CHAPTER 2

LITERATURE REVIEWS

In general, the yellowing of leaves, florets and fruit pericarp is an important factor, indicating quality deterioration of stored horticultural products. Obviously, in spinach (Yamauchi and Watada, 1991), parsley (Yamauchi and Watada, 1993), broccoli (Costa et al., 2005), and lime (Win et al., 1991; Srilaong et al., 2011), the most visible deterioration is the loss of sepal and peel greenness that usually occurs with chlorophyll (Chl) breakdown. On the other hand, in the fruit of an early-ripening cultivar, Wase Satsuma mandarin (*Citrus unshiu* Marc var. Tanaka), the peel is still green when the flesh matures and the fruit attains harvest maturity. To improve quality, ethylene is provided to the fruit to accelerate the degreening of the peel (Shimokawa et al., 1998). Thus, chlorophyll degradation is a characteristic symptom of leaf senescence and fruit ripening, and elucidating the mechanism of Chl degradation is important subject when considering the maintenance of the quality of harvested horticultural crops.

Quality in Tahitian lime (*Citrus latifolia* Tan.) deteriorates quickly after harvest. The most visible deterioration factor is the loss of peel greenness that usually occurs together with Chl degradation (Drazkiewice, 1994; Srilaong et al., 2011). Maintenance of the green color in the peel of lime is desirable during storage.

In Thailand lime (*Citrus aurantifolia* Swingle cv. Paan) is economically significant horticultural crop and the main production season is July to September (Pranamornkith et al., 2010). The lime fruits are harvest when the rind is still green. The price of lime fruit is strongly dependent on its availability and quality

characteristics. Postharvest quality in lime deteriorates quickly after harvest. The most visible deterioration factor is the loss of peel greenness that usually occurs together with Chl degradation (Srilaong et al., 2011; Kaewsuksaeng et al., 2011). For the maintenance of postharvest quality, it is necessary to retain the green color in the peel of lime as long as possible.

An early step of Chl *a* degradation seems to be the removal of the side chain attached to the tetrapyrrole macrocycle to form chlorophyllide (Chlide) *a* by chlorophyllase (Chlase). Chlide *a* formed still retains a green color (Shimokawa et al., 1978). The elimination of Mg^{2+} from Chlide *a* to produce pheophorbide (Pheide) *a* is induced by a Mg-dechelatase (MD) (Kaewsuksaeng et al., 2006, 2007) or Mg-dechelating substance (MDS) (Aiamla-or et al., 2010; Kaewsuksaeng et al., 2010), and the Pheide *a* formed loses its green color. Finally, Pheide *a* is decomposed to fluorescent Chl catabolites, which are primary colorless, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al., 1999). Chl-degrading peroxidase (POX) (Kaewsuksaeng et al., 2007) is also suggested to be involved in Chl degradation as the first step enzyme with oxidizes Chl *a* to form 13^2-hydroxychlorophyll ($C13^2$-OHChl) *a*. In addition, a new Chl degrading enzyme, pheophytinase (pheophytin pheophorbide hydrolase, PPH) which would dephytylate the Mg-free Chl pigment, pheophytin (Phein) *a* to give Pheide has been recently reported (Schelbert et al., 2009).

Chlorophyll structure

Chls are porphyrins containing basic tetrapyrrole rings, of which one is reduced. The four rings are coordinated with Mg^{2+} ion. A fifth isocyclic ring E, is found near the third pyrrole ring. At the fourth ring, the propionic acid substituent is esterified with diterpene alcohol phytol ($C_{20}H_{39}OH$), which is the hydrophobic side of the molecule, the rest of the molecule being hydrophilic. Chl *b* differs from Chl *a* only by having an aldehyde group (-CHO) in place of the methyl group at ring B position (Figure 2.1) (Hörtensteiner and Kräutler, 2000).

