QUANTIFYING ANTIOXIDANT ACTIVITY

OF LYCOPENE AND INFLUENCE OF

WATERMELON MATURITY ON TISSUE

ULTRASTRUCTURE AND LYCOPENE

DISTRIBUTION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I – Introduction and Review of Literature

Lycopene (gamma, gamma carotene), a lipophilic antioxidant, may play a number of key health-promoting roles in the human body. Among these are cancer prevention, reduction of cardiovascular risks, and regulation of the immune system (Giovannucci, 1999; Hassler, 1998; Rao and Agarwal, 1998). Currently, the most extensive use of lycopene is as a coloring agent for the food, cosmetic and pharmaceutical industries (Cadoni et al., 2000). However, the high antioxidant activity and bioavailability of lycopene make it very attractive as an ingredient in the functional food and nutraceutical products that are increasingly popular in today's food marketplace. Lycopene is the characteristic pigment in red tomato, red watermelon, and other fruits such as guava and pink grapefruit. Potential sources of lycopene and the levels present in different foods have been previously tabulated (Nguyen and Schwartz, 1999). The technology exists to extract and purify lycopene for use as a functional food ingredient. However, further research is needed to correlate lycopene concentration in a food product with antioxidant activity. Such knowledge is critical for determining effective and economical rates of lycopene addition.

Lycopene antioxidant activity and ORAC$_{FL}$ assay

Interest has grown in recent years in methods to measure antioxidant activity (Prior et al., 2003). This is attributed to overwhelming evidence of the role of reactive oxygen/nitrogen species (e.g. peroxyl radicals, hydroxyl radicals, singlet oxygen, super

oxide radicals and reactive nitrogen radicals) in aging and pathogenesis. Several popular in vitro assays are available, such as FRAP (ferric-reducing ability of plasma), TEAC (Trolox equivalent antioxidant capacity), TRAP (total radical-trapping antioxidant parameter) and the recent ORAC (oxygen radical absorbance capacity) (Wu et al., 2003). While the TRAP, TEAC, and FRAP methods are fast (5-6 minutes or less), ORAC is relatively more time intensive (30-70 minutes). TEAC and FRAP assays are similar in principle and measure the reducing power of the sample. TRAP operates similarly to ORAC except that TRAP calculations incorporate lag phase considerations rather than the "area under the curve" approach used with the ORAC assay. ORAC is considered more accurate, robust and, most importantly, reliable due to the fact that it measures the most common free radical (the peroxyl radical) in biological systems. This is in contrast to other methods that use radicals foreign to the human body. Further details on the principles, advantages, and disadvantages of each of these methods have been discussed in detail in the literature (Wu et al., 2003, Ou et al. (2001), Cao et al. (1993)).

One standardized and well established version of the ORAC method is the oxygen radical absorbance capacity-Fluorescein (ORAC$_{FL}$) assay. In the past, this method has been largely limited to testing water soluble antioxidants. However, a number of recent studies have tested the suitability of this assay for use with lipophilic antioxidants. The basic challenge to be overcome when using the ORAC$_{FL}$ assay with lipophilic antioxidants is poor solubilization and/or dispersion of the antioxidant. These solubility and/or dispersion problems help limit the usefulness of the ORAC$_{FL}$ assay for measuring the antioxidant activity of lycopene. Modifying the conventional ORAC$_{FL}$ assay to work with lycopene may provide a powerful and convenient tool for quantifying the

effectiveness of lycopene as an antioxidant in foods.

The ORAC assay originally was developed by Cao et al. (1993). Ou et al. (2001) enhanced the economy and repeatability of the method by substituting fluoresein for the phycoerythrin originally used as a florescent probe. This enhanced assay was termed $ORAC_{FL}$. Both the ORAC and $ORAC_{FL}$ assays employ hydrophilic reagents in an aqueous system, rendering the assay less than optimal for evaluating lipohilic antioxidants. Recently a number of studies have evaluated the suitability of this assay for use with lipophilic antioxidants. The basic challenge to be overcome when using the $ORAC_{FL}$ assay with lipophilic antioxidants is poor solubilization and/or dispersion of the antioxidant, which results in poor assay validity. The use of cyclodextrins as solubility enhancers was first described by Szente et al. (1993) during studies on fatty acid compounds. Cyclodextrins are ring molecules built from α-D-glucose units and, based on the number of glucose units, they are named as α, β or γ cyclodextrins. These doughnut-shaped compounds have the potential to bind a wide array of organic compounds into their central non-polar cavities through hydrophobic interactions. Further extensive studies (Szente et al., 1998, Pfitzner, 2000) on the application of cyclodextrins to carotenoid and fatty acid systems were carried out and various potential cyclodextrin derivatives such as methylated-β-cyclodextrin, hydroxyl propylated-β-cyclodextrin and branched β-cyclodextrin were identified as compounds that improved the solubilization of lipophilic antioxidants. Recently, the $ORAC_{FL}$ assay has been adapted by Huang et al. (2002) for use with vitamin-E and other phenolic antioxidants using randomly methylated β-cyclodextrin as a solubility enhancer. In theory, these lipophilic antioxidants present solubility and/or dispersion problems similar to lycopene. Thus, a modified $ORAC_{FL}$

assay could potentially meet the need for an effective and reliable method for measuring the antioxidant activity of lycopene in foods. One goal of our research was to correlate the antioxidant activity of lycopene with lycopene concentration using the ORAC$_{FL}$ as modified by the use of β-cyclodextrin as a solubility enhancer. Further, once the effectiveness of β-cyclodextrin was evaluated, we tested the efficacy of randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer for improving the ORAC$_{FL}$ assay for use with lycopene.

Watermelon ultrastructure, lycopene distribution and chromoplast biogenesis

Lycopene's high antioxidant activity and bioavailability as well as its potential as a food coloring make it very attractive as a possible ingredient in functional foods and nutraceuticals. Tomato has relatively high lycopene concentration and has been used as a source for natural lycopene extracts and concentrates. Watermelon is another candidate for such uses. Watermelon has been reported to have an average lycopene content of 45.1-53.2 μg/g compared to 30.2 μg/g found in tomatoes (USDA-NCC Carotenoid Database, 1998). Typical commercially harvested watermelons are reported to have an average lycopene content of 45.1-53.2 μg/g as compared to 30.2 μg/g in tomatoes (USDA-NCC Carotenoid Database, 1998). Current U.S watermelon production is estimated at 40 million tons with growth rate projections of 1.4%/year [NFAPP, 2003], and consumption is estimated at 2.3×10^9 kg/yr (USDA, 2001). Thus, watermelon has the potential to be an excellent source of lycopene in the U.S. diet and for the development of lycopene-rich functional foods.

Perkins-Veazie et al. (2001) demonstrated that lycopene levels in watermelon vary with cultivar and season. In particular, differences were typically observed between

seeded and seedless varieties; seedless melons usually exhibited markedly higher lycopene concentrations. Melon maturity is another factor that could influence both lycopene content and extractability. Some preliminary studies (Oikonomakos et al., 2004) have indicated that lycopene levels increase as melons mature and then drop as melons reach over-maturity. Identifying optimum melon maturity for lycopene content and functionality at harvest could therefore be critical for designing processes to maximize lycopene retention in watermelon-based extracts and/or functional foods. However in order to identify optimum maturity, we must understand the structural changes that occur in watermelon tissue as the fruits mature. These changes may influence lycopene content and distribution within the melon tissues. We know, for example, that lycopene may be converted to other carotenoid forms such as β-carotene, γ-carotene and phytofluene during maturation (Harris and Spurr, 1969a). But we do not know how changes in watermelon ultrastructure relate to these conversions. It is necessary, therefore, to understand the association of lycopene with various organelles in watermelon during melon maturation. Currently no research has been done on watermelon ultrastructure relating melon maturity to lycopene distribution within the watermelon mesocarp.

Lycopene in both tomato and watermelon is located in chromoplasts, which are non-senescent, carotenoid-containing plastids lacking in chlorophyll. Chromoplast development is one of the numerous biochemical changes (softening, development of flavor volatiles, starch to sugar conversion, ethylene production, respiratory rise and others) occurring in a ripening fruit. The pathway for chromoplast development (Thomson and Whatley, 1980; Marono et al, 1993; Vothknecht and Westhoff, 2001) can broadly be classified based on the plastid's ability to differentiate either from

photosynthetic or non-photosynthetic plastids. Chromoplast biogenesis is typically classified into two types: Type A and Type B (Marono et al., 1993). In Type A biogenesis, chromoplasts originate from fully developed photosynthetic chloroplasts. This transition is marked by loss of chlorophyll, accumulation of carotenoids, degradation of structured thylakoid membranes, and other physical and chemical changes (Heaton and Marangoni, 1996). In Type B chromoplast biogenesis, chromoplasts originate directly from several types of non-photosynthetic plastids *(*Marono et al, 1993*)*. These non-photosynthetic plastids may be amyloplasts (carrots, squash) or they may be proplastids (squash & mutant tomato, pepper and wild-type fruits grown in the dark). Apart from Type-A & B biogenesis, occasionally chromoplasts can further dedifferentiate & re-differentiate into chloroplasts (e.g. in orange peel).

Mechanisms by which photosynthetic/non-photosynthetic plastids develop into chromoplasts are determined by fruit genotype. If a plant has dominant gene B, proplastids will develop directly into chromoplasts (e.g. in squash). However, if a plant possesses the B+ gene, the developmental pathway takes the form proplastids➔chloroplasts➔chromoplasts (e.g. in tomatoes and peppers).

Chromoplasts are heterogeneous organelles with carotenoid components associated with different structural forms. These serve as the basis for the classification of chromoplasts into 5 groups: Crystalline (carotenoid bodies are in the form of crystals); Globular (carotenoids are dissolved in lipid globules); Fibrillar; Membranous; and Tubular (carotenes are bound to protein molecules). Predominant chromoplast types vary considerably among plant species (Bonora et al., 2000; Cheung et al, 1993; Frey-Wyssling and Schwegler, 1965; Harris and Spurr, 1969a; Harris and Spurr, 1969b;

6

Iwahori and Van-Steveninck, 1976; Knoth, 1981; Kooji et al, 2000; Lim and Boyer, 1994; Ljubesic et al., 2001; Mateos et al, 2003; Weston and Pyke, 1999; Whatley and Whatley, 1987).

Watermelon and tomato both accumulate lycopene within intracellular chromoplasts. However, the structure of these organelles may differ between watermelon and tomato. Tomatoes exhibit Type A chromoplast formation *(*Harris and Spurr, 1969a; Harris and Spurr, 1969b*)*. In watermelon the chromoplast biogenesis could possibly be Type B type due to the fact that rind covers the mesocarp, creating a dark environment. We might therefore expect to observe the conversion of proplastids directly into chromoplasts. However, the occurrence of both Type A and Type B chromoplast biogenesis cannot be ruled out. Watermelons may therefore exhibit a greater variety of chromoplast structures than do tomatoes because more biogenesis pathways may be active in watermelon. In addition, changes that occur during melon maturation/senescence may increase the variety of chromoplast structures observed. For example, ongoing breakdown of thylakoid structures may create a variety of intermediate structures and make it difficult to distinguish between chromoplasts derived from Type A biogenesis and those derived from Type B. Edwards et al. (2003) list chromoplast type as one factor that affects the absorption of carotenoids in the gut after plants are consumed as food. This suggests that chromoplast structure may well influence carotenoid characteristics during and after processing.

Other expected visible changes in melon ultrastructure that occur as melons mature and senesce include the disappearance of numerous structural organelles (e.g. mitochondria, endoplasmic reticulum's, ribosomes, vacuoles, and other plastid

structures). Piechulla et al., (1987) and Livne and Gepstein (1988) attribute these changes to an age-related decline in protein composition necessary for photosynthetic activity. This results in the termination of various metabolic processes necessary for the survival of these structures. Any or all of these various changes may account for the reported tendency for lycopene concentrations to decline in over-mature watermelons, though the mechanism for such decline remains undescribed.

