CHAPTER 2

LITERTURE REVIEWS

2.1 General information of Japanese bunching onion

Japanese bunching onion (*Allium fistulosum*) is a perennial onion. Other names that may be applied to this plant include welsh onion, green onion, salad onion and in some countries call spring onion which using condiments for food, fresh vegetable and cooking vegetable in most of countries in East Asia especially in Japan, China and Korea(Kumazawa et al., 1965; Ford-Lloyd and Armstrong, 1993).

In Japan have bunching onion more than 100 cultivars. Cultivars are identified according to growing season, hardiness, blanching characteristics and, degree of tillering. For example, major cultivar types Kaga and Kujyo are known for their growth and dormancy adaptation to coolest and warmest growing environments in Japan. Senju is a type with an intermediate adaptation. Kaga and Kujyo types are usually grown for pseudostem production, whereas Senju types are tiller producers. Nebuka is term that also identifies cultivars producing long pseudostem. Hanegi refers to cultivars generally grown for tiller production. Japanese bunching onion are plants vigorous and grown as annual herb which never produce enlarge bulb. Leaves are hollow and circular in cross section 15-30 cm in length. Shoots (tillers) increase by tillering. The number of tillers emerge from a seeding in growing season is mainly depend on the cultivar or group. Kujyo-negi group in Japan produce 20-30 tillers in a growing season. The bulbs are elongate and not much thicker than the stem. After a cold spell, bunching onions send up hollow stalks topped with little greenish flowers in round umbels. Japanese bunching

onion are grown in colder climates. Japanese bunching onion can be grown from seeds and more plants can be produced by division of established plants.

2.1.1 Foliage leaf structure of Japanese bunching onion

The Japanese bunching onion similar onion has similar anatomy of leaf compared with other onion that an outer epidermal layer coated with a waxy cuticle which contains many sunken stomata. Below this are three or four layers of columnar palisade cells. Within the palisade layer are numerous interconnecting elongated cells called laticifers. These contain the milky fluid which oozes when leaf is cut across. This fluid is rich in sulfur-containing flavor compounds (Hayward, 1938). Below the palisade cells are about two layers of larger and rounder cells surrounded by much intercellular air space. Many chloroplasts occur within these cells, close cell well adjacent to air spaces. Below these chlorophyll-dense layers lie the vascular bundles surrounded by large parenchymatous cell containing fewer chloroplasts than the outer layers. The leaf cavity is lined by senescent cells lacking in protoplasm. The vascular bundles are surrounded by a compact layer of elongated cell that form a bundle sheath. The chloroplasts in this layer of cells have been shown to contain starch (Wilson et al., 1985). During the early stages of onion foliage leaf growth, while the leaf is within the sheath of next older leaf, the leaf blade elongates but the sheath does not. When the leaf tip emerges from the pore of the older leaf the sheath begins to elongate also (Heath and Hollies, 1965). Initially cell division occurs throughout the length of onion leaf blade, but it continues for longest in the basal parts and gives rise to large proportion of fully grown blade. During enlargement, the cells in the central region do not keep pace with the

enlargement of outer layer, and the central cavities develop within the leaf. Later, some of the inner parenchyma cell decay, adding to size of cavity (Hayward, 1938).

2.1.2 Postharvest Senescence of Japanese bunching onion

Japanese bunching onion (*Allium fistulosum*) is a highly perishable crop. Harvesting causes severe stress condition of nutrient and water deficiency and changes in hormonal status, determining an inability to maintain homeostasis and the appearance of senescence symptoms. Perishable crop have relative short shelf life in terms of external appearance as well as other quality parameters such as microbial growth and nutritional content. Japanese bunching onion loss of quality during storage is due mainly to chlorophyll degradation leading to leaf yellowing which renders the produce unacceptable to consumers. Yellowing of Japanese bunching onion is one the main factors in the quality deterioration of stored Japanese bunching onion. Discoloration of Japanese bunching onion leaf surface started from leaf tips and progress towards the base as evidenced. The storage life of Japanese bunching onion at 5°C is only about 1 week and high temperatures cause more rapid yellowing and decay of the leaves. Utilizations of very low temperature storage at close to 0°C and controlled atmospheric conditions could extend the shelf-life by maintaining the green color of Japanese bunching onion (Hardenburg et al., 1986). The main factors of quality deterioration or discolorations of stored horticultural crops such as broccoli, spinach, parsley, green beans and Japanese bunching onion was occurred chlorophyll breakdown (Costa et al.,2005; Yamauchi and Watada, 1991; Yamauchi and Watada, 1993). Furthermore, Chlorophyll breakdown mostly occurs in the chloroplast. The changes of other organelles may take in part on chlorophyll catabolism. The chloroplast is the organelle

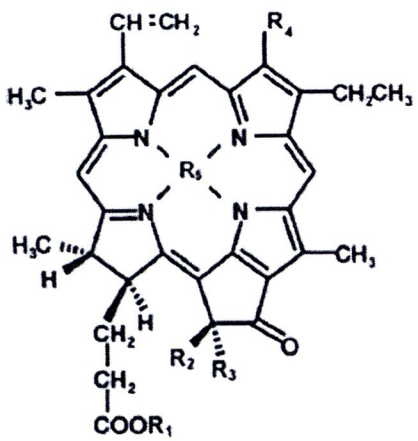
in green cells that exhibits senescence (Biswal and Biswal, 1988) through chloroplast transformation as a result of dissociation of grana, increase size and number of plastogloboli, and disruption of the chloroplast envelope (Zavaleta-Mancera et al., 1999). When chloroplast degradation has already started, thylakoid degradation takes place and plastoglobuli content increases. Plastoglobuli possibly containing chlorophyll were moved from chloroplast thylakoid to the vacuole. Earlier, chlorophyll degradation in bunching onion has been reported to occur in the chloroplast and vacuole.

2.2 Chlorophyll

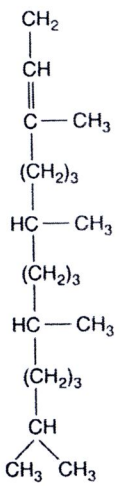
2.2.1 Chlorophyll Structure

Chlorophyll is porphyrins containing basic tetrapyrrole rings at the center of the porphyrins ring is a magnesium ion. A fifth isocylic ring, ring E is found near the third pyrrole ring. At the fourth, propionic acid substituent is esterifies with the diterpene alcohol phytol ($C_{20}H_{39}OH$).

(A)



(B)



Trivial	R_1	R_2	R_3	R_4	R_5
Chlorophyll *a*	Phytol(Fig B)	H	$COOCH_2$	CH_3	Mg
Chlorophyll *b*	phytol(Fig B)	H	$COOCH_2$	CHO	Mg

Figure 2.1 Structures formulae of chlorophyll (A) Tatrapyrrole rings and (B) phytol tail (modified from Roca et al., 2007)

2.2.2 Chlorophyll Derivative

Chlorophyll could be rapidly transformed both *in vivo* and *in vitro* then its change to a series of derivative.

2.2.2.1 Chlorophyllide *a* and Chlorophyllide *b*

Chlorophyllide is the product of the reaction catalyzed by chlorophyllase because the phytyl ester of chlorophyll could be easily hydrolyzed to give chlorophyllide and phytol. The hydrolysis takes place under mild conditions either by acid or alkali. Generally enzyme chlorophyllase found in green plant tissues. Leaves are especially rich in chlorophyllide *a* during senescence such as spinach leaves (Yamauchi and Watada, 1991) and radish cotyledons (Akiyama et al., 2000).

2.2.2.2 Pheophytins *a* and Pheophytins *b*

Pheophytin are the magnesium-free derivative of chlorophyll which the reaction catalyzed by Mg-dechelatase. The activity of Mg-dechelatase in yellow leaves was found to be higher than in green leaves. Tang et al. (2000) report that the direct removal of magnesium from chlorophyll *a* occurred in advance of dephytylation in the *Ginkgo*.

2.2.2.3 Pheophorbide *a* and Pheophorbide *b*

Pheophorbide *a* and Pheophorbide *b* are hydrolyzed chlorophyll without phytol (chlorophyllide) that have also loss the magnesium by enzyme Mg-dechelatase.

2.2.2.4 C13^2-hydroxy chlorophyll *a* and C13^2-hydroxy chlorophyll *b*

Chlorophyll *a* is oxidized with the oxygen atom being located at position C13^2 and hydroxychlorophyll being formed. C13^2-hydroxy chlorophyll *a* indentified in senescing excised leaves (Maunder et al., 1983) and broccoli (Yamauchi and Watada, 1998).

2.2.2.5 Pyrochlorophylls

Pyroderivative of chlorophyll or their derivative are compounds that have lost the carbomethoxy group –$COOCH_3$ at C-10 of the isocyclic ring, the group bring replaced by hydrogen. Chlorophyll *a*, methyl chlorophyllide *a*, pheophytin *a*, or methyl pheophorbide *a* when heated in pyridine at 100°C give rise to "pyro" derivative by decarbomethoxylation (Pennington et al., 1964).