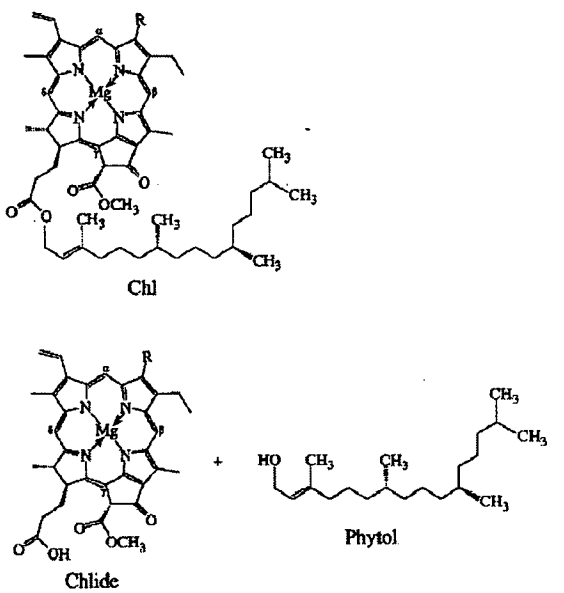

Figure 2.1 Structure formulae top: Chl *a* (R=CH_3), Chl *b* (R= CH=O); bottom: Chlide *a* (R=CH_3), Chlide *b* (R= CH=O) and phytol.

Chlorophyll derivatives

Chls can be readily transformed, both *in vivo* and *in vitro*, into a series of derivatives.

1. Chlorophyllides a and b

The phytyl ester can be easily hydrolyzed to give Chlide and phytol. The hydrolysis takes place under mild conditions either by acid or alkali. Chlides *a* and *b* are prepared enzymatically, the hydrolysis being catalyzed by Chlase, enzyme commonly found in green plant tissues. Leaves that are especially rich in Chlase such as sugar beet (Holden, 1961), common cocklebur (*Xanthium pennsylvanicum*) (Pennington et al., 1964), goosefoot (*Chenopodium album)* (Shioi et al., 1996) and *Citrus unshiu* fruit (Yamauchi et al., 2003) are used as enzyme sources.

2. Pheophytins a and b

Pheins are the magnesium-free derivatives of Chls. Pheins *a* and *b* are easily obtained from Chlase by the action of dilute acids, which remove the magnesium. The reaction lasts 1-2 min, and the concentration of HCl used is 13% (Pennington et al., 1964; Hynninen et al., 1973).

3. Pheophorbides a and b

Pheides *a* and *b* are hydrolyzed Chl without phytol (Chlides) that have also lost the magnesium. The reaction may be prepared from Chls treated with concentrated acid (30% HCl) or from acidified Chlides (Pennington et al., 1964; Hynninen et al., 1771).

4. C13^2-hydroxychlorophyll a and b

Chl *a* are oxidized with the oxygen atom being located at the position C-13^2 and hydroxychlorophyll being formed. C13^2-OHChl *a* was indentified in senescing excised leaves (Maunders et al., 1983) and broccoli, which was prepared by adding

peroxidase with the existence of H_2O_2 and *p*-coumaric acid to Chl *a* solution (Funamoto et al., 2002).

5. Pyrochlorophylls

Pyroderivatives of Chls or their derivatives are compounds that have lost the carbomethoxy group $-COOCH_3$ at C-10 of the isocyclic ring, the group being replaced by hydrogen. Chl *a*, methyl Chlide *a*, Phein *a*, or methyl Pheide *a* when heated in pyridine at 100°C give rise to 'pyro' derivatives by decarbomethoxylation (Pennington et al., 1964).

The chlorophyll degradation pathway

The generally accepted pathway of chlorophyll degradation comprises two stages, before (early stage) and after (late stage) cleavage of tetrapyrrole macrocyelic rings. 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), 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 thus essential in the degreening of the Chl molecule and therefore it determines Chl 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) (Takamiya et al., 2000).