Ultrastructure studies using transmission electron microscopy (TEM) have been useful for understanding the structural organization of plant and animal tissues. Numerous studies on tomato ultrastructure and lycopene formation have used this approach (Harris and Spurr, 1969a and 1969b; Cheung et al., 1993). Application of TEM therefore holds promise to study watermelon ultrastructure and to relate melon maturity with the distribution of lycopene and other structural organelles within the watermelon mesocarp.

Specific Study Objectives

1. To correlate the antioxidant activity of lycopene with lycopene concentration using the $ORAC_{FL}$ assay as modified by the use of β-cyclodextrin as a solubility enhancer.

2. To further study the effectiveness of randomly methylated β-cyclodextrin as a solubility enhancer in improving the lycopene concentration vs. $ORAC_{FL}$ correlations.

3. To study watermelon ultrastructure (with TEM technique) and to relate melon maturity to the distribution and organization of lycopene within structural organelles in watermelon mesocarp.

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Chapter II – Effect of β-cyclodextrin in improving the correlation between lycopene concentration and ORAC values

Abstract

Lycopene, a lipophilic antioxidant, plays a crucial role in biological systems. It may play an important role in human biological systems by providing protection against cardiovascular disease, some cancers and by boosting the immune system. Oxygen radical absorbance capacity (ORAC) has been validated as an index of antioxidant activity for many hydrophilic antioxidants but not for lycopene. This study validated the ORAC assay for different concentrations of lycopene in the presence of β-cyclodextrin, a water-solubility enhancer. Lyc-O-Mato^{TM} 6 % extract was used as a source of lycopene for the experiments. Lycopene was extracted by standard spectrophotometric assay procedure in the presence of β-cyclodextrin at concentrations of 0, 0.4, 0.8 & 1.6 % and the antioxidant activity of lycopene was measured with the ORAC assay. Experiments were conducted in quadruplicate and statistical pooled correlations analyzed. Statistical analysis showed a very high correlation (R^2=0.99) between ORAC and ascorbic acid concentrations validating our method. Lycopene concentration correlated poorly with ORAC (R^2=0.33) in the absence of β-cyclodextrin. Correlations improved with increasing levels of β-cyclodextrin (R^2=0.58 and 0.91 for 0.4 and 0.8 % β-cyclodextrin respectively). A very high β-cyclodextrin concentration (1.6%) decreased the correlation between ORAC and lycopene concentration. Inclusion of β-cyclodextrin in the ORAC assay improves correlation between ORAC and lycopene concentration, thus expanding

the scope of the ORAC assay to include an additional fat soluble antioxidant such as lycopene.

KEYWORDS: ORAC, lycopene, lipophilic antioxidants, β-cyclodextrin

Introduction

Lycopene, a lipophilic antioxidant, may play a number of key health-promoting roles in the human body. Among these are cancer prevention, reduction of cardiovascular risks, and regulation of the immune system (Giovannucci, 1999; Hassler, 1998; Rao and Agarwal, 1998). Currently, the most extensive use of lycopene is as a coloring agent for the food, cosmetic and pharmaceutical industries (Cadoni et al., 2000). However, the high antioxidant activity and bioavailability of lycopene make it very attractive as an ingredient in the functional food and nutraceutical products that are increasingly popular in today's food marketplace. Potential sources of lycopene and the levels present in different foods have been previously tabulated (Nguyen and Schwartz, 1999). The technology exists to extract and purify lycopene for use as a functional food ingredient. However, further research is needed to correlate lycopene concentration in a food product with antioxidant activity. Such knowledge is critical for determining effective and economical rates of lycopene addition. One standardized and well established method for analyzing antioxidant activity as a function of concentration is the oxygen radical absorbance capacity (ORAC) assay. In the past, this method has been largely limited to water soluble antioxidants. Modifying this method to work with lycopene and perhaps other fat soluble antioxidants provides a powerful and convenient tool for quantifying the effectiveness of lycopene as an antioxidant in foods.

The ORAC assay originally was developed by Cao et al, 1993. Ou et al., 2001, enhanced the economy and repeatability of the method by substituting fluoresein for the phycoerythrin originally used as a florescent probe. These enhanced assays were termed ORAC$_{FL}$. Both the ORAC and ORAC$_{FL}$ assays employ hydrophilic reagents in an aqueous system. Even so, a number of recent studies have tested the suitability of this assay for use with lipophilic antioxidants.

The basic challenge to be overcome when using the ORAC$_{FL}$ assay with lipophilic antioxidants is poor solubilization &/or dispersion of the antioxidant, resulting in poor assay validity. The use of cyclodextrins as solubility enhancers was first described by Szente et al, 1993 during studies on fatty acid compounds. Cyclodextrins are ring molecules built from α-D-glucose units and based on the number of glucose units, they are named as α, β or γ cyclodextrins. These doughnut-shaped compounds have the potential to bind a wide array of organic compounds into their central non-polar cavities through hydrophobic interactions. Further extensive studies (Szente et al, 1998, Pfitzner, 2000) on the application of cyclodextrins to carotenoid and fatty acid systems were carried out and various potential cyclodextrin derivatives such as methylated-β-cyclodextrin, hydroxyl propylated-β-cyclodextrin and branched β-cyclodextrin were identified that improved the solubilization of lipophilic compounds. Recently, the ORAC$_{FL}$ assay has been adapted by Huang et al, 2002 for use with vitamin-E and other phenolic antioxidants using randomly methylated β-cyclodextrin as a solubility enhancer. In theory, these lipophilic antioxidants present solubility &/or dispersion problems similar to lycopene. Thus, a modified ORAC assay could potentially meet the need for an effective and reliable method for measuring the antioxidant activity of lycopene in foods.

The goal of this study was to correlate the antioxidant activity of lycopene with lycopene concentration using the ORAC$_{FL}$ as modified by the use of β-cyclodextrin as a solubility enhancer.

Materials and Methods

Reagents.

Fluorescein, trolox, β-cyclodextrin, ascorbic acid, potassium phosphate, sodium phosphate and sodium azide were purchased from Sigma (St. Louis, MO). 2, 2'-Azobios (2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals (VA, USA). Lyc-O-Mato^{TM} 6 % extract was obtained from LycoRed Natural Products Industries Ltd. (Beer-Sheva, Israel) as a source of lycopene for our experiments. Preliminary testing showed that the fluorescein used in our experiments underwent a decay of >90% fluorescence within 45 min in the presence of 0.332 g of AAPH in 10 ml of phosphate buffer (PB).

Lycopene determination.

Concentration of lycopene in the Lyc-O-Mato^{TM} preparation was determined using a modification of the method of Sadler et al, 1990. Acetone (25 ml), ethanol (25 ml), and hexane (50 ml) and the lycopene concentrate were mixed in a foil-covered Erlenmeyer flask. An aqueous β-cyclodextrin solution (15 ml at 0, 0.4, 0.8 or 1.6%) was then added, the flask was sealed, and the mixture was agitated at approximately 25°C in a constant temperature water bath/shaker for 15 min. The solution was allowed to equilibrate at room temperature for 15 min, and then approximately 3 ml of the top hexane layer was sampled for spectrophotometric analysis. Absorbance at 503 nm was read on a Beckman DU series 500-spectrophotometer (Beckman Instruments, Inc.USA)

16

with a 4.5 ml glass cuvette. Lycopene concentration was calculated as:

$$C = (Ab_{503}/172) * (50) * (535.85)*(1/SW) \text{ where}$$

C = concentration of lycopene in mg/g concentrate.

Ab_{503} = absorbance of hexane/lycopene solution measured at 503 nm.

172 = extinction coefficient of lycopene in hexane in kmoles/cm.

50 = volume of hexane used for extraction in ml.

535.85 = molecular weight of lycopene in g/mole.

SW = sample weight of lycopene concentrate in mg.

The actual measured concentration of lycopene in the extract was approximately 166mg/g.

Lycopene solution preparation.

11mg of concentrate was weighed into a 250 ml foil covered Erlenmeyer flask and extracted according to the lycopene determination method described above, yielding a stock solution of about 68.0 µM lycopene in hexane. This was further diluted 2, 3, 4, 5, and 10 times in hexane to obtain approximately 34.0, 22.7, 17.0, 13.6, and 6.8 µM working lycopene solutions.

β-cyclodextrin solutions (0, 0.4, 0.8 and 1.6%) were prepared in a 1:1 water-acetone mixture (v/v) and kept on a shaker for at least 1 h with heat until the solutions were completely dissolved. These solutions were introduced into the lycopene solutions during the extraction procedure (Sadler et al, 1990) by replacing the water with the β-cyclodextrin solutions.

Ascorbic acid solutions (40 µM) were prepared by dissolving 0.705 mg of

ascorbic acid in 100ml PB and diluting with water to get 20, 10 and 5 µM solutions. This served as a standard to evaluate the equipment for correlation between ascorbic acid antioxidant activity and concentration.

ORAC assay.

The ORAC was conducted using a Perkin-Elmer HTS-7000 Microplate reader in Falcon brand 48-well clear plates at excitation and emission wavelengths of 485 and 535 nm respectively. The reaction temperature used was 37°C, as described in Ou et al, 2001. Other than the antioxidant samples, all other reagents were prepared using PB of pH 7 as a diluent. The volume and final concentrations of freshly prepared $ORAC_{FL}$ reagents used in the 48-well clear plate were as follows: Fluorescein-160 µL/ (0.125g in 1000ml PB➜2ml diluted to 125 ml of PB as diluted stock FL➜ 2ml diluted stock FL diluted in 19ml PB and stored before use), 20 µM Trolox-20 µL (standard wells), hexane-20 µL (Blank wells), lycopene extracts with β-cyclodextrin-20µL (sample wells) and AAPH-20 µL/ (0.332 g in 10ml PB). $ORAC_{FL}$ assays were run for 45 min (or 25 cycles) to generate relative fluorescence profiles over time. The analyzer was preset to record fluorescence every minute after the addition of AAPH. Results were expressed as relative fluorescence with respect to the initial reading. Final computation of results was made by taking the differences of areas under the decay curves between blank and sample & /or standard (trolox). The assay was repeated for each of the five tested lycopene concentrations (34.0, 22.7, 17.0, 13.6, and 6.8 µM) using three β-cyclodextrin levels (0, 0.4, 0.8 and 1.6%), and all assays were replicated four times. Average values were calculated for the 4 replicates and average ORAC values expressed as micromoles of Trolox equivalent (TE). During all experiments, the time gap between the addition of AAPH and the start of fluorescence

reading on the assay reader was kept as short as possible (~15-20 s).

Experimental Design and Statistical analysis.

Our experiment was designed as a completely randomized design with a factorial treatment structure (3x5 – five levels of lycopene concentration by 3 levels of added β-cyclodextrin). Experiments were conducted in quadruplicate; all extractions and analyses were replicated four times. For each replication, results from multiple $ORAC_{FL}$ assays were averaged. Means and standard deviations for $ORAC_{FL}$ values and Pearson's correlation coefficients for $ORAC_{FL}$ values and lycopene concentration were calculated using SAS (SAS, V.7., Cary, NC, 1999) General Linear Means (GLM) and Means procedures. Means were tested for significant differences using Duncan's means separation test within the GLM procedure. Regression analysis was conducted and linear model equations were generated using the SAS Regression procedure.

Results and Discussion

Method Validation for linearity.

With ascorbic acid as a standard antioxidant, relative fluorescence (RF) trends were profiled and correlations obtained between ascorbic acid concentration and $ORAC_{FL}$ values. As in other studies, we observed a very high correlation ($R^2 = +0.983$) between ascorbic acid concentration and $ORAC_{FL}$ values thereby validating our equipment and techniques (data not shown).

Stability of lycopene.

$ORAC_{FL}$ assays were run using both lycopene and, for comparison purposes, α-tocopherol without added AAPH in order to quantify possible intrinsic effects of our

19

lipophilic antioxidant preparations on the oxidative decay of fluorescein. Lycopene concentration was also measured over time using the spectrophotometric method in order to quantify the inherent oxidative stability of our lycopene extracts over the time frame of a typical ORAC assay.

