CHAPTER 4

RESULTS AND DISCUSSION

4.1 Experiment 1 Vacuole preparation from Japanese bunching onion leave

4.1.1. Preliminary of exaction protoplast from Japanese bunching onion leave

- **Protoplast takes for difference time of the digestion**

In this preliminary of experiment, extraction protoplasts was used to enzyme cellulase combine with macerozyme (pectinase) and then incubation for 30, 40, 50, 60 min. As show in the figure 4.1, the difference time of incubation found that difference of quantity protoplasts. The incubation for 60 min was shown higher protoplasts than that other of time digestion by observe from density of protoplasts (Fig 4.1). The first 30 min incubation contained cells of spongy parenchyma (Fig. 4.1A arrow), whereas that from 40 min incubations contained cells of palisade parenchyma (Fig. 4.1B arrow). After incubation for 50 and 60 min show contained mostly cell of protoplasts (Fig 4.1 C, D). Therefore, the increasing time of incubation had released mostly cell of protoplasts. Sinha et al., 2003 reported that several factors influence protoplast release, including the extent of temperature, duration of enzyme incubation, concentration of enzyme solution (Takebe et al., 1968). Therefore, time of incubation is another main factor for quantity and qualities of extraction protoplast form the Japanese bunching onion leaves

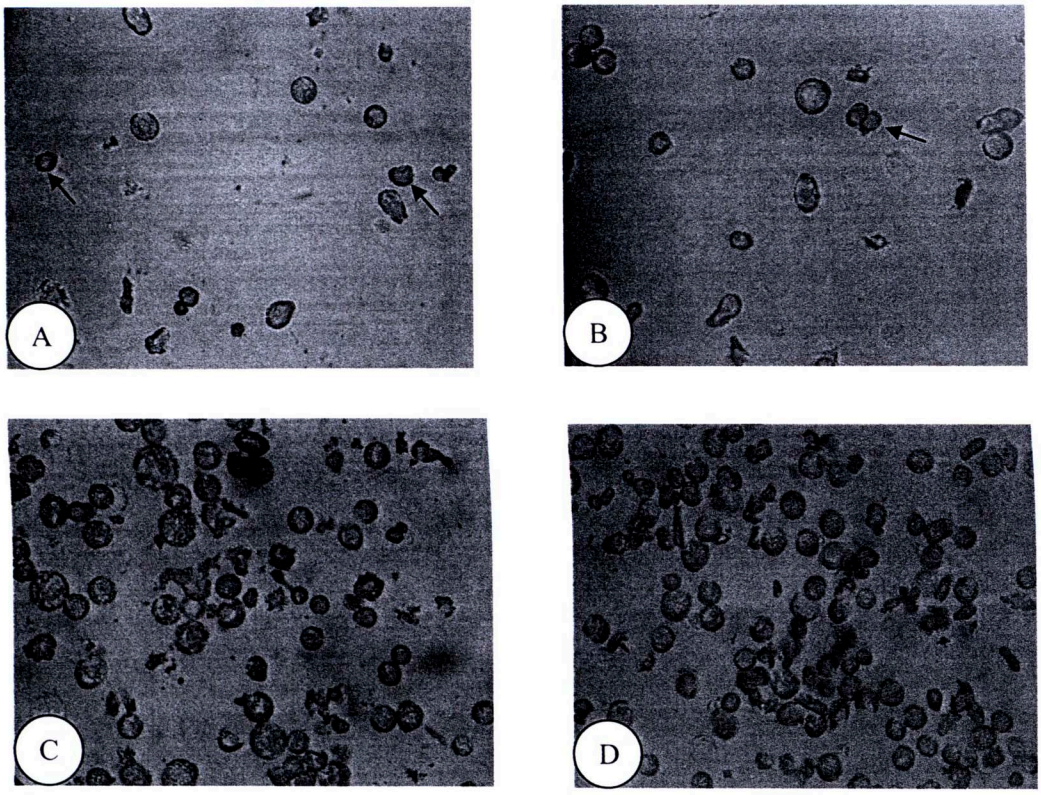

Figure 4.1 Photographs of the extraction protoplasts of Japanese bunching onion leaves: A, digestion for 30 min (×40); B, digestion for 40 min (×40); C, digestion for 50 min (×40); D, digestion for 60 min (×40)

- **Filtration protoplasts with difference layer of filter**

As shown in the figure 4.2 and figure 4.3, Filtration protoplasts used the one layer of Miracloth and then one layer of nylon net and two layer of Miracloth and then one layer of nylon net. Filtration protoplasts with one layer of Miracloth and then one layer of nylon net more protoplasts than the two layer of Miracloth and then one layer of nylon net by observer from density (Fig 4.2 and Fig 4.3 A). However, Protoplasts more fix in the nylon net of filtration from two layers of Miracloth than filtration from one layer Miracloth (Fig 4.2 and Fig4.3 B). These results, filtration protoplasts with one layer of Miracloth and one layer of nylon net had better efficiency separated protoplasts from cell debris. Filtration with Miracloth and nylon net is primary separated cell debris,

other organelles, and broken protoplasts. In generally, protoplasts from leaf tissues of plant have size range 20-50 μm in diameter (Michael et al., 2010). Mastrangelo 1979 reported that pre-moistened Miracloth is also a good filters the protoplasts because Miracloth have pore size of 22-25 μm. Therefore, Miracloth can be used separate the cell debris and other organelles because these have size range 0.05-12 μm (Stephanova et al., 2001). Moreover, nylon net pore size of 40 μm can be separated uniform of protoplast. Thus, one layer of Miracloth and one layer of nylon net were suitable for separate protoplasts from Japanese bunching onion leave.

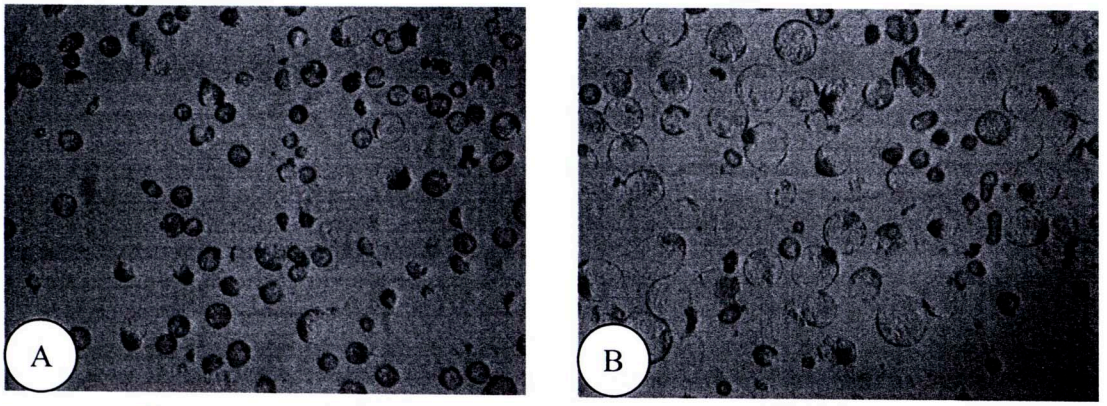

Figure 4.2 Photographs of filtration protoplast thought 1 layer of Miracloth then 1 layer 40 μm of nylon net : A, protoplasts after filtration(×40); B, Protoplasts fix in nylon net layer(×40)

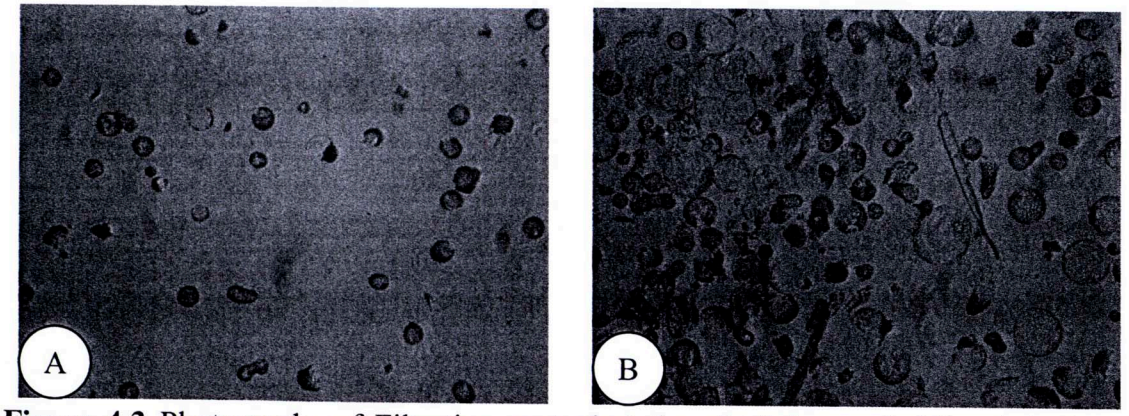

Figure 4.3 Photographs of Filtration protoplast thought 2 layer of Miracloth then 1 layer 40 μm of nylon net : A, protoplasts after filtration(×40); B, Protoplasts fix in nylon net layer(×40)

