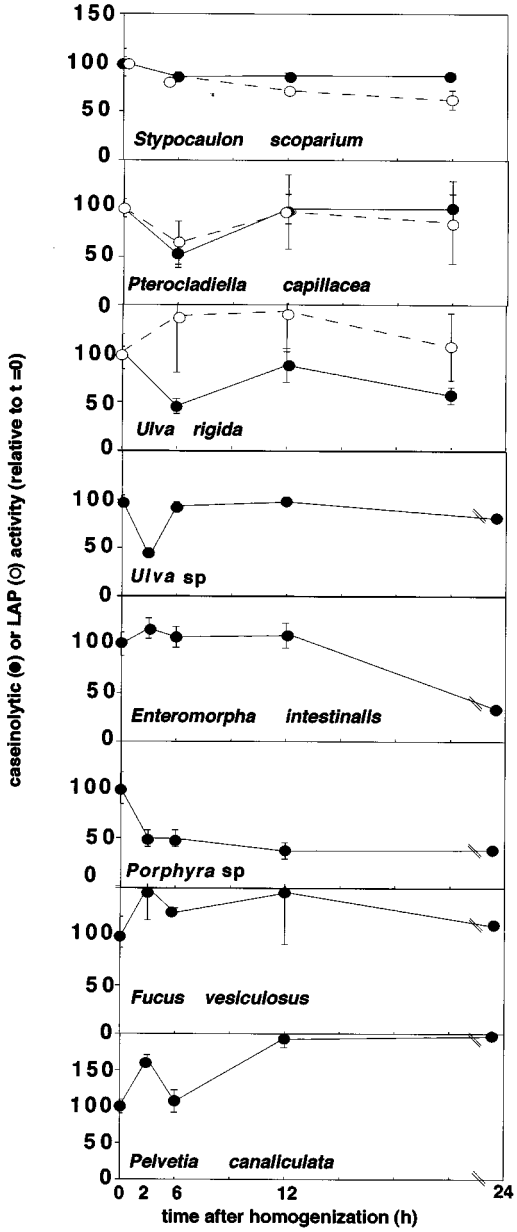

Fig. 3. Stability of caseinolytic activity (continuous lines) and LAP activity (broken lines) of eight species of marine macroalgae maintained at 24°C for 12 or 24 h. LAP activity was defined as the activity leading to the cleavage of Leu-AMC or Leu-pNA, regardless of the nature of the enzymes involved (Berges & Falkowski, 1996). Each point represents the mean of determinations from three homogenates, ± 1 SE, relative to activity immediately following homogenization in 50 mM Tris buffer at pH 7.5.

Discussion

Methodology and interspecific variability

Methodology developed by Berges & Falkowski (1996) to detect proteolytic activities in microalgae was quite successful when applied to macroalgae. Moreover, although certain differences were found between azocasein and FTC-casein assays, consistent relationships were found between spectrophotometric and spectrofluorometric assays. All the species surveyed, excepting the rhodophytes *Jania rubens* and *Gigartina* sp., showed some degree of caseinolytic activity. Although no clear trends were observed for the different taxa, Ulvales and some species of Fucales (*Fucus vesiculosus, Pelvetia canaliculata*) showed the highest values in qualitative terms (i.e. activity was not scaled to protein content) among chlorophytes and phaeophytes, respectively. LAP activity was also detected in all the chlorophytes tested. In contrast to caseinolysis, LAP activity was often not detected in Fucales and Laminariales.

Zymograms revealed both low ($\leqslant$20 kDa apparent molecular mass) and high (200 kDa) molecular mass proteases, depending on the species. The exact molecular weight of these proteins is difficult to specify since we used native gel electrophoresis and have no information about the conformation of the proteases in question. In general, the high-molecular-mass bands detected were poorly resolved. Attempts to resolve this band better using either gradient electrophoresis or deoxycholic acid and Triton X-100 while preparing the gels (Hames, 1990) were not successful. High-molecular bands have been observed in the diatom *Thalassiosira weissflogii* (Berges & Falkowski, 1996) and low-molecular bands in the microalgae *T. weissflogii, Dunaliella tertiolecta* (Berges & Falkowski, 1996) and *Chlamydomonas reinhardtii* (38 kDa; Hoober & Hughes, 1992). Kadokami *et al.* (1990) purified two low-molecular-mass proteases (38–39 kDa) in the seaweed *Codium fragile*. In a survey of brown and red algal species, Wang *et al.* (1998) used gelatin zymograms to identify proteases of about 18–19 kDa that were characteristic of *Laminaria* species. We have therefore established the means to investigate proteases in macroalgae, and documented substantial variations in proteases among different macroalgae.

