Examination of the Contribution of Vacuolar Proteases to Intracellular Protein Degradation in *Chara corallina*

Yuji Moriyasu*

Department of Biology, Faculty of International Relations, University of Shizuoka, 52–1 Yada, Shizuoka-shi, 422 Japan

The contribution of proteases in the central vacuole of *Chara corallina* internodal cells to overall cellular protein degradation was examined. I measured the decrease in the trichloroacetic acid (TCA)-precipitable radioactivity in the cell for a 6-d chase period after labeling cellular proteins with [3H]leucine. The kinetics of [3H]leucine-labeled protein disappearance showed that the half-life of the cellular soluble proteins was 4 to 5 d. This value did not change when cells were treated with (2S,3S)-trans-epoxysuccinyl-l-leucylamido-3-methyl-butane ethyl ester, a permeant inhibitor of cysteine proteases. This inhibitor mostly inhibited bovine serum albumin-degrading activity in the vacuole. I also measured the release of TCA-soluble radioactivity from the TCA-insoluble fraction in the cell. This experiment showed that 13% of [3H]leucine-labeled cellular proteins were degraded in 1 d. This value agreed well with the half-life obtained for soluble proteins in the above experiment. This value did not change even when both trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, a cysteine protease inhibitor, and pepstatin A, an aspartic protease inhibitor, were introduced into the vacuole. With this operation, bovine serum albumin-degrading activity in the vacuole was almost completely inhibited. These data suggest that the cytoplasmic but not the vacuolar proteases contribute to cellular protein turnover in *Chara* internodal cells.

Plant vacuoles have various kinds of hydrolytic enzymes including acid phosphatase, α-mannosidase, proteinase, carboxypeptidase, and RNase (Ryan and Walker-Simmons, 1983; Boller and Wiemken, 1986). In addition, mature plant vacuoles have sometimes, especially in the course of senescence, been observed to include cytoplasm containing organelles such as chloroplasts and mitochondria (Matile, 1975). From these two lines of evidence, Matile (1975) proposed that mature plant vacuoles are a kind of lysosome. However, direct evidence that mature plant vacuoles contribute significantly to intracellular protein degradation in a manner similar to mammalian lysosomes is still lacking. In mammalian cells, the contribution of lysosomes to intracellular protein degradation has been estimated by various investigators using various cells to be as high as 100% to as low as 10% (Dice, 1987). In yeast cells, the contribution of the vacuole (lysosome) to overall protein turnover was estimated to be about 40 and 86% under normal and nutrient-starved conditions, respectively (Teichert et al., 1989). In plant cells, examination of the involvement of the mature vacuole in cellular protein degradation has been done by Canut et al. (1986). Using cultured cells of *Acer pseudoplatanus*, they performed feeding experiments with [14C]Phe and [3H]Leu and showed that, when vacuolar proteases are inhibited by increasing the vacuolar pH with benzylamine, more labeled proteins accumulate in the cells. From these results, they concluded that the vacuole is involved in the degradation of intracellular proteins.

My associate and I have been investigating the mechanism of intracellular protein degradation using an alga, *Chara corallina*, as a model system of plant cells. The large size of its internodal cells allows us to perform various surgical operations to control the chemical composition of the vacuole and the cytoplasm (Tazawa et al., 1987). In a previous study, we reported that the central vacuole of *Chara* internodal cells contains an amount of proteases comparable to those of higher plants and fungi (Moriyasu and Tazawa, 1988). We further showed that these proteases are active in digesting exogenous proteins such as BSA and casein when these proteins are introduced into the vacuole (Moriyasu and Tazawa, 1988).

In the study described here, I examined the contribution of proteases in the central vacuole of *Chara* internodal cells to overall cellular protein degradation using a combination of pulse-chase experiments with [3H]Leu and protease inhibitors.

MATERIALS AND METHODS

Plant Material

*Chara corallina* was used. The alga was grown in a plastic bucket (90 L in volume) at 25 ± 1°C in 15-h/9-h light/dark cycles. Two fluorescent lamps (FL20SS; Toshiba, Tokyo, Japan) were set at the top of the bucket for illumination. Mature internodal cells were cut from adjacent internodal cells with scissors and kept in APW (0.1 mM KCl, 0.1 mM NaCl, 0.1 mM CaCl₂, and 5 mM Hepes-Na [pH 7.5]) at 25 ± 1°C under a dim light for more than 1 d before the experiment.