4.1.2. Accurate protoplast exaction from Japanese bunching onion leave

In this study, protoplasts were extracted from Japanese bunching onion leave and used for studied on the degradation of chlorophyll in vacuole. In order to obtain a high quality and quantity of protoplasts, many factors were involved such as maturity of leave, concentrations of enzymes, incubation time and extraction buffer (Sinha et al., 2003). The differences of incubation times for 30, 40 and 60 min combined with a different of enzyme concentrations (pectinase and cellulase) were used in the experiment. Pectinase is an enzyme that break down pectin to alpha-galacturonic acid while cellulase enzyme breakdown a cellulose to beta-glucose. Both pectin and cellulose are a polysaccharide substrate, these are found in the cell walls of plants (Takebe et al., 1968). For the first experiment to extract the protoplast, two different extraction methods (A and B) were used. The concentrations of cellulase and pectinase (macerozyme) in method B were higher than in method A for 2 times and 4 times, respectively. For the incubation time, the first digestion was approximately 30 min at 37 °C, and second digestion was approximately 1-3 hr at 37 °C. As results, the number of protoplast cells extracted from fresh Japanese bunching onion leave were obtained about $5.8\pm1.2\times10^{4}$cells (methods A) and $1.8\pm0.7\times10^{5}$cells (methods B), repectively. The numbers of protoplast extracted by method B were about three times higher than method A (Table 4.1). A higher number of protoplast cells from method B may involved in the optimum combination between enzyme concentration and incubation time. After enzyme incubation, crude protoplasts were centrifuged to removed debris and broken protoplasts. Boudet et al. (1981) found that the centrifugation of protoplasts suspension and the pellet protoplasts then wash for 3 time with the buffer, would be obtain the uniformity in size of protoplast cell (Fig 4.1A and B). In addition, the

maintenance of osmotic pressure of protoplast by using mannitol and sorbitol containing buffer could kept the natural shape of protoplasts suggesting that those compounds kept osmotic pressure of protoplasts. Smith et al. (1983) reported that when the protoplasts were placed in isotonic or hypotonic solution, cause protoplast shrink or swell. In this study, protoplasts were kept in 50 mM Mes-Tris buffer (pH 6.5) containing 0.6 M mannitol which is the most effective condition to keep the quality of protoplast.

Table 4.1 Number of protoplasts from Japanese bunching onions was extract by different methods

	Methods A (cells)	Methods B (cells)
Number of protoplasts	$5.8\pm1.2\times10^4$	$1.8\pm0.7\times10^5$

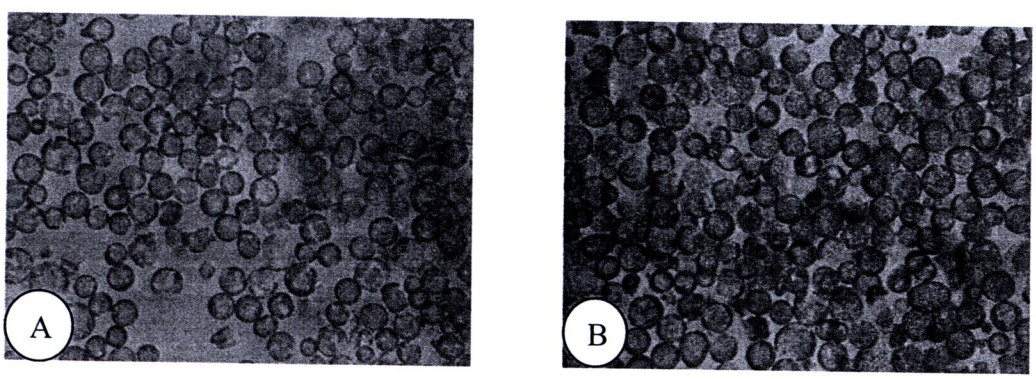

Figure 4.4 Photographs of: A, protoplasts preparation from method A (using 2% (w/v) cellulase and 0.3% (w/v) macerozyme and digestion for 1 hr 30 min)(×40) ; B, protoplasts preparation from method B (using 4% (w/v) cellulase and 1.5% (w/v) macerozyme and digestion for 3 hr 30 min)(×40)

4.1.3 Protoplast purification by Ficoll density gradient centrifugation

In the previous step, the isolation of vacuole resulted to get a crude protoplast. However, a crude protoplast was contaminated with sub-cellular, debris, undigested cell and some broken protoplasts. Thus, the purification of healthy protoplast was done by gradient centrifugation technique. The crude protoplasts were centrifuged discontinuously gradient and the separation of debris was occurred according to the differentiation of density between debris and protoplasts (Boller et al., 1979). In addition, the Ficoll solution was used for protoplasts separation. The concentration of Ficoll was varied from 0.8-15% for gradient extraction of protoplasts (Table 4.2). Larkin (1976) reported that the different concentrations of Ficoll solution would help the separation of protoplasts by gradient density property. Furthermore, Ficoll was chosen for using in the gradient purification because it has high density contributes minimally to osmotic pressure (Attree and Sheffied, 1986). From the result, using of 2, 5,10, and 15% Ficoll gradient purification, yielded a purified protoplast from 5 and 10% interface layer about 29% (48% viability) and 17.5% (72% viability) respectively (Fig 4.2 A). Although, the yield of purified protoplast from 5 and 10% interface gradient layer was higher than that of at 10 and 15% interface gradient layer but at 10 and 15% interface gradient layer showed higher percentage of viability than that of at 5 and 10% interface gradient layer. In this study, the percentage of viability was determined by Evan Blue method that can penetrate into non-viable cells but viable cell showed fluorescence under microscope (Kanai and Edward, 1973) (Fig 4.2 B). In addition, the difference gradient of Ficoll at 2, 3.5, 5, and 10% was examined the yield of purified protoplasts. The result found that at 3.5 and 5% interface gradient layer have 4% of yield and at 5 and 10% interface gradient layer have 7% of yield. For the Ficoll gradient

at 0.8, 2 and 3.5% Ficoll, the protoplasts were found in pellet. At 2, 5 and 10% Ficoll gradient layer also found the protoplasts at 5, 10% Ficoll interface and this layer have 1.07% of yield protoplast. Boudet et al. (1981) reported that protoplasts purification have 20% yield of initial protoplasts. Therefore, from result Ficoll gradient of purification protoplasts was used 2, 5, 10, 15%Ficoll. Using protoplast from 10, 15% interface gradient layers have 17.5% of yield and high percentage viability of protoplasts.

Table 4.2 The Ficoll gradients for purify the protoplasts

Gradient constitutions	Protoplasts location in gradient	% yield	% viable
0.8% Ficoll	ND	ND	ND
2% Ficoll	ND	ND	ND
3.5% Ficoll	pellet	-	-
2% Ficoll	ND	ND	ND
3.5% Ficoll	ND	ND	ND
5% Ficoll	pellet	-	-
2% Ficoll	ND	ND	ND
5% Ficoll	5-10%	1.07%	-
10% Ficoll	Pellet	-	-
2% Ficoll	ND	ND	ND
3.5% Ficoll	3.5 – 5%	4%	-
5% Ficoll	5-10%	7%	-
10% Ficoll	pellet	-	-
2% Ficoll	ND	ND	ND
5% Ficoll	5-10%	29%	48%
10% Ficoll	10-15%	17.5%	72%
15% Ficoll	ND	ND	ND

– means rare percentage yield and viable of vacuole

ND means not detected

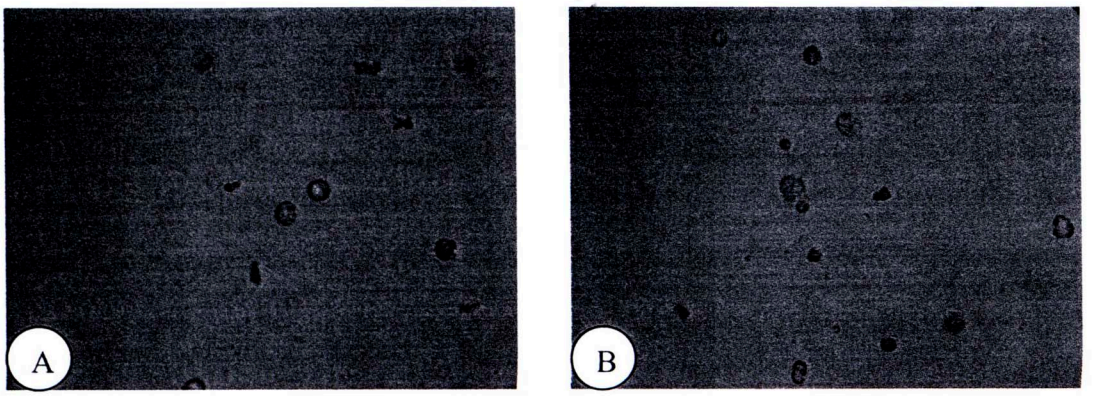

Figure 4.5 Photographs of: A, Protoplasts taken from the 2 and 5% Ficoll interface of the discontinuous gradient (×40); B, Protoplasts taken from the 5 and 10% Ficoll interface of the discontinuous gradient (×40)