2.2.3 Chlorophyll degradation pathway

Chlorophyll (Chl), the most abundant pigment on earth, is a key component of photosynthesis required for the absorption of sunlight. However, because of its light-absorbing properties Chl is a dangerous molecule and a potential cellular phototoxin. This is seen in situations where the photosynthetic apparatus of plants is overexcited, for example in high light conditions. Absorbed energy can then be transferred to oxygen, resulting in the production of reactive oxygen species (ROS). Likewise, inhibition of Chl biosynthesis or degradation can lead to ROS production and cell death. Because of this, metabolism of Chl is highly regulated during plant development. Whereas biochemistry and regulation of Chl biosynthesis are intensively studied, Chl degradation massively occurring during leaf senescence or fruit ripening, but also as a response to many biotic and abiotic stresses, is less well understood. For many years,

Chl degradation was considered a biological enigma. Only the identification and structure determination of several key Chl catabolites as natural breakdown products allowed the stepwise elucidation of a Chl degradation pathway which is common to higher plants. The generally accepted pathway of chlorophyll degradation (Fig 2.2) comprises two stages, before (early stage) and after (late stage) the cleavage of the tetrapyrrole macrocyclic ring. The products of the early stage are greenish, whereas those of the late stage are essentially colorless. The early stage includes modification of the side chain of the tetrapyrrole macrocycle hydrolysis of a phytol residue in ring IV (dephytylation) and then its release of Mg^{2+} from the tetrapyrrole macrocycle by displacement with $2H^{+}$ (dechelation) and some modifications of the macrocycle that are probably specific for the plant species. The late stage includes the cleavage of the tetrapyrrole macrocycle by an oxygenase and subsequent reactions, such as reduction to yield colorless fluorescent and further nonfluorescent catabolites.

The second stage is essential in the degreening of the chlorophyll molecule. Therefore it determines chlorophyll degradation in leaf senescence and fruit ripening. In most cases of leaf senescence and fruit ripening, degradation intermediates do not accumulate to an appreciable extent, suggesting that there is a series of degradation reactions.

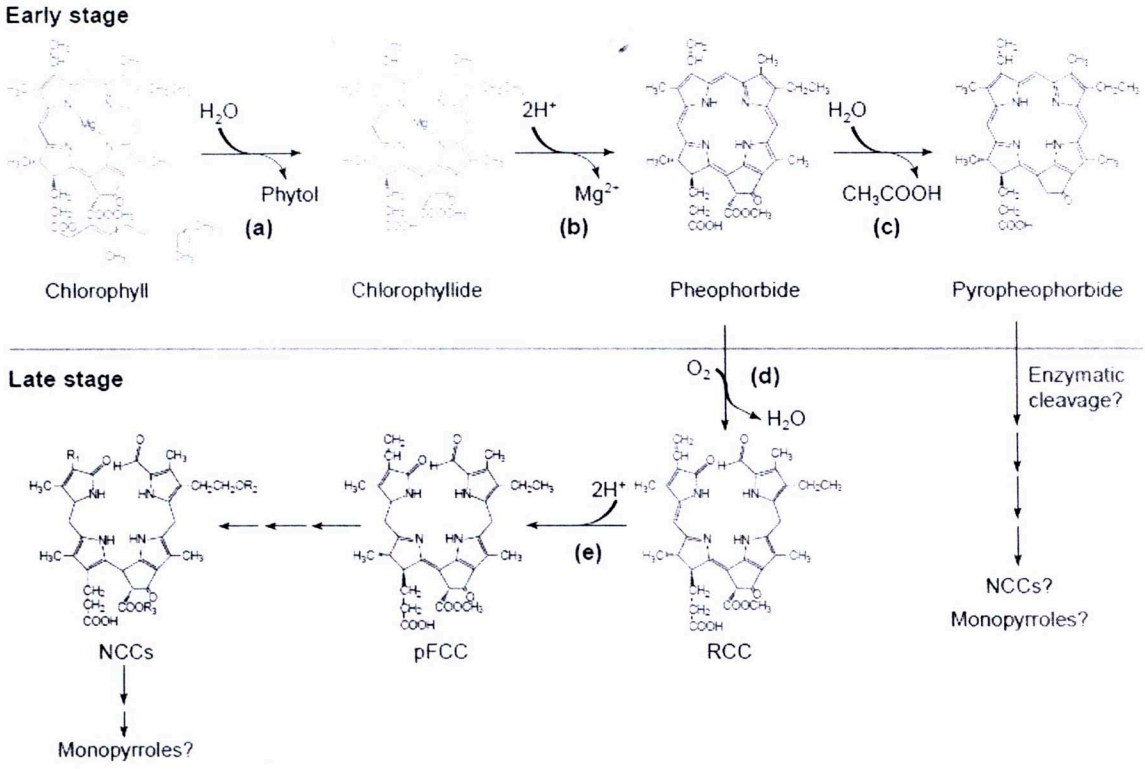


Figure 2.2 Chlorophyll degradation pathway in higher plants. The pathway is composed of two stages, an early stage before the cleavage rea ction of the tetrapyrrole macrocyclic ring and a late stage that includes the cleavage reaction and steps after the reaction. The products in the early stage are green, whereas those in the late stage are colorless. (a) Chlorophyllase, (b) Magnesium dechelatase, (c) Pheophorbidase, (d) Pheophorbide *a* Oxygenase, (e) Red chlorophyll catabolite reductase. Abbreviations: NCCs, nonfluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite(Takamiya et al., 2000).

2.2.3.1 Early stage of chlorophyll degradation pathway

2.2.3.1.1 Chlorophyllase

Commonly, the hydrolysis of Chlorophyll into Chlorophyllide *a* and phytol is regarded as the initial step of breakdown. Moreover, accepts chlorophyll *b* and pheophytins as substrates with oxidized pyrrole ring D (Hortensteiner, 1999). Chlorophyllase can be found in the chloroplast, thylakoid membrane and etioplast of at least higher plants.

Activity detection of chlorophyllase using chloroplast subfraction locatized chlorophyllase activity to envelop, probably in the inner membrane (Matile et al., 1997). Chlorophyllase is a hydrophobic protein of plastid membranes that is distinguished by its functional latency. While Chlorophyll breakdown in senescent leaves Chlorophyllase remains latent. The most problem of regulation of Chlorophyll breakdown at the level of Chlorophyllase concerns the mechanism which the interaction between Chlorophyllase and its substrates is achieved. Latency of the enzyme would be explained by the spatially separation between Chlorophyll in the thylakoid pigment-protein complexes and Chlorophllase, which appears to be located in the plastid envelope. The chlorophyll molecule or chlorophyll-protein complex released from plastoglobuli could be attacked by chlorophyllase in the vacuole more than the chloroplast (Parthier, 1988; Satoh et al., 1998).

2.2.3.1.2 Mg-decheratase

The enzymic release of Mg^{2+} from Chlorophyllide *a* in exchange for $2H^{+}$ has been demonstrated in as from higher plants. Under conditions preventing further catabolism of the reaction product Pheophorbide *a* the activity can be demonstrated by assessing Pheophorbide *a* accumulation in vivo as well as in isolated chloroplasts and chloroplast membranes (Langmeier et al., 1993). This activity is associated with chloroplast membranes and like Chlorophyllase seems to be constitutive. The activity was heat-stable and associated with a low-molecular-weight compound rather than with a protein (Shioi et al., 1996; Vicentini et al., 1997). Mg-dechelating substance is identical with the activity responsible for the release of Mg that occurs in the catabolic pathway of Chl

remains to be demonstrated. Furthermore, Mg-dechelatase can be release Mg atom from chlorophyll *a* to pheophytin *a* (Tang et al., 2000).

2.2.3.2 Late stage of chlorophyll degradation pathway

This stage is composed of three steps:

- Oxygenolytic cleavage of the macrocyclic ring of tetrapyrrole
- Conversion of the cleavage product to colorless fluorescent compounds
- Conversion of the colorless fluorescent compounds to nonfluorescent compounds

The identification and properties of the products and enzymes involved in this stage have been intensively investigated for several years.

2.2.3.2.1 Pheophorbide *a* Oxygenase

The ring-opening step of the catabolic pathway is decisive for the loss of green color. The activity was found in the envelope membrane but oxygenase has not yet been purified (Roodoni et al., 1997). The biochemical properties of pheophorbide *a* oxygenase as an envelope-localized nonheme iron monooxygenase allowed the identification of pheophorbide *a* oxygenase candidate genes from *Arabidopsis* (Gray et al., 2002). This activity was detected in senescent leaves of several plant species as well as in ripening fruits. Furthermore, pheophorbide a is only substrate for the oxygenase, which is consistent with the exclusive occurrence of final degradation products of chlorophyll *a*

2.2.3.2.2 Red Chlorophyll catabolite reductase

Closely coupled with oxygenese reaction is a reduction of the δ–methine bridge of red chlorophyll catabolites by stromal enzyme to yield a colorloss fluorescent produce (primary fluorescent chlorophyll catabolites (Rodoni et al., 1997). Red chlorophyll catabolite reductase was purified and cloned recently in barley and *Arabidopsis* (Wuthrich et al., 2000). Red chlorophyll catabolite reductase is a soluble constitutive component of plastids that occurs not only in green tissues but also in etiolated leaves and even in roots (Rodoni et al., 1997). Because the oxygenase activity is expressed only in gerontoplasts, the reductase might have another role in nonsenescent choloroplasts and root.