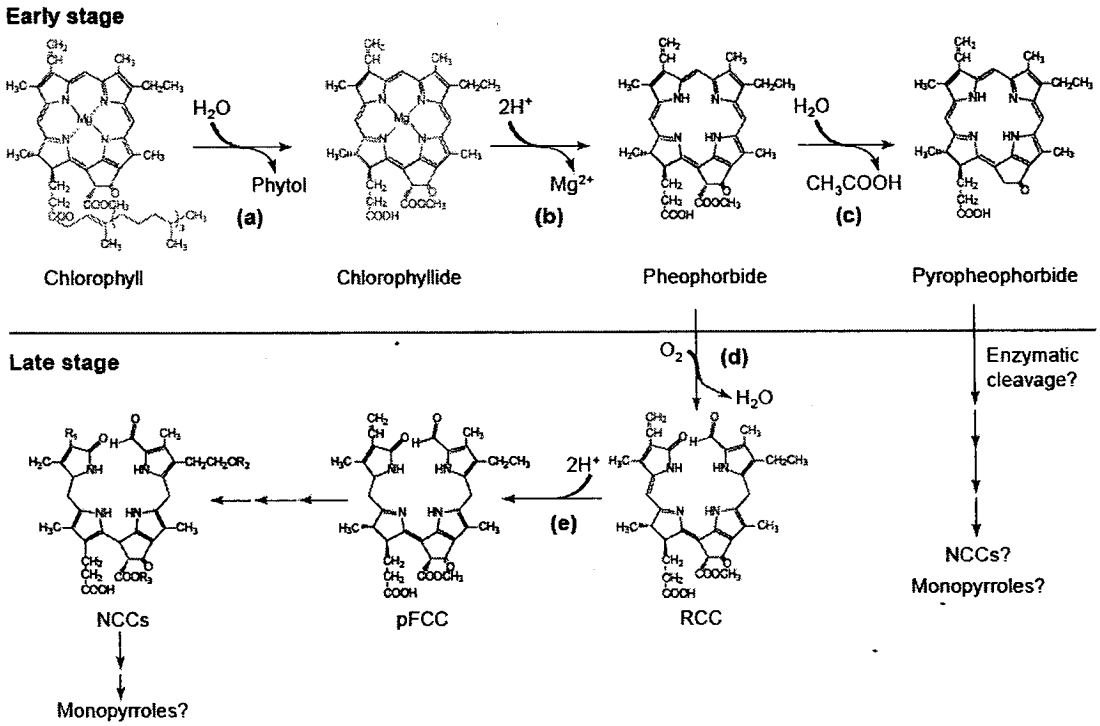

Figure 2.2 Chlorophyll degradation in higher plants (a) chlorophyllase (b) Mg-dechelatase (c) Pheophorbidase (d) Pheophorbide *a* oxygenase. (e) Red cholorphyll catabolite reductase. Abbreviations: NCCs, nonfluorescent chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolite; RCC, red chlorophyll catabolite.

Characterization of Chlorophyll-degrading enzymes in relation to chlorophyll degradation

1. Chlorophyllase

The enzyme catalyzing the dephytylation, Chlase was one of the first plant enzymes to be studied (Willstätter and Stoll, 1913). There are many reports on Chlase activity, including the properties of crude enzymes and the effect of internal

and external factors such as phytohormones and temperature stresses on the activity (Drazkiewice, 1994). In spite of repeated isolations of Chlase from various plants and algae (Trebitsh et al., 1993; Khalyfa et al., 1995; Tsuchiya et al., 1999), molecular properties such as the entire amino acid sequence, functional domain, homology among Chlase and regulation of the expression of Chlase were unclear. The Chlase reaction is the first step of Chl degradation and therefore the location of Chlase is a factor in determining the site of Chl degradation. There is more than one compartment for Chlase localization. In most cases, the Chlase activity was latent in chloroplasts and *in vitro* which was considered to be thylakoid bound. Activity detection of Chlase using chloroplast subfractions localized Chlase activity to the envelope, probably in the inner membrane (Matile et al., 1997). The latency appeared to be merely the result of the spatial inaccessibility of Chlase to Chl in the Chl–protein complex in thylakoid. Thus, based on the envelope location of Chlase, it was recently proposed that, *in vivo*, an as-yet unknown carrier protein for chlorophyll is synthesized in the senescent cell and transported to senescing chloroplasts and then it shuttles between the thylakoid and envelope membrane (Matile et al., 1997; 1999). The Chl molecule or Chl–protein complex released from such plastoglobuli could be attacked by Chlase in the vacuole more than the chloroplast (Parthier, 1988; Satoh et al., 1998).

2. Mg-dechelation activity

Mg-dechelation takes place after dephytylation to yield pheophorbide *a* and pheophorbin *a* using Chlide *a* and Chlin *a* as substrates (Kanieda et al., 2005). Initially, the *in vivo* and *in vitro* accumulation of pheopigments during Chl degradation of algae and higher plants suggested the presence of MD enzymes