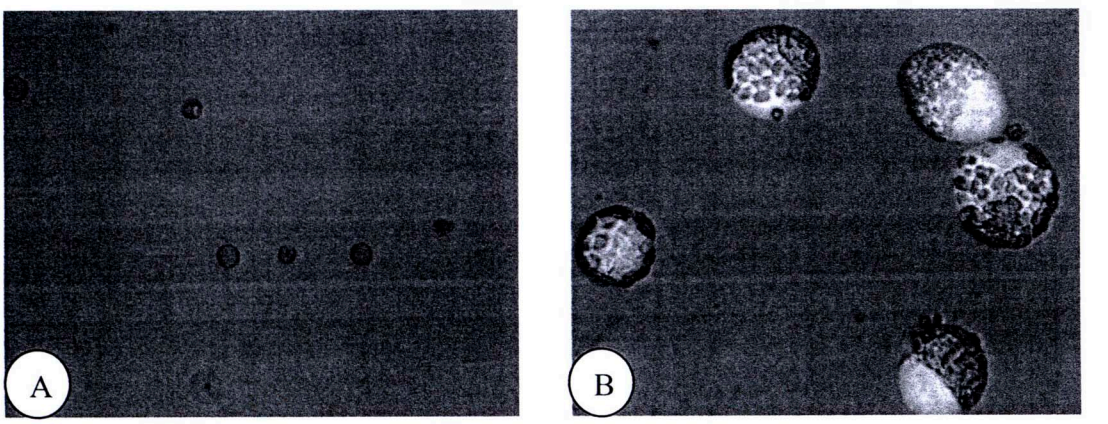

Figure 4.6 Photographs of: A, Protoplasts taken from the 10 and 15% Ficoll interface of the discontinuous gradient (×40); B, protoplasts were stained with Even Blue (×100)

4.1.4 Preliminary of isolation vacuole from purify protoplast

- **Isolated vacuole by sonicate technique**

As shown in figure 4.7, sonicate technique did not isolated vacuole from protoplasts, although the time of sonicate was increased. Sonicate for 10 min display more release vacuole than sonicate for 3 and 6 min observed from number of vacuole (Fig 4.7). Vacuole was shown pink color because vacuole was stain in neutral red with indicator for vacuole. Sonicate for 20 min shown least of vacuole compare with sonicate for 3, 6 and 10 min. The sonication is used to disrupt cells, particularly tissue cultures cells, and to release subcellular organelles (Stephanova et al., 2001). Sonicate is effect cell broken by high frequency sound. However, sonication can not released vacuole or least released vacuole form protoplast. Therefore, sonication cannot be isolate vacuole from protoplast of Japanese bunching onion.

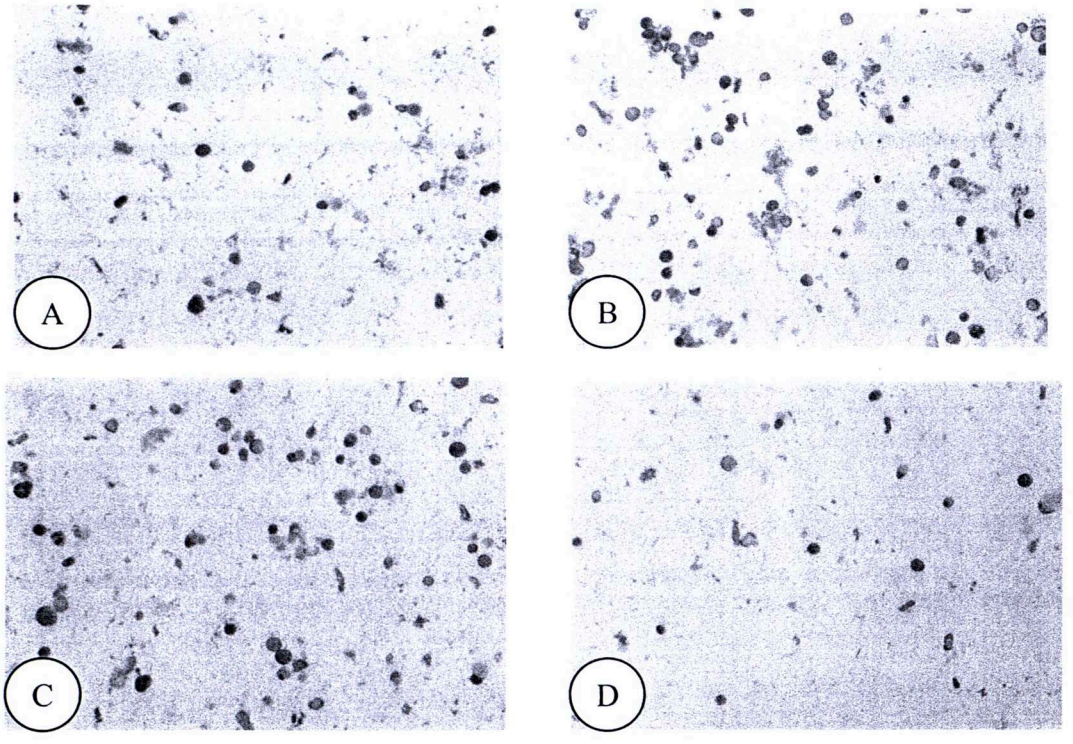

Figure 4.7 Photographs of: A, Sonicate for 3 min (×10); B, Sonicate for 6 min (×10) C, Sonicate for 10 min (×10); D, Sonicate for 20 min (×10) pink color is vacuole stain with neutral red dye

- **Method A (modified from Boudet et al., 1981) from 2, 5, 20% Ficoll gradient centrifugation at 2500 rpm for 40 min**

This method isolation of vacuole used mechanical technique combine with poly-base-induced lysis. These were released vacuole from protoplast by disrupting the plasma membrane with DEAE dextran and shearing forces of centrifugation through Ficoll gradients. Boudet et al., 1981 improved the method by adding the DEAE dextran and Dextran sulfate to gradients with increasing concentration of Ficoll. As shown in figure 4.8, Vacuoles were obtained by omitting the 5% Ficoll and 20% Ficoll layer in centrifuge tube (fig 4.8 A, B). However, vacuoles form this method was contaminated the unlysis of protoplasts and vacuoplasts. Vacuoplasts are spheres consis of vacuoles within adhering cytoplasm within a resealed plasma membrane; contribute to cytoplasmic contamination (Valk et al., 1987). Under this method, centrifugation of Japanese bunching onion protoplasts through polybase-containing gradients with increasing concentrations of Ficoll did not result in 100% lysis. Thus, isolation of these method pure vacuoles from Japanese bunching onion protoplasts was not possible by using the modify method of Boudet et al., (1981).