2.2.3.2.3 Reaction on primary fluorescent chlorophyll catabolite

The primary fluorescent chlorophyll catabolite is converted to fluorescent chlorophyll catabolites by several modifications such as demethylation and hydroxylation depending on the plants and some of them are exported from gerontoplast to cytosol by an ATP-dependent translocator in the envelope membrane (Matile et al., 1992). These reactions were increased in the water solubility of the catabolites. However, the enzymes involved in these reactions have not yet been identified. A pheophorbidase-like enzyme might be involved in the demethylation in some plants. The modified fluorescent chlorophyll catabolites would be transported to the central vacuole by an ATP-dependent translocatorin the tonoplast (Hinder et al., 1996) and non enzymatically converted to non-fluorescent chlorophyll catabolites by rearrangement of the double bonds in the pyrrole IV ring and adjacent δ-methine bridge.

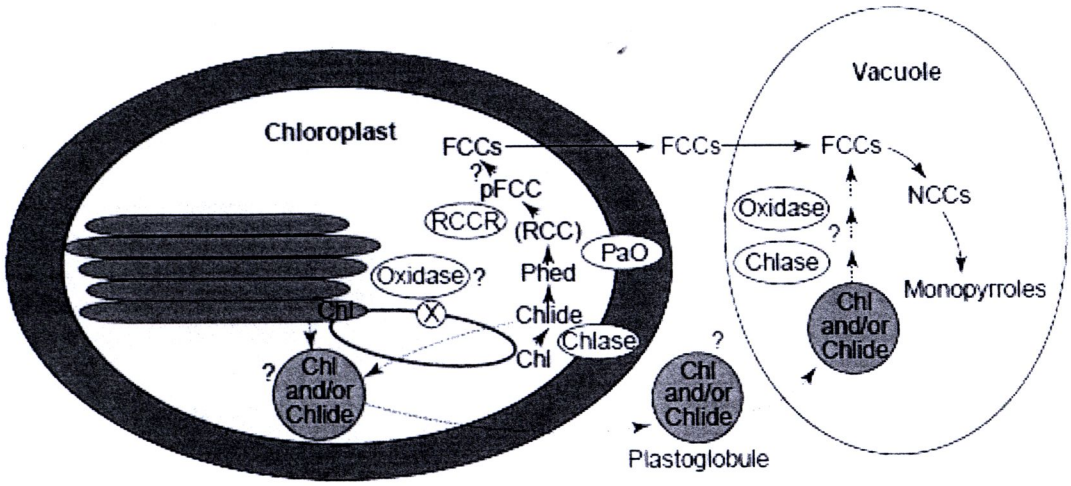


Figure 2.3 Hypothetical compartmentation of the chlorophyll (Chl) degradation pathway. Solid lines indicate the chlorophyll degradation pathway and broken lines indicate additional hypothetical pathways depending on plant species. Abbreviations: Chlase, chlorophyllase; Chlide, chlorophyllide; FCCs, fluorescent chlorophyll catabolites; NCCs, non-fluorescent chlorophyll catabolites; PaO, pheophorbide *a* oxygenase; pFCC, primary fluorescent chlorophyll catabolite; Phed, pheophorbide; RCC, red chlorophyll catabolite; RCCR, RCC reductase; X, a hypothetical chlorophyll carrier. Enzymes are represented by ellipses (Takamiya et al., 2000).

2.2.3.3 Other related reaction

2.2.3.3.1 Chlorophyll *b* degradation

The degradation of Chlorophyll *b* is occurred after converted to chlorophyllide *b* by Chlase. Chlorophyllide *b* is then converted to chlorophyllide *a* by chlorophyll *b* reductase (Ito et al., 1996; Folly and Angel, 1999; Scheumann et al., 1999).

2.2.3.3.2 Oxidative chlorophyll bleaching

Chlorophyll is readily bleached *in vitro* by the action of oxidative enzymes such as peroxidases in the presence of H_2O_2 and a phenolic compound or lipoxygenase in the presence of linolenic acid (Johnson-Flanagan and Spencer, 1996; Janave, 1997). Indeed,

in some plants, the rise in peroxidase activity or oxidase activity paralleled the degreening of seeds or cotyledons. Although these oxidative enzymes are certainly in the chloroplast, they are also present in the vacuole. Thus, oxidative and/or oxygenolytic cleavage of the macrocycle, whether it is might occur outside the chloroplast. The cleavage reaction has never been examined using a fraction completely lacking the extracts derived from chloroplasts. Peroxidase or Chlorophyll oxidase are also reported to be involved *in vitro*, oxidized the phenolic compound with hydrogen peroxide and forms phenoxy radical then, the phenoxy radical oxidizes chlorophyll and it derivatives to colorless through the formation of C13^2-hydroxychlorophyll *a*, fluorescent chlorophyll catabolite and bilirubin-like compound as an intermediate (Yamauchi et al., 2004). Furthermore, the chlorophyll degrading peroxidase is involved in chlorophyll degradation (Yamauchi and Watada., 1998).

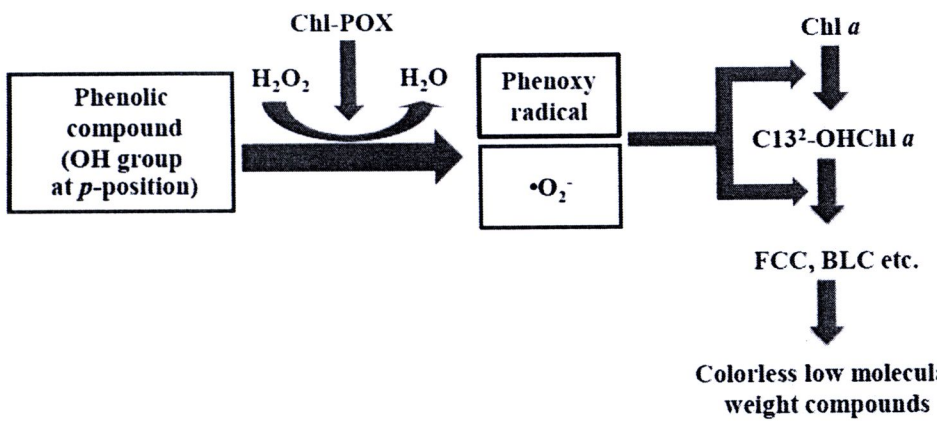


Figure 2.4 Putative pathway of peroxidase-mediated chlorophyll degradation. Chl-POX: Chlorophyll degrading peroxidase, Chl: chlorophyll, C13^2-OHChl: C13^2-hydroxychlorophyll, FCC: Fluoresencent chlorophyll catabolite, BLC: Bilirubin-like compounds (Yamauchi et al., 2004)

2.3 Change of plastid during leaf senescence

2.3.1 Senescence-related changes in the ultrastructure of plastid

Ultrastructure change in senescing chloroplasts are well documented by conventional electron microscopy techniques (Butler and Simon, 1971; Biwal and Biwal, 1988; Gepstein, 1988). They comprise a reduction in thylakoid membrane system, a loosening of the grana stacks, a swelling of intrathy- thylakoid space, shrinkage of the size of the organelle with a transition from ellipsoid to circular shape and an increase in size and number of plastoglobuli.

2.3.2 The degrading of thylakoid membrane

In most studies has been observed that stoma thylakoid loose their integrity earlier than grana thylakoid (Hurkman., 1979; Gepstein., 1988). Hashimoto et al., (1989) observed that senescing chloroplasts the organization of stoma and grana thylakoid does change, and grana stacks with a large number of thylakoid similar to those of shade chloroplasts become prevalent. This observation is consistent with studies reporting a decrease in the chlorophyll *a/b* ratio of senescing rice leaves (Kura-Hotta et al., 1987) and a high stability of light harvesting complex (LHC) during barley leaf senescence under field conditions (Humbeck et al., 1996). In a recent investigation with barley leaf segment kept in the light found grana disintegrated before stoma thylakoid (Spundova et al., 2003). The authors suggested that under these specific conditions the LHC is degraded to lessen the risk of photooxidative damage. Recent work on cucumber cotyledons indicates that the grana stacks may get reduced by lateral movement of the light harvesting complex to Photosystem (Prakrash et al., 2001, 2003). At later stages of

senescence, thylakoid become distended and dissociate into distinct vesicles (Hurkman, 1979). In the studies with rice coleoptiles, intergranal lamellae began to swell already at onset of senescence (Inada et al., 1998).