Abbreviations: APW, artificial pond water; AVS, artificial vacuolar sap; E-64, trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane; E-64-c, (2S,3S)-trans-epoxysuccinyl-l-leucylamido-3-methyl-butane; E-64-d, (2S,3S)-trans-epoxysuccinyl-l-leucylamido-3-methyl-butane ethyl ester; FCT-casein, fluorescein thiocarbamoyl-casein.

* E-mail moriyasu@momo1.u-shizuoka-ken.ac.jp; fax 81-54-264-5099.
Chemicals

L-[4,5-3H]Leu (2.63 TBq/mmol, 37 MBq/mL) was purchased from ICN. E-64, pepstatin A, and leupeptin were from Peptide Institute, Inc. (Minoh-shi, Osaka, Japan). BSA (fraction V) was from Nacalai Tesque, Inc. (Kyoto, Japan). L-Leu was from Wako Chemical Industries, Ltd. (Osaka, Japan). Casein (according to Hammarsten) was from Merk (Darmstadt, Germany). Liquid paraffin was from Kanto Chemical Co., Inc. (Tokyo, Japan). Fluorescein isothiocyanate (isomer I) and PMSF were from Sigma. E-64-c and E-64-d were generous gifts from M. Tamai (Taisho Pharmaceutical Co., Ltd., Oomiya, Japan). FTC-casein was prepared according to the method of Twining (1984).

Introduction of Protein and/or Protease Inhibitors into the Vacuole

To introduce an exogenous protein, BSA, and/or a protease inhibitor(s), I perfused the vacuole with 10 μL of AVS (80 mM KCl, 30 mM NaCl, 10 mM CaCl2, and 10 mM MgCl2) containing these chemicals (Moriyasu and Tazawa, 1988). The composition of AVS was designed to mimic the concentrations of K+, Na+, Ca2+, Mg2+, and Cl− and the osmotic value of the natural vacuolar sap isolated from the internodal cells (Moriyasu et al., 1984). As a result of the perfusion, the original vacuolar sap is diluted with AVS by 20 to 25%, since the volume of the vacuole in an internodal cell is 40 to 50 μL.

Electrophoresis and Sample Preparation for Electrophoresis

Each internodal cell was homogenized with 333 μL of the homogenization buffer (0.1 M Hepes-Na [pH 7.5], 100 μM leupeptin, 1 mM PMSF, and 1 mM EDTA) using a mortar and pestle. The homogenate was centrifuged at 15,000 g for 10 min. The resulting supernatant was mixed with one-fifth volume of the SDS-PAGE sample-preparing solution, consisting of 6% (w/v) SDS, 0.24 M DTT, 0.012% (w/v) bromophenol blue, and 50% (w/v) Suc. The mixture was boiled at 100°C for 90 s, cooled, and stored at −20°C until electrophoresis. SDS-PAGE was done with Laemmli’s discontinuous buffer system (Laemmli, 1970) on 10% gels, which were purchased as ready-to-use gel sandwiches (Multi Gel 10; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) or prepared by me. Separation of proteins on gels from these two sources was essentially the same. After electrophoresis, gels were stained with silver. Phosphorylase b (97 kD), BSA (66 kD), egg albumin (45 kD), and carbonic anhydrase (29 kD) were used as molecular mass markers.

Measurement of the Decrease in TCA-Precipitable Radioactivity in the Cells

Internodal cells (about 30 cells) were incubated in 6 mL of APW containing 60 μCi of [3H]Leu for 1 d in darkness at 25 ± 1°C. They were then washed in 100 mL of APW for 5 min and kept in fresh APW until the AVS (10 μL) with and without protease inhibitors was introduced. Subsequently, each cell was incubated in 2.5 mL of APW containing 1 mM Leu in darkness at 25 ± 1°C. Immediately (time = 0) and 1 d after the start of the chase period, the cell was homogenized with 500 μL of the homogenization buffer using a mortar and pestle. The homogenate was centrifuged at 15,000g for 10 min. The supernatant (50 μL) was spotted onto a glass filter (glass microfiber filter GF/A 2.1 cm, Whatman). The filter was dried and transferred into a scintillation glass vial. It was washed twice with 5 mL of 10% (w/v) TCA for 10 min each and twice with 5 mL of ethanol for 10 min each. After the filter was dried again, 5 mL of scintillator (Econofluor, NEN) were added, and the radioactivity of the filter was measured using a liquid scintillation counter (LSC-3100; Aloka, Mitaka, Japan).

