CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Material

Japanese bunching onions (*Allium fistulosum* L. cv. Kujyo) were harvested from the experimental farm of Yamaguchi University. Uniformity of leaves was selected by the length of its stem. Then, they were transported to the laboratory of horticultural science, at Yamaguchi University. After harvest, Japanese bunching onion leaves were thoroughly rinsed with flowing tap water to eliminate any impurities on their surfaces and well dried using moisture-absorbing paper.

3.2 Experimental Designs

3.2.1 Experiment 1 Vacuole preparation from Japanese bunching onion leave

3.2.1.1 Preparation of protoplasts

The regular method for plant protoplasts extraction is osmotic balance. In this experiment, the method was modified from Boudet, et al. (1981) to enhance the quality and quantity of protoplasts.

Method A

Japanese bunching onion leaves were cut into 5 mm length and soaked into the 50 mM Mes-Tris buffer (pH5.5) containing 0.6 M mannitol and then incubated at 37 °C for 5 min. After incubation, the sample was transferred into 50 mM Mes-Tris buffer (pH 5.5),

containing 0.6 M mannitol, 2% (w/v) cellulase and 0.3% (w/v) macerozyme. The digestion was performed for 1.5 hr at 36 °C. The solution was filtered thought one layer of Miracloth (Calbiochem) then one layer of nylon net. The filtrate was centrifuged at 100 ×g for 5 min. The pellet was washed twice with 50 mM Mes-Tris buffer (pH 5.5). The obtained pellet was added with 1 mL of 50 mM Mes-Tris buffer (pH 6.5). The number of protoplasts was checked by using the calculating board for blood cell.

Method B

Tissue from Japanese bunching onion leaves (1 gram) was cut with a razor blade. Then, it was placed in a petri dish contained 0.6 M mannitol in 50 mM Mes-Tris buffer (pH 5.5) and incubated at 37 °C for 5 min. Then, the solution was replaced by 5 mL of 0.6 M mannitol 50 mM Mes-Tris buffer (pH 5.5) contained 4% cellulase and 1.5% Macerozyme. The digestion was performed for 30 min at 36 °C in the shaking incubator at 45 rpm. After this period, the digestion solution containing some debris and cells was removed and then added 10 mL of 50 mM Mes-Tris buffer (pH 6.5) containing 0.6 M mannitol contained 4% cellulase and 1.5% Macerozyme to the remaining material and digestion continued at the same temperature and shaking rate as described above for 3 hr. Then filtrate through two layers of Miracloth and one layer of a 40 μm nylon net, respectively. The filtrate was centrifuged at 100 ×g for 5 min and the pellet was washed twice with 10 mL of 50 mM Mes-Tris (pH 6.5) buffer and finally re-suspended in 20 mL of 50 mM Mes-Tris buffer (pH 6.5). The protoplasts were allowed to settle for 1.5 hr at 4 °C. The supernatant was removed and the protoplasts were re-suspended in 50 mM Mes-Tris (pH 6.5) buffer and checked the number of protoplasts using the calculating board for blood cell.

3.2.1.2. Purification of protoplast

Crude protoplasts were centrifuged on discontinuous gradient. Crude protoplasts (1 mL) were put on top of four layer gradients (each 2 mL) composed ficoll concentrations of 2%, 5%, 10%, and 15% (w/v) in 50 mM Mes-Tris buffer (pH 6.5) contain 0.6 M mannitol from bottom to top and then centrifugation at 2000 rpm for 5 min. Purified protoplasts were deposited at the interface of 10% and 15% ficoll gradients (Fig 3.1).

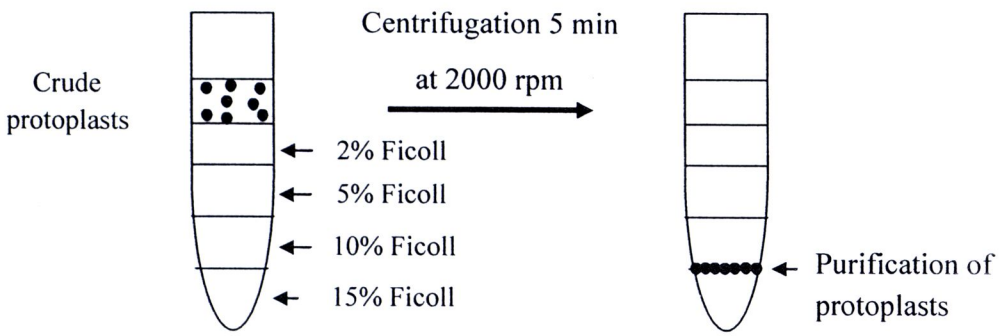

Figure 3.1 Procedure for purification of protoplasts (Boudet et al., 1981).

