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A novel calcium-activated protease in *Chara corallina*

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A protease that is activated by Ca^{2+} has been discovered in the alga *Chara corallina*. The activity of the protease was measured using the substrates succinyl-bovine serum albumin (BSA) and [^{14}C]methyl-BSA. In the absence of $CaCl_2$, the optimal activity of the homogenate was found to be in the acidic range. However, in the presence of $CaCl_2$, the activity in the alkaline range increased. The concentration of $CaCl_2$ that gave half-maximal activation of the enzyme was 100–500 μM at pH 7.5. The effect of Ca^{2+} could be partially replaced by Sr^{2+}, but not by Mg^{2+}. The molecular mass of the Ca^{2+}-activated protease was estimated by gel permeation chromatography to be about 40 kD. Cell fractionation, using the vacuolar perfusion technique, showed that the enzyme is localized in the cytoplasm. The Ca^{2+}-activation at pH 7.5 was inhibited by p-chloromercuribenzoic acid. The partially purified enzyme had negligible activity in the absence of Ca^{2+}, showing that this enzyme has an absolute requirement for Ca^{2+} for its activity. This protease could be separated into two protein isoforms by activity-staining on polyacrylamide gels containing gelatin as the substrate.

Key words: calcium, calpain, *Chara*, protease, proteinase

Introduction

Intracellular proteases are involved in various cellular activities, including protein turnover, the elimination of abnormal proteins, and the modulation of proteins in cellular metabolic and signal transduction pathways (Spremulli, 2000). In mammalian cells, two major proteases exist in the cytoplasm; one is the proteasome and the other is calpain. The 20S proteasome is a ubiquitous protease that is distributed in all eukaryotic cells. It associates with a 19S adapter protein to form the 26S proteasome, which contributes to protein turnover and other cell-specific functions (Coux *et al*., 1996). Calpains are proteases that are activated by Ca^{2+} (Suzuki, 1987; Pontremoli & Melloni, 1986) and are thought to occur in all vertebrates and in some invertebrates. In *in vitro* experiments, calpains tend to hydrolyze proteins that are involved in signal transduction pathways, such as protein kinases and receptor proteins (Wang *et al*., 1989), suggesting that they are involved in the regulation of signalling rather than in generalized protein turnover.

In comparison to mammalian cells, knowledge of protease structure and function in plant cells is limited. It has been reported that proteasomes are present in the cytoplasm of plant cells and that the properties of the 20S plant proteasome are similar to those of the mammalian counterpart (Vierstra, 1993). However, proteases that correspond to conventional animal calpains have not been previously reported in plant cells (Wolfe *et al*., 1989) although calpain-like proteases exist widely in plant and yeast cells as well as in animal cells (Ono *et al*., 1999).

We have investigated the mechanism of intracellular protein degradation in plant cells using the giant alga *Chara corallina*. In the course of these studies we found a protease that is activated by Ca^{2+} and hypothesized that it may be calpain (Moriyasu & Tazawa, 1987). In the present study, however, we have characterized it and found that this protease, while being Ca^{2+}-activated, is distinct from calpains.

Materials and methods

Plant material

Chara corallina Klein ex. Wild (= *Chara australis* R. Brown) was cultured as previously described (Moriyasu, 1995). The whole body of the plant, including the nodal, internodal and leaf cells was typically used to prepare the enzyme. However, when the vacuolar/extravacuolar distribution of the enzyme activity was examined, only internodal cells were used. To remove the vacuolar sap from an internodal cell, the vacuole was perfused with an

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artificial vacuolar sap consisting of 80 mM KCl, 30 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (Moriyasu *et al.*, 1984). Almost all the vacuolar proteins including vacuolar marker enzymes such as carboxypeptidase and acid phosphatase can be removed by this procedure (Doi *et al.*, 1975; Moriyasu & Tazawa, 1986).

Chemicals

Casein was purchased from Merck, bovine serum albumin (BSA) from Armour, and p-chloromercuribenzoic acid (pCMB) from Wako Chemical Industries, Ltd. (Osaka, Japan). Iodoacetic acid, phenylmethylsulphonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) were purchased from Sigma. Leupeptin, E-64 and pepstatin A were obtained from the Peptide Institute Inc. (Minoh-shi, Osaka, Japan), DEAE-Toyopearl and Toyopearl HW-55F from Tosoh Corp. (Japan) and [^{14}C]formaldehyde from ICN Radiochemicals.

BSA was succinylated according to Mellgren *et al.* (1979). Briefly, BSA (1 g) was dissolved in 33 ml of 6 M urea containing 0.5 mM dithiothreitol. Succinic anhydride (5 g) was added to this solution while keeping the pH at 7–8 by adding NaOH. The resulting solution was dialyzed against water.

[^{14}C]Methyl-BSA was prepared according to Rice & Means (1971). BSA (0.1 mg) was dissolved in 0.1 ml of 0.2 M borate-Na (pH 9.0) and mixed with [^{14}C] formaldehyde (4 mCi). To this solution, 2 μl of sodium borohydride (5 mg ml^{-1}) was added four times at 30 s intervals. After 1 min, a further 10 μl of sodium borohydride solution was added. The resulting solution was dialyzed against water.

Preparation of the enzyme solution

Either the whole algal body or the internodal cells were homogenized with a mortar and pestle in 0.1 M Tris-Cl (pH 7.5 at 4°C) containing 28 mM 2-mercaptoethanol (2-ME). One ml of the buffer was used for 1 g of fresh material. The homogenate was filtered through 2 layers of gauze and centrifuged at 20 000 *g* for 10 min. The supernatant was passed through a column of Sephadex G-25 (6.5 cm diameter, 28 cm long) equilibrated with 50 mM Hepes-Na (pH 7.5) containing 2.8 mM 2-ME except when pH dependency of the enzyme was examined, and the supernatant was passed through the same column equilibrated with 5 mM Hepes-Na (pH 7.5) containing 2.8 mM 2-ME. The desalted protein fraction was used directly for the enzyme assay or for electrophoresis.