(Kaewsuksaeng et al., 2006, 2007). Furthermore, *in vitro* assays of the dechelatase activity revealed that it was associated with thylakoid membranes in a latent form of rape cotyledon (Langmeier et al., 1993). By contrast, in *Chenopodium album*, the activity could still be detected in a soluble with a low molecular mass (900 Da.) fraction after gel filtration of the enzyme, and it was heat stable. This activity was thus designated MDS (Shioi et al., 1996). Costa et al. (2002) reported that the Mg-dechelation activity was associated with a compound with a low molecular weight substance of 2180 ± 20 Da. Suzuki et al. (2005) and Kunieda et al. (2005) demonstrated that the low molecular weight substances in radish cotyledons and mature leaves of *Chenopodium album* play a role in catalysis of the Mg-dechelation reaction using Chlide *a* as a substrate. Specifically, the low molecular weight substances in the mature leaves of *Chenopodium album* were found to be the molecular masses of 3.3 and 1.1 KDa (Kunieda et al., 2005). Lastly, the characterization of Mg-dechelation activity of stored broccoli florets was more investigated to clarify the mechanism of Chl degradation (Kaewsuksaeng et al., 2010). Only one peak of the activity was detected in fresh broccoli extract and no other isozyme with Mg-dechelating action was found in the yellow broccoli extract and molecular mass was about 70 KDa.

3. Pheophorbidase

Chl derivatives are found with modified side chains of a tetrapyrrole macrocycle. Recently, an enzyme named pheophorbidase (28–29 KDa) has been purified from *Chenopodium album*, which catalyzes the hydrolysis of the methyl ester bond of the isocyclic ring of Pheide to yield C13^2-carboxypyropheophorbide (Watanabe et al., 1999). This is not stable and therefore is nonenzymatically converted to

pyropheophorbide (Pyropheide). Interestingly, pheophorbidase is located outside the chloroplast (Kunieda et al., 2005). If Pheide *a* is a true substrate of the enzyme, it suggests that there is another degradation pathway whose early steps occur outside chloroplast. Because the pheophorbidase activity is found in several, but not in all species of higher plants tested, this reaction might be specific for certain plants. $C13^2$-OHChl *a* is reported to be accumulated in ethylene-treated *Citrus* species and other plants (Janave, 1997; Maeda et al., 1998) and might be an intermediate in the oxidative Chl bleaching pathway as well (Janave, 1997).

4. Chlorophyll degrading-peroxidase

The mechanism of *in vitro* Chl degradation by peroxidase can be summarized as shown in Figure 2.3 Peroxidase oxidizes the phenolic compounds, which have the hydroxyl group at the *p*-position, to form the phenoxy radical and superoxide anion; then, the radical and/or superoxide anion attacks Chl *a* to form $C13^2$-OHChl *a*. Chl *a* may be ultimately degraded in sequence to colorless low molecular weight compounds through Chl catabolites such as $C13^2$-OHChl *a* and bilirubin-like compounds (Yamauchi et al., 2004) . Martinoia et al. (1982) demonstrated that peroxidative Chl bleaching activity was present in the thylakoid membrane of barley seedlings. Abeles et al. (1988) reported that in cucumber (*Cucumis sativus* L.) cotyledons treated with ethylene, cationic peroxidase (33KD, pI=8.9), which degrades Chl *in vitro*, increased. Yamauchi and Watada (1998) reported the involvement of peroxidase in Chl degradation of stored broccoli florets. The activity of peroxidase, which is involved in Chl degradation, showed a sharp increase concurrently with floret yellowing (Funamoto et al., 2002). By the method of native-PAGE, six anionic and two cationic isoperoxidases were detected in fresh broccoli

florets. In these isoperoxidases, only one cationic isoperoxidase (Rf 0.3) was related to Chl degradation. The cationic isoperoxidase was further purified by means of molecular exclusion chromatography and cationic exchange chromatography. Two Chl-degrading peroxidase isozymes (Type I and Type II) were contained in the cationic isoperoxidase (Funamoto et al., 2003).