3.2.1.3 Isolation of vacuole

The vacuoles were isolated from protoplasts of Japanese bunching onion tissue. Various methods were isolated vacuole including mechanical technique, chemical technique and structure damage technique. Therefore, this method was applied chemical technique by using the polybase (cationic) chemical for isolation of vacuole. These were modified method of Boudet et al. (1981) and Asami et al. (1985).

Method A (modified from Boudet et al. (1981))

The vacuoles were released from predicate protoplasts of Japanese bunching onion. The lysis of protoplasts and the purification of vacuoles occur during the same centrifugation step. The vacuoles released during the centrifugation through the

discontinuous layer. These were included 1mg/mL of DEAE Dextran in 50 mM Mes-Tris buffer (pH 6.5) contain 0.6 M mannitol, 1mg/mL of Dextran sulfate in 50 mM Mes-Tris buffer (pH 6.5) contain 0.6 M mannitol, 2% of Ficoll in 50 mM Hepes-tris (pH 8.0) contain 0.6 M mannitol and 20% of Ficoll in in 50 mM Hepes-tris (pH 8.0) contain 0.6 M mannitol (Figure 3.2). The vacuoles banded at the interface between the solutions containing 2% and 20% Ficoll were collected with a micropipette. A pellet at the bottom of the tube consisted mainly of chloroplasts and some damaged protoplasts. Vacuoles and protoplasts were examined by light microscopy and counted using a hemocytometer. Test integrity and viability of protoplasts and vacuoles were used 2.5% (w/v) Evans blue and 0.05% (w/v) neutral red in 50 mM Hepes-tris (pH 8.0) buffer contain 0.6 M mannitol, respectively.

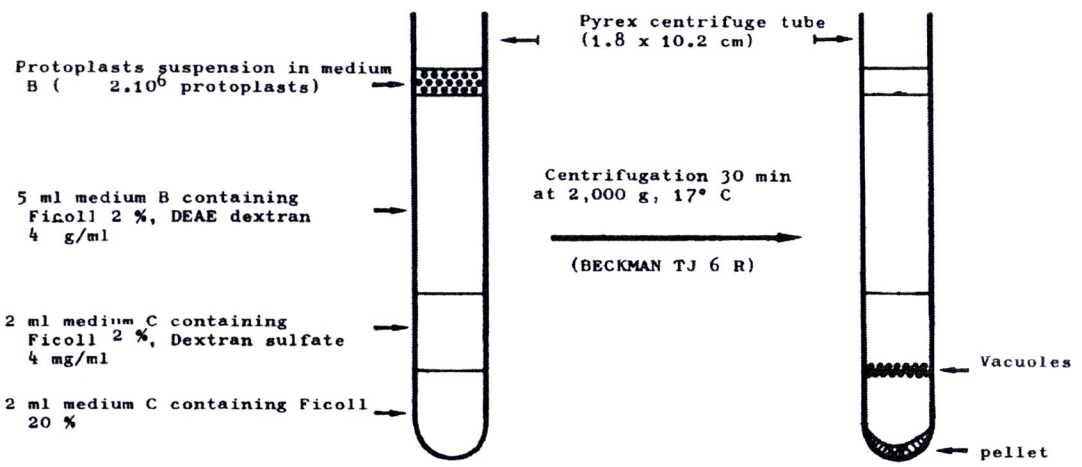

Figure 3.2 Procedure for preparation and purification vacuole (Boudet et al., 1981).

Method B (modified from Asami et al. (1985))

The vacuoles were isolated from protoplasts by using 100 µl of 1×10^6 protoplasts. These were added to 1 ml of 1 mg/ml DEAE in 50 mM Mes-Tris buffer (pH 6.5) containing 0.6 M mannitol and then gentle swirling for 5 min. After that, adding 0.5%

(w/v) dextran sulfate in 50 mM Mes-Tris buffer (pH 6.5) containing 0.6 M mannitol was swirled for 1 min. Then, the solution as above was put in layered on top of gradient 5% ficoll in 50 mM Hepes-tris (pH 8.0) buffer contain 0.6 M mannitol and centrifuged at 10,000×g for 25 min at 15 °C. The vacuoles were recovered at the interface of two ficoll layer. Vacuoles were examined by light microscopy and the neutral red was used for checking the integrity of vacuole.