When the molecular mass of the enzyme was examined, the supernatant following centrifugation was passed through a column of Sephadex G-25 equilibrated with buffer A (50 mM Tris-Cl (pH 7.5) containing 5.6 mM 2-ME) and thereafter the protein fractions were concentrated by ammonium sulphate precipitation. Ammonium sulphate was added to 70% saturation, and precipitated proteins were collected by centrifugation at 19 000 *g* for 30 min. The precipitate was dissolved in buffer A and the resulting solution was passed through a small column of Sephadex G-25 (PD-10, Amersham Pharmacia Biotech) equilibrated with buffer A. The protein fraction was then applied to a column of Toyopearl HW-55F (2.5 cm diameter, 77 cm long) equilibrated with buffer A containing 0.1 M NaCl. Thyrogloblin (mass 669 000), apoferritin (443 000), β-amylase (200 000), BSA (66 000), carbonic anhydrase (29 000) and cytochrome *c* (12 400) were used as the molecular mass standards.

Measurement of protease activity

When succinyl-bovine serum albumin (succinyl-BSA) or BSA was used as a substrate, protease activity was measured as an increase in trichloroacetic acid (TCA)-soluble, ninhydrin-positive substances. When the crude enzyme solution was immediately used for enzyme assays, 1 ml of the reaction mixture was prepared by mixing 0.5 ml of crude enzyme solution in 50 mM Hepes-Na (pH 7.5) containing 2.8 mM 2-ME, 25 μl of 0.1 M $CaCl_2$ or 4 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA), 0.3 ml of 1% (w/v) succinyl-BSA and 0.175 ml of water. Thus the final concentrations of $CaCl_2$ and EGTA were 2.5 mM and 0.1 mM, respectively. The effects of Ca^{2+}, Sr^{2+} and Mg^{2+} on the enzyme activity were examined by using various concentrations of $CaCl_2$, $SrCl_2$ and $MgCl_2$ instead of 0.1 M $CaCl_2$. When the pH dependency of the enzyme was investigated, 0.5 ml of the crude enzyme was replaced with 0.25 ml of crude enzyme in 5 mM Hepes-Na (pH 7.5) containing 2.8 mM 2-ME and 0.25 ml of 50 mM Mes-Na from 5.5 to 6.5, with 50 mM Hepes-Na from 6.5 to 8.0 or 50 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid (Taps)-Na from 8.0 to 9.0. Reaction mixtures were shaken at 37°C. After various time intervals, 0.2 ml of 60% (w/v) TCA was added to the reaction mixture to stop the reaction. After centrifugation at 2000 *g* for 20 min, the supernatant was subjected to the ninhydrin reaction (Moore & Stein, 1954). One unit of succinyl-BSA degrading activity was defined as the production of TCA-soluble substances equivalent to 1 μmole of L-alanine by ninhydrin reaction per 1 h under the assay condition.

When the protease was assayed following column chromatography, 0.5 ml of reaction mixture was prepared by mixing 0.25 ml of 0.1 M Hepes-Na (pH 7.5), 12.5 μl of 0.1 M $CaCl_2$ or 4 mM EGTA, 0.125 ml of 1% (w/v) succinyl-BSA, 50 μl of enzyme solution and 62.5 μl of water. After incubation at 37°C for 2 h, the reaction was stopped by adding 0.5 ml of 10% (w/v) TCA. The mixtures were centrifuged at 17 000 *g* for 10 min and 0.6 ml of the supernatant was processed to ninhydrin reaction. One unit of protease activity was defined as an increase in A_{570} for 2 h under the assay condition.

When [^{14}C]methyl-BSA was used as a substrate, the enzyme activity was measured as a time-dependent increase in TCA-soluble radioactivity. Usually, 200 μl of the reaction mixture contained 25 mM Hepes-Na (pH 7.5), 1.4 mM 2-ME, [^{14}C]methyl-BSA (15 000 cpm), enzyme solution, and 0.1 mM EGTA or various concentrations of $CaCl_2$. The reaction mixture was incubated at 30°C. After various time intervals, 25 μl of 10% (w/v) BSA and 575 μl of 10% (w/v) TCA were added to the reaction mixture. The mixture was kept at 4°C for 60 min, and was then centrifuged at 17 000 *g* for 10 min. The supernatant (0.4 ml) was mixed with 5 ml of

scintillation cocktail to measure radioactivity. When the pH dependency was investigated, the pH was adjusted in the same way as for measuring succinyl-BSA degrading activity. One unit of [^{14}C]methyl-BSA degrading activity was defined as the production of TCA-soluble radioactivity equivalent to 1% of [^{14}C]methyl-BSA added in the assay tube per mg protein per 1 h under the assay condition.

Electrophoresis and detection of protease on the gel

Polyacrylamide gel electrophoresis (T = 7.5%) was performed according to Davis (1964) with some modifications using gels (85 mm wide, 60 mm long, 1 mm thick) containing 0.4% (w/v) gelatin. After electrophoresis, gels were preincubated for 1 h at room temperature in 50 mM Tris-Cl (pH 7.5) and 2.8 mM 2-ME containing either 0.1 mM EGTA or various concentrations of $CaCl_2$ (1 μM to 2.5 mM). During the preincubation, gels were constantly shaken and the buffers were changed three times. After preincubation, the gels were incubated at 37°C for 12 h without shaking. Thereafter they were stained with Coomassie Brilliant Blue R-250.

Protein assay

Protein was assayed according to Lowry *et al.* (1951) as modified by Bensadoun & Weinstein (1976) using BSA as a standard.