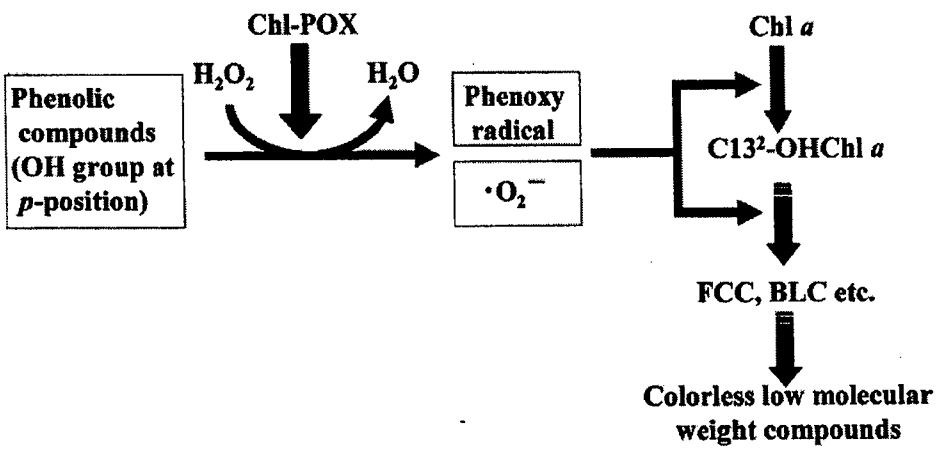

Figure 2.3 Pathway of peroxidase-mediated chlorophyll degradation. Chl-POX: Chlorophyll-degrading peroxidase, Chl: Chlorophyll, $C13^2$-OHChl: $C13^2$-Hydroxychlorophyll, FCC: Fluorescent chlorophyll catabolite, BLC: Bilirubin-like compounds

5. Pheophytinase

A new Chl degrading enzyme, PPH which would dephytylate the Mg-free Chl pigment, Phein *a* to give Pheide *a* has been recently reported (Schelbert et al., 2009). They identified PPH, a chloroplast-located and senescence-induced hydrolase widely distributed in algae and land plants. *In vitro*, *Arabidopsis* PPH specifically dephytylates the Mg-free chlorophyll pigment, phein, yielding Pheide *a*. An *Arabidopsis* mutant deficient in PPH (*pph-1*) is unable to degrade chlorophyll during senescence and therefore exhibits a stay-green phenotype. Furthermore, *pph-1*

accumulates Phein during senescence. Therefore, PPH is an important component of the Chl breakdown machinery of senescent leaves, and we propose that the sequence of early Chl catabolic reactions be revised. Removal of Mg most likely precedes dephytylation, resulting in the following order of early breakdown intermediates: Chl → Phein → Pheide. Chlide, the last precursor of Chl biosynthesis, is most likely not an intermediate of breakdown. Thus, Chl anabolic and catabolic reactions are metabolically separated.

Postharvest irradiation

The UV treatments have been reported to maintain the postharvest quality of horticulture crops. UV-C seems to be especially effective in maintaining the postharvest quality of strawberries (Erkan et al., 2008) and in inhibiting Chl degradation in stored broccoli florets (Costa et al., 2006a). Aiamla-or et al. (2010) found that UV-B treatment effectively delayed Chl degradation of broccoli during storage. Srilaong et al. (2011) also reported that UV-B treatment effectively suppressed Chl degradation and retarded the reduction of the Chl derivative levels in mature green lime during storage.

Postharvest heat treatment

Heat treatment substitutes a non-damaging physical treatment for chemical prevention. There are three methods in use to heat commodities; hot water, vapor heat and hot air. Hot water is originally used for fungal control, but has been extended to disinfestation of insects. Vapor heat is developed specifically for insect control, and hot air has been used for both fungal and insect control and to study the

response of commodities to high temperature. The last two methods (vapor heat and hot air) have subdivisions in that sometimes the air is relatively static, and sometimes air flow is quite high; additionally, hot air can have humidity control or not. All of these permutations may affect the response of the commodity to the heat treatment and affect the length of time of exposure needed to achieve a desired effect (Lurie, 1998).

Hot water dips and sprays

Hot water dips are effective for fungal pathogen control, because fungal spores and latent infections are either on the surface or in the first few cell layers under the peel of the fruit or vegetable. Postharvest dips to control decay are often applied for only a few minutes, at temperatures higher than heat treatments designed to kill insect pests located at the interior of a commodity, because only the surface of the commodity requires heating. Many fruits and vegetables tolerate exposure to water temperatures of 50–60°C for up to 10 min, but shorter exposure at these temperatures can control many postharvest plant pathogens (Barkai-Golan and Phillips, 1991). In contrast, hot water dips for fruit require 90 min exposure at 46°C. The extension of the hot water treatment has been the development of a hot water spray machine (Fallik et al., 1996a). This is a technique designed to be part of a sorting line, whereby the commodity is moved by means of brush rollers through a pressurized spray of hot water. By varying the speed of the brushes and the number of nozzles spraying the water, the commodity can be exposed to high temperatures for 10-60 s. The water is recycled, but because of the temperatures used (50–70°C), organisms which are washed off the product into the water do not survive. This machine is in

use both to clean and to reduce pathogen presence on a number of fruits and vegetables, such as mangos (Prusky et al., 1997) and peppers (Fallik et al., 1996b).