2.3.3 Plastoglobuli

Plastoglobules are plastid-localized lipoprotein particles that contain tocopherols and other lipid isoprenoid derived metabolites, as well as structural proteins named plastoglobulins (Claire et al., 2007).

2.3.3.1 Increase in size and number of plastogluli

One of the most obvious changes occurring in chloroplasts during senescence is the increase in number and size of plastoglobuli (Butler and Simon, 1971; Biswal and Biswal, 1988; Gepstein, 1988; Guiamet et al., 1999; Gosh et al., 2001). During leaf senescence of rape (*Brassica napus*) aggregation and enlargement of plastoglobuli was accompanied by the loss of photosystem II activity and grana compactness (Gosh et al., 2001). It is common opinion that these senescence associated plastoglobuli contain the breakdown products of the thylakoid membranes (Tevini and Steinmüller, 1985; Matile, 1992). Indeed, the composition of plastoglobuli changes during senescence (Lichtenthaler., 1969). In chloroplasts of rice coleoptiles, the plastoglobuli was observed to increase in size and number just at the time when intergranal membranes began swell (Inada et al., 1998). An association of osmiophilic globules with thylakoid and vesicle formation from the distention of the thylakoid was observed by electron microscopic investigations (Butler and Simon, 1971; Hurkman, 1979).

2.3.3.2 Blebbing of plastoglobuli into the cytosol

Rupture of chloroplast envelopes and release of plastoglobuli to the cytoplasm at late stages of leaf senescence was described early by electron microscopy (Butler and Simon, 1971; Hurkman, 1979). Indeed, osmiophilic deposits resembling plastoglobuli have often been reported to occur in the cytoplasm of senescing leaves (Guiamét et al., 1999). Until recently, it has been assumed that these lipids were derived from membranes outside the plastid. The remarkable ultrastructural study on soybean leaf senescence of Guiamét et al. (1999), however, provided evidence these lipids are derived from senescing chloroplasts. It have been report that globules specific for senescing cells protrude through the chloroplast envelope and emerge into the cytoplasm. Judging from the protrusions in the chloroplast envelope, however, postulate of the plastoglobuli was pressed against and squeeze through the outer membrane of the chloroplasts. The fluorescence characteristics of the senescence associated globules indicated that they contain chlorophyll or chlorophyll degradation products. The fluorescing globules described by Guiamet et al., (1999) may be similar to the colored vesicles observed in the cytoplasm of senescing broccoli florets (Terai et al., 2000). These vesicles are derived from globules inside the chloroplasts and become larger when the grana thylakoid degenerate. Both by electron microscopy and light microscopy the colored vesicles or globules derived from the senescing chloroplast of broccoli were observed to emerge on outside of the plastid and then to spread into the cytosol (Terai et al., 2000). So, discuss that the vesicles may be either transferred into the cytosol through the intact plastid envelope or may be exposed to cytosol by destruction of envelope. Although the mechanisms of globule formation from plastid not know, the final destination of the globule in any case could be the vacuole (Inada et

al., 1998). Indeed, in their comprehensive study on chloroplast senescence in rice coleoptiles observed oil drops sequestered in the vacuole.

2.3.4 Change the chloroplast of Japanese bunching onion during senescence

The chloroplasts of higher plants are lens-shaped organelles with a diameter of ~5 μm and a width of ~2.5 μm (Fig 2.5). Each chloroplast is delineated by two envelope membranes, which surrounding an aqueous matrix by the stroma and the internal photosynthetic membranes. The envelope membranes control the transport of metabolites, lipids and proteins into and out of chloroplasts. Components of the stroma include the enzymes involved in carbon fixation, circular DNA anchored to the thylakoids, ribosomes, starch granules and plastoglobuli. The plastoglobuli are osmophilic and spherical bodies rich in lipid (Tevini and Steinmuller, 1985). When chlorophyll breakdown lead to the yellowing of leaf which the main symptom of senescence in plant and Japanese bunching onion. The chloroplast is first organelle in green cell to show signs of decay (Biswal and Biswal, 1988) by the dissociation of grana, increase in size and number of plastoglobuli and disruption of the chloroplast envelop (Zavaleta-Mancera et al., 1999). Moreover, protruding of enlarged plastoglobuli from chloroplasts trough cytoplasm to vacuole was observed in the soybean chloroplasts (Guiamet et al., 1999). Dissanayake (2009) found that moving of plastoglubuli from chloroplasts to vacuole was evident in Japanese bunching onion leaf cell storage at 25°C. Grouping of small droplets of plastoglubuli were observed in chloroplast and large bodies of plastoglobuli were clearly in cytoplasm and especially in vacuole (Fig 2.7) which plastoglubuli seem to be high in the leaf cells with yellowing of

Japanese bunching onion leaf and degradation of thylakoid. It could be assumed that the chlorophyll was likely to be localized in plastoglubuli (Keskitalo et al., 2005) or in other structures. The finding of Guiamet et al. (1999) have confirmed that chlorophyll contained in isolated plastoglobuli in soybean leaves. Therefore, content that chlorophyll in plastoglobuli of Japanese bunching onion leaves is also possible. The presence of numerous plastoglobuli within vacuole explained a mass exodus from senescing choloplasts (Guiamet et al., 1999). Accordingly, chlorophyll in plastoglobuli seems to be partly degrading in vacuole.

Figure 2.5 Thin section electron micrograph of a young tobacco chloroplast. Two envelope membranes (EM) surround the chloroplast stroma (S), within which stacked grana thylakoids (GT) and unstacked stroma thylakoids (ST) can be recognized. Plastoglobuli (PG) and DNA-containing regions (arrows) are also seen. Reproduced from Staehelin. (1986).

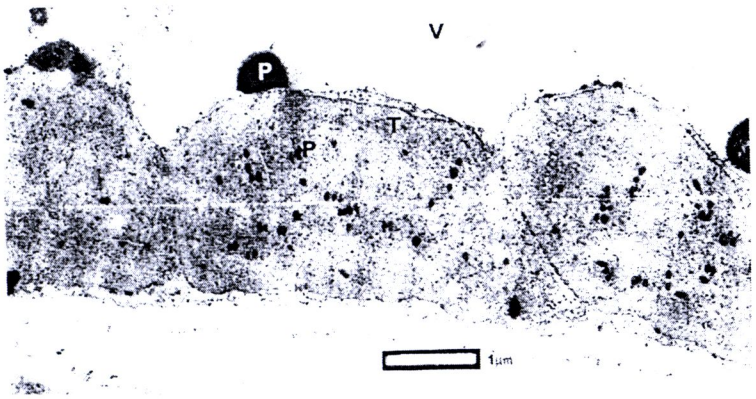


Figure 2.6 Degrading chloroplast in Japanese bunching onion in day 0. Numerous plastoglobuli (P) can be seen in chloroplasts.Also, protruding of enlarged plastoglobuli from cytosole to vacuole (V) is visible (Dissanayake, 2009).

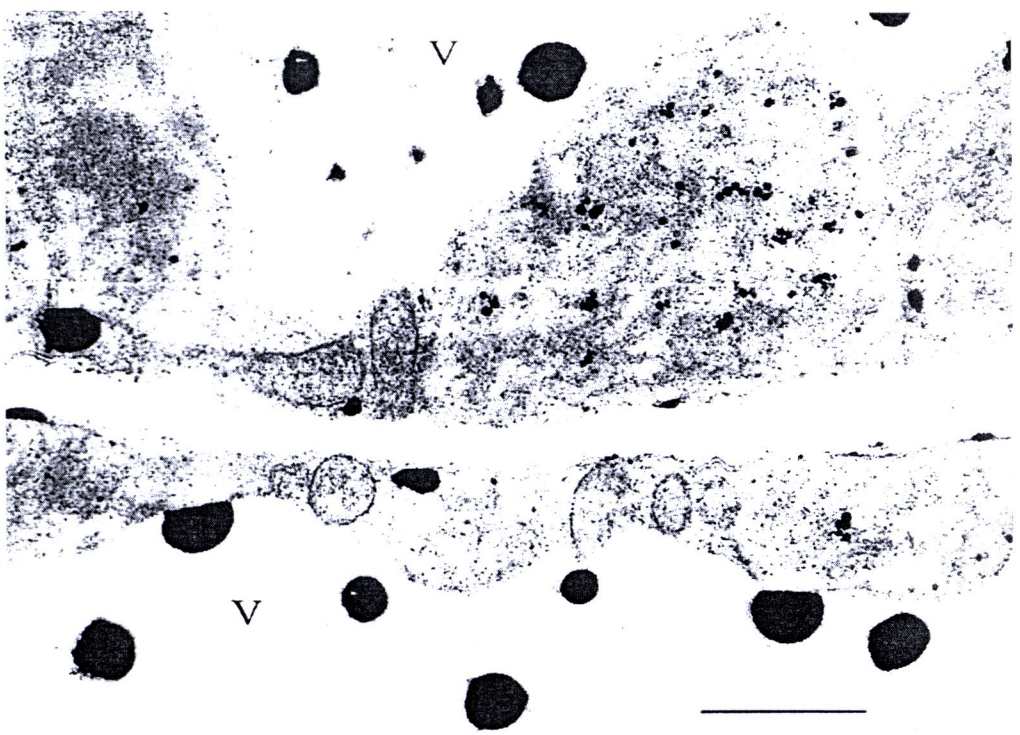


Figure 2.7 Degrading chloroplasts and enlarged plastoglobuli in vacuole (V). The bar represents1 µm (Dissanayake, 2009).