Partial purification of the enzyme

Whole plants (190 g fresh weight) were washed with water and blotted on filter paper. The plants were frozen in liquid nitrogen, and then pulverized using a mortar and pestle. The powder was further homogenized in a mortar and pestle with 190 ml of 0.1 M Tris-Cl (pH 7.5) containing 14 mM 2-ME. The homogenate was centrifuged at 19 000 *g* for 30 min. The supernatant (260 ml) was passed through a column of Sephadex G-25 (void volume = 350 ml) equilibrated with buffer A. The fraction containing cellular proteins (300 ml) was applied to a column of DEAE-Toyopearl (bed volume = 85 ml) equilibrated with buffer A. After the column was washed with buffer A, proteins were eluted with a linear gradient (0–0.4 M) of NaCl in 480 ml of buffer A. The eluate was fractionated into 6 ml aliquots. Those containing Ca^{2+}-activated protease (Nos. 18–34) were combined and applied to a second column of DEAE-Toyopearl. After washing with buffer A, the Ca^{2+}-activated protease was eluted with a linear gradient (0–0.2 M) of NaCl in 160 ml of buffer A. The eluate was fractionated and the fractions containing Ca^{2+}-activated protease (Nos. 14–21) were combined. Ammonium sulphate was added to the combined fractions so that the concentration of ammonium sulphate reached 70% saturation. The Ca^{2+}-activated protease was precipitated by centrifugation at 31 000 *g* for 20 min. The precipitate was dissolved in buffer A to a final volume of 3 ml. The resulting protein solution was passed through a column (2.5 cm diameter, 77 cm long) of Toyopearl HW-55F equilibrated with buffer A containing 0.1 M NaCl. The eluate was fractionated into 1.5 ml fractions and those with Ca^{2+}-activated protease (#40–52, total 19 ml) were combined. The combined fractions were used for the characterization of the partially-purified Ca^{2+}-activated protease.

Results

Presence of Ca^{2+}-activated protease activity in the homogenate of Chara

Protease activity was measured using the ninhydrin reaction to quantify the degradation products from succinyl-BSA. When using succinyl-BSA, the ninhydrin-positive amino groups in BSA are eliminated by the succinylation reaction. Although we used succinyl-BSA in order to minimize the background activity, we found by chance that the degradation of succinyl-BSA was activated by Ca^{2+}, whereas the degradation of BSA was not. In order to determine if the activation of the protease by Ca^{2+} was an artifact dependent on the use of succinyl-BSA as a substrate and the ninhydrin technique, we measured the protease activity using a radioactive substrate, [^{14}C]methyl-BSA, and monitoring the radioactivity of the degradation products. The protease in the homogenate was activated by Ca^{2+} (Fig. 1). In the presence of 2 mM $CaCl_2$, the TCA-soluble radioactivity increased almost linearly with time for at least 90 min. In contrast, there was no [^{14}C]methyl-BSA degrading activity in the presence of 0.1 mM EGTA, which chelates calcium. Together with our previous observations (Moriyasu & Tazawa, 1987),

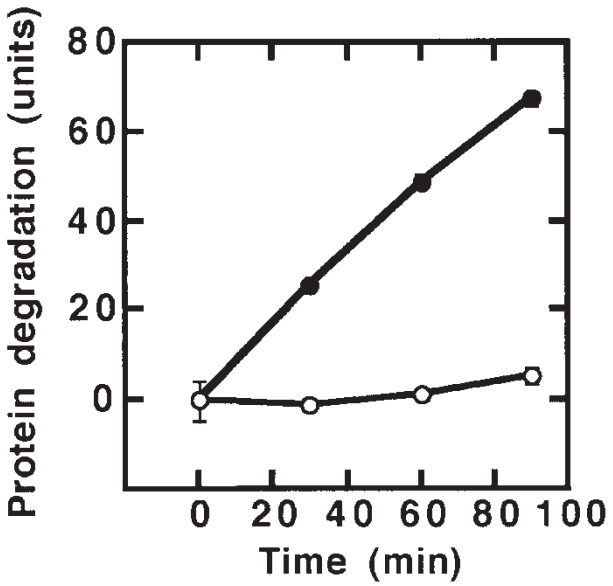

Fig. 1. Effect of Ca^{2+} on degradation of [^{14}C]methyl-BSA in homogenates of *Chara corallina*. Protein degradation was measured using [^{14}C]methyl-BSA in the presence of 2.0 mM $CaCl_2$ (closed circles) or 0.1 mM EGTA (open circles). Results are means ± SD (n = 3).

these results show that the crude homogenate of *Chara* plants exhibits a Ca^{2+}-activated protease activity, which can be observed using two independent assays.

Dependence of protease activity on pH

To characterize the Ca^{2+}-activated protease activity, we examined the dependence of protease activity on the pH of the reaction mixture in the presence and absence of Ca^{2+} (Fig. 2). Without Ca^{2+} (Fig. 2A, open circles), the optimal pH of the succinyl-BSA degrading activity was about 6.0, which is consistent with the data in which the protease activity was measured using casein as a substrate (Moriyasu & Tazawa, 1986). The majority of the activity in the acidic range is likely to result from vacuolar proteases. However, when 2.5 mM $CaCl_2$ was added into the reaction mixture (Fig. 2A, closed circles), the optimal pH shifted in the alkaline direction and the maximal activity increased about three times. Similar results were obtained using [^{14}C]methyl-BSA (Fig. 2B). The degradation of [^{14}C]methyl-BSA in the absence of Ca^{2+} was high in the acidic range, but very low in the alkaline region, with an optimal pH between 5.5 and 6.0. In the presence of 2.0 mM $CaCl_2$, however, the optimal pH shifted to the alkaline region, and the maximal activity was amplified by 2–3 times. These results suggest that the Ca^{2+}-activated protease works optimally in the neutral to alkaline region. The difference in the pH-dependence observed in the crude homogenate using these two methods may be caused by exopeptidases that are active in an acidic range. These exopeptidases further hydrolyze the degradation products and, as a result, the amount of TCA-soluble, ninhydrin-positive substances is enhanced.