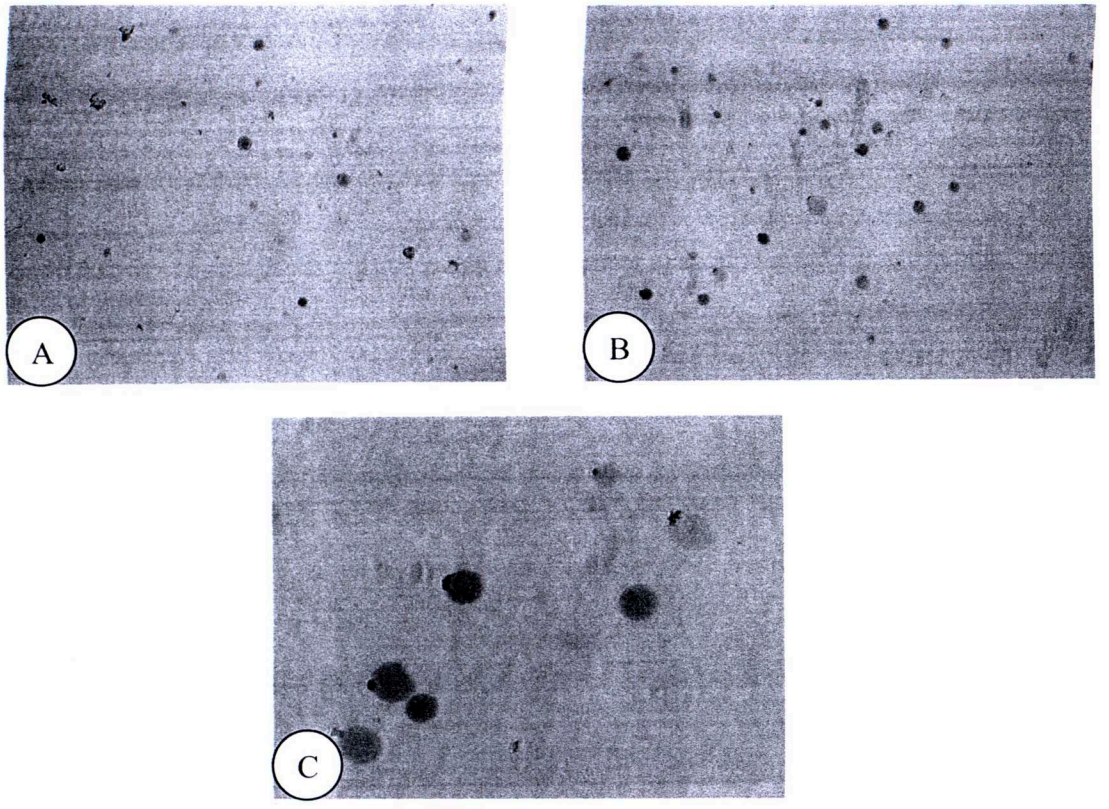

Figure 4.8 Photographs of: A, vacuoles take from 2, 5% Ficoll interface layer of discontinuous gradient (×40); B, vacuoles take from 5, 20% Ficoll interface layer of discontinuous gradient (×40); C, vacuoles take from 5, 20% Ficoll interface layer of discontinuous gradient (×100) pink color is vacuole stain with neutral red dye

- **Method B (modified from Asami et al., 1985)**

This method of isolation vacuole was used polybase-induce lysis technique. Polybase using in this method was polycationic DEAE dextran and dextran sulfate dissolve in osmoticum (0.6 M manitol). The strong (ionic) detergents can solubilize the most hydrophobic membrane proteins (Stephanova et al., 2001). As shown in figure 4.9, isolation vacuole by treating protoplasts with DEAE dextran gentle swirled for 1, 5, 10 and 20 min. Protoplasts with DEAE dextran swirling for 5 min more released vacuole than swirling for 1, 10 and 20 min. Whereas, increasing time of swirling protoplast in DEAE dextran found least released vacuole from protoplast. DEAE dextran

(polycationic) disrupted the tonoplasts (membrane of vacuole) and it is affect to the vacuoles (Alain et al., 1981). Therefore, isolation of vacuole with DEAE dextran gentle swirling for 5 min was good released vacuoles from protoplasts of Japanese bunching onion.

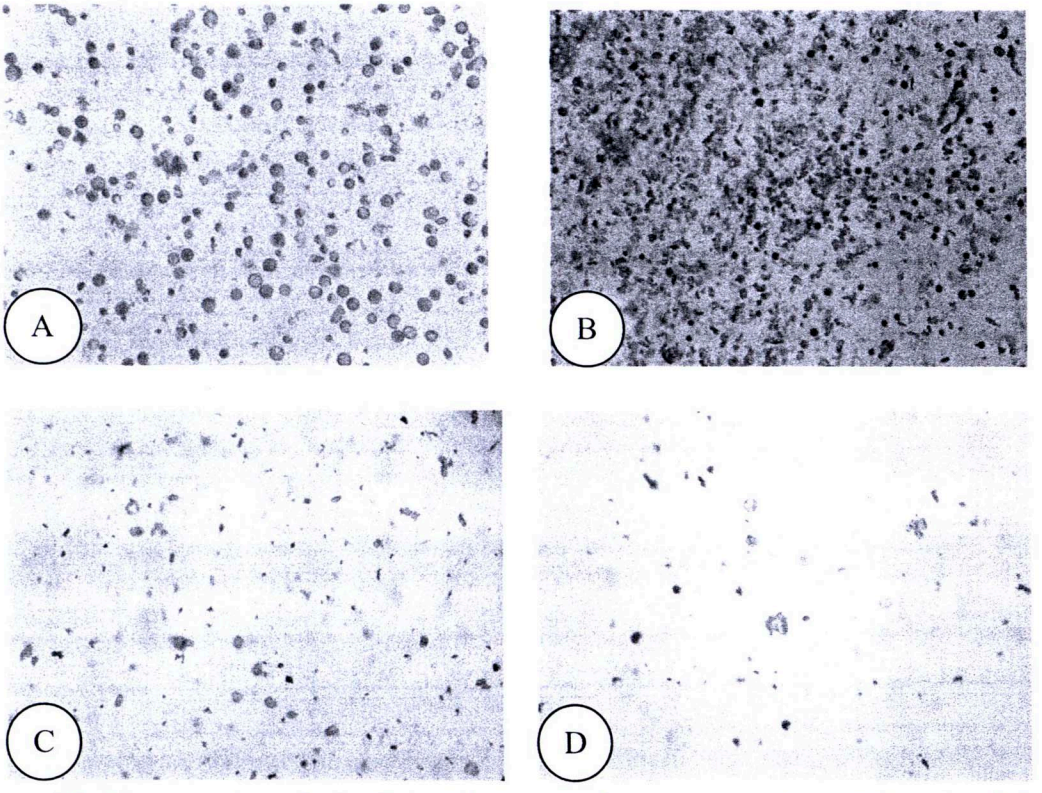

Figure 4.9 Photographs of: A, Extraction vacuoles were gentle swirling in DEAE dextran for 1 min (×10); B, Extraction vacuoles were gentle swrling in DEAE dextran for 5 min(×10); C, Extraction vacuoles were gentle swirling in DEAE dextran for 40 min (×10); D, Extraction vacuoles were gentle swrling in DEAE dextran for 20 min (×10) pink color is vacuole stain with neutral red dye

4.1.5 Accurate vacuole isolation and purification from purify protoplast

In this experiment the vacuoles were prepared according to a modified procedure from Alain et al. (1981) and Asami et al. (1984) (method A and B). The method A, lysis of protoplasts and purification of vacuoles occur in the same time during the centrifugation. While, the method B, vacuoles were extracted and purified step by step with Ficoll gradient. The vacuoles were obtained from the lysis of protoplasts with DEAE dextran (polybase). Polybase efficiently ruptures the plasma membrane, the cytosolic were isolated and then adjust DEAE dextran to be neutralize by dextran sulfate for protection the disruption of vacuoles membranes by polybase which is a polycation. The vacuole isolation by methods A used to purify protoplasts by centrifugation through discontinuous gradient contain DEAE dextran and dextran sulfate. The efficiency of protoplast extraction at the optimal condition (centrifugation speed, time and concentration of gradient) was investigated. In this experiment, the protoplasts 1×10^6 cells was centrifuged through the gradient of 2% Ficoll containing DEAE dextran, 5% Ficoll containing dextran sulfate, and 20% Ficoll, and then the vacuole banded at the interface between 2 and 5% Ficoll, and 5 and 20% Ficoll. As a result, this extraction condition was not effective to isolated and purified of vacuole due to the vacuoles found in interface between DEAE dextran solution layer and in the pellet. Thus, the increase of gradient layer and change of gradient volume for isolation and purification of vacuole were used (Table 4.4). The result found that the vacuoles were sedimented in the interface layer between DEAE dextran solution and in the pellet as in the previous extraction condition and also got a small amount of vacuole (Table 4.4). Normally, the vacuoles were not stability in DEAE dextran solution due to DEAE dextran solution disrupts the tonoplasts, the vacuole membrane (Alain et al., 1981). The

vacuole isolation method B was released from 1×10^5 protoplasts by using DEAE dextran/dextran sulfate (polycationic) then the isolated vacuole were purify by Ficoll gradient extraction. The isolated vacuole was added with Ficoll at concentration of 5% and the amount of purified vacuole was obtained the yield of 10 to 20%. Increasing the gradient of layer, concentration of gradient, speed and time of centrifugation have an effect on decreasing yield of vacuole or not appear the vacuole in Ficoll gradient. Because the protoplasts have similar size and density to vacuole which difficulties separated vacuole by Ficoll gradient and centrifugations (Alain et al., 1981). The purity of vacuoles can be assessed by staining with neutral red and then observe under microscopy (Fig 4.4). Neutral red diffuses into the vacuole and be stain bright pink to red depending on the pH in the vacuole (Buser-Suter et al., 1982). From result, the isolation and purification by method B got 10-20% yield of initial number of protoplasts. It can be obtained vacuole for verify of purification vacuole.