Measurement of the Release of TCA-Soluble Radioactivity

This measurement allows us to assay the degradation of all proteins, including both soluble and insoluble proteins. Internodal cells (about 16 cells) were incubated in 5 mL of APW containing 50 μCi of [3H]Leu for 1 d in darkness at 25 ± 1°C. They were then washed in 100 mL of APW for 5 min and kept in fresh APW until the AVS (10 μL) with and without protease inhibitors was introduced. Subsequently, each cell was incubated in 2.5 mL of APW containing 1 mM Leu in darkness at 25 ± 1°C. Immediately (time = 0) and 1 d after the start of the chase period, the cell was homogenized with 500 μL of the homogenization buffer. The homogenate (50 μL) was spotted onto a glass filter. The filter was washed with TCA and ethanol, and its radioactivity, which is regarded as the radioactivity in the total cellular proteins (P), was measured. The remaining homogenate was centrifuged at 15,000g, and the supernatant (50 μL) was spotted onto each of two glass filters. The radioactivity of one filter was measured directly. The radioactivity of the other filter was measured after it was washed with TCA and ethanol. The difference between these two radioactivities was regarded as TCA-soluble radioactivity in the cell (Sout). The external solution (50 μL) was also spotted onto a filter for the measurement of TCA-soluble radioactivity in it (Sout). Protein degradation for 1 d (PD) can be defined as in Equation 1a:

\[
PD = \frac{(S_{out} - S_{in}) + (S_{out}^1 - S_{in}^1)}{I_{out}^1},
\]

where \(I_{out}^1\) is TCA-insoluble radioactivity in the cell at 0 d, \(S_{out}^1\) is TCA-soluble radioactivity in the external solution at 0 d, \(S_{out}^1\) is TCA-soluble radioactivity in the external solution at 1 d, \(S_{in}^1\) is TCA-soluble radioactivity in the cell at 0 d, and \(S_{in}^1\) is TCA-soluble radioactivity in the cell at 1 d. Thus,

\[
PD = \frac{S_{out}^1 + S_{in}^1 + S_{out}^1 + S_{in}^1}{I_{out}^1}.
\]
In this experiment, $I^{in}$, $S^{in}$, and $S^{out}$ varied greatly between the cells, and as a result, the PD had large deviations. However, the ratios of various radioactivities in each individual cell were relatively constant. To use the ratios from each individual cell in our calculation, Equation 1b was transformed to Equations 4, 5, and 6 as follows: Because the total radioactivity does not change in 1 d for the individual cell,

$$\frac{I^{in}}{I^0} = 1 - PD,$$

where $I^{in}$ is TCA-insoluble radioactivity in the cell at 1 d.

Applying Equation 2 to the first term of Equation 1b, Equation 1b can be transformed to

$$PD = (1 - PD) \frac{S^{out} + S^{in}}{I^{in}} - \frac{S_0^{out} + S_0^{in}}{I_0^{in}}.$$ (3)

Thus, the PD can be expressed as

$$PD = \frac{A - B}{1 + A},$$ (4)

where $A = \frac{S^{out} + S^{in}}{I^{in}}$ (5)

and $B = \frac{S_0^{out} + S_0^{in}}{I_0^{in}}$. (6)

The individual values of $A$ and $B$, which represent the ratios of the total TCA-soluble radioactivity to the TCA-insoluble radioactivity at 1 and 0 d, respectively, can be obtained from each individual cell. Thus, their deviations are minimized. The means ($\bar{A}$) and $\sigma_\bar{A}$ values of $A$ were calculated with the data obtained from five inhibitor-treated and five control cells; the mean ($\bar{B}$) and $\sigma_\bar{B}$ of $B$ were from another five cells. $S^{out}$ was considered to be 0. The means of the PD were calculated by applying $A$ and $B$ to Equation 4. The $\sigma_\bar{A}$ of PD ($\sigma_{PD}$) was estimated according to the following equation:

$$\sigma_{PD}^2 = \left(\frac{\partial PD}{\partial A}\right)^2 \sigma^2_{A} + \left(\frac{\partial PD}{\partial B}\right)^2 \sigma^2_{B}.$$ (7)