2.4 Postharvest treatment for retarding chlorophyll degradation in plant

2.4.1 Heat treatment

Heat treatment substitutes anon-damaging physical treatment for chemical prevention. During the past few years, it has been increasingly interested in heat-treatment methodologies for control of insect pests, prevention of fungi rots, and modification of ripening or response to temperature extremes of commodities (Lurie, 1998). Heat treatment is frequently used to maintain the postharvest quality of many horticultural crops (Tian et al, 1996; Costa et al., 2005; Funamoto et al., 2002, 2003). The advantages of postharvest treatment that result a reduction of yellowing of broccoli florets and suppression of activities of chlorophyll-degrading enzymes, such as chlorophyllase, Mg-dechelation activity and chlorophyll-degrading peroxidase have been reported (Funamoto et al. ,2002, 2003; Kaewsuksaeng et al., 2007).

2.4.2 Ultraviolet irradiation treatment

UV light can be divided into three parts following the range of electromagnetic spectrum; UV-A (315-400 nm), UV-B (280-315 nm), and UV-C (less than 280 nm) radiation, as well as electromagnetic radiation of shorter and longer wavelength and particles such as neutrinos (Björn, 2004). UV-C radiation does not penetrate to the Earth's surface and is not part of the natural environment in the biosphere. UV-B penetrates to an appreciable but variable extent. It is absorbed by ozone, mainly present in the stratosphere, the amount of which is high variable. Although less biologically active than UV-C, it still has an appreciable effect, and so the amount of ozone present

in the atmosphere is of great biological importance. UV-A is only weakly absorbed by ozone, and many respects have biological effect similar to photosynthetic active radiation (400-700 nm). UV treatments have been report to maintain postharvest quality of many horticulture crops. UV-C seem to be especially effective in maintaining postharvest quality of strawberries (Erkan et al., 2008) and in inhibiting chlorophyll degradation in stored broccoli florets (Costa et al., 2006) Aiamla-or et al. (2010) found that UV-B treatment effectively delayed chlorophyll degradation of broccoli during storage. Srilaong et al. (2011) also report that UV-B treatment effectively suppressed chlorophyll degradation and retarded the reduction of levels of chlorophyll derivatives in mature green lime during storage.

2.5 Storage temperature

Temperature is the characteristic of postharvest environment that has the greatest impact on the storage life of horticultural crops. Horticultural crops deteriorate are after harvested. Thorne and Alvarez (1982) have pointed out that it is well established that the deterioration of most agricultural product is a direct function of temperature. Within the range of temperature bounded on the lower end by chilling injury or freezing and on the upper by heat injury, deterioration of agricultural product caused by physiological, pathological, or physical factor is function of time and environment (Holt et al., 1983). Postharvest losses of horticultural crops are estimated to be as high as 25% to 50% of the production due to poor postharvest handling techniques, mainly poor temperature management, especially in some regions of the globe such as tropical and subtropical regions and where refrigeration facilities are not available (Desai and Salunkhe, 1991; Harvey, 1978; Rippon, 1980). For example the large quantity of onion (*Allium cepa* L.)

is lost between the field and the consumer in India due to lack of adequate postharvest fact, the most important and simplest procedure for delaying product deterioration. In addition, optimum storage temperature retards the aging of horticultural crops, softening, textural and color changes as well as slowing undesirable metabolic changes, moisture loss, and losses due to pathogen invasion. Temperature is also the factor that can be most easily and promptly controlled. Optimum preservation of horticultural crops quality can only be achieved when the produce is promptly cooled to optimum temperature as soon as possible after harvest.

2.5.1 Storage temperature on quality of vegetable

The visible quality of the product is the appearance of product which the most important factor that determines the market value of fresh vegetable. When consumers were asked about fresh fruit and vegetable, ripeness, freshness, and taste were named by 96% as the most important selection criteria, while appearance and condition of the product came in second in order of important (94%) (Zind, 1989). Although not visually perceptible, nutritional value was considered by about 66% of consumer to be the decisive factor for buying the product.

2.5.1.1 Appearance of vegetable

Color, One of the major factors of product appearance is a primary indicator of maturity of ripeness and is due to presence of particular pigments in the product. Undesirable changes in the uniformity and intensity of color can be observed when vegetable are not stored at recommended temperatures. Temperature can therefore have a direct effect on color change during storage of fresh vegetable. For example, while loss of chlorophyll is a desirable process in few vegetable such as tomato and some sweet pepper cultivars,

yellowing of green vegetable such as broccoli or Brussels sprouts (*Brassica oleracea* L. Gemmifera group) is considered undesirable. Subjective visual observations combined with CIE L* a* b* uniform color space (CIE LAB) determination, and total chlorophyll and carotenoid content constitute a very good indicator of color changes in many vegetable during storage. Yellowing of broccoli is very often due to storage above the recommended temperature and it is major cause of product rejection. Several studies show that temperature can be important effect on color changes of broccoli during storage (Makhlouf et al., 1991; Tovivonen, 1997; Zhuang et al., 1997). Makhlouf et al. (1991) studied the effect of temperature on chlorophyll content of broccoli florets storage for 5 day at 25 or 1°C. They concluded that storage at 1°C greatly reduces chlorophyll losses compared to 25°C storage. In another study, reduction in broccoli quality was associated with the degree of yellowing (Tovivonen, 1997). Moreover, it has been reported that storage temperature a significantly effected on the color change of stored broccoli. Broccoli stored for 10 day at 10°C became fully yellow by the fourth day after being transferred to 13°C, while broccoli stored at 1°C for 10 days remained fully dark green in color for 5 days at 13°C. Zhuang et al. (1997) observed no significant change in the total chlorophyll content of broccoli stored at 2°C for 6 days. However, chlorophyll content declined in broccoli stored at 13 or 23°C for same period of time. And it have report that after a 6 days storage period at 13 or 23°C, a 42% or 86% reduction, respectively, was observed in total chlorophyll content of broccoli. At the end of the storage period, they observed that the broccoli stored at 13°C contained significantly lower levels of total chlorophyll than that stored at 12°C. Dissanayake (2009) studied of storage temperature at 4 and 25°C during storage of green Japanese bunching onion and found that green Japanese bunching onion storage at 25° turned

progressively yellow starting from the leaf tip towards the base during 3 days of storage whereas leaves stored at 4°C maintained their green color apart from slight yellowing at the tip.

2.6 Isolation and purification of cellular organelles

The eukaryotic cells have significant application in current biotechnology. Well known is the widespread application of yeasts in traditional biotechnologies such as brewing, baking, wine making and distiller's fermentations, as well as their significance for cell and genetic engineering, which define them as a basic object. However, apart from yeasts appreciable progress has been made applying mammalian cells in production of monoclonal antibodies (hybridoma technologies) and in production of important proteins as interferones, interleukines etc. (using cell cultures). Some general problems exist, when we study the native structure of cell organelles and the activity of their components (for example preparation of extract for purposes of enzyme activity). Successful preparation of subcellular organelles, their isolation and purification requires that we pay attention to conditions that may alter the properties and integrity of both – cellular organelles and their molecules. Over the last 40 years many techniques for isolation of subcellular organelles have been developed, commonly based on size or density differences (hydrodynamic properties of an organelle population in a centrifuge).

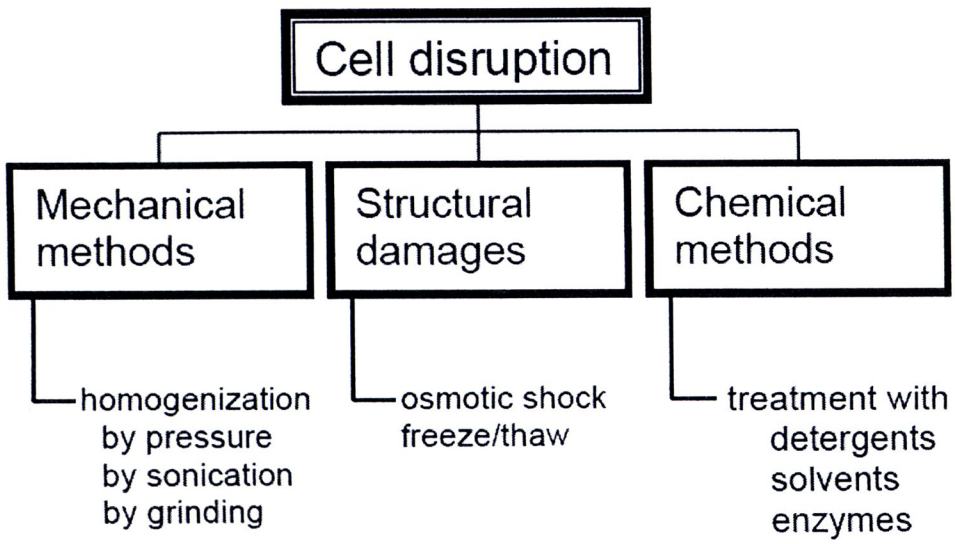


Figure 2.8 Techniques of cell disruption for isolated cellular organelles (modified from Stephanova and Topouzova, 2001)

2.6.1 Cell disruption

The initial step of subcellular organelle purification is to rupture the cell wall, the plasma membrane or both, as this is a prerequisite to fractionation techniques. Cells can be disrupted in various ways, which include mechanical methods – homogenization (shear), cell rupture by pressure, sonication, grinding; structural damage,osmotic shock, freeze/thaw; chemical methods – treatment with detergents, solvents and enzymes.

