Characterization of a VLK-protease from *Symbiodinium* sp. strain KB8

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Abstract. A cysteine protease was characterized from *Symbiodinium* sp. strain KB8 isolated from *Cassiopea ornata*. The protease was purified 36-fold with a yield of 1.8%, by four chromatographic steps using DE-52, DEAE-Toyopearl, MonoQ, and Superdex 200 HR. Among the six substrates tested, the enzyme showed highest activity toward *t*-butyloxycarbonyl-Val-Leu-Lys-4-methylcoumaryl-7-amide (Boc-VLK-MCA), and Z-LLE-MCA (approximately 60% of Boc-VLK-MCA). Other substrates were hydrolyzed at lower rates (less than 25%), indicating that this enzyme uses substrates specific for Boc-VLK-MCA. This enzyme activity was almost completely inhibited by the cysteine protease inhibitors, leupeptin and E64, as well as moderately inhibited by serine protease inhibitors. These results suggest that this enzyme is not metallo and aspartic protease, but likely belongs to cysteine protease. pH optimum of the enzyme was estimated to be pH 4.5 by the analysis in the pH range from 3 to 6. From the triplicate data at six concentrations of the substrate from 0 to 200 μmol, the K_m and V_{max} values were calculated to be 140 μM and 79 μM min^{-1}, respectively, for Boc-VLK-MCA. As far as we know, this is the first report on the isolation and characterization of a protease from *Symbiodinium.*

Key words: Cysteine protease, *Symbiodinium*, *Cassiopea ornata*, Dinoflagellate, Protease purification.

Introduction

Proteases are a widely distributed class of enzymes that function in major physiological and developmental processes such as protein turnover, enzyme modification, regulation of gene expression, and nutrition. These processes are essential and ubiquitous in all organisms and are tightly regulated to ensure proper activation in the required cell at the appropriate moment. One of the most important roles of proteases is the removal of misfolded proteins. Abnormal proteins are continuously produced by a variety of mechanisms including mutation, biosynthesis errors, spontaneous denaturation, free-radical-induced damage, environmental perturbation and disease (Viestra 1993). Protein degradation is accomplished by a number of complex proteolytic pathways (Viestra 1996). There have been many reports on biochemical characterization of proteases involved in these proteolytic pathways.

Recently, plant genome sequencing has revealed more information on genes encoding proteases in plants (Majeran et al. 2000; Rüfenacht and Boschetti 2000). However, in algae, proteases have not been well characterized, particularly at the protein level (Usui et al. 2007). Indeed, there is no information on the biochemical characterization of protease in dinoflagellates, to complement the several physiological studies of proteases. Proteolytic activity of photosynthetic dinoflagellates was measured using the artificial substrate for leucine aminopeptidase (LAP) (Stoecker and Gustafson 2003). LAP associated with the cell surface was found in axenic and xenic species and this enzyme may play a role in nutrition of mixotrophic dinoflagellates by providing amino acids for assimilation. It is reported that the induction and excretion of specific extracellular protease activity, leupeptin-sensitive thiol protease, in ageing cultures of *Peridinium gatunense* (Vardi et al. 2007). More recently, the results of inhibitor studies experimentally demonstrate that a caspase-induced apoptotic pathway initiated by the production of reactive oxygen species plays a key role in symbiosis dysfunction, bleaching and subsequent death in several coral species symbiotic with *Symbiodinium* spp. (Tchernov et al. 2011).

Pilot studies in *Symbiodinium* found the presence of protease activities. We were interested, therefore, in examining these putative proteases in more detail. Here we report the purification and characterization of a VLK-protease from *Symbiodinium* sp. strain KB8 isolated from the jellyfish *Cassiopea ornata*. We show that this enzyme was more sensitive to cysteine protease inhibitors than serine protease inhibitors,

suggesting that it probably belongs to the cysteine protease group of enzymes.