Table 4.3 Polybase procedure for various Japanese bunching onions (method A)

Volume of layers(ml)	Gradient constitution	DD (mg/mL)	DS (mg/mL)	pH	Time and speed of centrifugation	Vacuoles location in gradient
5	2%Ficoll	4	0	6.5	30min, 2500 rpm	pellet
2	3.5%Ficoll	0	4	8		
2	20%Ficoll	0	0	8		
5	2%Ficoll	4	0	6.5	30min, 2000 rpm	2-5%Ficoll
2	5%Ficoll	0	4	8		
2	20%Ficoll	0	0	8		
5	2%Ficoll	4	0	6.5	30min, 2500 rpm	2-5%Ficoll
2	5%Ficoll	0	4	8		
2	20%Ficoll	0	0	8		
5	2%Ficoll	4	0	6.5	40min, 2500 rpm	2-5%Ficoll,
2	5%Ficoll	0	4	8		5-20%Ficoll
2	20%Ficoll	0	0	8		
5	2%Ficoll	4	0	6.5	30min, 4000 rpm	2-5%Ficoll
2	5%Ficoll	0	4	8		
2	20%Ficoll	0	0	8		

Table 4.3 (Cont.) Polybase procedure for various Japanese bunching onions (method A)

Volume of layers(ml)	Gradient constitution	DD (mg/mL)	DS (mg/mL)	pH	Time and speed of centrifugation	Vacuoles location in gradient
3	2%Ficoll	4	0	6.5	30min, 2000 rpm	3.5-5%Ficoll, pellet
2	2%Ficoll	0	4	8		
2	3.5%Ficoll	0	0	8		
2	5%Ficoll	0	0	8		
3	2%Ficoll	4	0	6.5	30min, 2000 rpm	2-3.5%Ficoll,
2	3.5%Ficoll	0	4	8		3.5-5%Ficoll
2	5%Ficoll	0	0	8		
2	7%Ficoll	0	0	8		
3	2%Ficoll	4	0	6.5	30min, 2000 rpm	2-3.5%Ficoll,
2	3.5%Ficoll	0	4	8		3.5-5%Ficoll
2	5%Ficoll	0	0	8		
2	10%Ficoll	0	0	8		

Table 4.4 Polybase procedure for various Japanese bunching onions (method B)

Volume of layers(ml)	Gradient constitutions	pH	Time and speed of centrifugation	Vacuoles location in gradient	% yield
6	5% Ficoll	8	25 min, 10,000×g	0-5%Ficoll	10-20%
4 4	2% Ficoll 5% Ficoll	8 8	30min, 10,000×g	2-5%Ficoll	>1%
4 3	2% Ficoll 5% Ficoll	8 8	30 min, 5,000×g	ND	ND
4 3	2% Ficoll 5% Ficoll	8 8	30 min, 10,000×g	ND	ND
3 3	1% Ficoll 5% Ficoll	8 8	60 min, 15,000×g	1-5%Ficoll	>1%
1 4	2% Ficoll 5% Ficoll	8 8	60 min, 10,000×g	2-5%Ficoll	>1%
1 5	2% Ficoll 5% Ficoll	8 8	60 min, 10,000×g	2-5%Ficoll	1.6%

ND means not detected

Table 4.4 (Cont.) Polybase procedure for various Japanese bunching onions (method B)

Volume of layers(ml)	Gradient constitutions	pH	Time and speed of centrifugation	Vacuoles location in gradient	% yield
1 5	2% Ficoll 5% Ficoll	8 8	60 min, 12,000×g	2-5%Ficoll	6.5%
1 5	2% Ficoll 5% Ficoll	8 8	60 min, 15,000×g	2-5%Ficoll	0.6%
1 6	2% Ficoll 5% Ficoll	8 8	60 min, 15,000×g	2-5%Ficoll	1.2%
1 6	2% Ficoll 5% Ficoll	8 8	30 min,10,000×g	2-5%Ficoll	8%
1 6	2% Ficoll 5% Ficoll	8 8	40 min, 10,000×g	2-5%Ficoll	4%
1 7	2% Ficoll 5% Ficoll	8 8	60 min, 10,000×g	2-5%Ficoll	2%
1 7	2% Ficoll 5% Ficoll	8 8	60min, 15,000×g	2-5%Ficoll	1%

Table 4.4 (Cont.) Polybase procedure for various Japanese bunching onions (method B)

Volume of layers(ml)	Gradient constitutions	pH	Time and speed of centrifugation	vacuoles location in gradient	% yield
2 3 3	2% Ficoll 3.5%Ficoll 5% Ficoll	8 8 8	30 min, 10,000×g	ND	ND
3 1 2	2% Ficoll 3.5%Ficoll 5% Ficoll	8 8 8	30 min, 10,000×g	ND	ND
2 2 2	2% Ficoll 3.5%Ficoll 5% Ficoll	8 8 8	30 min, 10,000×g	ND	ND
3 1 2	2% Ficoll 3.5%Ficoll 5% Ficoll	8 8 8	30 min, 10,000×g	pellet	-
3 1 2	2% Ficoll 3.5%Ficoll 5% Ficoll	8 8 8	30 min, 10,000×g	pellet	-

– means rare percentage yield and viable of vacuole

ND means not detected

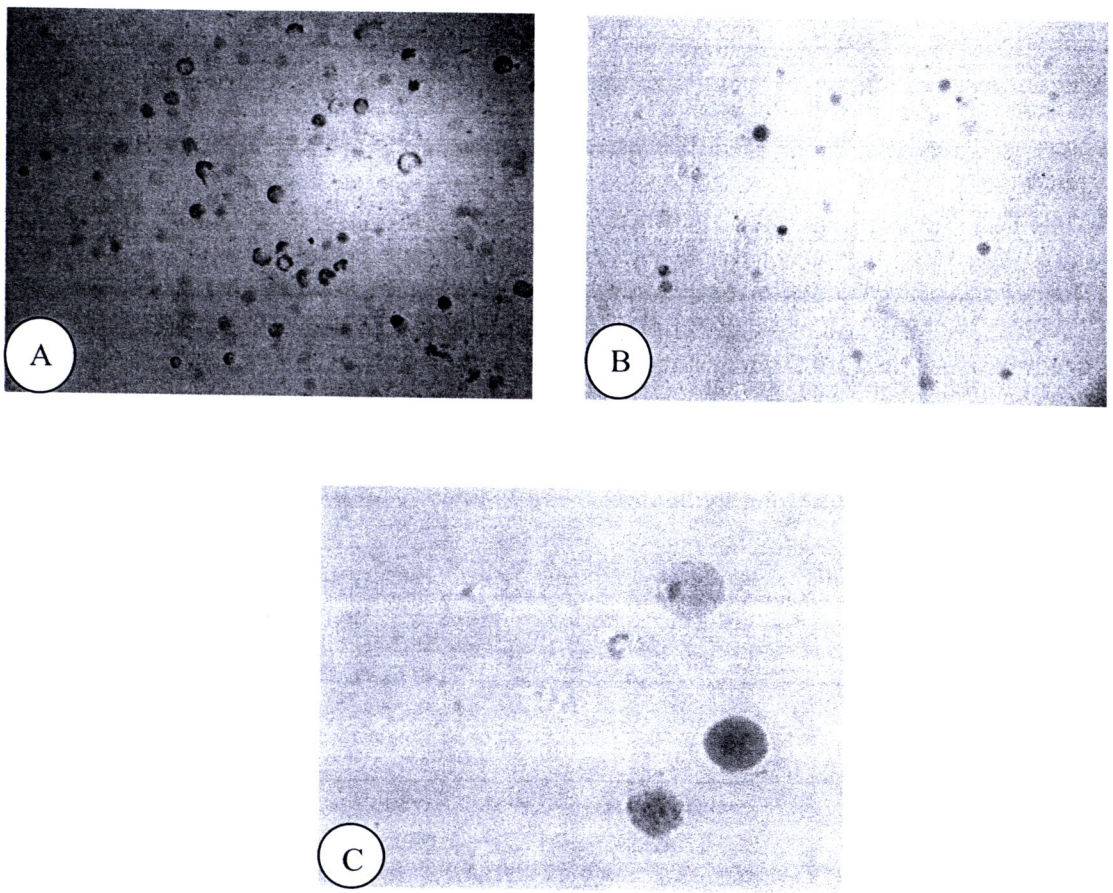

Figure 4.10 Photographs of: A; The solution obtain after DEAE dextran/dextran sulfate lysis without purification (×40); B (×40) and C (×100), Vacuoles after purification by vacuoles were stained with neutral red

4.1.6 Study of hydrolytic enzyme in purification vacuole

The vacuole from the previous step were used for checking a purity. The contamination of other organelles in the vacuole was investigated by using hydrolase maker enzyme including catalase, NADH cytochrome C reductase, NADH malate dehydrogenase, and alcohol dehydrogenase which were the maker enzyme that found in other organelle or cytosol. Acid phophatase and β-glucosidase were used as an indicator enzyme for vacuole. The activities of catalase, NADH cytochrome C reductase, NADH malate dehydrogenase and alcohol dehydrogenase were evaluated in a purify of vacuole. A high activity of these enzymes referred to a high contamination of other organelles in the purified vacuole. In contrast, a high activity of acid phophatase and β-glucosidase presented a high purity of vacuole. The activities of catalase, NADH cytochrome C reductase and NADH malate dehydrogenase (maker enzymes in organelles peroxisome, mitochondria and microsome or endoplasmic reticulum (ER)) were lowered in the purified vacuole, this could be explain a less cytoplasmic contamination (Nishimura et al., 1978). Moreover alcohol dehydrogenase (maker enzyme in cytosol) activity was not detected. Furthermore, acid phophatase and β-glucosidase activities in vacuoles were higher than that in the protoplasts. Therefore, the purified vacuoles were low contamination from other organelle or cytosol. Acid phosphatase has been shown to be located in vacuoles isolated from various plant materials (Butcher et al., 1977; Mettler et al., 1979; Thom et al., 1982; Walker-Simmon, 1977).