2.6.2 Methods for cell disruption

Cell could be disrupted in various ways, which include:

- Mechanical methods
- Structural damage and

- Chemical methods

All of these methods allow receiving a mixture of subcellular organelles, which is very convenient for further separation

2.6.2.1 Mechanical methods

- Homogenization

Disruption of cells is a process known as homogenization. First the cells are suspended in solution of appropriate pH and salt content (usually isotonic sucrose buffer) or combination of salts similar in composition to those in the cell interior. Then the cells are broken by special pressurized tissue homogenizer, in which the cells are forced through a very narrow space between the plunger and the vessel wall. The cell solution must be kept at 0°C in order to preserve enzymes and other constituents, after they release from the stabilizing forces of the cell.

- Cell rupture by pressure

This way of disruption is particularly useful to disrupt microbial cells but also for other cell types. The cells are forced through a narrow extrusion by applying high pressure. A kind of disruption by repeated high velocity compression and expansion is known as a "French press". This device is very powerful and is applicable for molecular dissociation.

- Sonication

Many cells are broken by exposing a cell suspension to high frequency sound (ultrasonic oscillations). The sonication is used to disrupt cells, particularly tissue cultures cells, and to release subcellular organelles. It must be taken into account that considerable heat is generated, which is the main disadvantage using this method. The

ultrasonic waves commonly break open the cell periphery and leave the cellular organelles intact.

- Grinding

Some types of cells (such as plant cells, microbes and yeasts), especially those which cell periphery include in addition to their plasma membrane, complex layer (e.g. chitin or other poly-saccharides), must be disrupted first by grinding. This method includes simple mortar and pestle grinding using sand or glass beads. Great efficiency can be achieved if cells are undergone rapid freezing in liquid nitrogen.

2.6.2.2 Structural damage

- Osmotic shock

The advantages of this method are its simplicity, easiness of performance and absence of shearing forces. The method is useful for osmotic sensitive cells and it is a good for non-osmotic organelles such as ribosomes, etc. Essentially, the cells are transferred from one medium to another hypotonic and it leads to swelling and finally the cells burst open, because of passing the water into the cells in a effort to balance the osmotic pressure. However there are some disadvantages: the osmotic sensitive organelles such as mitochondria, lysosomes, Goldgi apparatus, endoplasmic reticulum, etc might also burst. On the other hand bacteria and some tissues don't undergone to osmotic lysis and for these reasons the method is inapplicable.

- Freeze/thaw

This approach includes freezing, followed by thawing, which procedure is repeated several times, e.g. 8-10 times. The method is based on the formation of ice crystals during freezing, which broke the cell membranes.

2.6.2.3 Chemical methods

- Detergents

The most useful chemical reagents for disrupting the cell membranes are detergents, which are small amphipathic molecules that tend to form micelles in water. Because of the properties of the membrane compounds the hydrophobic ends of detergents bind to the hydrophobic regions of the membrane proteins, thereby displacing the lipid molecules and disrupting the membranes. Mainly two types of detergents are used: ionic (such as SDS – sodium dodecyl sulfate) and nonionic (such as Triton X-100 and NP 40). The strong (ionic) detergents can solubilize the most hydrophobic membrane proteins, but at the same time they unfold them. In this respect such strong detergents are unusable for functional studies and for isolation of subcellular membrane structures. In such case mild (nonionic) detergents are predominantly used in a low concentrations (To affect only the plasma membrane).

- Organic solvents

Besides detergents there are other agents capable of solubilizing membrane structures, but their usage is not so widely spread. Many organic solvents like toluene, acetone, ethyl acetate, etc. are applied to dissociate membrane compounds. As they affect other intracellular membrane-bound structures, they are considered to be less useful in practice.

- Enzymes

This approach is mainly used to digest the extracellular matrix structure (e.g. animal tissues) and the cell walls (bacteria, fungi and plants). The choice of enzyme depends on the specificity of the object. The aims of the treatment are directed to obtain cell suspension from solid tissues or protoplasts from cells possessing rigid walls. In the first

case different proteolytic enzymes (collagenase, trypsin, elastase, etc.) are applicable for animal tissues and others (cellulase, pectinase, etc) for plant tissues. In the second case we use enzymes to digest some specific components of the cell wall without (or less) effecting the membrane, e.g. cellulase for plant cells, glucanase (Zymolyase, Lyticase or Gluzulase) for yeasts and lysozyme for bacteria.

2.6.3 Methods for cell separation and purification

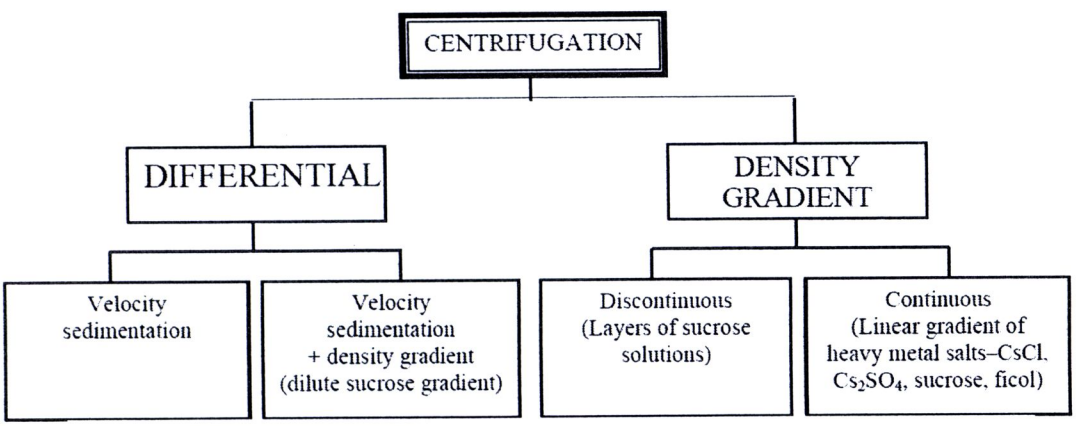


Figure 2.9 Methods for purification of cellular organelles (modified from Stephanova and Topouzova, 2001)

2.6.3.1 Centrifugation

Generally fractionation of subcellular organelles is performed using centrifugal forces, according to a number of basic procedures. Organelles may be separated in a centrifuge depending on their behavior in a centrifugal field, according to their size, density and shape. Essentially the heavier and/or denser particles will sediment faster in a

centrifugal field. In the case of two particles with similar weight and density, the one with the more compact shape will sediment faster, because of retarding of another, due to the frictional resistance. Subcellular structures can be separated at an interface or can be part of a moving boundary, a moving zone, classical sedimentation equilibrium, a performed gradient isodensity and an equilibrium isodensity. Commonly used in practice are "analytical" procedures, applying high speed with lower volume capacity and "preparative" techniques, applying low speed with greater volume capacity. The term "ultracentrifuge" means a centrifuge working at speed higher than 20,000 rpm.

2.6.3.2 Differential centrifugation

The most widely used method for cellular components fractionation is differential centrifugation. Differential centrifugation is used for the first steps in cell fractionation because it rapidly separates large membrane organelles from smaller one. It is based on differences in speed at which organelles sediment to the bottom of a centrifuge tube. The selection of velocity and duration of time depends on different size weight, density and shape of studying organelles. This type of centrifugation is known as velocity sedimentation.

2.6.3.2.1 Velocity sedimentation

Sedimentation velocity (so called zone centrifugation) has the advantage of low speed centrifugation and short time, but results in incomplete separation. Each particle has terminal velocity, when subjected to a centrifugal field. It is determined by above mentioned particle characteristics, as well as by the viscosity of the medium they have to pass through and the relative centrifugal force (RCF). It is possible to estimate the

terminal velocity by mathematical factor, the sedimentation coefficient, given in Svedberg (S) units. T. Svedberg is well known Swedish scientist, a leader of centrifugation theory and practice. The S units are determined by dividing terminal velocity by the centrifugal force field strength.

$$S = v/\omega 2\ x$$

Where:

ω = angular velocity of the rotor in radians/sec calculated as 0.10472 x RPM

x =the distance between the particle and the center of rotation in cm

v = the rate of movement of the particle in cm/sec 1S = 1x10-13

Thus, a particle whose sedimentation coefficient is measured at 10-13 sec = 10 x 10-13 sec is said to have a value of 10 S. The terminal velocity may used to measure the size, weight and density of the cellular organelles and particles.