Measurement of Caseinolytic Activity in the Vacuolar Sap Isolated from Internodal Cells

Vacuolar sap was collected from internodal cells as follows (Moriyasu et al., 1984). When the cell lost turgor in the air, both cell ends were cut and liquid paraffin was introduced into the vacuole from one open cell end. The vacuolar sap exuded from the other open end was collected in a glass capillary tube. The collected vacuolar sap was pooled and kept on ice until protease was assayed. The reaction mixture contained 40 $\mu$L of 0.1 m acetic-Na (pH 5.0), 10 $\mu$L of vacuum sap, 10 $\mu$L of H$_2$O$_2$, and 40 $\mu$L of a substrate solution (0.5% [w/v] FTC-casein). The reaction was started by the addition of the substrate and the mixture was incubated at 37°C. When the effects of protease inhibitors on the reaction were examined, 10 $\mu$L of H$_2$O were replaced with the solutions containing inhibitors. When pepstatin A and PMSF were used, the final concentrations of DMSO and methanol were less than 1% (v/v). These solvents did not have any effect on the reaction. The reaction was stopped by the addition of 100 $\mu$L of 10% (w/v) TCA. After the mixture was kept on ice for about 1 h, it was centrifuged at 15,000g for 10 min. The supernatant (150 $\mu$L) was mixed with 2 mL of 0.5 m Tris-Cl (pH 8.5) and its fluorescence at 525 nm was measured following excitation at 490 nm. Under this condition, the time-dependent increase in fluorescence was almost linear for at least 2 h.

Treatment of the Cells with Chloroquine, Protein Assay of Vacuolar Sap, and Measurement of Vacuolar pH

Internodal cells were kept in APW containing 0.2 m chloroquine under a dim light at 25 ± 1°C. After various time intervals, vacuolar sap (10 $\mu$L) was isolated as described above and its protein was measured with the Pierce BCA Protein Assay Reagent according to the manufacturer's instructions. BSA was used as a standard protein.

The pH of the isolated vacuolar sap was measured with a pH microelectrode (SE170GC; Fuji Chemical Measurement, Mitaka, Japan). The effect of chloroquine on BSA-degrading activity in the vacuole was examined using the cells that had been kept in APW containing 0.1 or 0.2 m chloroquine for 1 d. After BSA was introduced, the cells were kept in the same solutions for another 1 d until they were homogenized.

RESULTS

Inhibition of BSA-Degrading Activity in the Vacuole by a Permeant Cys Protease Inhibitor, E-64-d

After I introduced an exogenous protein, BSA, into the central vacuole of a Chara internodal cell, I analyzed the degradation of BSA with SDS-PAGE (Fig. 1). The BSA disappeared in 1 d (Fig. 1, Control versus $t=0$). This result is consistent with that reported in a previous paper (Moriyasu and Tazawa, 1988). We have already shown that BSA is degraded within the central vacuole, not outside the vacuole (Moriyasu and Tazawa, 1988).

When I treated the cell with a permeant Cys protease inhibitor, E-64-d, 1 d before the introduction of BSA, degradation was mostly inhibited. However, there was still some degradation, since I observed two products of BSA degradation with molecular masses of approximately 50 kD (Fig. 1, E-64-d before loading). One of these bands has been shown to cross-react with an antibody against BSA (Moriyasu and Tazawa, 1988). E-64-d was less effective in inhibiting BSA degradation when the cells were treated with this inhibitor after the BSA introduction (Fig. 1, E-64-d after loading).