Figure 1 shows the RF versus time profiles obtained for ORAC assays conducted without the radical generator AAPH added. For assays containing lycopene; the net degradation seen over the course of an experimental run was ~8.5 % and this remained consistent over several experiments. Compared to both blank and α-tocopherol samples, lycopene samples exhibited a slightly higher degree of degradation (~8.5% versus <4.5 %). But the relative fluorescence in all samples decayed by less than 10% during the 45 min ORAC assay period. Therefore, we concluded that lycopene did not significantly influence the oxidative decay of fluorescein in the absence of AAPH.

Results of spectrophotometric quantification of lycopene concentration in prepared extracts over time showed that degradation of lycopene during the time frame of our ORAC assay was insignificant (data not shown).

Lycopene concentration and ORAC$_{FL}$ correlation.

Table I gives the mean and standard deviation information for lycopene concentration vs. ORAC$_{FL}$ values obtained at different levels of β-cyclodextrin. The relative antioxidant activities of lycopene extracted with varying levels of β-cyclodextrin, expressed as RF versus time, are shown in Figures 2-4. We observed a similar pattern in all these profiles; an increase in lycopene concentrations reduced the rate of decay in the RF profiles. The pooled correlation of ORAC$_{FL}$ and lycopene concentration with β-cyclodextrin at 0, 0.4 and 0.8 % is shown in Figure 5. Lycopene extracts had a very poor

correlation with ORAC$_{FL}$ values in the absence of β-cyclodextrin (R^2= 0.331). With inclusion of β-cyclodextrin in at 0.4 %, the correlation improved significantly (R^2= 0.58, p<0.001). This trend continued with increased β-cyclodextrin levels up to 0.8 % (R^2= 0.91, p<0.001). At a β-cyclodextrin addition level of 1.6%, the correlation between lycopene concentration and ORAC$_{FL}$ declined significantly (R^2= 0.72, data not shown) possibly due to the fact that the lycopene-cyclodextrin complexation had reached its optima for solubilization or dispersion at a lower concentration. When averaged over all lycopene concentrations, there was no significant difference (p>0.05) between mean ORAC$_{FL}$ values in the presence of 0.4 and 0.8% added β-cyclodextrin (0.0120 and 0.0077 equivalent μmoles Trolox respectively). However, mean ORAC$_{FL}$ values obtained in the absence of added β-cyclodextrin (029407 equivalent μmoles Trolox) were significantly different (p<0.05) than those measured when β-cyclodextrin was added.

Our demonstrated optimum level of β-cyclodextrin addition corresponded with the practical upper limit of β-cyclodextrin solubility that we observed. There was no problem dissolving the β-cyclodextrin at 0.4 % in water: acetone mixture with simple agitation, heat was required in addition to agitation at the 0.8 % level, and at higher levels some degree of haze or sedimentation was seen.

One interesting result observed was that while the correlation between lycopene concentration and ORAC$_{FL}$ values improved significantly with the addition of β-cyclodextrin, overall relative fluorescence decay rates also increased, particularly at the higher lycopene concentrations (Figures 2-4). Previous studies have not shown that β-cyclodextrin functions as a pro-oxidant (Huang et al, 2002). This was supported by our observation of a proportionally greater change in decay rates between 0 and 0.4% β-

cyclodextrin than between 0.4 and 0.8% β-cyclodextrin. Therefore it seems likely that the apparent change in the rate of fluorescein oxidation was a result of the more complete dispersal of lycopene in the solution brought about by the addition of β-cyclodextrin. It may be that, in the absence of β-cyclodextrin, the non-polar lycopene was forming micelles that served to entrap and protect the fluorescein from oxidation. This would explain both erratic and erroneously high $ORAC_{FL}$ values in the absence of β-cyclodextrin.

Our results demonstrating the effectiveness of β-cyclodextrin as a solubility enhancer for lycopene are similar to results reported by other researchers using other lipophilic antioxidant compounds. Huang et el, 2002 saw similar effects using randomly methylated β-cyclodextrin (7% in a (1:1) water-acetone mixture and in a 75mM phosphate buffer at pH 7.4) as a solubility enhancer in $ORAC_{FL}$ assays on the lipophilic phenolic compounds α-tocopherol, δ-tocopherol, γ-tocopherol and γ-oryzanol. Very high correlations (R^2>0.97-0.99) were reported for these compounds in the presence of β-cyclodextrin. This study was a sequel to that of Szente et al, 1998 who ranked the solubilizing effectiveness of β-cyclodextrin and its derivatives in the following order: methylated-β-cyclodextrin> hydroxypropylated-β-cyclodextrin = branched β-cyclodextrin. Similar studies on the effectiveness of β-cyclodextrin in solubilizing carotene/fatty acid compounds have been conducted with comparable results (Pfitzner et al, 2000, Szente et al, 1998, Szente et al, 1993). Szente et al, 1998 found that randomly methylated-β-cyclodextrin added at a concentration of 10-40% enhanced the aqueous solubility of lipophilic antioxidants by as much as 1000 fold.

Conclusions

Our results demonstrate that inclusion of β-cyclodextrin in the $ORAC_{FL}$ assay improves the correlation between $ORAC_{FL}$ and lycopene concentration, thus expanding the scope of the assay to include fat soluble antioxidants. From our studies we infer that ~0.8 % β-cyclodextrin is optimal for obtaining the highest correlation between $ORAC_{FL}$ and lycopene concentration. This opens avenues for using the $ORAC_{FL}$ assay as a tool for evaluating lycopene antioxidant activity, thus enabling a better understanding of the relationship between lycopene concentration and antioxidant activity in various food systems. Avenues for continued research include evaluating the effectiveness of randomly methylated-β-cyclodextrin as a lycopene solubilizing agent and attempting to correlate $ORAC_{FL}$ values with lipophilic antioxidant compound concentrations in actual foodstuffs.

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Chapter III – Effect of randomly methylated β-cyclodextrin in improving the correlation between lycopene concentration and ORAC values

Abstract

Lycopene, a lipophilic antioxidant, has a crucial role in biological systems as an anti-cancer agent, lowering cardiovascular risks and in regulating the immune system. Oxygen Radical Absorbance Capacity (ORAC) as an index for antioxidant activity has been validated extensively for hydrophilic antioxidants, but its applicability to lycopene has only recently been validated in our earlier studies with the incorporation of β-cyclodextrin, a water solubility enhancer. The study investigated extending the ORAC assay to measure the antioxidant activity of different concentrations of lycopene in the presence of randomly methylated β-cyclodextrin (RMCD), a derivative of cyclodextrin with higher solubilizing power. Lyc-O-Mato^{TM} 6 % extract was used as a source of lycopene for our experiments. Lycopene was extracted by a standard spectrophotometric assay procedure in the presence of RMCD at concentrations of 0, 0.4, & 0.8 % and the antioxidant activity of lycopene at different levels was measured with the ORAC assay. Experiments were conducted in quadruplicates and pooled correlations were calculated. Statistical analysis showed a very high correlation (R^2=0.99) between ORAC and ascorbic acid concentrations. Lycopene concentration correlated poorly with ORAC (R^2=0.821) in absence of both β-cyclodextrin and RMCD. Correlations improved with

increasing levels of RMCD (R^2=0.9284 and 0.9959 for 0.4 and 0.8 % RMCD respectively). Further these correlations improved considerably relative to β-cyclodextrin in our earlier studies (R^2=0.51 and 0.9 for 0.4 and 0.8 % β-cyclodextrin respectively). Inclusion of RMCD in the ORAC assay improves correlation between ORAC and lycopene concentration compared to no added cyclodextrin or β-cyclodextrin alone, thus expanding the scope of the ORAC assay to better measure the antioxidant activity of this fat soluble antioxidant.

KEYWORDS: ORAC, lycopene, lipophilic antioxidants, β-cyclodextrin and randomly methylated β-cyclodextrin

Introduction

Lycopene, a lipophilic antioxidant, has numerous key health-promoting roles in human body such as cancer prevention, reduction of cardiovascular risks, and regulation of the immune system (Giovannucci, 1999). Currently, lycopene is used as a coloring agent in the food, cosmetic and pharmaceutical industries. However, the high antioxidant activity and bioavailability of lycopene make it a very attractive ingredient in functional foods, which are gaining increasing importance in today's food marketplace. Potential sources of lycopene and the levels present in different foods have been previously tabulated (Nguyen and Schwartz, 1999). The technology exists to extract and purify lycopene for use as a functional ingredient. However, research is needed to correlate lycopene concentration in foods with antioxidant activity. This is critical for determining effective and economical rates of lycopene addition.

The Oxygen Radical Absorbance Capacity (ORAC) assay has been validated

extensively as an index of antioxidant activity for hydrophilic antioxidants, but its applicability to lycopene via the incorporation of β-cyclodextrin, a water solubility enhancer, was only recently validated (Bangalore et al., 2005). Our group developed a modified assay for improving the Lycopene and ORAC correlation using β-cyclodextrin and observed that the optimum addition rate for best correlation was at 0.8% (W/V) β-cyclodextrin. In earlier work, Huang et al., 2002 adapted the ORAC$_{FL}$ assay for tocopherol and other phenolic antioxidants using RMCD as a solubility enhancer. The goal of this study was to evaluate the effect of RMCD as a solubility enhancer in improving the correlation between lycopene concentration and ORAC assay values.

Materials and Methods

Reagents.

Fluorescein, trolox, ascorbic acid, potassium phosphate, sodium-phosphate, and sodium azide were purchased from Sigma (St. Louis, MO). We purchased 2, 2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) from Wako Chemicals (VA, USA) and RMCD (technical grade with trade name TRAPPSOL and CAT-No# TRMB-T) from cyclodextrin technologies Development Inc. (FL, USA: www.cyclodex.com). Lyc-O-Mato^{TM} 6 % extract was obtained from LycoRed Natural Products Industries Ltd. (Beer-Sheva, Israel) as a source of lycopene for our experiments. Preliminary testing showed that the fluorescein used in our experiments underwent a decay of >90% fluorescence within 45 min in the presence of 0.332 g of AAPH in 10 ml of phosphate buffer (PB).

Lycopene analysis.

Lycopene concentration in Lyc-O-Mato^{TM} preparation was determined using a

modification of the method of Sadler et al. (1990). Acetone (25 ml), ethanol (25 ml), hexane (50 ml), and the lycopene concentrate were mixed in a foil-covered Erlenmeyer flask. An aqueous RMCD solution (15 ml at 0, 0.4, or 0.8% W/V) was then added, the flask was sealed, and the mixture was agitated at about 30°C in a constant temperature water bath/shaker for 15 minutes. The solution was allowed to equilibrate at room temperature for 15 minutes, and then about 3 ml of the top hexane layer was sampled for spectrophotometric analysis. AB_{503} was read on a Beckman DU series 500-spectrophotometer with a 4.5 ml glass cuvette. Lycopene concentration was then computed as described by Sadler et al. (1990). Lycopene concentration in the extract was calculated to be about 166mg/g.

Lycopene solution preparation.

About 11mg of concentrate was weighed into a 250 ml foil covered Erlenmeyer flask and extracted according to Sadler et al. (1990). This yielded a stock solution of about 68.0 µM lycopene in hexane. This was further diluted 2, 3, 4, 5, & 10 times in hexane to obtain approximately 34.0, 22.7, 17.0, 13.6, & 6.8 µM working lycopene solutions. RMCD solutions (0, 0.4, & 0.8%) were prepared in a 1:1 water-acetone mixture (v/v) and agitated with a shaker for at least ½ hour till the solutions were completely dissolved. Ascorbic acid solutions (40 µM) were prepared by dissolving 0.705 mg of ascorbic acid in 100ml Phosphate Buffer (PB) and diluting with water to get 20, 10 & 5 µM solutions. This served as a standard to evaluate the equipment for correlation between ascorbic acid antioxidant activity as measured in ORAC values and ascorbic acid concentration.

ORAC assay.