• **Separation on the basis of different size and shape**

In the homogenate cell organelles are mixture of particles with different size, weight and shape and they will be separated on the basis of those parameters. The larger particles will sediment faster than the smaller one. From the total homogenate we can obtain different cell fractions (known as pellets) following several steps of centrifugation with appropriate velocity and duration of time. In this way the first fraction (at low centrifugal force) will be nuclear fraction, comprising not only the nuclei, but also intact cells and cell debris. The second (mitochondrial) fraction, consisting mainly of tree different subcellular components (such as mitochondria, lysosomes and peroxisomes), is obtained after further centrifugation at an increased centrifugal force of the supernatant from the first fraction. Following this description,

the next step will result in another mixed pellet so-called microsomal fraction consisting of membrane vesicles derived from disrupted membranes (e.g. plasma membrane, endoplasmic reticulum and Goldgi apparatus). All of the above mentioned fractions have to be rehomogenized and undergone to the additional procedures, in order toobtain purified organelles.

2.6.3.2.2 Velocity sedimentation through sucrose gradient

The basic fractions obtained by differential centrifugation are not enough pure to perform fundamental investigations. They can be purified further applying another approach: separation through density gradient. For example, a pure nuclear fraction will be achieved by sedimentation through the sucrose cushion (a hyperosmotic sucrose solution layered on the bottom of the centrifuge tube). The "cushion", a small volume of high-density solution, placed on the bottom of the tubes, acts as a step in the gradient and may be useful as effective part of the separation. A cushion makes easier to resuspend the sedimented material at the end of the run, and prevents from damage the particles, that may not withstand pelleting. In particular, some viruses lose viability when pelleted. This approach permits the nuclei to pass through the sucrose layer, retaining at the same time all other contaminations on its surface. In fact it is better to combine both, re-homogenization followed 2-3 time centrifugation with or without sucrose cushion.

2.6.3.3 Density gradient centrifugation

Despite the different behaviors of the particles in mixed fractions it is possible both - to sediment and float them simultaneously. As the cell organelles usually have different

densities (mg/ml) it is easy to separate them through a medium, which slow down organelles with one density but allows others with higher density to pellet. Commonly the re-homogenized fraction is overlaid on the density gradient medium. Two main protocols exist including step and linear gradients.

2.6.3.3.1 Discontinuous (step) density gradient

Discontinuous or step gradients are best suited for separating whole cells or subcellular organelles from plant or animal tissue homogenates, and for purifying some viruses. In this technique we form a liquid density gradient of different media, the choice of which depends on the type of sample. The performance of such gradients is achieved by layering a lighter solution over a denser one. Because of instability of gradients due to the diffusion process, they should be prepared just before use and placed on ice! Examples of useful gradient media are listed below:

a) Sugars (e.g. sucrose) – more frequently used for separation of organelles as well as for isolation of viruses. If such a gradient is applied for separation of cells, it is necessary to wash the cell fraction with iso-osmotic solution, immediately after centrifugation. The appropriate sucrose concentrations (correlation molarity/density) are varied in the range from 0.029 M/ 1.002 g/cm^3 to 2.5 M/1.316 g/cm^3 at 20°C. Note that the one and the same concentration corresponds to the different density varying the temperature (e.g. 0.7 M sucrose has density of 1.0899 at 20°C, but 1.0918 at 5°C).

b) Polysaccharides (e.g. Ficoll) – good for some applications. Multiple steps are sometimes used with high-viscosity gradients made with Ficoll solution. Gradients of Ficoll are applicable for isolation either cells or organelles and viruses with the same quality. The appropriate Ficoll concentration (correlation with density) is varied in the

range from 3.03 %/ 1.0106 g/cm^3 to 46.52 % /1.1629g/cm^3 at 4°C. Specific characteristic of Ficoll solutions is their grate viscosity.

c) Colloidal silica (e.g. Percoll) – such kind of media are applied with success for cells and organelles separation. Percoll is composed of polyvinylpyrrolidone-coated silica particles (20 nm). It is often use as a hybrid gradient with metrizamide. Pay attention that Percoll has pH 9.0 and precipitates in acid. The maximum density of Percoll is 1.3g/cm^3 but for separation of organelles is recommendable 1.0408 g/cm^3 (6 %concentration). Maximum density of metrizamide is 1.45 g/cm^3, but the appropriate concentration (correlation morality or % / density) is varied in the range from 0.127 M or 10 % / 1.0512 g/cm^3 to 0.887 M or 70 % /1.3812 g/cm^3 at 20°C.

• Separation on the basis of density

Usually the sample solution is layered on the top or on the bottom of the gradient and, under a centrifugal force, the particles floated through the medium in separate zones until they reached a layer with equal or higher density. At this point the particles can not move down through the next layer with higher density. At the end of centrifugation a set of discrete bands at the interface of density layers may be observed. Each zone comprises particles characterized by their sedimentation rate. Interfaces are sequentially removed from the top of the gradient with a Pasteur pipette connected to a pipette pump or using needle to puncture the plastic centrifuge tube and collecting drops from the bottom. Step gradients are formed prior to centrifugation, either by over layering the different concentrations into the tube by pipette or under-layering the solutions by syringe and needle, long enough to reach the bottom of the tube. In the second case the lighter solution is placed first.

2.6.3.3.2 Continuous (linear) density gradient

This method is characterized by sedimentation through a steep density gradient that contains very high concentration of carbohydrates or heavy metal salts. Two types of continuous gradients are known: self-forming and preformed gradients. On the other hand they can also be normal and iso-osmotic, the latter are important for separating cells. With the exception of Percoll, self-forming gradients cannot be iso-osmotic. In the case of Percoll gradient, as it is a colloid not a true solution, its osmolarity depends on the diluents solutions (such as 0.25 M sucrose or 0.15 M NaCl). Thus the major advantage is that it is possible to prepare either preformed or self-forming iso-osmotic gradients.

(a) Preparation of preformed gradients

Continuous gradients can be prepared easily, allowing solutions with different density to diffuse. The duration of diffusion is about 12-18 hr if the tube is vertical but the time can be reduced if the tube is laid horizontally. For example, to prepare a 5 – 20% gradient, equal amounts of 20, 15, 10 and 5 % sucrose can be layered into a tube, starting from the bottom Alternatively four aliquots, for example solutions with densities of: 1.025, 1.050, 1.075 and 1.100 g/cm^3 can be underlaid carefully into a centrifuge tube, then the tube is securely capped, laid in a horizontal position and left undisturbed for 45 min at room temperature. During this time an almost linear gradient will be formed by diffusion. This method is effective with the most diffusible gradient solutions. Another way of performing linear gradient is by using a simple two-chamber gradient maker, to mix the solutions having two final concentrations, chosen for the top and bottom of the gradient. Depending on the speed of gradient preparation, it may be sharper or smooth. The cells or organelles can be placed throughout the gradient by

mixing them with one of the solutions, or loaded on the top, immediately prior to centrifugation.

(b) Self-forming gradients

Isopycnic experiments with cesium salt solutions can be made using such type of gradients. To make such gradients is easy, since they involve selecting only one concentration of salt solution calculated so that, when redistributed under centrifugal force, the resultant gradient will have a density range that encompasses the particle or particles of interest. It is easier to start with a uniform solution of the sample and a gradient material. Under the influence of centrifugal force, sample particles sediment or float to their isopycnic position. This self-generating gradient technique often requires long hours of centrifugation (e.g. for DNA it takes 36 to 48 hours in cesium chloride gradient). It is important to note that the run time cannot be reduced increasing the rotor speed, this only results in changing the position of the zones in the tube. The length of time can be shortened by preformed gradient like a "step" gradient and overlaying the sample on the top. During centrifugation the layers become linear in 2–3 hours, depending on the concentration. With such gradients, most isopycnic experiments are completed in as little as 2 to 8 hours, although the gradient may not yet have reached equilibrium.

• Separation on the basis of density

In linear gradient centrifugation the cellular organelles are moved until they reach a position where the density of the solution is equal to its own density. At this point the components float and cannot move further. At the end of centrifugation a series of distinct bands is produced in the tube. The bands near to the bottom of the tube contain the components of the highest density. For this reason the method is known as

equilibrium density separation and is so sensitive, that it is possible to separate macromolecules having incorporated heavy isotopes, from the same macromolecules that have not. Nucleic acids have been studied extensively by linear density gradient using heavy metal salts, such as CsCl and Cs2SO4. DNA and RNA, as well as synthetic polynucleotides can be resolved permitting genetic studies. The sedimentation characteristics of native DNA compared with denatured or damaged DNA may also be studied and the guaninecytosine content can be determined from buoyant densities.