Activity staining of Ca^{2+}-activated protease on polyacrylamide gels

In order to determine if the Ca^{2+}-activated protease activity can be attributed to a single protease, we separated proteins from the crude homogenate by electrophoresis on polyacrylamide gels containing gelatin as a substrate and stained for protease activity (Fig. 3). Two bands showing protease activity were detected only in the presence of $CaCl_2$ (Fig. 3, arrowhead). Either there are two Ca^{2+}-activated proteases or one that is composed of two isoforms that have distinct mobilities on polyacrylamide gels. More than 100 μM Ca^{2+} (pCa 4) was needed for the activation of both protease bands and we could not detect any activity at or below 10 μM Ca^{2+} (pCa 5; Fig. 3). These results suggest that these proteins are

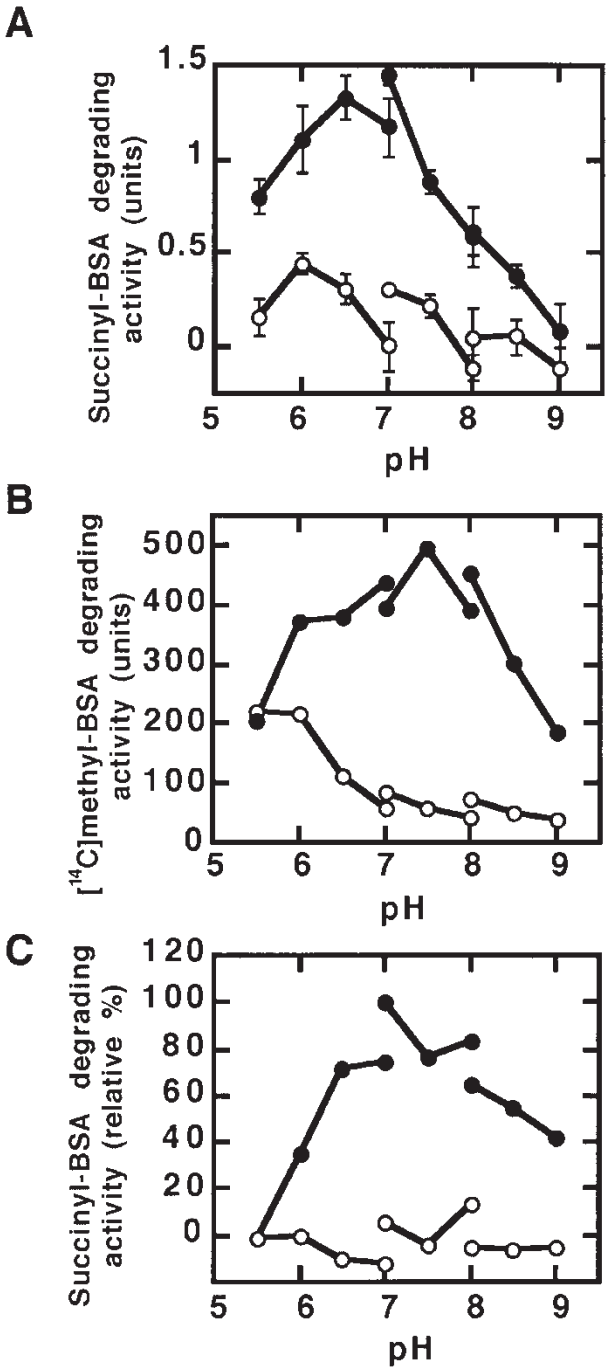

Fig. 2. Effect of pH on succinyl-BSA and [^{14}C]methyl-BSA degrading activities in *Chara corallina*. (*A*, *B*) Protease activity in crude enzyme solutions using succinyl-BSA (*A*) or [^{14}C]methyl-BSA (*B*) as substrates with 2.5 mM (*A*) or 2.0 mM (*B*) of $CaCl_2$ (closed circles) and 0.1 mM of EGTA (open circles). (*C*). pH-dependence of activity of partially purified Ca^{2+}-activated protease using succinyl-BSA as a substrate in the presence of 2.5 mM $CaCl_2$ (closed circles) or 0.1 mM EGTA (open circles). Experiments were done at least twice and similar results were obtained; in (*A*), results are means $\pm$ SD ($n = 3$); in (*B*) and (*C*), typical results are shown.

responsible for protease activity that is activated by Ca^{2+} and that the Ca^{2+}-activated protease in *Chara* needs Ca^{2+} for its activation. A Ca^{2+}

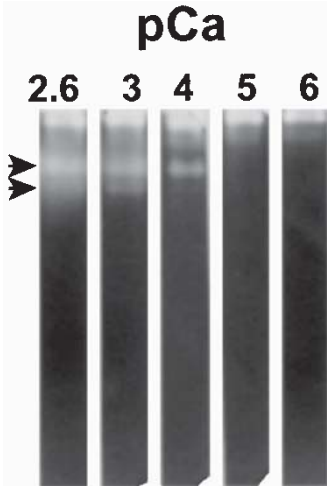

Fig. 3. Activity staining of crude protease on a non-denaturing polyacrylamide gel. Protease activity visualized on gels at pH 7.5 at different concentrations of free Ca^{2+}. Arrowheads indicate two bands of protease activity that exhibit Ca^{2+}-dependency. pCa = $-\log [Ca^{2+}]$.

insensitive protease was also detected near the top of the gels (Fig. 3).