Vapor heat

Vapor heat is a method of heating fruit with air saturated with water vapor at temperatures of 40–50°C to kill insect eggs and larvae as a quarantine treatment before fresh market shipment (Animal and Plant Health Inspection Service, 1985). Heat transfer is by condensation of water vapor on the cooler fruit surface. This procedure was first used to kill Mediterranean (*Ceratitis capitata* Wiedemann) and Mexican (*Anastrepha ludens* Loew) fruit fly (Hawkins, 1932; Baker, 1952) in a chamber without forced air. However, once ethylene dibromide and methyl bromide came into use as inexpensive chemical fumigants. In modern facilities the vapor heat includes forced air which circulates through the pallets and heats the commodity more quickly than vapor heat without forced air. Commercial facilities operate in many countries, mainly for use on subtropical fruits, particularly on mango and papaya (Paull and McDonald, 1994).

Hot air

Hot air can be applied by placing fruits or vegetables in a heated chamber with a ventilating fan, or by applying forced hot air where the speed of air circulation is precisely controlled. Hot air, whether forced or not, heats more slowly than hot water immersion or forced vapor heat, although forced hot air will heat produce faster than a regular heating chamber. The hot air chamber has been utilized to study physiological changes in fruits and vegetables in response to heat (Klein and Lurie, 1991, 1992). Forced hot air, however, has been used to develop quarantine procedures (Gaffney and Armstrong, 1990). One reason is that the high humidity in

vapor heat can sometimes damage the fruit being treated, while the slower heating time and lower humidity of forced hot air can cause less damage. A high temperature forced air quarantine treatment to kill Mediterranean fruit fly, melon fly and oriental fruit fly on papayas has been developed (Armstrong et al., 1989; Hansen et al., 1990). This procedure may require rapid cooling after the heat treatment to prevent fruit injury, as may the forced hot air treatment for citrus (Sharp and Gould, 1994; Sharp and McGuire, 1996).

Effect of heat treatment on chlorophyll degradation

Heat treatment leads to an accelerated rate of degreening in apples (Liu, 1978; Klein et al., 1990). Chl content in apple peel, plantain peel and tomato pericarp decreased during a hot air treatment of 35–40°C (Seymour et al., 1987; Lurie and Klein, 1991, 1992). Hot water immersion at 45°C for 30–60 min can also induce yellowing of cucumbers (Chan and Linse, 1989), as does forced vapor heat for 30 min at 45°C for zucchini (Jacobi et al., 1996) (zucchini are known as courgettes in some countries). Color changes in papaya skin or flesh were not affected by hot water immersion at 42°C for 30 min followed by 49°C for 90 min (Paull and Chen, 1990) and the same hot air treatment which stimulated degreening of plantains failed to degreen bananas (Seymour et al., 1987). Hot water dips at 43–55°C for up to 10 min delayed yellowing of broccoli (Forney, 1995; Tian et al., 1996, 1997). The difference in responses of different commodities may be an indication of whether new enzymes must be synthesized to effect the color changes or not. In the case of apples chlorophyll degradation reveals the yellow of the underlying carotenoids already present, while other fruits may require synthesis of carotenoids. For example, it has

been found that hot air at 38°C or higher inhibits lycopene synthesis in tomatoes (Cheng et al. 1988). The inhibition of lycopene is due to the inhibition of transcription of mRNA for lycopene synthase, a key enzyme in the pathway, and this recovers after removal from heat (Lurie et al., 1996). In bananas the inhibition of degreening during the heat treatment appears to be due to the absence of the Chl oxidase enzyme resulting in the retention of Chl in the peel (Blackbourn et al., 1989). In broccoli hot water-treated at 45°C for 14 min, floret yellowing was apparently retarded for 2 to 3 days at 20°C storage (Kazami et al., 1991). Tian et al. (1996) observed that hot water treatment at 47°C for 7.5 min was the best treatment for retarding floret yellowing. Terai et al. (1999) reported that hot air treatment at 50°C for 2 or 3 h effectively inhibited floret yellowing, but the treatment for 3 h strongly retarded enzyme activity, such as that of ACC oxidase. Funamoto et al. (2005) showed that hot air treatment at 50°C for 2 h. with broccoli could reduce Chl degradation due mainly to the suppression of Chl degrading enzyme activities such as Chlorophyllase, Chl-degrading peroxidase and Chl oxidase. Moreover, Costa et al. (2006a) also reported a treatment at 48°C for 3 h delayed Chl *a* catabolism in broccoli during postharvest senescence and decreased the activities of chlorophyllase, Mg-dechelatase and peroxidase.