2.6.4 Assessment of samples purity

To estimate the quality of organelles purification, both biochemical and morphological criteria can be used. To be confident that the applied methods ensure preservation of the subcellular organelles integrity as well as their morphological parameters, the microscopic techniques can be applied. These techniques allow investigator to make quick and clear visualization of obtained fraction for estimation of purity. Considering the organelles of interests, light or electron microscopy observations can be used. When the purpose is the enzyme purification or physiological studies of enzyme activity, it is essential to obtain native structures for biochemical assays. During the fractionation the enzyme markers, specific for the organelles in question, should be verify at each step.

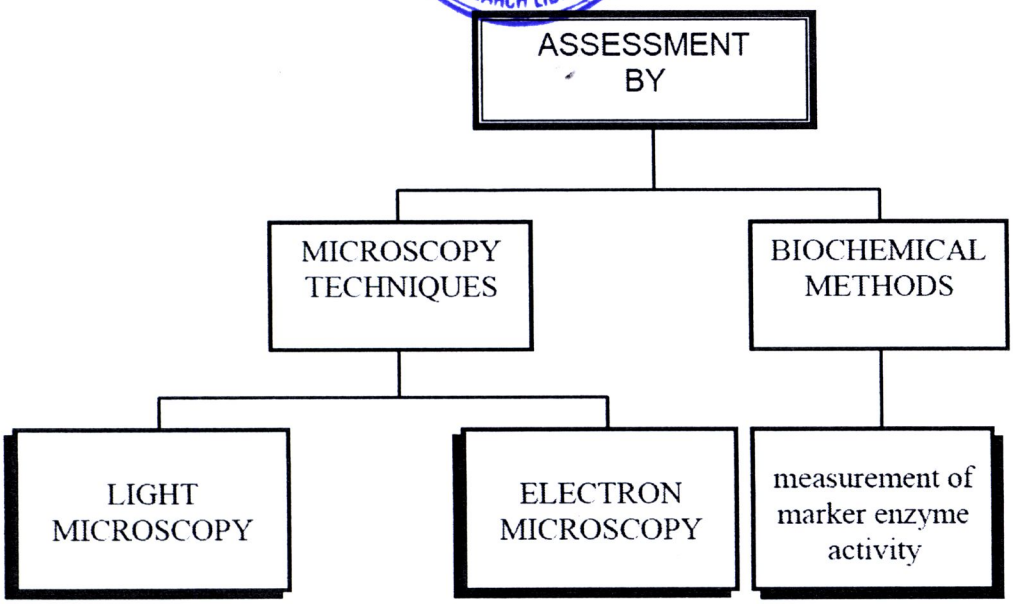


Figure 2.10 Methods for measurement of purification organelles (modified from Stephanova and Topouzova, 2001)

2.6.4.1 Microscopy techniques

Microscope preparations are extensively used to examine the morphological features of samples. In special cases, during isolation direct examination of fractions is required for observing their natural state. In this case the rapid and easy way for assessment is light microscopy. Electron microscopy provides an alternative methodology for assessment of the homogeneity of organelle preparations.

• Light microscopy

For some fractions (including nuclear and nucleolar) it is easier to control the purification steps under the phase-contrast microscopy. Both phase-contrast and interference microscopy are based on the amplification of phase differences, caused by differences in refractive index, within various structures of the observed specimen.

Phase optic allows estimating of unstained native cell organelles, without harming them. For others, additional specific staining (using dyes that selectively stain the required structures or compartments of the cell) should be applied in order to increase the contrast.

- **Electron microscopy**

The most precise criteria for purity and homogeneity of isolated subcellular fractions and macromolecular architecture of the cell are provided by electron microscopy. Electron microscopy of the fractions thought to contain organelles of interest allows determining if the organelles are free of contaminating particles and if their morphology is preserved. Gradient fractions (e. g., the mitochondrial and lysosomal preparations) can easily be prepared for electron microscopic examinations.

2.6.4.2 Biochemical methods

A suitable approach to proof the subcellular organelles infractions is assessment of specific enzyme activities. It is known that each subcellular structure possess marker molecules, which distinguished it from others. The enzymatic assessment of the purity of an organelle preparation requires careful tabulation of marker enzyme recoveries, specific activities and enrichment. During the separation and purification, the enzyme markers specific for the organelle should be enriched at each step. That means that, the specific activity of the enzyme marker should increase, but at the same time the abundance of other organelle markers should decrease. The purity of nuclear preparation can be examined by the absence of cytoplasmic organelle marker activities.

• **Measurement of marker enzyme activities**

Intact organelle membranes prevent substrate access to luminal enzymes. Thus, organelle-specific enzyme activity is generally measured in the presence of nonionic detergent. Comparison of enzyme activity in the presence and absence of detergent provides a quick method to assess organelle intactness. In order to determine the recovery of the marker enzyme activities, total enzyme activities (sample activity/sample volume x total ml) should be estimated. If the preparations are well done, the recoveries should range between 80 and 120 %. Assessment of marker enzyme-specific activities (total activity/total protein) in each gradient fraction should be done to provide the fold enrichment values (the ratio of specific activity of the gradient fraction to specific activity of initial suspension).

2.7 Cell protoplast and extraction of protoplast

Protoplasts are cells (plant, fungal or bacterial) that have had their cell walls removed. This can be done mechanically, or by enzymic digestion. The 'naked' cells are surrounded only by a cell membrane and can be used in a variety of ways. Protoplasts are spherical, clearly 3-dimensional and float freely within the solution. Chloroplasts may be pushed against a small portion of the membrane by the vacuole (the vacuole membrane cannot be seen). The sizes of the protoplasts are generally consistent within a particular preparation, but different tissue sources and different osmotic solutions may result in varying sizes of protoplasts. Compare the size of the protoplasts with the size of cells in the untreated leaf squares. Various plant tissues can provide the cells for protoplast production. Protoplasts have also been isolated from suspension cultures

(single cells floating and growing in solution), callus cultures (a callus is undifferentiated tissue), embryos, shoots and seedlings. Tissue with a thin cuticle works best (Karen, 1993). Plant protoplasts can be produced by two procedures: (1) mechanically slicing of chopping of plant tissues (2) enzymatically digesting the cell wall using various hydrolytic enzymes. To isolate protoplasts by mechanical slicing, numerous cuts are made through plant tissue. This eventually allows some of the protoplasts to slip out of tangentially sectioned cell. This procedure produces protoplasts rapidly, but the yields are low and technique like potato tubers or apple fruit (Leigh and Branton, 1976). Protoplasts isolation by the move common procedure of enzymatic cell wall digestion involves the use of cellulase, hemicellulase and pectinase, which are extracted from various sources including fungi, snail gut and termite gut. These hydrolytic enzymes are available commercially in differing formulations of varying purity. Digestion by a combination of these three enzymes is generally conducted at a pH 5.5-5.8 over period of 3-16 h. No matter which procedure is used, protoplasts can be collected and purified by centrifugation techniques designed to separate broken and damaged cell from intact protoplasts by taking advantage of their differing buoyant densities.

2.8 Vacuole and Isolation of vacuole

The vacuole is the most conspicuous organelle of mature plant cells and plays an important role in the control of a variety of cellular processes. These include the regulation of cell turgidity, the modulation of cytoplasmic component concentrations and cellular functions through cytoplasm/vacuole exchanges across the tonoplast (Boudet et al., 1984). Most of the present knowledge on the biochemical characteristics

of the plant vacuole and its membrane comes from studies on isolated vacuoles. Vacuoles, which are relatively large in size and surrounded by a single membrane, are very fragile organelles. This explains why they were the last cellular organelles to be isolated in a pure state in the mid-1970s and that most of the methods developed include gentle disruption of the protoplasts to release the intact vacuoles. There is no single or preferable way to isolate vacuoles. The choice of a procedure may depend on the nature of the plant material or on the purpose of the scientist. Dürr et al. (1975) originally designed the polybase induced lysis of protoplasts for yeast and modified by us for plant vacuoles. In a recent comparison of different experimental procedures potentially suitable for vacuole isolation from *Petunia hybrida* protoplasts, Aerts and Schram 1985 conclude that the polybase procedure results in the best vacuole preparation. When vacuoles are released from protoplasts, whatever the specific procedure used, certain principles must be observed: (1) rapid protoplast isolation procedure to avoid prolonged exposure to wall-degrading enzymes, (2) gentle lysis of the protoplasts in order to avoid rupture of the released vacuoles, (3) efficient lysis in order to limit the occurrence of residual protoplasts which exhibit similar size and density to the vacuoles and are very difficult to discard by centrifugation methods, and (4) rapid vacuole isolation procedure and limitation of the purification steps in order to reduce bursting of the released vacuoles and leakage of solutes through the tonoplast.