3.2.1.4 Enzyme activity

3.2.1.4.1 Measurement of catalase activity (The peroxisome maker enzyme)

Catalase activity was determined by modification method of Evelyn et al. (1987). The reaction mixture contained 2 mL of 50 mM K-phosphate (pH 7.0), 0.5 mL of 0.3% H_2O_2 in 50 mM K-phosphate (pH 7.0), 0.1 mL of cell extraction. The activity was determined spectrophotometrically by measuring the decrease of H_2O_2 at 240 nm per min per mg protein at 30 °C.

3.2.1.4.2 Measurement of NADH-Malate dehydrogenase activity (The mitochondria maker enzyme)

NADH-Malate dehydrogenase was determined by modification of the method of Leegood et al. (1982). The reaction mixture contained 2 mL of 25 mM Tris-HCl buffer (pH 8.0), 0.5 mL of 3 mM oxaloacetic dissolved in 0.6% EDTA, 0.05 mL of 6 mM NADH in 25 mM Tris-HCl buffer (pH 8.0), 0.5 mL of H_2O, 0.05 mL of cell extract. The activity was determined spectrophotometrically by measuring the decrease of NADH at 350 nm per min per mg protein.

3.2.1.4.3 Measurement of NADH-Cytochrome C reductase activity (The endoplasmic reticulum maker enzyme)

NADH-Cytochrome C reductase was determined by modification method of John et al. (1969). The reaction mixture contained 0.2 mL of 0.1 M K-phosphate (pH 7.5), 0.02 mL of 2% Cytochrome C, 0.08 mL of 1 mM NADH, 0.1 mL of cell extract The activity was determined spectrophotometrically by measuring the increase of NAD^+ at 550 nm per min per mg protein.

3.2.1.4.4 Measurement of alcohol dehydrogenase activity (The cytosol maker enzyme)

Alcohol dehydrogenase was determined as described by Yamashita et al. (1976). The reaction mixture contained 0.1 mL of 15 mM NAD, 0.1 mL of 0.5% Triton X-100, 1.4 mL of 0.1 M Tris-HCl (pH9.0), 5 µL of Ethanol, 0.4 mL of cell extract. The activity was determined spectrophotometrically by measuring the decrease of NAD at 340 nm per min per mg protein at 25 °C.

3.2.1.4.5 Measurement of acid phosphatase activity (The vacuole maker enzyme)

Acid phosphatase was referred to method of Nishimura et al. (1978) by Acid phosphatase was assay with *p*-Nitrophenyl Phosphate (PNPP) substrate. The reaction mixture contained 0.5 mL of 50 mM K-acetate (pH 5.0) and 2.5 µmol PNPP. The reaction mixture was incubated in water bath at 37 °C for 5 min. The enzyme reaction was stopped the free PNP color developed by adding 2.5 mL of 0.2 N NaOH. The absorbance at 400 nm was measuring and PNP concentration determined from standard curve.

3.2.1.4.6 Measurement of β-Glucosidase activity (The vacuole maker enzyme)

β-Glucosidase was determined as refer to method of Nishimura et al (1978). β-Glucosidase was assay by hydrolysis of *p*-Nitrophenyl Phosphate-β -D-glucopyranoside (PNP-β-d-Glu). The reaction mixture contained 0.5 mL of 50 mM K-acetate (pH 5.0) and 1.25 µmol PNP-β-d-Glu. The reaction mixture was incubated in water bath at 37 °C for 5 min. The enzyme reaction was stopped the free PNP color developed by adding 2.5 mL of 0.2 N NaOH. The absorbance at 400 nm was measuring and PNP concentration determined from standard curve

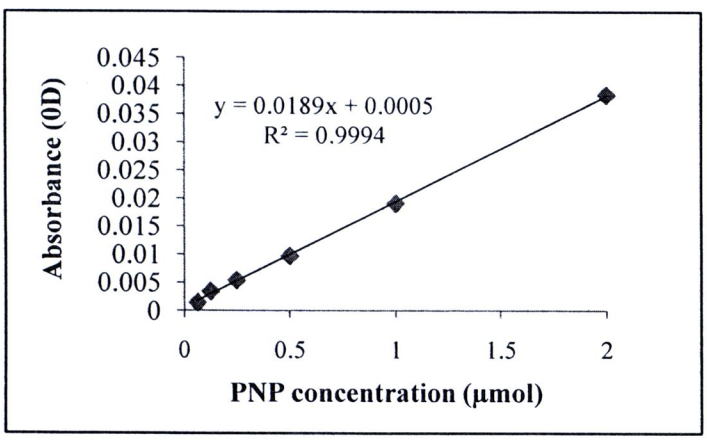

Fig 3.3 The standard curve of PNP concentration for determined acid phosphatase and β-Glucosidase activity.