ORAC assays were conducted using a Perkin-Elmer HTS-7000 Microplate reader and Falcon brand 48-well clear plates at excitation and emission wavelengths of 485 & 535 nm respectively. The reaction temperature used was 37°C, as described in Ou et al. (2001). Other than the blanks and antioxidant samples, all other reagents were prepared using PB at pH 7 as a diluent. The volume and final concentrations of freshly prepared $ORAC_{FL}$ reagents used in the various wells of the 48-well clear plate were as follows:

Fluorescent Probe:

Fluorescein – 160 µL of 0.56 µM solution.

Test Solution:

Trolox (Standard) – 20 µL of 10 µM solution.

OR

Lycopene Blank – 20 µL of Hexane.

OR

Lycopene Sample – 20 µL of 34.0, 22.7, 17.0, 13.6, or 6.8 µM solution in hexane.

OR

Ascorbic Acid Blank – 20 µL of PB.

OR

Ascorbic Acid Sample – 20 µL of 5, 10 or 20 µM solution.

Peroxyl Radical Generator:

AAPH – 20 μL of 122.4 mM solution.

For each $ORAC_{FL}$ assay there were 12 blank wells, 12 standard well, and 24 sample wells per plate. Assays were run for ~70 min (or 45 cycles) to generate relative fluorescence profiles over time. The analyzer was preset to record fluorescence every minute after the addition of AAPH. Results were expressed as relative fluorescence with respect to the initial reading. Final computation of results was made by taking the differences of the areas under the decay curves between sample wells (Lycopene or Ascorbic Acid) or standard wells (Trolox) and blank wells (Hexane or PB). ORAC values were expressed as micromoles of Trolox equivalent (TE). The assay was repeated for each of the five tested lycopene concentrations (34.0, 22.7, 17.0, 13.6, & 6.8 μM) using three RMCD levels (0, 0.4, & 0.8%), and all assays were replicated four times. During all experiments the time gap between the addition of AAPH and start of fluorescence reading on assay reader was kept as short as possible.

Statistical analyses.

$ORAC_{FL}$ values were averaged for the duplicate wells on each plate in each run. Mean $ORAC_{FL}$ values were calculated from the four replication of each run. Correlations of $ORAC_{FL}$ and lycopene in presence of RMCD at 0, 0.4, & 0.8% levels were obtained for the four replications. Results of $ORAC_{FL}$ and lycopene concentration assays were compared and presented on a Lycopene vs. ORAC graph.

Results and Discussion

Method Validation for linearity with ascorbic acid.

$ORAC_{FL}$ values and ascorbic acid concentration had a very high correlation (R^2= +0.983) as described in Bangalore et al. (2005).

Stability of lycopene over the duration of the assay.

Lycopene did not by itself significantly influence the oxidative decay of fluorescein in the absence of AAPH during the ORAC assay (Bangalore et al., 2005).

Lycopene concentration and $ORAC_{FL}$ correlation.

The relative antioxidant activities of lycopene extracted with varying levels of RMCD, expressed as RF versus time, are presented in Figures 6-8. We observed a similar pattern in all these profiles; an increase in lycopene concentrations reduced the rate of decay in the RF profiles. Pooled correlation of $ORAC_{FL}$ and lycopene concentration with RMCD at 0, 0.4 & 0.8 % is shown in Figure 9. Lycopene extracts had a poor correlation with $ORAC_{FL}$ values in the absence of RMCD (R^2= 0.821). Correlations improved with increasing levels of RMCD (R^2=0.9284 and 0.9951 for 0.4 and 0.8 % RMCD respectively). Further these correlations represent an improvement relative to those observed with β-cyclodextrin in our earlier studies (Bangalore et al., 2005) (R^2=0.51 and 0.9 for 0.4 and 0.8 % β-cyclodextrin respectively). Higher correlations were observed at lower RMCD concentrations, thus demonstrating the greater solubilizing power of RMCD. Inclusion of RMCD in the ORAC assay improved correlation between ORAC value and lycopene concentration compared to no added cyclodextrin or β-cyclodextrin alone, thus expanding the scope of the ORAC assay to allow better measurement of the

antioxidant activity of this fat soluble antioxidant. There were no problems observed in dissolving RMCD with simple agitation at the concentrations studied.

One observation was that while the correlation between lycopene concentration and ORAC$_{FL}$ values improved significantly with the addition of RMCD, overall relative fluorescence decay rates also increased, particular at the higher lycopene concentrations (Figures 6-8). Previous studies have not shown that β-cyclodextrin functions as a pro-oxidant (Huang et al, 2002). This was supported by our observation of a proportionally greater change in decay rates between 0 and 0.4% RMCD than between 0.4 and 0.8% RMCD. It is possible that the apparent change in the rate of fluorescein oxidation in the presence of RMCD was a result of the more complete dispersal of lycopene in the solution brought about by the addition of RMCD. It may be that, in the absence of RMCD, the non-polar lycopene was forming micelles that served to entrap and protect the fluorescein from oxidation. This would explain both erratic and erroneously high ORAC$_{FL}$ values in the absence of RMCD.

Our results demonstrating the effectiveness of RMCD as a solubility enhancer for lycopene are similar to results reported by other researchers using other lipophilic antioxidant compounds. This corresponds to our earlier studies (Bangalore et al., 2005) on lycopene-ORAC correlation using β-cyclodextrin as a solubility enhancer. Huang et al. (2002) saw similar effects using RMCD (7% in a (1:1) water-acetone mixture and in a 75mM phosphate buffer at pH 7.4) as a solubility enhancer in ORAC$_{FL}$ assays on the lipophilic phenolic compounds α-tocopherol, δ-tocopherol, γ-tocopherol and γ-oryzanol. Very high correlations (R^2>0.97-0.99) were reported for these compounds in presence of β-cyclodextrin. Indeed, Szente et al, 1998 found that RMCD at a concentration of 10-

40% enhanced aqueous solubility of lipophilic antioxidants by ~ 1000 fold.

Conclusions

Our results indicate that the inclusion of RMCD in ORAC$_{FL}$ assay improves the correlation between ORAC$_{FL}$ & lycopene concentration and thus improves the accuracy of the assay for measuring lycopene antioxidant activity. Our studies show 0.8% RMCD to be optimum among the concentrations tested for obtaining the highest correlation between ORAC$_{FL}$ and lycopene concentration.

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Chapter IV – Watermelon chromoplasts and ultrastructure

Abstract

Watermelon, a natural source of the antioxidant lycopene (~48.7mg/g), could be a useful ingredient in functional foods. An understanding of the structure of watermelon chromoplasts (lycopene-containing organelles), along with other cellular structures, is critical to correlate melon maturity with maximum lycopene content and extraction efficiency. This study employed Transmission Electron Microscopy (TEM) to conduct research on the ultrastructure of watermelon mesocarp. Watermelon samples (cultivar: HAZARA SW-1) of different maturities were sectioned for ultrastructure studies and TEM micrographs were prepared. Micrographs from immature melons showed incompletely formed chromoplasts. A combination of clear and distinct pigment bearing chromoplasts and incompletely formed chromoplasts were observed in mature melon micrographs. Growth-related organelles were also observed in both immature and mature but not in over-mature melons. Electron micrographs showed chromoplasts changing from less organized globular form in immature to a definite, symmetrical form in mature and finally to an asymmetrical structure in over-mature melons. Results of this study will further our understanding of watermelon physiology and the effect of maturity on compartmentalization of lycopene in the fruit. Future studies will relate observed changes in watermelon cellular ultrastructure to processing techniques and lycopene recovery.

KEYWORDS: watermelon ultra structure, melon maturity, lycopene bearing chromoplasts, Transmission Electron Microscopy.

Introduction

Lycopene (gamma, gamma carotene), a lipophilic antioxidant, plays a number of key health-promoting roles in the human body. Among these are cancer prevention, reduction of cardiovascular risks, and regulation of the immune system (Giovannucci, 1999; Hassler, 1998; Rao and Agarwal, 1998). Lycopene is the characteristic pigment in red tomato, red watermelon, and other fruits such as guava and pink grapefruit. Nguyen and Schwartz (1999) have tabulated potential sources of lycopene and levels present in different foods. Lycopene's high antioxidant activity and bioavailability as well as its potential as a food coloring make it very attractive as a possible ingredient in functional foods and nutraceuticals.

Tomato has relatively high lycopene concentration and has been used as a source for natural lycopene extracts and concentrates. Watermelon is another candidate for such uses. Watermelon has been reported to have an average lycopene content of 45.1-53.2 μg/g compared to 30.2 μg/g found in tomatoes (USDA-NCC Carotenoid Database, 1998). Current U.S watermelon production is estimated at 40 million tons with growth rate projections of 1.4%/year [NFAPP, 2003], and consumption is estimated at 2.3×10^9 kg/yr (USDA, 2001). Even so, up to half of the watermelon crop may remain unharvested in a given year due to depressed fresh market prices. These fruit can serve as an excellent source of lycopene that can be used in antioxidant-rich functional foods.

Perkins-Veazie et al. (2001) have shown that lycopene levels in watermelon vary with cultivar and season. In particular, differences were typically observed between seeded and seedless varieties: seedless melons usually exhibited markedly higher lycopene concentrations. Melon maturity is another factor that could influence both

lycopene content and extractability. Some preliminary studies (Oikonomakos et al., 2004) have indicated that lycopene levels increase as melons mature and then drop as melons reach over-maturity. Identifying optimum melon maturity for lycopene content and functionality at harvest may prove to be critical for designing processes to maximize lycopene retention in watermelon-based extracts and/or functional foods.

In order to identify optimum maturity, we must understand the structural changes that occur in watermelon tissue as the fruits mature. These changes may influence lycopene content and distribution within the melon tissues. We know, for example, that lycopene may be converted to other carotenoid forms such as β-carotene, γ-carotene and phytofluene during maturation (Harris and Spurr, 1969a). But we do not know how changes in watermelon ultrastructure relate to these conversions. It is necessary, therefore, to understand the association of lycopene with various organelles in watermelon during melon maturation. Currently no research has been done on watermelon ultrastructure relating melon maturity to lycopene distribution within the watermelon mesocarp.

Lycopene in both tomato and watermelon is located in chromoplasts, which are non-senescent, carotenoid-containing plastids lacking in chlorophyll. Chromoplast biogenesis is classified into two types: Type A and Type B (Marono et al, 1993). In Type A biogenesis chromoplasts originate from fully developed photosynthetic chloroplasts. This transition is marked by loss of chlorophyll, accumulation of carotenoids, degradation of structured thylakoid membranes, and other physical and chemical changes. In Type B chromoplast biogenesis, chromoplasts originate directly from several types of non-photosynthetic plastids (Marono et al, 1993).

Fully developed chromoplasts are further classified into 5 groups based on structure. These are Crystalline (Carotenoid bodies are in the form of crystals), Globular (Carotenoids are dissolved in lipid globules), Fibrillar, Membranous, and Tubular (Carotenes are bound to protein molecules). Predominant chromoplast types vary considerably among plant species (Bonora et al., 2000; Cheung et al, 1993; Frey-Wyssling and Schwegler, 1965; Harris and Spurr, 1969a; Harris and Spurr, 1969b; Iwahori and Van-Stevenick, 1976; Knoth, 1981; Kooji et al, 2000; Lim and Boyer, 1994; Ljubesic et al., 2001; Mateos et al, 2003; Weston and Pyke, 1999; Whatley and Whatley, 1987).

Watermelon and tomato both accumulate lycopene within intracellular chromoplasts, but the structure of these organelles may differ between watermelon and tomato. Tomatoes exhibit Type A chromoplast formation (Harris and Spurr, 1969a; Harris and Spurr, 1969b). Intuitively one might expect watermelon to undergo Type B chromoplast biogenesis due to the fact that a comparatively thick rind covers the watermelon mesocarp and creates a dark environment. However, the occurrence of both Type A and Type B chromoplast biogenesis in watermelon cannot be ruled out. Watermelons may therefore exhibit a greater variety of chromoplast structures than do tomatoes because more biogenesis pathways may be active in watermelon. In addition, changes that occur during melon maturation/senescence may increase the variety of chromoplast structures observed. For example, ongoing breakdown of thylakoid structures may create a variety of intermediate structures and make it difficult to distinguish between chromoplasts derived from Type A biogenesis and those derived from Type B. Edwards et al. (2003) list chromoplast type as one factor that affects the

40

absorption of carotenoids in the gut after plants are eaten. This suggests that chromoplast structure may well influence carotenoid disposition during processing.