Effect of E-64-d on the Half-Life of the Cellular Soluble Proteins

Based on the above result that E-64-d can effectively inhibit protease activity in the vacuole, I investigated
whether or not the turnover of the native soluble proteins is affected by E-64-d. After labeling the overall cellular proteins with $[^3]$H]Leu, I measured the radioactivity in the cellular soluble protein fractions at various times during the chase period. The kinetics showed that the half-life of $[^3]$H]Leu-labeled soluble proteins was 4 to 5 d (Fig. 2). Although this measurement does not consider the reincorporation of $[^3]$H]Leu, this value is consistent with the half-lives of *Lemna* soluble proteins obtained by various methods (Davies, 1982). The kinetics did not change significantly when the cells were kept in 0.1 mM E-64-d and Cys protease activity was inhibited (Fig. 2). I ascertained that BSA-degrading activity in the vacuole was still inhibited after 6 d. These results suggest that a vacuolar Cys protease(s) does not contribute to the turnover of soluble proteins in *Chara* internodal cells.

**Effects of Various Inhibitors on Caseinolytic Activity in the Isolated Vacuolar Sap**

There remained a possibility that a protease(s) other than a Cys protease(s) in the vacuole contributes to protein turnover. Thus, I tried to characterize proteases in the isolated vacuolar sap using a substrate, FTC-casein, and various protease inhibitors. A Cys protease inhibitor, E-64 (10 $\mu$M), and an Asp protease inhibitor, pepstatin A (10 $\mu$M), inhibited 35 and 86% of the total activity, respectively (Table I). In contrast to these two inhibitors, a Ser protease inhibitor, PMSF (2 mM), had no inhibitory effect (Table I). These results suggest that not only a Cys protease(s) but also an Asp protease(s) is localized in the vacuole and that the Asp protease(s) has a higher activity than the Cys protease(s) in the degradation of FTC-casein.

**Effects of E-64 and Pepstatin A on BSA-Degrading Activity in the Vacuole**

Table I suggests that an Asp protease(s) also contributes to vacuolar protease activity. Thus, I tried to test the hypothesis that the degradation of BSA is also inhibited by pepstatin A. Since pepstatin A does not penetrate the cell, I introduced pepstatin A simultaneously with BSA. This Table I. The effect of E-64 and pepstatin A on FTC-casein-degrading activity in the isolated vacuolar sap

Vacular sap was collected from *Chara* internodal cells. The effects of various protease inhibitors on its protease activity were measured using FTC-casein as a substrate. The remaining activities (percentage) relative to the control are shown.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Remaining Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$\mu$M</td>
<td>%</td>
</tr>
<tr>
<td>E-64</td>
<td>10</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>$1$</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>$10$</td>
<td>14</td>
</tr>
<tr>
<td>PMSF</td>
<td>2000</td>
<td>111</td>
</tr>
</tbody>
</table>
Intracellular Protein Degradation in Chara corallina

Intracellular Protein Degradation in Chara corallina

Effect of E-64 and Pepstatin A on Intracellular Protein Degradation

Based on the above results showing that both BSA-degrading and caseinolytic activities can be inhibited almost completely by E-64 and pepstatin A, I further examined whether or not the inhibition of both activities affects the cellular protein turnover. I measured the release of TCA-soluble radioactivity from [3H]Leu-labeled cellular proteins and calculated the protein degradation according to Equations 4, 5, and 6 in "Materials and Methods." The protein degradation mainly reflected the release of TCA-soluble radioactivity to the external solution.

In the cells, 13.3% of the total cellular proteins were degraded in 1 d (Table II, Control). With this treatment, however, protein concentration in the vacuole began to increase by about 60 h (Fig. 5). In contrast, I did not observe such an increase with E-64-d for 6 d (data not shown).

The release of TCA-soluble radioactivity from [3H]Leu-labeled Chara internodal cells was measured, and protein degradation (percentage) for 1 d was calculated. In the cells with inhibitors (+Inhibitors), vacuolar proteases were inhibited by the introduction of E-64 and pepstatin A into the vacuoles. The means ± SD of five cells are shown.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Protein Degradation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Inhibitors</td>
<td>13.9±4.9</td>
</tr>
<tr>
<td>Control</td>
<td>13.3±3.7</td>
</tr>
</tbody>
</table>

the half-life of the total cellular proteins is 4.9 d, assuming that the rate of protein degradation is constant. Therefore, the half-life for the total cellular proteins is not much different from that for the soluble proteins shown in Figure 2.