Estimation of the molecular mass of Ca^{2+}-activated protease

To confirm whether the Ca^{2+}-activated protease visualized on polyacrylamide gels is the same as the protease that degrades succinyl-BSA in a Ca^{2+}-dependent manner, we separated proteins in the homogenate of *Chara* by gel permeation chromatography and measured protease activity in the presence and absence of Ca^{2+} in various fractions using succinyl-BSA (Fig. 4). In the presence of Ca^{2+}, the activity eluted as a single peak, but the fractions from the peak did not have any measurable protease activity against succinyl-BSA in the absence of Ca^{2+} (Fig. 4A). Both protease isoforms co-eluted through a Toyopearl HW-55F column and showed Ca^{2+}-activated gelatin-degrading activity when assayed by activity staining (Fig. 4B). This result shows that isoforms of the Ca^{2+}-activated protease on polyacrylamide gels correspond to the Ca^{2+}-activated protease that degrades succinyl-BSA. The molecular mass of the isoforms, estimated by gel permeation chromatography, was approximately 40 kD.

Localization of Ca^{2+}-activated protease in the cytoplasm of Chara *cells*

In order to localize the Ca^{2+}-activated protease, we prepared a cell homogenate from the cells whose vacuolar contents had been removed by vacuolar perfusion (Moriyasu & Tazawa, 1986). Almost all vacuolar marker enzymes such as acid phosphatase

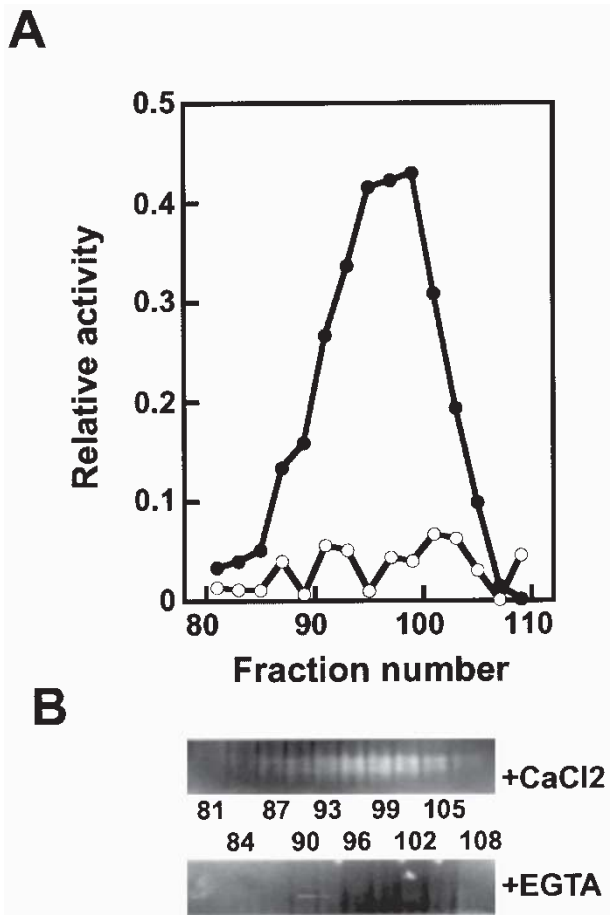

Fig. 4. Gel permeation chromatography of Ca^{2+}-activated proteases of *Chara corallina*. Crude homogenate of whole plants of *Chara corallina* was applied to the gel permeation column. (*A*) Succinyl-BSA degrading activity in each fractionated eluate in 2.5 mM $CaCl_2$ (closed circles) or 0.1 mM EGTA (open circles). (*B*) Electrophoresis of each fraction on polyacrylamide gels containing gelatin, stained for protease activity in 2.5 mM $CaCl_2$ (+$CaCl_2$) or 0.1 mM EGTA (+EGTA). Fraction numbers are shown between upper and lower panels.

and carboxypeptidase were removed by this procedure (Doi *et al.*, 1975; Moriyasu & Tazawa, 1986). Almost all of the Ca^{2+}-activated protease activity remained in the cytoplasmic fraction after vacuolar perfusion (Table 1). This result clearly shows that the Ca^{2+}-activated protease is a cytoplasmic enzyme and not a vacuolar one.

Other characterizations of Ca^{2+}-activated protease

We also examined the sensitivity of the protease activity to Ca^{2+} concentration. Succinyl-BSA degrading activity increased as the concentration of Ca^{2+} increased and was saturated at 1 mM (Fig. 5A). The concentration of Ca^{2+} needed for half-maximal activity was about 0.5 mM (Fig. 5B). However, when [^{14}C]methyl-BSA was used, the degradation activity was saturated at a lower Ca^{2+} concentration and half-maximal activity was ob-

Table 1. Vacuolar/extravacuolar distribution of the Ca^{2+}-activated protease in *Chara corallina*

	Ca^{2+}-activated protein degradation[a]	
	succinyl-BSA (μmol Ala equivalent h^{-1} g^{-1} fresh weight)	[^{14}C]methyl-BSA (% degradation h^{-1} g^{-1} fresh weight)
Whole cells	0.105 (100%)	26 (100%)
Perfused cells[b]	0.095 (90%)	34 (130%)

[a] Ca^{2+}-activated protein degradation was defined as the difference between protein degradation in the presence of 2.5 mM $CaCl_2$ (for succinyl-BSA) or 2.0 mM $CaCl_2$ (for [^{14}C]methyl-BSA) and that in the presence of 0.1 mM EGTA.[b] Vacuolar proteins were removed by vacuolar perfusion.

served at about 0.1 mM (Fig. 5C). The Ca^{2+} requirements found here are similar to the requirements found by activity staining of polyacrylamide gels (Fig. 3).

In some Ca^{2+}-sensitive reactions, Ca^{2+} can be replaced with Sr^{2+} but not with Mg^{2+} (Hepler & Wayne, 1985), and this was tested for the Ca^{2+}-activated protease. Sr^{2+} activated the protease activity, but less effectively than Ca^{2+}, and half-maximal activity was observed at 2 mM Sr^{2+} (Fig. 6A, circles). Moreover, the maximal activity in the presence of Sr^{2+} was lower than in the presence of Ca^{2+}. In contrast, Mg^{2+} did not have any significant effect on the activity (Fig. 6A, triangles). Similar results were obtained using [^{14}C]methyl-BSA as a substrate (Fig. 6B), but lower concentrations of Sr^{2+} and Ca^{2+} were needed for half-maximal activation. These results suggest that Sr^{2+} but not Mg^{2+} can partially substitute for Ca^{2+} in activating the protease of *Chara*.