Other expected visible changes in melon ultrastructure that occur as melons mature and senesce include the disappearance of numerous structural organelles (e.g. mitochondria, endoplasmic reticulum's, ribosomes, vacuoles, and other plastid structures). Piechulla et al., (1987) and Livne and Gepstein (1988) attribute these changes to an age-related decline in the protein composition necessary for photosynthetic activity. This results in the termination of various metabolic processes necessary for the survival of these structures. Any or all of these various changes may account for the observed tendency for lycopene concentrations to decline in over-mature watermelons, though the mechanism remains unknown.

Ultrastructure study using transmission electron microscopy (TEM) has been useful for understanding the structural organization of plant and animal tissues. Numerous studies on tomato ultrastructure and lycopene formation have used this approach (Harris and Spurr, 1969a and 1969b; Cheung et al., 1993). Our study applies TEM to study watermelon ultrastructure and to relate melon maturity to the distribution of lycopene and other structural organelles within the watermelon mesocarp.

These ongoing studies of watermelon ultrastructure are aimed at gaining a better understanding of lycopene organization within watermelon tissue with an eye toward delivering lycopene-rich functional products derived from watermelon.

Materials and Methods

Reagents.

All reagents for this study were sourced through the Electron Microscopy Lab, Department of Veterinary medicine, Oklahoma State University.

Melon Samples.

Watermelon samples for ultrastructure studies (cultivar: HAZARA SW-1) at different maturities "immature", "mature", and "over-mature" stages) were collected from research plots at the Oklahoma State University Bixby Research Station. Melon maturity determinations were based on field observations at time of harvest as described in Table 2. Whole melons were stored at refrigeration temperatures (~ 8°C) prior to study; refrigerated storage intervals did not exceed 15 days. Melons were warmed to room temperature (~25°C) before ultrastructure studies.

Sample preparation for TEM study.

Fresh whole watermelons were sliced into four equal parts and approximately 5 cm cubes were cut from each core piece.. Sections were taken at random from the middle portion of each of these blocks and placed in a 2% phosphate buffered glutaldehyde solution for about 2 hours. Sections were then washed for twenty minutes in 0.1M phosphate buffer, removed from the solution, and then washed for another 20 minutes in fresh buffer. The sections were then mounted on glass slides and examined at 100x magnification to select areas of interest. Selected slides were then washed in 0.1M phosphate buffer and fixed for 2 hours in 1% aqueous osmium. Three 20 minute washing treatments in 0.1M phosphate buffer followed, allowing ample time for tissue fixation.

Samples were then dehydrated using an ETOH series (30, 50, 70, 90, 95, 100% - repeated 3 times) Finally, samples were immersed in vials containing (1:1) 100% ETOH/LR White, capped tightly and placed into refrigerated storage (5°C). The prepared sections were subsequently removed from storage and examined using TEM at different magnifications to identify structures of interest. Micrographs were examined and structures were identified and labeled. The structures identified and labeled on our micrographs are based on our interpretation of structures identified in reported TEM ultrastructure studies on tomatoes, peppers, squash and flowers. Thorough research would be needed to conclusively verify the identity of the structures we observed in our watermelon tissue samples.

Results and Discussion

Ultrastructure of watermelon chromoplasts.

Electron micrographs depicting the ultrastructure of typical mature watermelon mesocarp showed numerous structural organelles (Figures 10 and 11). Watermelon chromoplasts consist of a distinct plastid envelope (PE); packed with wide range of structural organelles. The main constituents are carotenoid containing osmophilic globules (G), undulating thread-like structures (C), large dilated thylakoid grana (Th), a number of small individual thylakoid sacs (t) which result from the deterioration of Th, thylakoid plexi (ThP) which consist of networked pigments and grana, and stroma (S) which are open spaces within pigmented globules. In addition there were numerous other structural organelles such as the cell wall (cw), tonoplasts (T), mitochondria (M), nucleus (N), endoplasmic reticula (Er), ribosomes (R), vacuoles (V) and other indeterminate structures.

43

Chromoplasts in watermelon have very few pigment globules in comparison to those in tomato. This may be due to the differences in moisture content; watermelons typically have higher moisture contents than tomatoes (Harris and Spurr, 1969a & b, Cheung et al., 1993).

Effects of melon maturity on chromoplast structure.

Electron micrographs showing ultrastructure of chromoplast for "immature" melons are presented in figures 12 – 14. These micrographs showed the ultrastructure of a watermelon cell with numerous chromoplasts (figure 12) that are not fully developed and/or organized but seem to have a distinct cell wall. In addition we observed a tonoplast, large intracellular spaces, and numerous other structural organelles such as mitochondria (figure 13). The process of pigmentation in chromoplasts generally starts with the carotenoid containing pigments developing and coalescing together into a clearly visible, intensely dark, and symmetrical globular structures showing distinctly formed stroma within them. These globular structures are reported to grow in size as the fruit ripens and an average diameter of 255mµ has been reported in a mature tomato chromoplast (Harris and Spurr, 1969a). In all our micrographs for immature melons the chromoplasts showed the presence of globular structures within the chromoplasts (figures 12 – 14). The pigment components in immature melons seemed to consist mostly of globular structures within the chromoplasts. A closer examination of the immature melon ultrastructure (figure 13) showed light colored carotenoid-containing pigments associating into a globular structure. Upon further magnification we saw these globular structures exhibiting greater internal structure (figure 14); the pigment components form a circular structure with gaps of incomplete coloration generally referred to as stroma.

44

The stroma consistently appeared within these globular structures forming a pattern similar to that seen in the cross-section of an Okra pod (http://www.istockphoto.com/file_thumbview_approve/689358/2/istockphoto_689358_sliced_okra_backlit.jpg). However these stroma were not well defined and remained incompletely formed.

Thus we conclude that in immature melons the process of pigmentation formation had begun within the chromoplasts, which showed a number of incompletely formed carotenoid containing globules beginning to align into a symmetrical structure. We also saw other cellular organelles reflective of the active metabolic processes underway as a part of fruit maturation.

Electron micrographs presenting the ultrastructure of chromoplasts in "mature" melons are shown in figures 10 and 11 and figures 15 – 19. Mature melons showed all the structural organelles seen in "immature" melons but had better defined chromoplast structures and a more densely packed organelles (figures 10 and 11), likely indicative of a higher degree of metabolic activity in these melons. Pigment-bearing carotenoid-containing globular structures were circular in shape and exhibited greater symmetry than those seen in "immature" melons. A combination of distinct globular structures of varying degrees of darkness was seen in these "mature" chromoplasts (figure 11). Further magnification of these globular structures (figures 15 – 17) showed clearly visible asymmetrical stroma with defined boundaries in both lightly and heavily pigmented globules. Thread-like stranded structures contributing to pigmentation were also observed in large numbers in these samples (figures 18 and 19). Harris and Spurr, (1969a) also reported the formation of thread like structures (in addition to globular forms) and

referred to them as lycopene deposits. These may derive from thylakoid grana formed during the process of chromoplast evolution. Also visible were regions of color agglomeration and grana that could have been intertwined with incompletely formed thylakoid plexi (figure 18) or clearly formed thylakoid plexi as seen in figure 16.

Thus we conclude that in "mature" watermelon the pigment formation process was almost complete within the chromoplasts. As a result we saw well defined circular carotenoid-containing globules that appeared with varying degrees of color intensity, presumably as a result of varying internal lycopene concentrations. Other structural organelles were also present in a rather dense distribution reflecting the higher activity of the cells in mature fruit tissues. The formation of well defined stroma may be one of the possible indices for identifying the optimal degree of maturity in watermelon for lycopene harvest.

Electron micrographs presenting ultrastructure of chromoplast in an "over-mature" melon are shown in figures 20 – 22. Our "over-mature" melons presented the same set of structural organelles seen in "immature" and "mature" melons but the organelles were much less densely packed within the cells (figure 20). This likely is a function of a decline in metabolic activity in these melons due to senescence. Electron micrographs showed chromoplasts changing from a definite, symmetrical form (figure. 10) in "mature" fruit to a less organized, asymmetrical structure (figure. 20) in "over-mature" fruit, likely as a function of maturity. A combination of well retained globular structures with fading pigmentation and darkly pigmented asymmetrical globules could be seen in these melons (figure 20). Harris and Spurr (1969a) reported similar effects of maturity in over-ripened tomatoes in which in the formation of such irregular or

elongated structures were observed in addition to the well ordered globular structures. On magnification of these globular structures (figures 20 and 21) we observed that the pigments appeared to be diffusing out of these globules and either agglomerating outside into future thylakoid plexus formations or depositing towards the walls of the plastid envelope. It seems that the globules were unable to retain their structures, leading to the release of pigment components into the cellular stream. Also the absence of stroma was very evident from these micrographs because of this loss of structural integrity. A further effect of ripening seen in "over-mature" melons was the loss of pigment-bearing thread-like carotenoid structures; we saw only a few traces of them remaining in the chromoplasts (figure 21).

Thus we conclude that in "over-mature" watermelon the breakdown cellular ultrastructure was well underway. Chromoplasts were losing their structural integrity and pigments were being released into the cellular matrix. Other structural organelles were also present in fewer numbers. The loss of well defined stroma within the chromoplasts may be a possible index for identifying as to when watermelon is becoming over-mature and less suitable for lycopene harvest.

Key process insights.

Currently additional studies are underway to develop efficient and economical processes for extracting and concentrating lycopene from watermelon. The general sequence of unit operations involved in lycopene extraction involves a combination of blending/grinding, heat treatment, filtration/centrifugation perhaps followed by air or freeze drying and subsequent extraction. These studies of watermelon ultrastructure could provide   insights   into   determining   the   optimum   maturity   for   lycopene

extraction/concentration. They may also allow us to develop hypotheses concerning differences in the ways that watermelon of varying maturity respond to different processing techniques. Lycopene organized in well defined chromoplasts may behave very differently during processing than lycopene contained in older, asymmetrical chromoplasts or than lycopene that has been released into the cellular matrix.

The shift from symmetrical to asymmetrical structures as watermelon senesces may be a key finding. This shift may play a role in the observed decrease in lycopene extraction efficiency (through a combination of grinding, filtration and centrifugation process) seen as melons become "over-mature" (Oikonomakos et al., 2004). These structures may be harder to rupture than the symmetrical structures seen in mature fruit. Therefore our results suggest that lycopene extraction from "over-mature" watermelon may be boosted from the use of higher shear force and/or longer shearing treatment times during grinding/blending to breakdown these asymmetrical structures and release the lycopene.

Another possible explanation for decreased lycopene extraction from "over-mature" fruit is that the breakdown in ultrastructure seen in "over-mature" watermelon may expose lycopene to the action of intracellular enzymes that catalyze the breakdown of lycopene into β-carotene and other products.

Conclusions

Ultrastructure of watermelon was studied using TEM and the internal structures and intracellular organization of lycopene-bearing chromoplasts in relation to various other structural organelles was revealed. Electron micrographs showed chromoplasts changing from rudimentary globular form in "immature" to a definite, symmetrical form

in "mature" and to a less organized, asymmetrical structure in "over mature" melons, likely as a function of maturity. Results of this study will further our understanding of watermelon physiology and the effect of maturity on compartmentalization of lycopene in the fruit. Future studies will relate observed changes in watermelon cellular ultrastructure to processing techniques and lycopene recovery. These efforts should pave the way for the economical creation of lycopene-rich extracts and concentrates from watermelon suitable for use in functional foods.

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CHAPTER. V – CONCLUSIONS

The following are the findings and conclusions that we have drawn from this study entitled *"Quantifying Antioxidant Activity of Lycopene And Influence Of Watermelon Maturity On Tissue Ultrastructure And Lycopene Distribution"*:

1. Lycopene concentration correlated poorly with ORAC values (R^2=0.38) in absence of both β-cyclodextrin and RMCD. Our results demonstrate that inclusion of β-cyclodextrin in the $ORAC_{FL}$ assay improved the correlation between $ORAC_{FL}$ and lycopene concentration, thus expanding the scope of the assay to include fat soluble antioxidants. From our studies we infer that about 0.8 % β-cyclodextrin is optimal for obtaining the highest correlation between $ORAC_{FL}$ and lycopene concentration.