Materials and Methods

Algae and culture

Symbiodinium sp. strain KB8 isolated from a jellyfish *C. ornata* was obtained from Dr. M. Hidaka (University of the Ryukyus) and used in this study. The zooxanthellae were cultured in f/2 medium (Guillard and Ryther 1962) at 24ºC in the light for 21 days. To increase in growth rate, the algal cultures were continuously illuminated by fluorescent lamps (daylight) at an irradiation of 16.2 µmol photon $m^{-2}s^{-1}$.

Protease assay

Protease activity was assayed by cleavage of a synthetic fluorogenic peptide, Boc-VLK-MCA as described previously (Tajima et al. 2011; Nakamura et al. 2011). The reaction mixture consisted of 50µl of 50 mM succinate-borate (SB) buffer (pH 4.0), 10 µl of enzyme solution and 1µl of 10 mM Boc-VLK-MCA dissolved in dimethyl sulfoxide (DMSO), and distilled water in a total volume of 100 µl. The reaction was started by adding 10 µl of enzyme solution and incubated for 30 min at 37ºC. After incubation, the reaction was stopped by adding 2 ml of 1% (w/v) sodium dodecyl sulfate (SDS) in 100 mM borate-NaOH (pH 9.0), and the fluorescence was measured with a Hitachi fluorescence spectrophotometer, model F-2500 (Tokyo, Japan). The hydrolysis was monitored by fluorescence emission at 460 nm with excitation at 360 nm. The activity was expressed as the amount of 7-amino-4-methylcoumarin (AMC) released from the fluorogenic peptide of 1 mg protein per h at 37ºC. AMC concentrations were estimated by its fluorescence intensity using the authentic AMC.

Purification of VLK-protease

All procedures were carried out at 4ºC unless otherwise noted. The algal cells were suspended with 20 mM tris (hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 8.0), and disrupted by ultrasonication at an output of 140-W. Unbroken cells and large debris were removed by centrifugation at 20,000 × g for 30 min at 4ºC. The supernatant was filtered through a Miracloth (Calbiochem, Darmstadt, Germany). The resulting clear supernatant was diluted with 2 volumes of 20 mM Tris-HCl buffer (pH 8.0) and applied to a column (1.6 × 4.5 cm) of DE52 (Whatman, Kent, England) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). Unbound proteins were washed with 20 mM Tris-HCl buffer (pH 8.0), and the bound proteins were eluted with the same buffer containing a linear gradient of NaCl from 0 to 0.4 M. A typical elution profile is shown in Fig. 1. The fractions with high protease activity (0.2 M NaCl) were collected and diluted with 2 volumes of 20 mM Tris-HCl buffer (pH 8.0), and applied to a column (1 × 3 cm) of DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with the same buffer. The column was washed and eluted in the same manner as described above. The fractions with high activity (0.15 M NaCl) were collected and dialyzed with a Centriplus YM-30 (Millipore, Bedford, MA). The sample was diluted with 2 volumes of 20 mM Tris-HCl buffer (pH 8.0), and applied to a column (0.5 × 5 cm) of Mono Q equilibrated with the same buffer using ÄKTA FPLC system (GE Healthcare, Tokyo, Japan). Unbound proteins were washed with 20 mM Tris-HCl buffer (pH 8.0), and the bound proteins were eluted with the same buffer containing a linear gradient of NaCl from 0 to 0.5 M. The fractions with high activity (0.2 M NaCl) were collected, concentrated and charged to Superdex 200 HR 16/60 equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl. The column was eluted with the same buffer and the fraction with high activity was collected and used for characterization.

Molecular weight determination

Molecular weight was determined by gel filtration with a Superdex 200 HR 10/30 using ÄKTA FPLC system. The column was equilibrated and eluted with 20 mM. Tris-HCl (pH 8.0) containing 0.15 M NaCl at a flow rate of 0.5 ml per min. Fractions (0.3 ml) were collected. The column was calibrated with the following molecular weight markers: blue dextran (M_r 2,000 kDa), thyroglobulin (M_r 669 kDa), β-amylase (M_r 200 kDa), alcohol dehydrogenase (M_r 150 kDa), bovine serum albumin (BSA) (M_r 66 kDa), carbonic anhydrase (M_r 29 kDa), and cytochrome c (M_r 12.4 kDa).

Electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970) using 10% (w/v) polyacrylamide gel under reducing conditions. Heat treatment was performed at 95ºC for 3 min. Gels were fixed and stained in aqueous methanol (25%, v/v) containing acetic acid (7.5%, v/v) in 0.25% (w/v) Coomassie brilliant blue R-250 or in aqueous silver nitrate (0.1%, w/v). Molecular markers used were SDS-PAGE molecular weight standards, broad range (Bio-Rad, Tokyo, Japan).

Protein determination

Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with BSA as a standard. For column chromatographic

fractions, protein was assayed spectrophotometrically by measuring at 280 nm at room temperature.

Results and Discussion

The enzyme was purified through four steps of successive chromatography using DE52, DEAE-Toyopearl 650M, MonoQ, and Superdex 200 HR gel filtration. A typical elution profile of DE52 chromatography is presented in Fig. 1. At the MonoQ step, purification of the enzyme typically led to a >50-fold increase in specific degrading activity as estimated by hydrolysis of Boc-VLK-MCA, and the overall yield was 10%. However, further purification by Superdex 200 gel filtration led to a decrease in activity. This is likely due to a loss of the activity by self digestion. A summary of the typical purification is shown in Table 1.

The molecular weight of the protease was determined by Superdex 200 HR gel filtration with an ÄKTA FPLC system. The molecular weight of the protease was calculated to be 29.9 kDa. This value is almost the same molecular weight of the protein band determined by SDS-PAGE of 29.2 kDa (data not shown), suggesting that this enzyme is likely a monomer.

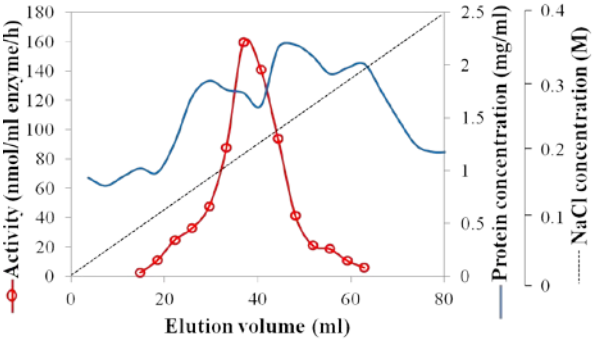

Figure 1: A typical elution profile of DE52 anion-exchange chromatography. Crude extract was applied to a column. Proteins were eluted with 20 mM Tris-HCl buffer (pH 8.0) containing a linear gradient of NaCl from 0 to 0.4 M. Solid line, absorbance at 280 nm as an amount of proteins; circle, activity of VLK-protease; dotted line, NaCl concentration.

The pH optimum of the purified enzyme was examined with 50 mM succinate-borate and MES-HEPES-Tricine buffers in the pH range from 3.0 to 9.0. The enzyme showed an asymmetrical curve giving a single peak of activity at pH 4.0-5.0 (Fig. 2). The optimum temperature of the purified enzyme was tested in the temperature range from 10 to 60ºC. This enzyme showed an optimum temperature of 40ºC as shown in Fig. 3. The temperature at which the activity of the enzyme was decreased by half, after a 10 min incubation at that temperature was approximately 40ºC (Fig. 4).

The substrate specificity of the enzyme was examined by fluorogenic substrates under the assay conditions described above (Fig. 5). Among the six substrates tested, the enzyme showed highest activity toward Boc-VLK-MCA, and also toward Z-LLE-MCA (the substrate for proteasome and V8 protease-like). Other substrates, however, were hydrolyzed at lower rates (less than 25%), indicating that this enzyme uses substrates specific for Boc-VLK-MCA.