Effects of several protease inhibitors on the Ca^{2+}-activated protease activity of the *Chara* extract were investigated using succinyl-BSA as a substrate. Among the inhibitors tested, only p-chloromercuribenzoic acid, a modifier of SH-group, was effective at 0.25 mM. However, inhibitors relatively specific to cysteine proteases such as E-64 (10 μM) and leupeptin (10 μM) did not have any effect. Neither the serine protease inhibitors, diisopropyl fluorophosphate (1 mM) and phenylmethylsulphonyl fluoride (1 mM), nor the aspartic protease inihibitor, pepstatin A (1 μM) had any effect.

Partial purification of Ca^{2+}-activated protease

The Ca^{2+}-activated protease was partially purified using DEAE ion-exchange and gel permeation chromatography (Fig. 7). After passage through the first DEAE ion-exchange column, the Ca^{2+}-activated protease seemed to be separated from other proteases, since the fractions exhibiting Ca^{2+}-activated protease activity did not have any protease activity in the absence of Ca^{2+} (Fig. 7A). Although the second DEAE ion-exchange column, which was based on the same principle of separation as the first, did not increase the specific activity (Fig. 7B), the last gel permeation chromatography was effective in increasing the specific activity (Fig. 7C). The increase in specific activity that occurred during the purification is summarized in Table 2.

The partially purified enzyme had protease activity from pH 6.5 to 8.5 with an optimum near pH 7 in the presence of Ca^{2+} (Fig. 2C). In the absence of Ca^{2+}, it exhibited no activity from pH 5.5 to 9.0. The partially-purified enzyme showed similar inhibitor sensitivities and Ca^{2+} requirements to the homogenate (data not shown).

Discussion

In a previous study, we investigated the contribution of vacuolar proteases to protein turnover in the cells of *Chara corallina* (Moriyasu, 1995). The results showed that vacuolar proteases do not contribute to protein turnover under normal physiological conditions. Consequently, we started to search for cytoplasmic proteases. We found two cytoplasmic proteases in *Chara*; one is the 20S proteasome (Moriyasu & Malek, unpublished) and the other is a Ca^{2+}-activated protease (Moriyasu & Tazawa, 1987).

In the present work, we have further characterized this Ca^{2+}-activated protease. Although the degree of purification attained in this study was low and further purification of the enzyme is needed to confirm some of the properties reported here, the results strongly suggest that the Ca^{2+}-activated protease of *Chara* is a novel protease that has not been reported in green plant cells thus far. The relative molecular mass of this protease is approximately 40 kD. Since the conventional calpains of mammalian cells are heterodimers consisting of an 80 kD large subunit and a 30 kD small subunit (Suzuki, 1987), we assume that the protease of *Chara* is distinct from the conventional calpains found in mammalian cells.

Ca^{2+}-activated proteases have been found and purified in several species, including the fungus

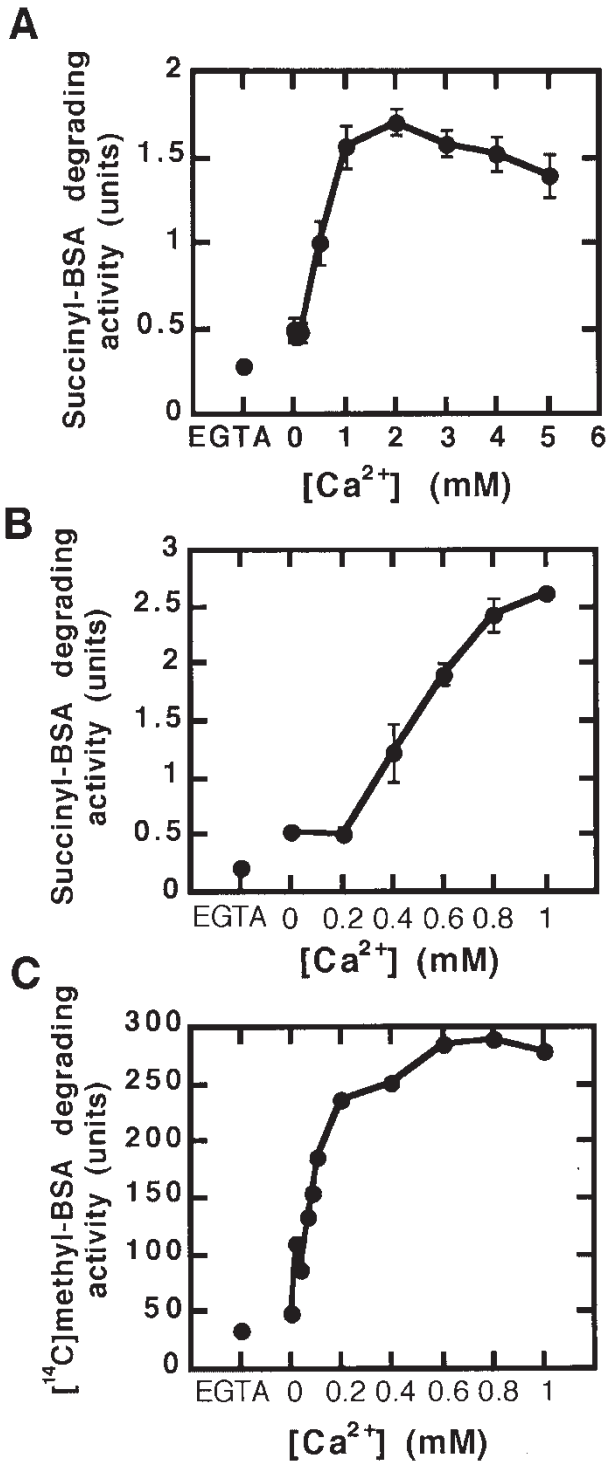

Fig. 5. Ca^{2+} dependence of protease of *Chara*. (*A*, *B*) Succinyl-BSA degrading activity in crude enzyme solution at 37°C in relation to $CaCl_2$ concentration. Results are means ± SD ($n = 3$). (*C*) [^{14}C]methyl-BSA degrading activity of crude enzyme at 30°C in relation to $CaCl_2$ concentration. 'EGTA' indicates replacement of $CaCl_2$ with 0.1 mM EGTA.