2. Further inclusion of RMCD in the $ORAC_{FL}$ assay resulted in improved correlations with increasing levels of RMCD (R^2=0.9284 and 0.9959 for 0.4 and 0.8 % RMCD respectively). Further these correlations improved considerably relative to β-cyclodextrin in our earlier studies (R^2=0.51 and 0.9 for 0.4 and 0.8 % β-cyclodextrin respectively). We saw improved correlations at lower RMCD concentrations, thus demonstrating the greater solubilizing power of RMCD. Inclusion of RMCD in the ORAC assay thus improves the correlation between ORAC values and lycopene concentration compared to no added cyclodextrin or

β-cyclodextrin alone. This opens up new avenues for using the $ORAC_{FL}$ assay as a tool for evaluating lycopene antioxidant activity, thus enabling us to better understand the relationship between lycopene concentration and antioxidant activity in various food systems.

3. Ultrastructure of watermelon was studied using Transmission Electron Microscopy (TEM). We observed the internal structures and intracellular organization of lycopene-bearing chromoplasts in relation to various other structural organelles. Electron micrographs showed chromoplasts changing from rudimentary globular form in "immature" to a definite, symmetrical form in "mature" and less organized, asymmetrical structure in "over mature" melons, likely as a function of maturity. Results of this study will further our understanding of watermelon physiology and the effect of maturity on compartmentalization of lycopene in the fruit.

Overall, we expect this study to aid in our efforts to develop better ways to evaluate lycopene antioxidant activity, understand the ultrastructural organization of lycopene in watermelon, and identify the optimum watermelon harvest maturity for efficient and effective lycopene extraction. These goals will assist our ongoing efforts to enhance the value of the watermelon crop and foster a healthier diet for consumers by developing lycopene-rich, value-added functional food products containing watermelon-derived ingredients.

Tables

Table 1: Mean and Standard Deviation information for lycopene concentration vs. ORAC$_{FL}$ values obtained at different concentrations of added β-cyclodextrin

Added β-cyclodextrin Concentration:	Lycopene Concentration (μM):	Mean ORAC value (Equivalent μmoles of Trolox):	Standard Deviation of ORAC value:	% Relative error of ORAC value:	n:
0% β-cyclodextrin	6.8	0.523	0.0949	34.9	4
	13.6	0.675	0.1904	15.3	4
	17.0	0.846	0.3469	46.9	4
	22.7	0.902	0.0634	30.7	4
	34.1	0.977	0.1097	23.3	4
0.4 % β-cyclodextrin	6.8	0.290	0.0360	20.67	4
	13.6	0.378	0.0325	21.8	4
	17.0	0.408	0.0249	6.1	4
	22.7	0.462	0.1007	18.6	4
	34.1	0.464	0.0145	14.7	4
0.8 % β-cyclodextrin	6.8	0.310	0.0229	29.2	4
	13.6	0.350	0.0001	22.5	4
	17.0	0.395	0.0227	20.9	4
	22.7	0.408	0.0364	26.9	4
	34.1	0.492	0.0319	12.9	4

Table-2: Visual and horticultural indices for harvested watermelons of different maturities

Maturity Ranking	Visual Attributes	Horticultural maturity indices
Under Mature or Immature	• Flesh substantially but not uniformly red. • Flesh lighter red than Mature melons.	• Clear echo heard with a thump on the melon. • Small fresh green tendrils. • Shining waxy rind.
Mature	• Flesh largely or uniformly bright red.	• Less clear echo heard with a thump on the melon • Longer, less greener tendrils • Shining waxy rind, but duller than immature melons • Light yellowish pink ground spot (point of contact between the watermelon and the soil) • Crispy fruit texture.
Over-mature	• Flesh uniformly dark red.	• Dull sound heard with a thump on the melon • Longer, dried tendrils, sometimes separated from the vine. • Dull rind. • Darker, pinkish ground spot. • Grainy and sticky fruit texture.