Kinetic analysis was applied to the purified enzyme to elucidate the action mechanism. A substrate-velocity plot was measured from triplicate data at six concentrations of the substrate from 0 to 200 µM. The K_m and V_{max} values were estimated to be 140 µM and 79µM min^{-1}, respectively, for Boc-VLK-MCA.

The effects of inhibitors on the enzyme activity were examined as shown in Table 2. This enzyme activity was almost completely inhibited by the cysteine protease inhibitor, leupeptin and E64, as well as moderately inhibited by serine protease inhibitors. Pepstatin and EDTA had slightly inhibitory effects. These results suggest that this enzyme is not metallo and aspartic protease, but likely belongs to cysteine protease.

Table 1: Summary of purification of VLK-protease from *Symbiodinium* sp. strain KB8. Crude extract was obtained from 10.7 g (fresh weight) of algal cells. The enzyme activity was assayed as described in the text. The activity corresponds to the amount of hydrolyzed Boc-VLK-MCA.

Purification step	Total protein (mg)	Total activity ($nmol \cdot h^{-1}$)	Specific activity ($nmol \cdot h^{-1} \cdot mg\ protein^{-1}$)	Purification (-fold)	Yield (%)
Crude extract	1113	1774	1.5	1.0	100
DE52	125	2582	20.6	12.9	145
DEAE-Toyopearl 650M	24	722	30.2	18.9	40
Mono Q 5/50	2.2	183	83.1	52.1	10
Superdex 200 HR	0.55	31	57.6	36.1	1.8

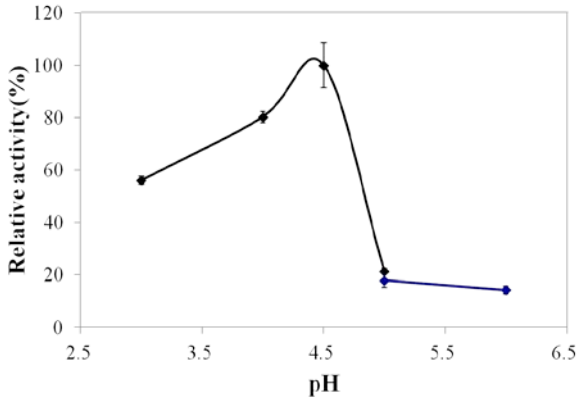

Figure 2: pH optimum of VLK-protease from *Symbiodinium* sp. strain KB8. The enzyme assay was performed with 50 mM succinate-borate buffer (pH 3 - 5) and 50 mM MES-HEPES-Tricine buffer (pH 5 - 6) at the indicated pH value from 3 to 6, as described in the text. n = 3 ± SE.

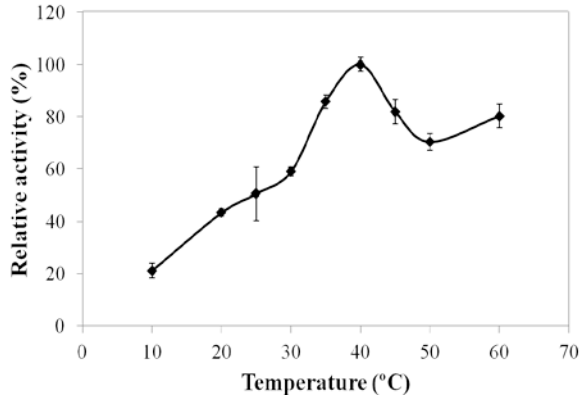

Figure 3: Optimum temperature of VLK-protease from *Symbiodinium* sp. strain KB8. The enzyme assay was tested in the temperature range from 10 to 60ºC, as described in the text. n = 3 ± SE.