Protease characterization

The kinetic parameters for caseinolysis activities (V_{max} and K_s) measured in macroalgae under

standard conditions (i.e. pH 7.5, 25°C) were generally one to two orders of magnitude higher than those found in terrestrial plants (e.g. pea leaves; Skoda & Malek, 1992) or in marine microalgae (Berges & Falkowski, 1996). Values of V_{max} for LAP activities were comparable to those measured in pea leaves (Liu & Jagendorf, 1986) but one to two orders of magnitude higher than those found for microalgae (Berges & Falkowski, 1996). K_s values for LAP activities in *Codium decorticatum, Ulva rigida* and *Pterocladiella capillacea* were comparable to those for certain marine microalgal species (Berges & Falkowski, 1996) and similar to those measured for an aspartic proteinase from wheat leaves (Galleschi & Felichioli, 1994). In contrast, *C. decorticatum* displayed K_s values two orders of magnitude higher than the K_s recorded under optimal conditions in two purified trypsin-like proteases from *C. fragile* (Kadokami *et al.*, 1990). There are real difficulties in trying to examine kinetic constants determined in crude homogenates; other proteins in crude homogenates may act as substrates (and thus compete with the synthetic ones) or, alternatively, there may be compounds that act as inhibitors of proteases. In fact, lower proteolytic activities and overestimation of kinetic parameters from crude extracts have been recorded, compared with those of purified proteases (DeMartino, 1989; Voet & Voet, 1992). Nonetheless, it seems clear that macroalgae have substantially higher protease activities than microalgae.

It is important to recognize that protease measurements in the present study were made on material freshly collected from the field and, thus, from an unspecified set of environmental conditions. In contrast, many of the previous measurements of microalgal protease activities have been made under ideal conditions supporting exponential growth. Thus, elevated protease activities in macroalgae might reflect higher levels of environmental stress. This seems unlikely to be the complete explanation, however. Multicellular organisms have potentially greater requirements for proteases than unicells. In contrast to phytoplankton, macroalgae must process and degrade intercellular signals, coordinate tissue growth and development through proteolysis (Ellis *et al.*, 1991), and reprocess tissues to develop reproductive structures and to renew damaged tissues (Lee, 1989). Higher protease levels may simply be a consequence of multicellular structure.

The brown algae (*Stypocaulon scoparium* and *Dictyota dichotoma*) displayed the lowest affinity and V_{max} values for LAP activity, and this corresponds with the low and often absent activity in the majority of phaeophytes studied. This may be due to compounds found in crude extracts of these species, as has been previously reported. For example, Davison & Pearson (1996) were able to separate proteins extracted from *Fucus evanescens* germlings using SDS-PAGE but were unable to do so with adult plants – problems they attributed to interference from large quantities of mucus and/or polyphenolic compounds. The difficulties experienced with BCA assays in the present study also support this interpretation.

Profiles of proteolytic activity versus pH gives some information about the cellular location of the protease involved (Vierstra, 1996). No sharp peaks of activity (either in LAP or caseinolysis) in the neutral to alkaline pHs were observed in *Pterocladiella capillacea,* suggesting that cytosolic and chloroplastic proteases are mainly involved. This pattern has also been observed in crude soybean extracts (Ragster & Chrispeels, 1981), and in some species of microalgae (Berges & Falkowski, 1996). *Ulva rigida* displayed a clear peak for LAP activity in the neutral range, suggesting a possible cytosolic origin of the proteases involved. A similar response has been observed for *Codium fragile* and other *Codium* species, with optima between pH 7 and 9 (Kadokami *et al.*, 1990; Matsubara *et al.*, 1998). A peak for LAP and caseinolysis was observed at pH 7–8 in *U. rigida* and *Stypocaulon scoparium* with minima in the acidic and alkaline regions. Similar responses have been observed in higher plant tissues (Tully & Beevers, 1978; Malik, 1983) as well as in some microalgal species (Hoober & Hughes, 1992; Langheinrich, 1995; Berges & Falkowski, 1996). Wang *et al.* (1998) surveyed 11 species of *Laminaria, Undaria* and *Porphyra,* and concluded that optimum pH for protease activities was 8.0. Overall, our findings suggest that the dominant proteases in marine macroalgae tend to be active at neutral and alkaline pHs, indicating proteases of cytoplasmic or plastid origins rather than vacuolar forms active at acidic pH (Callis, 1995).