Figures
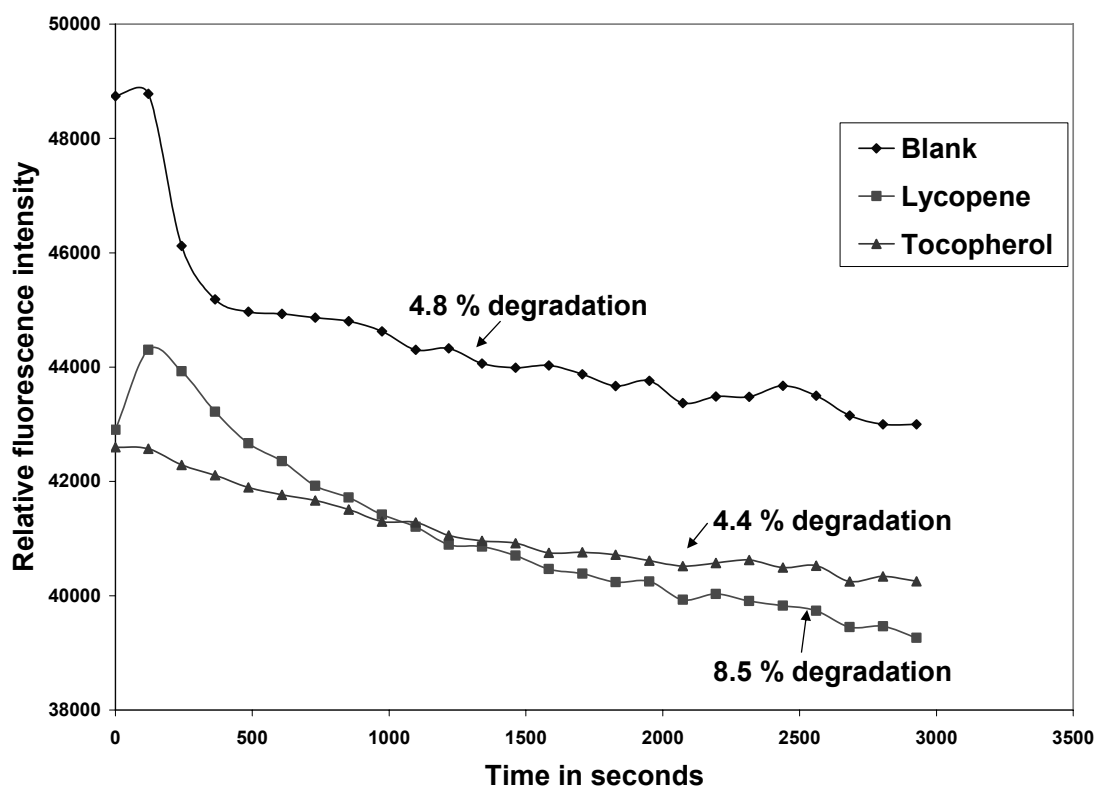
Figure 1. – Fluorescence Degradation kinetic profiles for Lycopene and α-Tocopherol without 2, 2'-Azobios (2-amidino-propane) dihydrochloride (AAPH).
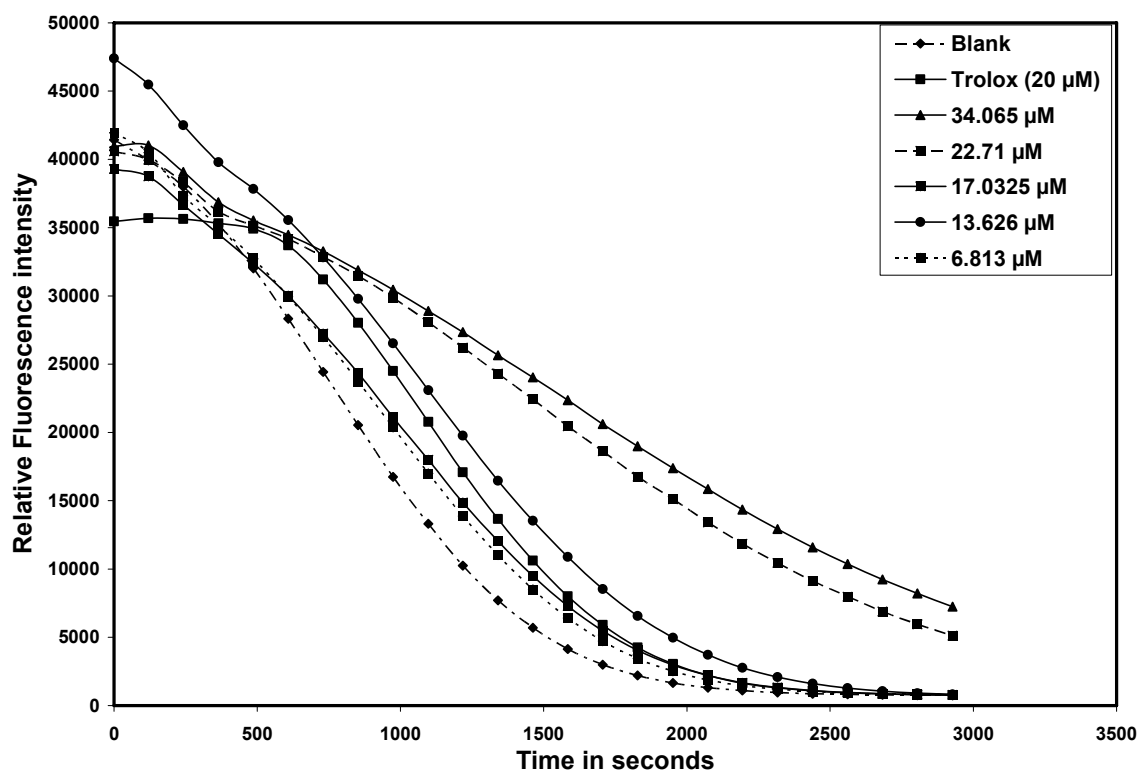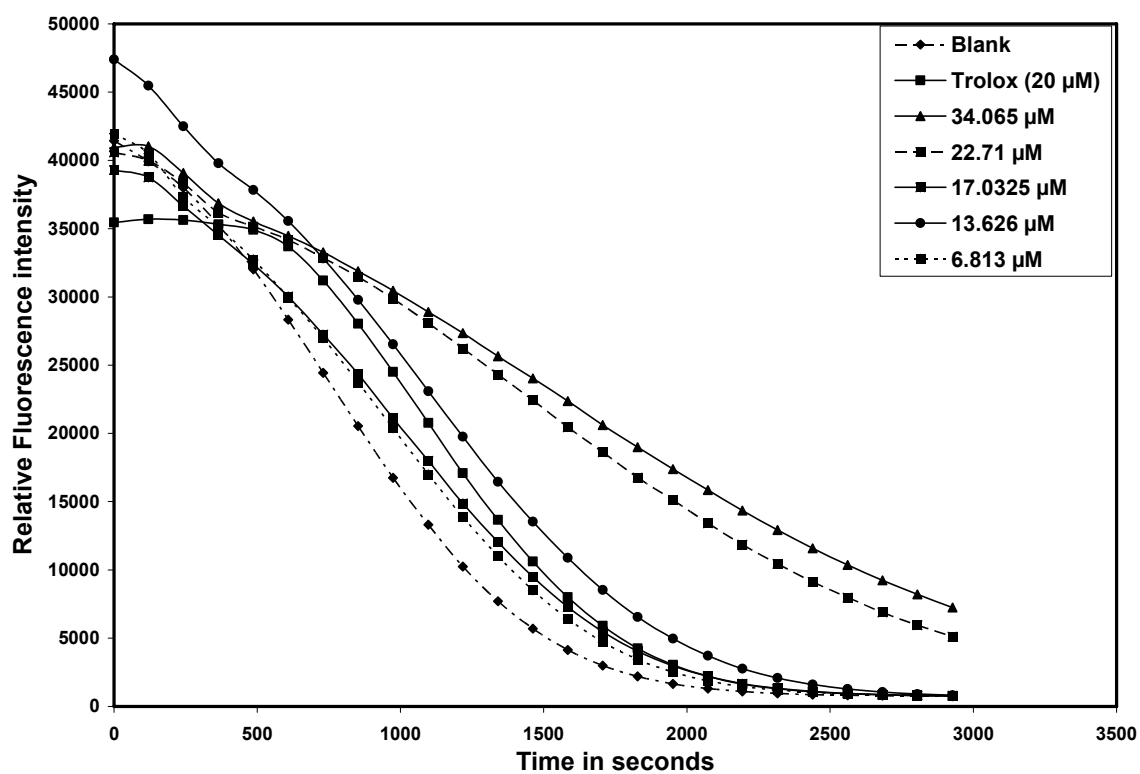
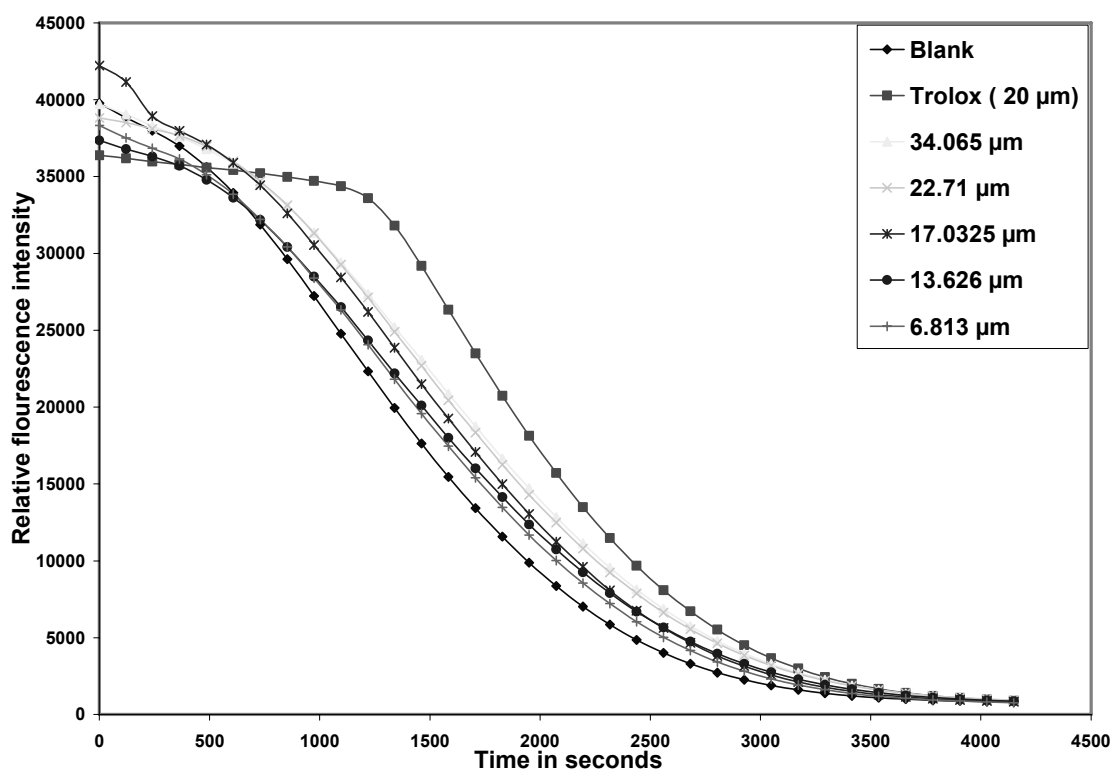**Figure 2. – Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the absence of β-cyclodextrin.**
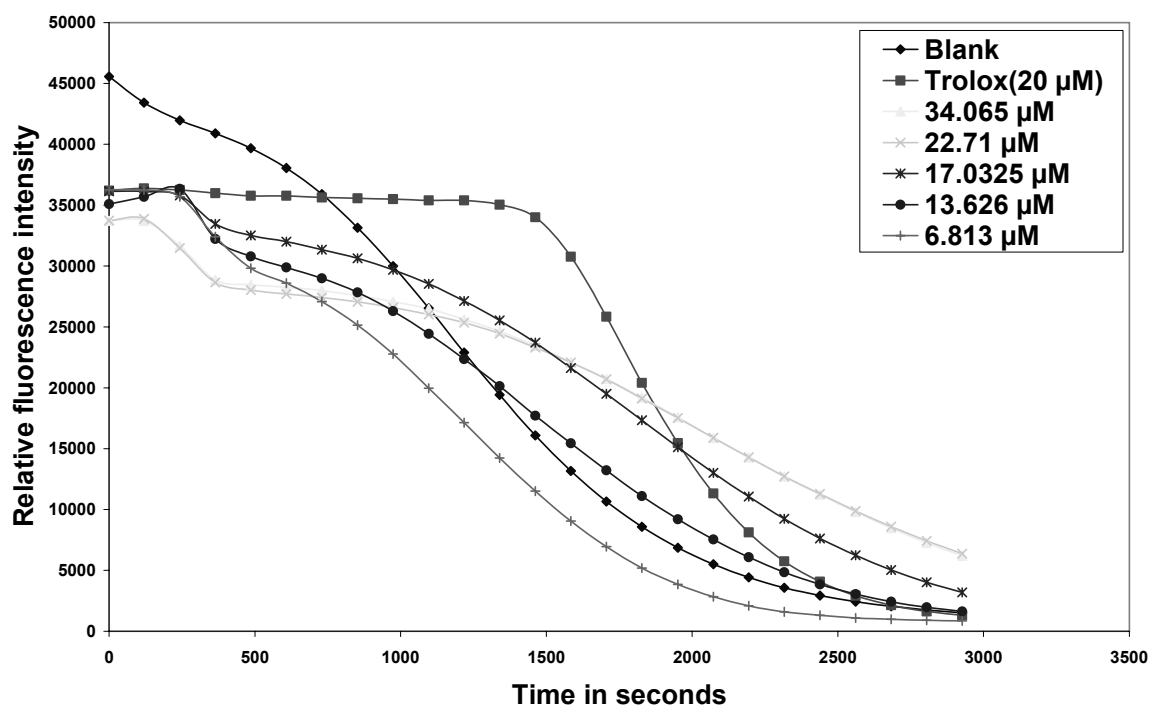
Figure 3. – Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the presence of 0.4 % β-cyclodextrin.
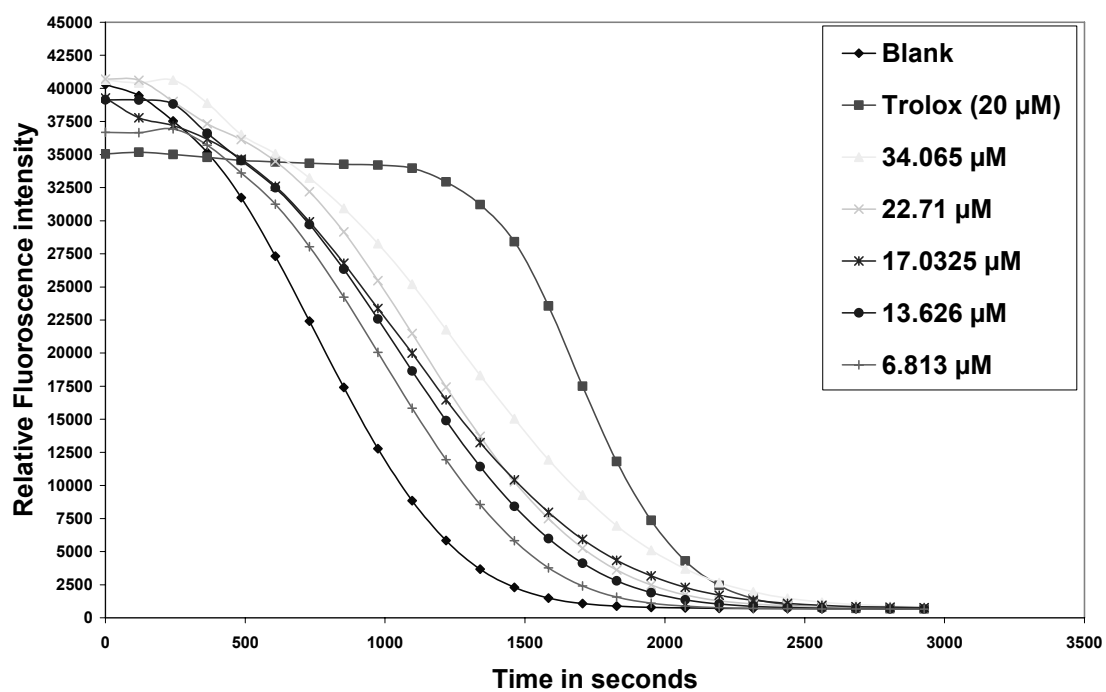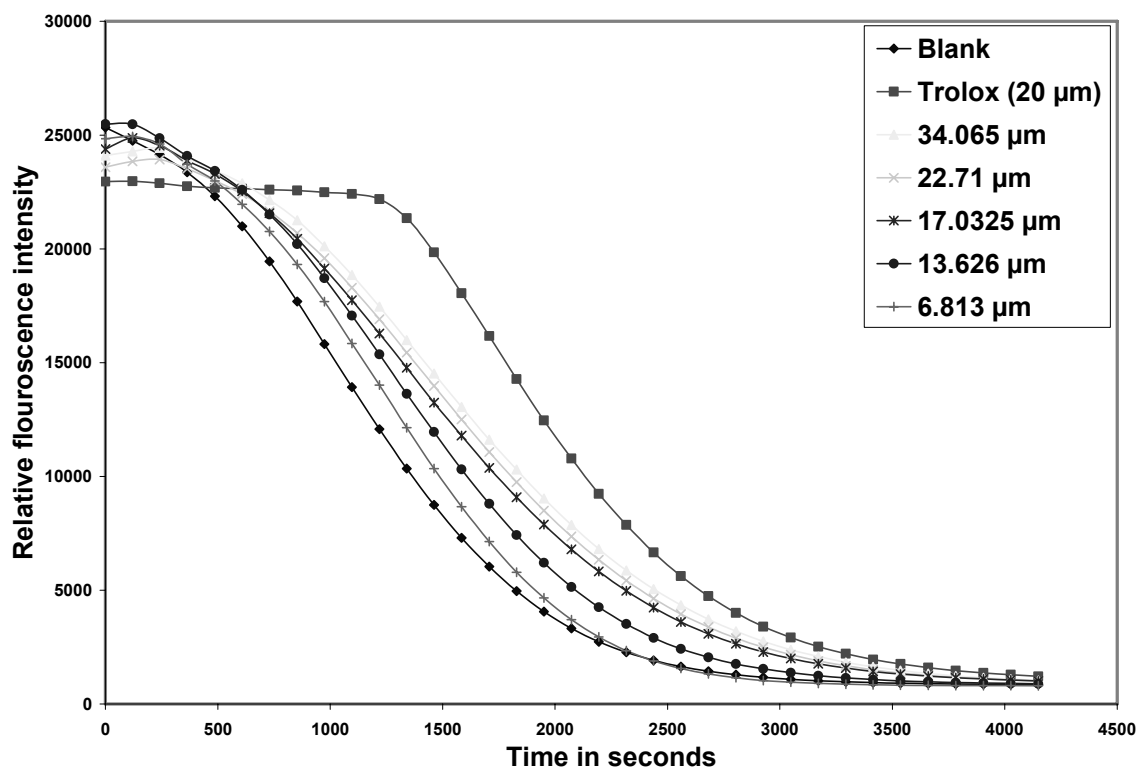
Figure 4. – Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the presence of 0.8 % β-cyclodextrin.
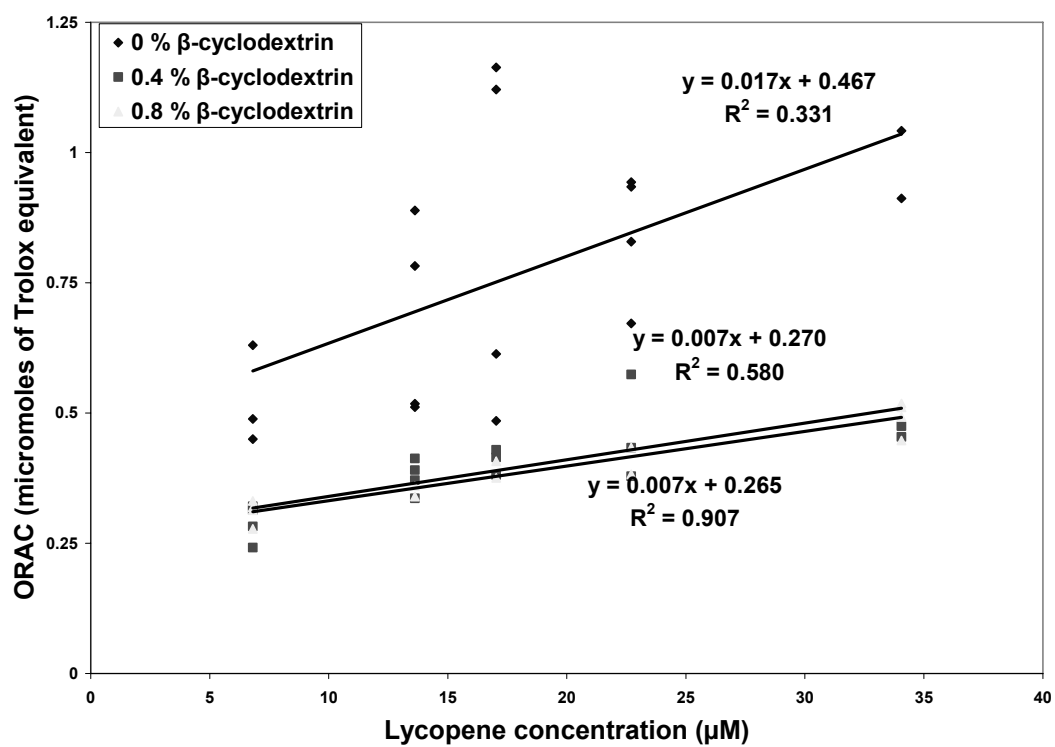
**Figure 5. – Lycopene and ORAC correlation derived for lycopene extractions with different levels of β-cyclodextrin.**