3.2.1.5. Protein content

Protein content of vacuoles and protoplasts were determined based on the Bradford method (Bradford, 1976) with bovine serum albumin as a standard by using 50 µl of a Coomassie protein assay reagent.

3.2.2 Experiment 2 Analysis of chlorophyll derivative in protoplasts and vacuoles of Japanese bunching onion

3.2.2.1 Analysis of chlorophyll derivatives

Vacuoles or protoplasts (0.8 mL) were pre-incubated for 20 min, and mixed with the reaction mixture containing 0.2 mL of chlorophyll *a* in acetone (Chl *a* 500 μg/0.1 mL), 1.5 mL of 0.1 M phosphate buffer (pH 7.0). The mixture was incubated in the dark for 0, 3, 6 and 24 hr at 25 °C. The reaction was stopped by adding 2 mL of acetone. Subsequently, the aliquots were filtered through a DISMIC filter (0.45 μm, AVANCETEC, Japan) and used for HPLC analysis. Chlorophyll and its derivatives were analyzed by HPLC using fluorescence spectrophotometer. The absorption spectrum of the pigment was excitated and emissed at the wavelengths of 440 and 660 nm. Pigments were separated on a LiChropher C18 column (MERCK), 4×250 mm, using two solvents; A, 80% of methanol (methanol: milipore water, 80:20, (v/v)) and B, 100% ethyl acetate in gradient. Solvent B was added to solvent A at a linear rate until a 50:50 mixture was attained at the end of 20 min. The 50: 50 mixture was then used isocratically for an additional 20 min, as described by Angelo and Gian (2004). The flow rate was 1.0 mL/min, and injection volume was 100 μL. The identification of Chlorophyll and its derivatives was base on the retention time and the visible absorption spectra.

3.2.3 Experiment 3 Chlorophyll degradation in vacuoles of Japanese bunching onion

3.2.3.1 Plant material preparation

After harvest, Japanese bunching onion leaves were thoroughly rinsed with flowing tap water to eliminate any impurities on their surfaces and well dried using moisture-absorbing paper. Japanese bunching onion leaves were kept in perforated polyethylene bags with two 6 mm holes, and stored at 25 and 4 °C for 3 days.

3.2.3.2 Surface color and chlorophyll assays

Surface color of Japanese bunching onion expressed as a hue angle value was measured using a color difference meter (Nippon-Denshoku NF 777). Hue angle value of 0, 90, 180, and 270 degree refer to red-purple, yellow, bluish-green and blue color, respectively. Chlorophyll from Japanese bunching onion leave was extracted using *N,N*-dimethylformamide according to the Moran's method (Moran, 1982). Japanese bunching onion (0.5 g) were extracted in 20 mL of *N,N*-dimethylformamide and kept overnight at 4 °C in dark condition. The chlorophyll content was measured spectrophotometrically by reading absorbance at 664 and 647 nm. Chlorophyll *a* and *b* were calculated with the following equations.

Chlorophyll *a* (μg/ml) = $12.64OD_{664}$-$2.99OD_{647}$

Chlorophyll *b* (μg/ml) = $-5.6OD_{664}$+$23.26OD_{647}$

The unit of μg/mL is converted into mg/100FW of Japanese bunching onion using the following equations.

$$\text{Chlorophyll } a \text{ (mg/100FW)} = (\text{Chlorophyll } a \text{ (μg/ml)} \times 20.5 \times \frac{100}{0.5} \times \frac{1}{1000}$$

$$\text{Chlorophyll } b \text{ (mg/100FW)} = (\text{Chlorophyll } b \text{ (μg/ml)} \times 20.5 \times \frac{100}{0.5} \times \frac{1}{1000}$$

3.2.3.3 Analysis of chlorophyll derivatives

Vacuoles (0.8 mL) were added with 2 mL of 80% acetone. Subsequently, the aliquots were filtered through a DISMIC filter (0.45 μm, AVANCETEC, Japan) and used for HPLC analyses. Chlorophyll and its derivatives were analyzed by HPLC using fluorescence spectrophotometer. The absorption spectrum of the pigment was excitated and emissed wavelengths of 440 and 660 nm. Pigments were separated on a LiChropher C18 column (MERCK), 4×250 mm, using two solvents; A, 80% of methanol (methanol: Millipore water, 80:20, (v/v)) and B, 100% ethyl acetate in gradient. Solvent B was added to solvent A at a linear rate until a 50:50 mixture was attained at the end of 20 min. The 50: 50 mixture was then used isocratically for an additional 20 min, as described by Angelo and Gian. (2004). The flow rate was 1.0 mL/min, and injection volume was 100 μL. The identification of Chlorophyll and its derivatives was based on the retention time and the visible absorption spectra.