Table 4.5 The maker enzyme activity of vacuole from the Japanese bunching onion (The maker enzyme in other organelles)

Enzyme	Activity in Vacuole	
	/mg protein	10^4 vacuoles
Catalase (unit/min)	0.0066±0.02	$14±2.6\times10^{-5}$
NADH cytochrome C reductase (unit/min)	0.0012±0.02	$25±0.1\times10^{-6}$
NADH malate dehydrogenase (unit/min)	0.00076±0.02	$16±0.1\times10^{-6}$
Alcohol dehydrogenase (unit/min)	ND	ND

ND means not detected

Table 4.6 The maker enzyme activity of vacuole and protoplasts from the Japanese bunching onion (The maker enzyme in vacuoles)

Enzyme	Activity in protoplasts		Activity in vacuoles	
	/mg protein	10^4 protoplasts	/mg protein	10^4vacuoles
Acid phosphatase (μmole/min)	14.0±0.001	0.70±0.16	18.0±0.001	0.72±0.00
B-Glucosidase (μmole/min)	0.026±0.002	0.021±0.00	0.52±0.02	0.12±0.00

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

4.2.1 Change of chlorophyll derivatives level in protoplasts and vacuoles of Japanese bunching onion

Catabolites of chlorophyll *a* including chlorophyllide *a*, pheophytin *a*, pheophorbide *a*, pyropheophorbide *a* and C13^2-hydroxychlorophyll *a* were identified by detection with HPLC. The level of each chlorophyll catabolite in protoplasts and vacuoles was very different. The protoplasts and vacuole were incubated in a reaction solution for 0, 3, 6, 18 and 24 hr at 25 °C and than detected the content of chlorophyll derivative. The level of chlorophyllide *a* in the protoplasts was increased and it was significantly higher than that of in the vacuole (Fig 4.5A). Pheophytin *a* level started to increment in 3 hr of incubation in both vacuole and protoplasts. The level of pheophytin *a* in protoplasts shown no significant difference throughout of incubation period (Fig 4.5 B). The formation of pheophytin *a* indicated that the Mg-dechelatase in vacuole possibly involved in removing of Mg atom from chlorophyll *a* to form pheophytin *a*. The level of pheophorbide *a* in the protoplasts increased in 3 hr of incubation and it was higher than that of in the vacuole (Fig 4.5 C, D). The level of pyropheophorbide *a* in both vacuoles and protoplasts was remained constant during 6 hr of incubation and then the content of pyropheophorbide *a* in protoplasts was dramatically increased to a level higher than that of in vacuole (Fig 4.5 D). There was no change of pyropheophorbide *a* in vacuoles during incubation for 24 hr. The level of C13^2-hydroxychlorophyll *a* in the protoplasts was increased and it was higher than that of in the vacuole throughout of incubation period (Fig 4.5 E). From the results showed that the high chlorophyll

derivatives may caused by the activity of chlorophyll degrading enzyme in both organelles. However, the level of chlorophyllide *a*, pheophorbide *a*, pyropheophorboie *a*, and C13^2-hydroxychlorophyll *a* in protoplasts were higher than in vacuoles. The previous studied found that chlorophyll degrading enzymes activity was high in the chloroplasts compared with other organelles (Matile et al., 1997). In addition, Taiz (1992) reported that the high level of pheophytin *a* lead to form pheophytin *a* from chlorophyll *a* was found in vacuole. From the results of the experiment, the accumulation of chlorophyll derivatives was also found in vacuole of Japanese bunching onion and these was agreed with the above studied.

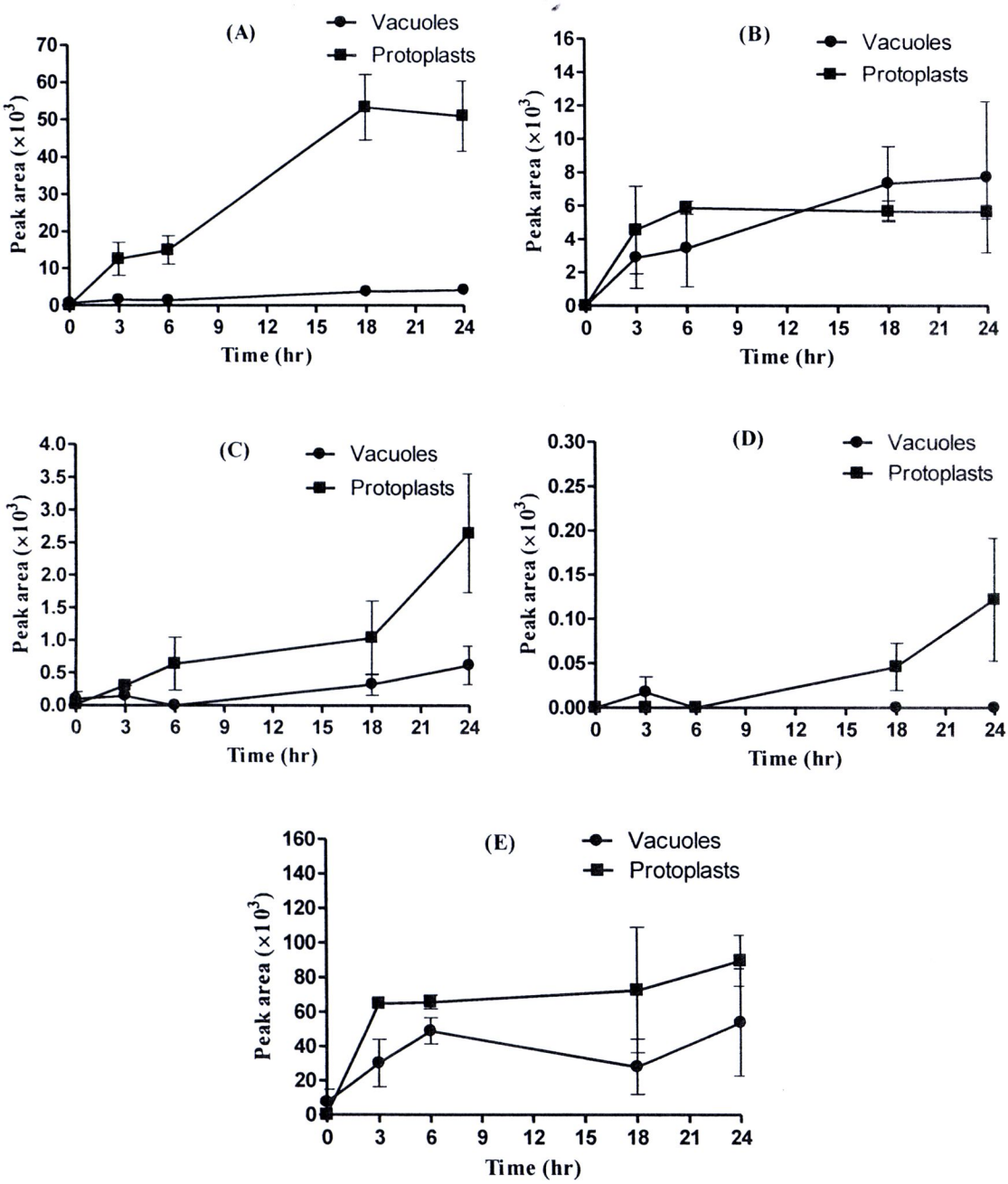

Figure 4.11 Change of chlorophyll catabolites in non boiled vacuoles and protoplasts of Japanese bunching onion during incubation for 0, 3, 6, 18 and 24 hr at 25 °C A: Chlorophyllide *a*; B:Pheophytin *a*; C: Pheophorbide *a*; D: Pyropheophorbide *a* ; E: C13^2-hydroxychlorophyll *a*