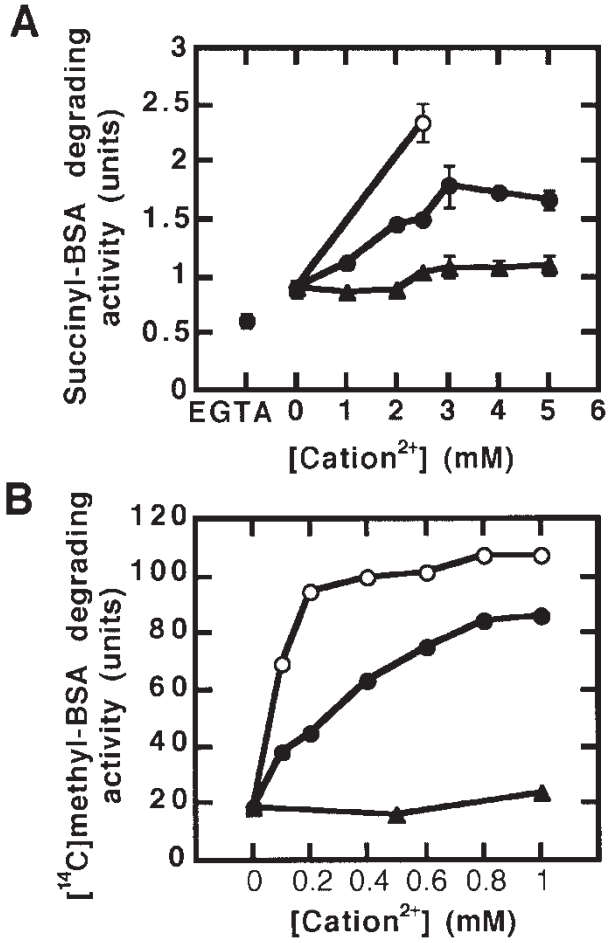

Fig. 6. Dependence of Ca^{2+}-activated protease in crude homogenate of *Chara* on Mg^{2+} and Sr^{2+}. (*A*) Succinyl-BSA degrading activity at various concentrations of $MgCl_2$ (triangles) or $SrCl_2$ (closed circles); activation by $CaCl_2$ (open circle) shown for comparison. 'EGTA' indicates replacement of $CaCl_2$ with 0.1 mM EGTA. Results are means ± SD ($n = 3$). (*B*) [^{14}C]Methyl-BSA degrading activity at 30°C at various concentrations of $MgCl_2$ (triangles) or $SrCl_2$ (closed circles); activation by $CaCl_2$ (open circles) shown for comparison.

Allomyces (Ojha & Wallace, 1988) and the cyanobacterium *Anabaena* (Lockau *et al*., 1988). A Ca^{2+}-activated protease was also detected in the higher plant *Arabidopsis* (Reddy *et al*., 1994). However, its activity was only measured in crude homogenates and consequently it is not known whether this protease, like the one from *Chara*, has an absolute requirement for Ca^{2+}. Moreover, the activation of the protease of *Arabidopsis* by Ca^{2+} was observed in a range from 1 to 5 mM, which was much higher than that for the Ca^{2+}-activated protease of *Chara* (Fig. 1 in Reddy *et al*., 1994 vs. Fig. 5 in this study).

The Ca^{2+} concentration necessary for activation of the Ca^{2+}-activated protease of *Chara* was 0.1 to 0.5 mM (Figs. 3 and 5). These concentrations are higher than the physiological Ca^{2+} concentrations in the cytoplasm, which vary between 0.1–0.4 μM in the resting state (Okazaki *et al*., 2002) and 6–40 μM during an action potential (Williamson & Ashley, 1982; Thiel *et al*., 1997). Thus, the concentration of Ca^{2+} needed for the activation of the Ca^{2+}-activated protease of *Chara* is higher