The rate of protein degradation did not change significantly even when vacuolar proteases were inhibited by both E-64 and pepstatin A (Table II). This result suggests that vacuolar proteases do not contribute significantly to the cellular protein turnover in Chara internodal cells under the experimental conditions.

Effects of Chloroquine on Vacuolar pH, Vacuolar BSA-Degrading Activity, and Vacuolar Protein Concentration

Lysosomotropic reagents can be expected to have an effect similar to vacuolar protease inhibitors because these reagents may inhibit vacuolar proteinolyis by increasing vacuolar pH. I therefore examined the effect of a lysosomotropic reagent, chloroquine, on BSA-degrading activity in the vacuole of Chara internodal cells.

With 0.2 mM chloroquine, vacuolar pH increased from 5.0 to 5.6 (Table III) and BSA-degrading activity in the vacuole was indeed inhibited (Fig. 4, 0.2 mM Chloroquine versus Control). With this treatment, however, protein concentration in the vacuole began to increase by about 60 h (Fig. 5). In contrast, I did not observe such an increase with E-64-d for 6 d (data not shown).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Vacuolar pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM Chloroquine</td>
<td>5.59±0.03(4)</td>
</tr>
<tr>
<td></td>
<td>4.95±0.08(5)</td>
</tr>
</tbody>
</table>

DISCUSSION

I examined the contribution of vacuolar proteases to cellular protein turnover in Chara internodal cells by inhibiting vacuolar proteases with specific protease inhibitors. I first checked the effect of several protease inhibitors, given from outside the cell, on vacuolar proteases. Leupeptin,
lysosomes to intracellular protein degradation increases and these factors may explain the difference between the degradation was affected by some environmental factors, the contribution of plant vacuoles to intracellular protein formation during vacuole formation. Second, it is possible that the contribution of vacuolar proteases to protein degradation (Marty, 1978), the data from Canut et al. (1986) may include whereas the vacuole in the internodal cells used in *Chara* cells were used, the cells were dividing and Acer cells. In contrast, Canut et al. (1986) reported that the contribution of marker proteins are on the left.

antipain, E-64-c, E-64, or pepstatin A had no effect; they may not penetrate the cell. I found that a Cys protease inhibitor, E-64-d, could reduce the vacuolar protease activity. It effectively inhibited the degradation of BSA introduced into the vacuole (Fig. 1), but it did not affect the half-life of [3H]Leu-labeled cellular soluble proteins (Fig. 2).

The central vacuole of a *Chara* internodal cell contains both a Cys protease(s) and an Asp protease(s) (Table I). The introduction of both Cys and Asp protease inhibitors, E-64 and pepstatin A, almost completely inhibited the degradation of BSA in the vacuole (Fig. 3). This operation, however, did not have any effect on the degradation rate of [3H]Leu-labeled cellular proteins (Table II).

These results suggest that cytoplasmic but not vacuolar proteases contribute to protein turnover in *Chara* internodal cells. In contrast, Canut et al. (1986) reported that the vacuoles in *Acer* cultured cells do degrade cellular proteins. There are some explanations for the difference between the experiments of *Acer pseudoplatanus* cultured cells and those of *Chara corallina* internodal cells. First, in the experiments in which *Acer* cells were used, the cells were dividing and growing and vacuole genesis was occurring in the cells, whereas the vacuole in the *Chara* internodal cells used in the present study were mature. Because autophagy is known to occur during the formation of plant vacuoles (Marty, 1978), the data from Canut et al. (1986) may include the contribution of vacuolar proteases to protein degradation during vacuole formation. Second, it is possible that the contribution of plant vacuoles to intracellular protein degradation was affected by some environmental factors, and these factors may explain the difference between the data. In mammalian and yeast cells, the contribution of the lysosomes to intracellular protein degradation was not affected by some environmental factors, whereas such accumulation of cellular proteins in the vacuole did not occur during 6 d of treatment with E-64-d.