Most of the diagnostic inhibitors used had surprisingly weak effects on proteolysis, making the classification of the enzymes responsible difficult. Although inhibitor concentrations used should provide some degree of specificity toward individual protease classes (Bond & Butler, 1987), no attempt has been made to distinguish specific from non-specific protease inhibition. Combined with pH data, the ineffectiveness of pepstatin A suggests that aspartic proteases are not a large component of the activity detected in all species, except perhaps *Stypocaulon scoparium*. PMSF and AEBSF were effective inhibitors, but there was considerable variability; in *Pelvetia canaliculata,* AEBSF appeared to stimulate caseinolysis. The effectiveness of these two inhibitors suggests the relative importance of serine and some cysteine

proteases. This finding is similar to the results of Kadokami (1990) and Matsubara *et al*. (1998), who demonstrated that proteases from various *Codium* species were inhibited by PMSF, leupeptin and diisopropyl fluorophosphate. However, leupeptin should also effectively inhibit serine and cysteine proteases, yet it had no effect in any species tested in the present study. It may be that the concentrations of leupeptin used here (1.6 μg ml^{-1}) are lower than required; some authors have recommended considerably greater levels (e.g. Beynon & Salvensen, 1989). The absence of reducing agents (e.g. mercaptoethanol or dithiothreitol) during homogenization might also have led to the inactivation of cysteine proteases, which would explain the ineffectiveness of leupeptin, although some cysteine proteases show reasonable activity without reducing agents (Chapman *et al*., 1997). However, the use of mercaptoethanol or DTT can be also problematic since the activity of many proteases can be inhibited (including some cystein proteases; e.g. the cysteine protease cathepsin B is destabilized in presence of sulphydryl reagents, see DeMartino, 1989).

EDTA was effective against LAP in all species tested, but caseinolysis was less affected. Since leucine aminopeptidases are generally zinc-dependent (e.g. Matsushima *et al*., 1991), this result is not unexpected, and similar results have been recorded in marine microalgae (Berges & Falkowski, 1996). Once again there was wide variation; EDTA was highly effective against caseinolysis in *Ulva* sp. and *P. canaliculata*. The existence of metal-dependent serine and cysteine proteases has been found in several organisms, for example, a Ca^{2+}-dependent serine protease has been measured in the cyanobacterium *Anabaena variabilis* (Wood & Hasselkorn, 1979; Lockau *et al*., 1988).

Our work shows that the stability of the proteolytic activity in crude homogenates was species-specific. Caseinolysis was quite stable in *Stypocaulon scoparium, Pterocladiella capillacea* and *Ulva* sp., and this agrees with previous findings in several species of marine phytoplankton (Berges & Falkowski, 1996) as well as in the seaweed *Codium fragile* (Kadokami *et al*., 1990). In fact, proteases appear to be among the most stable enzymes known, retaining their activity even after boiling crude extracts (Miller & Huffaker, 1981; Adams, 1993; Berges & Falkowski, 1996). In contrast, caseinolytic activity in *Ulva rigida, Enteromorpha intestinalis* and *Porphyra* sp. showed a 50% decrease after being kept for 12 or 24 h at room temperature. Bushnell *et al*. (1993) recorded a complete loss of activity for a partially purified endopeptidase from pea chloroplasts. They suggested that the endopeptidase itself was degraded by a serine protease present in the extract, since activity levels recovered after addition of an inhibitor of serine proteases (AEBSF). Clearly, there is wide scope for interactions between proteases in crude extracts. In some of the brown algal species, transient (*Fucus vesiculosus*) or continuous (*Pelvetia canaliculata*) increases in protease activities over time were observed.

Our results for BSA degradation suggest that the breakdown of proteins could be quite rapid. In vacuoles of *Chara corallina,* complete degradation of BSA (10 μg in 50 μl vacuolar volume) has been observed in 1 day (Moriyasu, 1995). This is only a slightly higher rate than we observed although it is difficult to make accurate comparisons since protein content was not determined for *C. corallina*. The fast degradation of BSA and the apparent lack of large breakdown products suggest that proteasomal degradation pathways exist within macroalgal cells. Coupled with the very high activities of proteases found for macroalgae in the present study, it appears that the turnover rate of proteins could be relatively high. Testing this hypothesis would require labelling studies or use of endogenous proteins in degradation experiments.

Our results suggest that proteases from macroalgae are easily measurable and highly variable. The source of this variation is not yet clear, but may be due to responses to different environmental conditions. The next step will be to understand what proteases in macroalgae are responding to, using controlled laboratory experiments and seasonal monitoring. In terms of their characteristics, we have documented that macroalgal proteases are rather different from those found in other organisms. It has long been appreciated that seaweed extracts have both agricultural and therapeutic value (e.g. Beckett *et al*., 1994; van Netten *et al*., 2000), and more recently work has begun to examine the nature of the active compounds (e.g. Nishino *et al*., 1991; Depix *et al*., 1998). Given the novel properties of macroalgal proteases and (by implication) their associated inhibitors, examining their properties may be particularly useful in applied research.

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