4.2.2 Change of chlorophyll derivatives level in boiled protoplasts and vacuoles of Japanese bunching onion

Chlorophyll derivative (chlorophyllide *a*, pheophytin *a*, pheophorbide *a*, pyropheophorboie *a* and C13^2-hydroxychlorophyll *a*) were determined in protoplasts and vacuoles boiled by detection with HPLC. In this experiment, the boiled protoplasts and vacuole were incubated in reaction solution for 0, 3, 6, 18 and 24 hr at 25 °C and than determined the content of chlorophyll derivatives. The level of chlorophyllide *a* in the protoplasts was increased in 18 hr and reached a value higher than the initial level in 24 hr of incubation and it was similar with that of in the vacuole (Fig 4.6A). Pheophytin *a* level in vacuoles and protoplasts was remained constant during 18 hr of incubation and then the level of pheophytin *a* in vacuoles was increased to higher level than that in protoplast (Fig 4.6B). The level of pheophorbide *a* in the protoplasts increased in 18 hr of incubation but it was not changed in the vacuoles during incubation period (Fig 4.6C). The level of pyropheophorbide *a* in both vacuoles and protoplasts was no significant change during incubation period (Fig 4.5D). The level of C13^2-hydroxychlorophyll *a* in the protoplasts was increased in 3 hr of incubation then it was decreased and maintained constant until the end of incubate time (Fig 4.6E). In contrast, the level of C13^2-hydroxychlorophyll *a* in vacuole increased in 6 hr and then decreased (Fig 4.6E). From the results showed the least level of chlorophyll derivatives in boiled vacuole and protoplast. Moreover, boiled vacuole and protoplast showed lower level of chlorophyll derivatives than that non boiled vacuoles and protoplasts. The vacuoles and protoplasts were boiled to denature the protein in vacuole and protoplast. The activity of enzymes in vacuoles and protoplasts were also inhibited by heat treatment thus the level of the chlorophyll derivatives in boil vacuoles and protoplasts were lowered.

Dissanayake (2009) reported that *in vitro* test of Japanese bunching onion by using boiled crude enzyme extract was not found a formation of chlorophyllide *a* and pheophytin *a* during incubation. This indicated that chlorophyllase, Mg-dechelatase and chlorophyll degrading peroxidase were degraded in vacuole of Japanese bunching onion. The results of Dissanayake (2009) is concomitant with the finding of this experiment.

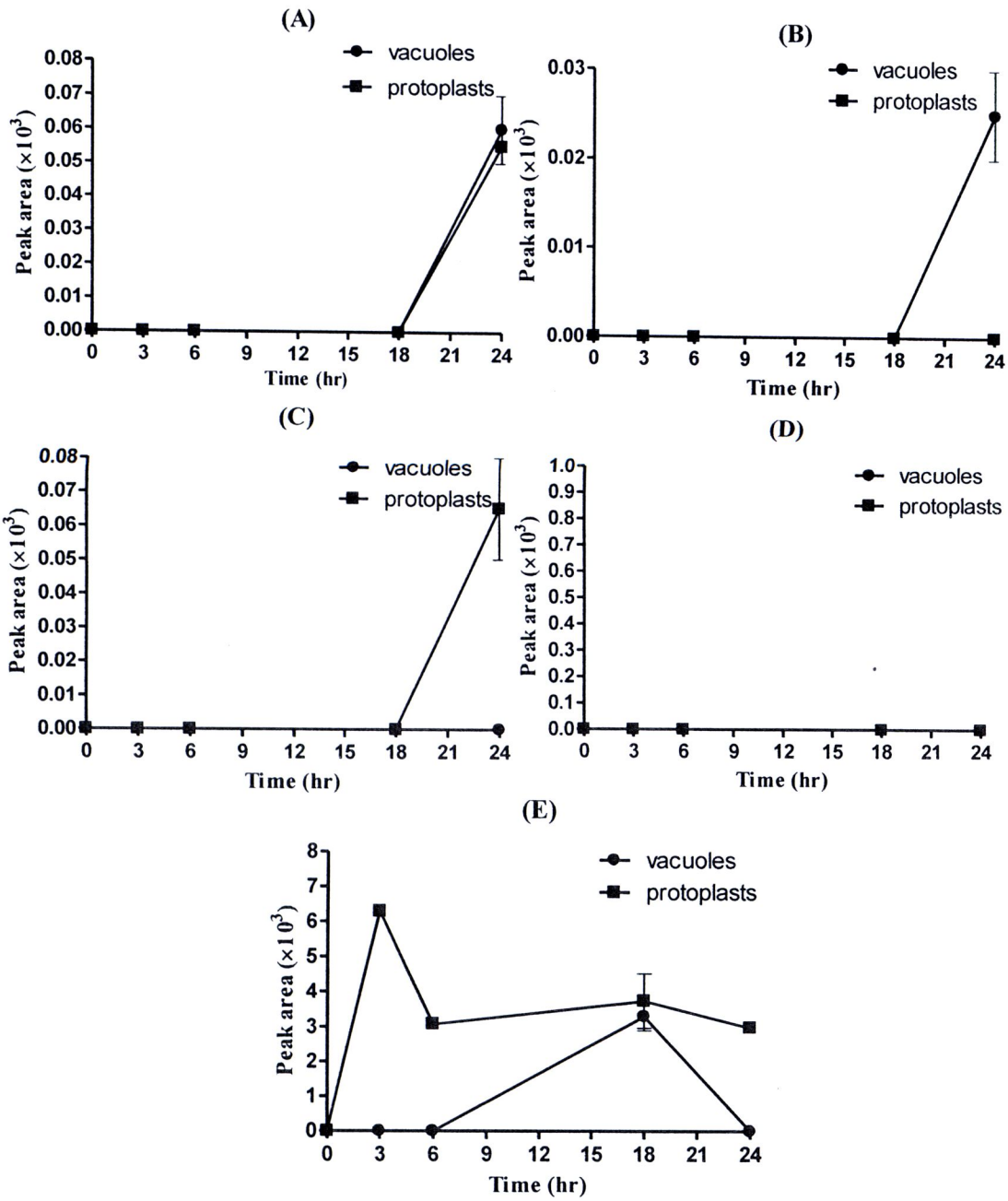

Figure 4.12 Change of chlorophyll catabolited in boiled vacuoles and protoplasts of Japanese bunching onion during incubation for 0, 3, 6, 18 and 24 hr at 25 °C A: Chlorophyllide *a*; B:Pheophytin *a*; C: Pheophorbide *a*; D: Pyropheophorbide *a* ; E: C13^2-hydroxychlorophyll *a*

4.3 Experiment 3 Chlorophyll degradation in vacuoles of Japanese bunching onion

4.3.1 Changes of leaf color, hue angle and chlorophyll content

Japanese bunching onion stored at 25 °C turned progressively to yellow starting from the leaf tip toward to the base during 3 days of storage, whereas leaves stored at 4 °C maintained the green color. Leaf colour reported by hue angle value was greater reduced in leaves stored at 25 °C compared with leaves stored at 4 °C (Fig 4.6). Chlorophyll *a* and chlorophyll *b* contents were decreased which accordant to green color reduction. The rate of reduction of chlorophyll *a* in leaves stored at 25 °C was higher than that in leaves stored at 4 °C. The chlorophyll *b* decreasing in leaves stored at 25 °C was faster than that stored at 4 °C (fig 4.7). The marketability of fresh Japanese bunching onion depends mainly on the appearance of the leaves, especially their green color. Yellowing of leaf during display in market detracts the consumer preference. The change in hue angle value of leaves stored at 25 °C was related with the reduction of chlorophyll content. Leaves stored at 4 °C showed the same trend of change in hue angle value and reduction of chlorophyll content, however it found in lower rate. This result indicated the degradation of chlorophyll is the main cause factor of yellowing in stored Japanese bunching onion and the results were similar with other leafy vegetables such as a yellowing in parsley, spinach (Yamauchi and Watada., 1991; Yamauchi and Watada., 1993) and broccoli florets (Yamauchi et al., 1999)

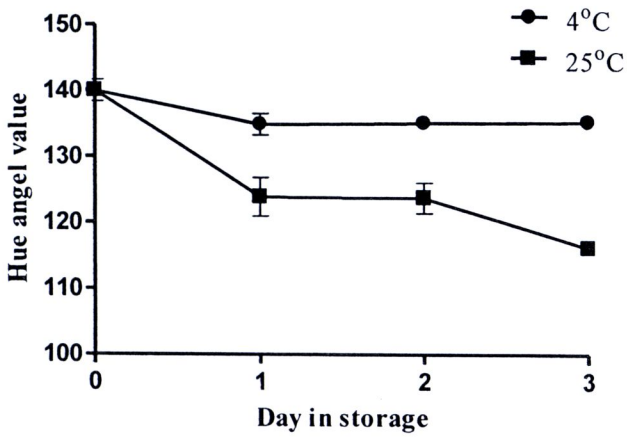

Figure 4.13 Change of hue angle value in Japanese bunching onion leaves during storage at 4 and 25 °C. Vertical bars represent average values with standard deviation (n=3).