A Cys protease inhibitor, E-64, and an Asp protease inhibitor, pepstatin A, but not a Ser protease inhibitor, PMSF, inhibited FTC-casein-degrading activity in the isolated vacuolar sap. E-64 and pepstatin A also inhibited the degradation of BSA introduced into the vacuole. These results suggest that the vacuole of *Chara* cells has at least one Cys protease and one Asp protease. Mammalian lysosomes have two major Cys proteases, cathepsins B and L, and a major Asp protease, cathepsin D (Bohley and Seglen, 1992), whereas yeast vacuoles contain a Ser protease, proteinase B, an Asp protease, proteinase A, and no Cys proteases (Jones, 1991). Thus, the composition of proteases in the vacuole of *Chara* internodal cells is more akin to that in the lysosome of mammalian cells rather than to that in the vacuole of yeast cells.

There still remain two important questions. One question is what proteases in the vacuole of *Chara* cells do. It is difficult to suppose that vacuolar proteases are merely left over from the early developmental phase of vacuoles, in which autophagy is known to occur, because vacuolar proteins may turn over like other cellular proteins (Canut et al., 1985). There may be some situations in which vacuolar proteases work in the cells. As mentioned above, a lysosomal/vacuolar contribution to cellular proteolysis is

Figure 4. The inhibition of protease activity in the vacuole of *Chara internodal cells* by chloroquine. After *Chara* internodal cells were kept in APW containing 0.1 or 0.2 mM chloroquine or in APW (Control) for 1 d, 10 µg of BSA were introduced into the vacuole of each internodal cell. After the introduction, cells were returned to the original solutions. The degradation of BSA (arrowhead) was analyzed 1 d after the introduction by SDS-PAGE. One-twentieth of the water-soluble proteins extracted from an internodal cell was loaded onto each lane. Different lanes represent different cells. Molecular masses of marker proteins are on the left.

Figure 5. The effects of chloroquine on protein concentration in the vacuoles of *Chara* internodal cells. *Chara* internodal cells were kept in APW containing 0.2 mM chloroquine (●) or in APW (●). At various intervals, vacuolar sap was isolated and its protein content was measured. The protein concentrations (mg protein/mL vacuolar sap) were measured as the means ± sd.
known to be activated upon nutrient starvation in mammalian and yeast cells (Schworer and Mortimore, 1979; Teichert et al., 1989; Takeshige et al., 1992). A similar situation can be expected in plant cells and in Chara cells as well. Journet et al. (1986) reported that net protein degradation occurs upon Suc starvation in Acer cultured cells, which grow heterotrophically. Such proteolysis is likely to occur in vacuoles. Furthermore, a possibility that vacuolar proteases act as defensive enzymes against pathogens must be considered (Boller, 1986).

The other question is what kind of proteases contribute to protein turnover in Chara cells. In this study, I showed that vacuolar proteases do not contribute significantly to the cellular protein turnover and suggested that extravacuolar proteases are involved. In mammalian and yeast cells, proteasomes are known to work in the degradation of several proteins with short half-lives (Hershko and Ciechanover, 1992). In addition, calpains, cytosolic proteases found in various animal cells (Pontremoli and Melloni, 1986), are thought to contribute to some specific proteolysis (Wang et al., 1989). Also, in higher plant cells, a proteasome is shown to be involved in the degradation of proteins such as phytochrome (Vierstra, 1993). Furthermore, chloroplasts and mitochondria are thought to have their own proteolytic systems. In Chara internodal cells, we can easily wash vacuolar proteases, which may interfere the assay of extravacuolar proteases, out of the cell by vacuolar perfusion. Taking advantage of this material, we are searching for extravacuolar proteases. So far, we have found a proteasome and a protease activated by Ca2+ (Moriyasu and Tazawa, 1987). Such an approach may help us to understand the mechanism of protein degradation in plant cells.

ACKNOWLEDGMENTS

I thank Dr. Tetsuro Mimura (Hitotsubashi University) and Dr. Randy Wayne and Dr. Mark Staves (Cornell University) for critical reading of the manuscript and Dr. Masaharu Tamai (Taisho Pharmaceutical Co., Ltd.) for providing E-64-c and E-64-d. I also thank Prof. Yasuhiro Miyoshi for constant encouragement.

Received July 10, 1995; accepted September 12, 1995.
Copyright Clearance Center: 0032-0889/95/109/1309/07.

LITERATURE CITED


Matile P (1975) The Lytic Compartment of Plant Cells. Springer-Verlag, Vienna, Austria