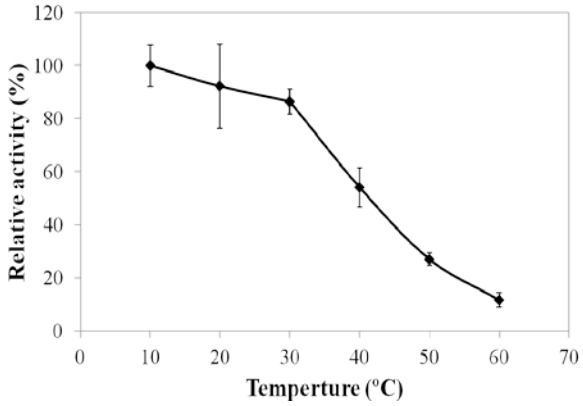

Figure 4: Thermal stability of VLK-protease from *Symbiodinium* sp. strain KB8. The enzyme assay was examined at temperatures from 10 to 60ºC. Residual activity of the enzyme was assayed in the standard conditions after incubation at the indicated temperatures for10 min in the absence of substrate. n = 3 ± SE.

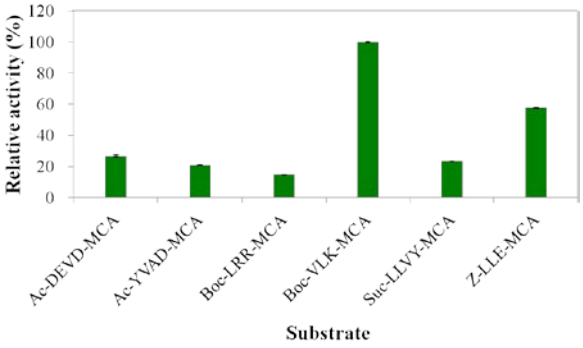

Figure 5: The substrate specificity of the enzyme was examined by six fluorogenic substrates under the assay conditions described in the text. n = 3 ± SE.

Table 2: Effects of inhibitors on the VLK-protease from *Symbiodinium* sp. strain KB8. The results are averages of three experiments, and SE is shown.

Inhibitor	Preferred Protease Target	Concentration (mM)	Relative activity (%)
Control		-	100 ± 1.3
PMSF	Ser	1	35.1 ± 2.6
		0.1	61.3 ± 4.4
Leupeptin	Ser and Cys	1	3.0 ± 0.9
		0.1	2.8 ± 0.2
		0.01	2.0 ± 1.0
TPCK	Ser	1	3.0 ± 1.1
		0.1	6.0 ± 0.2
		0.01	17.7 ± 2.4
E-64	Cys	0.1	2.8 ± 0.1
		0.01	2.8 ± 0.2
		0.001	2.0 ± 1.1
NEM	Cys	1	57.3 ± 3.1
		0.1	76.1 ± 3.4
Antipain	Cys	1	5.7 ± 0.3
		0.1	5.6 ± 0.5
		0.01	9.0 ± 0.4
Pepstatin	Asparate	0.1	63.1 ± 4.4
EDTA	Metallo	1	67.8 ± 8.9
		0.1	63.6 ± 7.2
EGTA	Metallo	1	88.2 ± 3.1
		0.1	85.6 ± 5.7

In preliminary experiments, a similar cysteine protease with VLK hydrolyzing activity was purified from another *Symbiodinium* sp., strain Y106, isolated from a mantle lobe of *Tridacna crocea* by three steps of chromatography (data not shown). The biochemical and enzymatic properties were similar, including an acidic pH optimum, however, the molecular weight was approximately 60 kDa.

Conclusion

In this study, we purified a cysteine protease with Boc-VLK-MCA hydrolyzing activity from the

Symbiodinium sp. strain KB8 isolated from *C. ornata* (Table 1, Fig. 1). The purified enzyme utilized Boc-VLK-MCA, which is a substrate for plasmin and calpain, and also to a slightly lesser degree, Z-LLE-MCA, a substrate for proteasome and V8 protease-like (Fig. 5). In addition, the purified enzyme was completely inhibited by the cysteine protease inhibitor E64 and slightly by the serine protease inhibitor PMSF (Table 2), as in the case of other cysteine proteases. As far as we know, this is the first report on the isolation and characterization of a protease from *Symbiodinium*.

Acknowledgements

We thank to Ms. Keiko Wakamatsu for technical assistance. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 19255002) (to YS) and the Japan Science and Technology Agency (to KA).

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