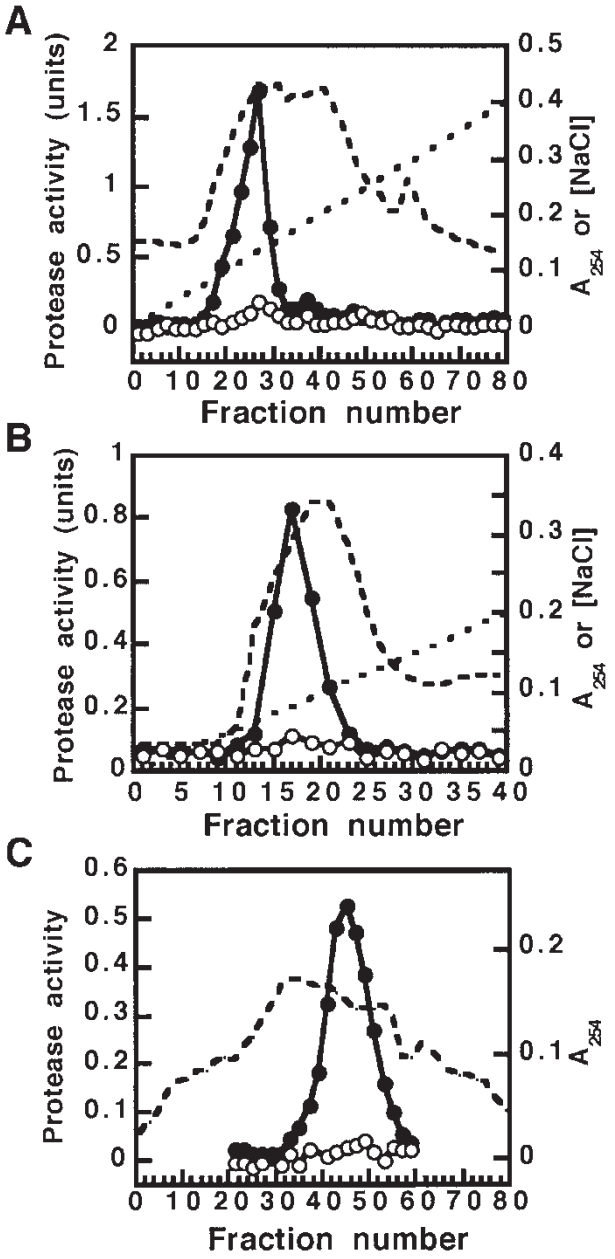

Fig. 7. Protease activity in the presence (closed circles) or absence (open circles) of 2.5 mM $CaCl_2$ of Ca^{2+}-activated protease from *Chara corallina* after different stages of partial purification. (*A*) Crude enzyme solution prepared from whole plants of *Chara corallina* was concentrated by ammonium sulphate precipitation, and applied to a DEAE-ion exchange column. (*B*) Fractions with Ca^{2+}-activated protease activity (#18–34) were combined and applied to second similar column. (*C*) Fractions with Ca^{2+}-activated protease activity (#14–21) were combined, concentrated again by ammonium sulphate precipitation and applied to a gel permeation chromatography column. Fractions with Ca^{2+}-activated protease activity (#40–52) were combined and used as partially purified enzyme solution for inhibitor assay and for examining pH-dependency (Fig. 2C). In ion exchange chromatography (*A* and *B*), proteins absorbed on the resin were eluted with a linear gradient of NaCl (dotted lines). In all chromatography, protein concentrations were monitored as A_{254} (broken lines).

than the concentration found in the cytoplasm, even at an excited state. In order for this protease to be physiologically significant, a mechanism that is able to attenuate the Ca^{2+} requirement is necessary.

Two classes of calpains in mammalian cells, m-calpain and μ-calpain, require higher concentrations of calcium (approximately 1 mM and 10 μM, respectively) *in vitro* than those encountered in the cell. It has been proposed that phosphatidylinositol reduces the calcium requirement (Fox and Saido, 1999). By analogy, it is conceivable that Ca^{2+}-sensitivity of the *Chara* enzyme may be modulated by some endogenous factors. It is also possible that the Ca^{2+}-activated protease of *Chara* is localized in some compartment of the cytoplasm whose Ca^{2+} concentration is higher than the general concentration in the cytosol.

The physiological function of the Ca^{2+}-activated protease in *Chara* cells is unknown. One of the functions of the conventional calpains of mammalian cells is thought to be the modulation of Ca^{2+} signal transduction pathways because the conventional calpains have a tendency to degrade the protein components in such pathways (Wang *et al.*, 1989). Similar Ca^{2+} signal transduction pathways also occur in plant cells (Trewavas, 2000), but modulation of these pathways by intracellular proteases has not been reported. Thus one may speculate that proteases that are regulated by Ca^{2+} are widely distributed in plant cells and may contribute to such modulation. Recently, calpain-like proteases have been found to be present in many eukaryotic cells. In such calpain-like proteases, at least the cysteine protease domain of mammalian conventional calpains is conserved (Ono *et al.*, 1999). There seems to be the only one calpain-like protease in *Arabidopsis* and corn (Lid *et al.*, 2002). In such putative proteases, the cysteine protease and C2 domains of conventional calpains are conserved, but the Ca^{2+}-binding EF-hand domain is lacking (for C2 domain, see Kopka *et al.*, 1998). A mutant of the gene for this enzyme was isolated in corn and described as the mutant *dek1* (Lid *et al.*, 2002). The phenotype of this mutant suggests that this protease functions to maintain and restrict the aleurone cell fate during seed formation.

It is conceivable that the Ca^{2+}-activated protease of *Chara* is a member of calpain-like proteases and plays some role in cellular developmental processes. It may be involved in regulating Ca^{2+}-dependent intracellular signal transduction pathways. Since the internodal cells of Charophyta are so large, we can easily inject inhibitors and/or antibodies of the enzyme into the cell to inhibit the activity of the protease. By this method, we will be able to test whether the protease is involved in

Table 2. Purification of Ca^{2+}-activated protease from *Chara corallina*.

Fraction	Volume (ml)	Enzyme activity[a]	Protein (mg)	Specific activity[a]	Purification (-fold)	Enzyme yield (%)
Crude extract	300	1720	67	26	1	100
1st DEAE-Toyopearl	79	1060	11	96	3.8	62
2nd DEAE-Toyopearl	33	261	3.3	79	3.1	15
Toyopearl HW-55F	19	128	1.0	130	5.0	7.5

[a]One unit of enzyme was the amount of enzyme required to change A_{570} by 1 under the assay conditions; specific activity = enzyme units mg^{-1} protein.

some of the reactions that are known to be modulated by Ca^{2+} in *Chara*. In the future, we will also purify the enzyme to obtain its amino acid sequence and then perform a genome-wide search for its homologues in plant cells.

We conclude that (1) *Chara* cells contain a protease that has an absolute requirement for Ca^{2+} for its activity; (2) its molecular mass is about 40 kD, suggesting that this protease may not correspond to the calpains in animal cells; (3) the enzyme is located in the cytoplasm; (4) its optimal activity occurs between pH 7 and 8; (5) the Ca^{2+} concentration at which half the maximal activity occurs is 0.1–0.5 mM; and (6) it is partially inhibited by the SH-modifying reagent, pCMB.

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