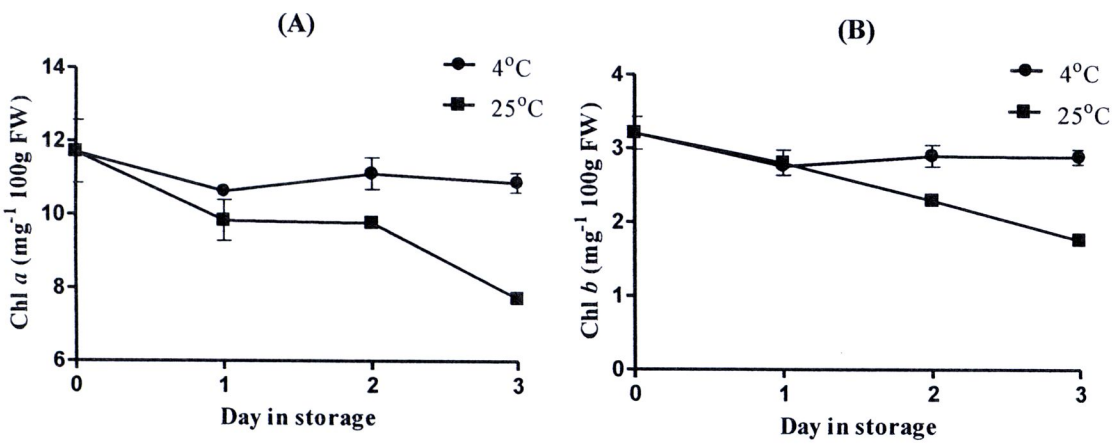

Figure 4.14 Changes in chlorophyll *a* and chlorophyll *b* content in Japanese bunching onion leaves during storage at 4 and 25 °C. Vertical bars represent average values with standard deviation (n=3).

4.3.2 Changes of Chl *a* and its derivatives levels in vacuole of Japanese bunching onion

Chlorophyll *a* and its catabolites such as chlorophyllide *a*, pheophytin *a*, pheophorbide *a*, pyropheophorboie *a* and C13^2-hydroxychlorophyll *a* were detected by HPLC. The results showed that chlorophyll *a* decreased on day 2 at 4 °C and on day 1 at 25 °C (Fig 4.8). The decrease of chlorophyll *a* at 25 °C was accompanied with the increase of chlorophyllide *a*, Pheophytin *a*, Pheophorbide *a*, and pyropheophorbide *a* levels (Fig 4.8E) but the C13^2-hydroxychlorophyll *a* was unchanged and had lower level than that at 4 °C (Fig 4.8F). In contrast, at 4 °C, the decrease in chlorophyll *a* corresponded with the increase of C13^2-hydroxychlorophyll *a* (Fig 4.8A, 4.3F). Yamauchi (1999) report that the accumulation of C13^2-hydroxychlorophyll *a* was observed in broccoli stored at low temperature. This implied that C13^2-hydroxychlorophyll *a* is an indicator of chlorophyll catabolism in plant during storage at low temperature. The results demonstrated that chlorophyll breakdown in the vacuole contributed to yellowing in Japanese bunching onion, particularly during storage at 25 °C. Chlorophyll degradation at high storage temperature (25 °C) followed the same pathway as reported in other green vegetables, such as parsley, spinach (Yamauchi and Watada 1991,1993) and green beans (Monreal, 1999) as evidenced by the accumulation of chlorophyll a derivatives. In this experiment shown the high contents of pheophytin *a* and C13^2-hydroxychlorophyll *a*. They were the main chlorophyll catabolites in Japanese bunching onion leaves. In contrast, at low storage temperature (4 °C), chlorophyll degradation seemed to follow the peroxidase or chlorophyll oxidase mediated pathway which conforms to earlier observations by Yamauchi (1999). During storage at 4 °C, a different mechanism for chlorophyll loss may operate and should be proof in the study

future. From the results the high contents of chlorophyll derivatives were shown in vacuole of Japanese bunching onion during storage at 4 °C and 25 °C. Moreover, content of chlorophyll derivatives in vacuole of Japanese bunching onion stored at 25 °C was higher than that vacuole of Japanese bunching onion stored at 4 °C. Dissannayake (2009) reported that Japanese bunching onion storage at 25 °C for 3 day shown the increase of chloroplast size and number, and found a movement of plastoglobuli into vacuole. During leaf senescence of soy bean leaf (Guiamét et al., 1999), broccoli florets (Terai et al., 2000) and leaf of Japanese bunching onion (Dissanayake, 2009), plastoglobuli increased the size and number (Matile et al., 1999). Therefore, chlorophyll degradation in Japanese bunching onion could be degraded in both organelles, chloroplast and vacuole. This basic information is vital to manipulate the genetic and environmental factors to control yellowing and improve shelf life of Japanese bunching onion.

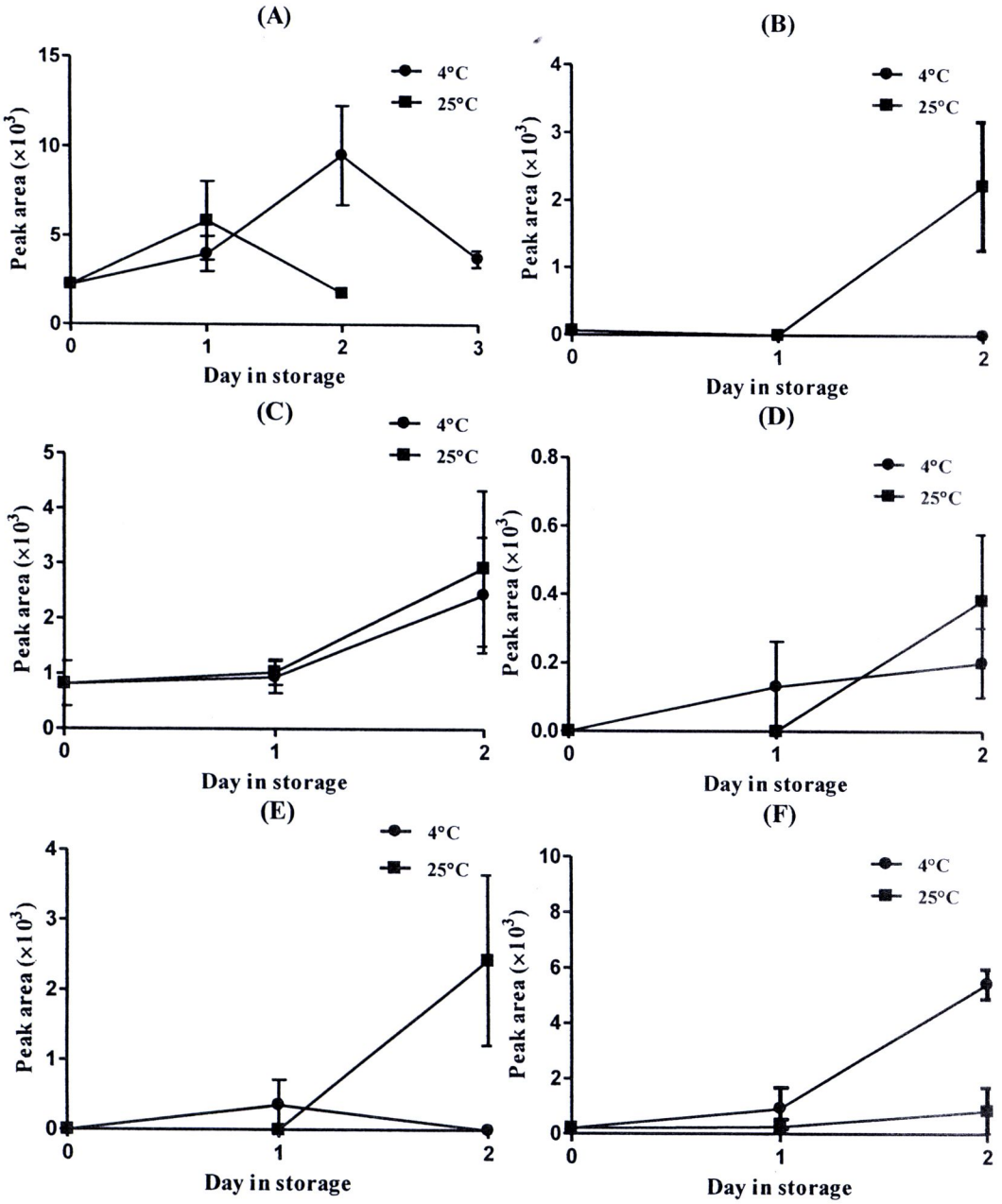

Figure 4.15 Changes of chlorophyll derivatives in Japanese bunching onion during storage at 4 and 25°C. A: Chlorophyll *a*; B: Chlorophyllide *a*; C: Pheophytin *a*; D: Pheophorbide *a*; E: Pyropheophorbide *a* and F: C13^2-hydroxychlorophyll *a*