Figure 6. – Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the absence of β-cyclodextrin and randomly methylated β-cyclodextrin.

Figure 7. – Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the presence of 0.4 % randomly methylated β-cyclodextrin.

Figure 8. – Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the presence of 0.8 % randomly methylated β-cyclodextrin.
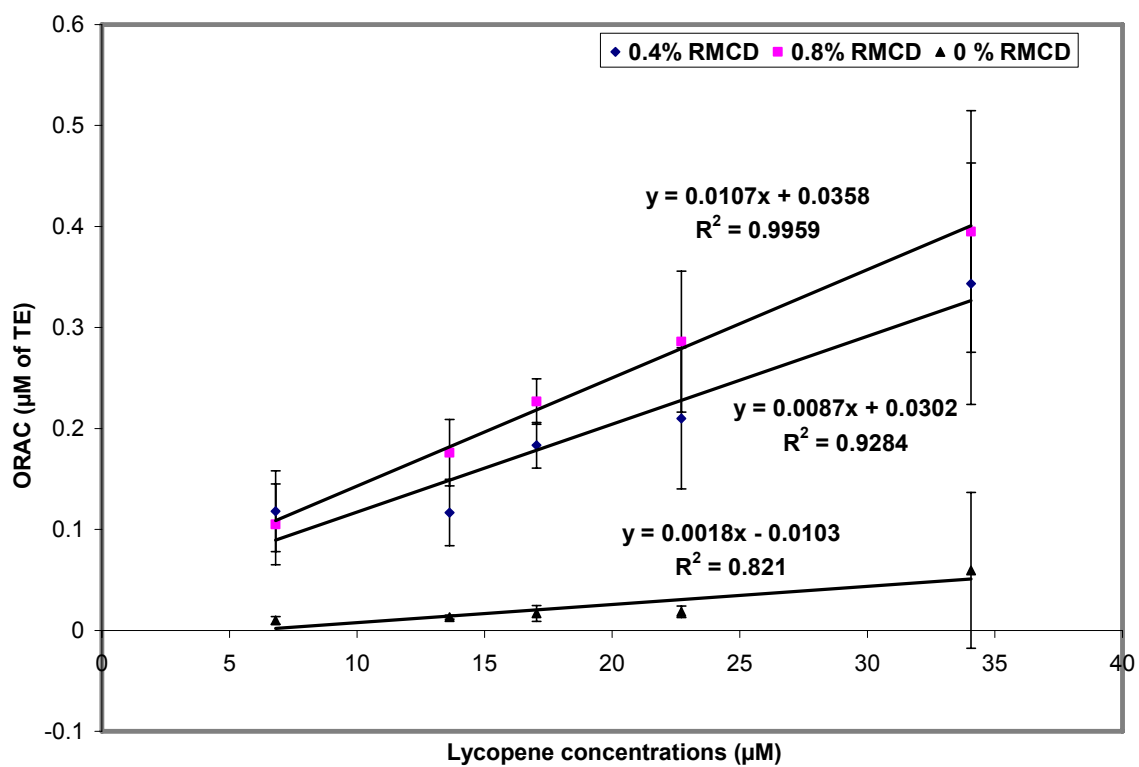
Figure 9. – Lycopene and ORAC correlation derived for lycopene extractions with different levels of randomly methylated β-cyclodextrin.
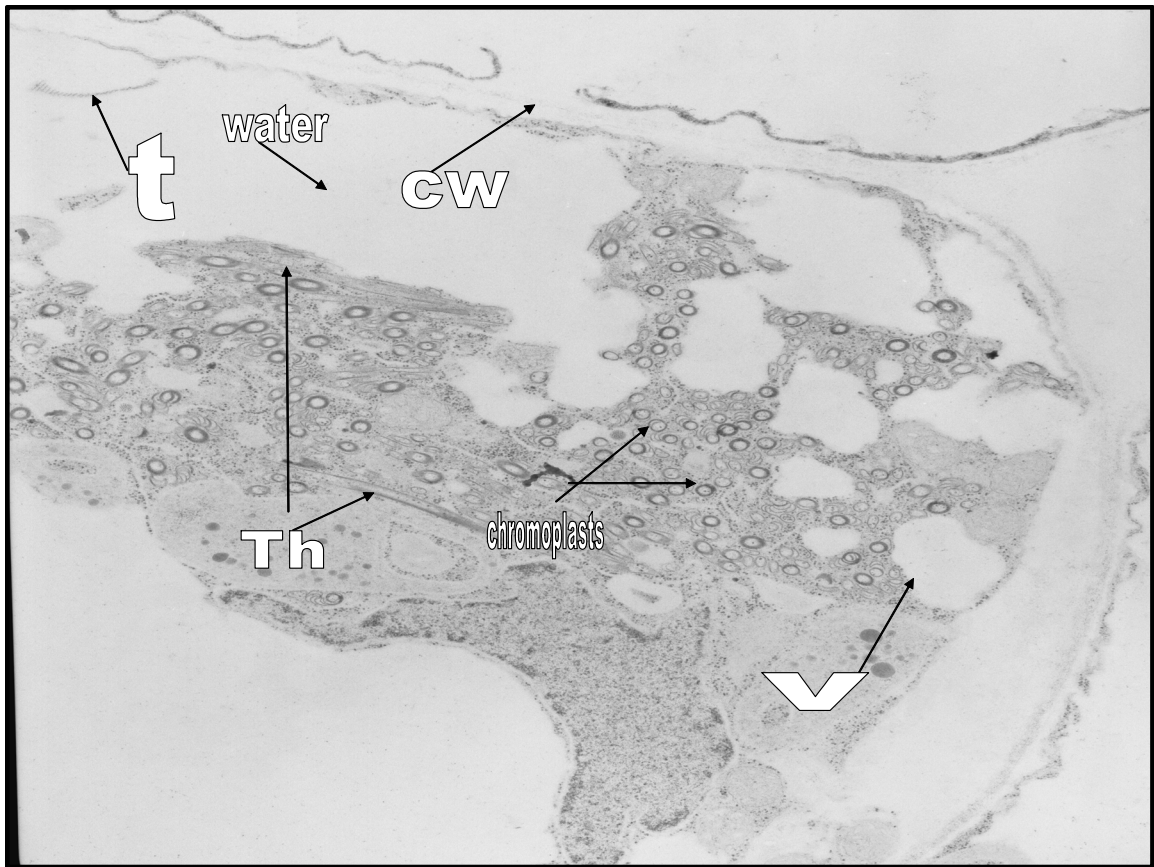
Figure 10. – 14X magnification micrograph of a mature watermelon showing a watermelon cell with cell wall, pigmented globules, residual thylakoid structures, vacuoles, and other structural organelles.

Structures are identified as follows:

(Th)   Dilated Thylakoid Granum
(t):   Individual Thylakoid Sac
(cw):   Cell Wall
(V):   Vacuoles
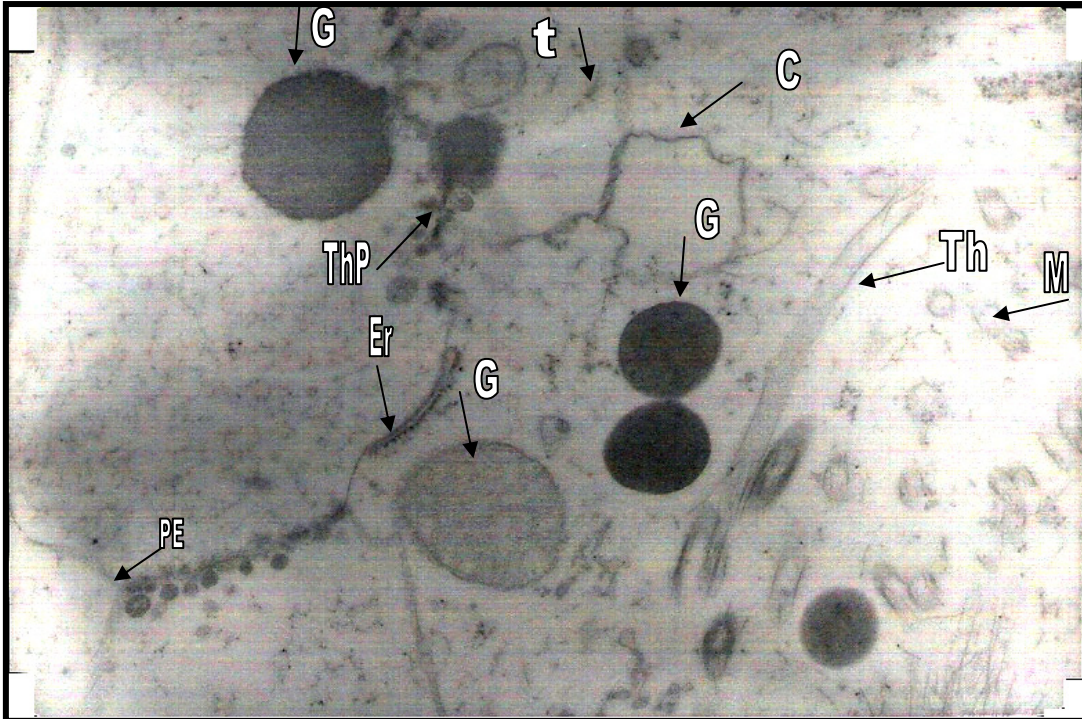
Figure 11. – 19X magnification micrograph of a mature watermelon showing various structural organelles.

Structures are identified as follow:

(PE):   Plastid Envelope

(G):   Carotenoid-containing Osmiophilic Globule

(C):   Undulating Thread-like Structure

(Th)   Dilated Thylakoid Granum

(t):   Individual Thylakoid Sac

(ThP): Thylakoid Plexus

(M):   Mitochondrion

(Er):   Endoplasmic Reticulum

IMMATURE
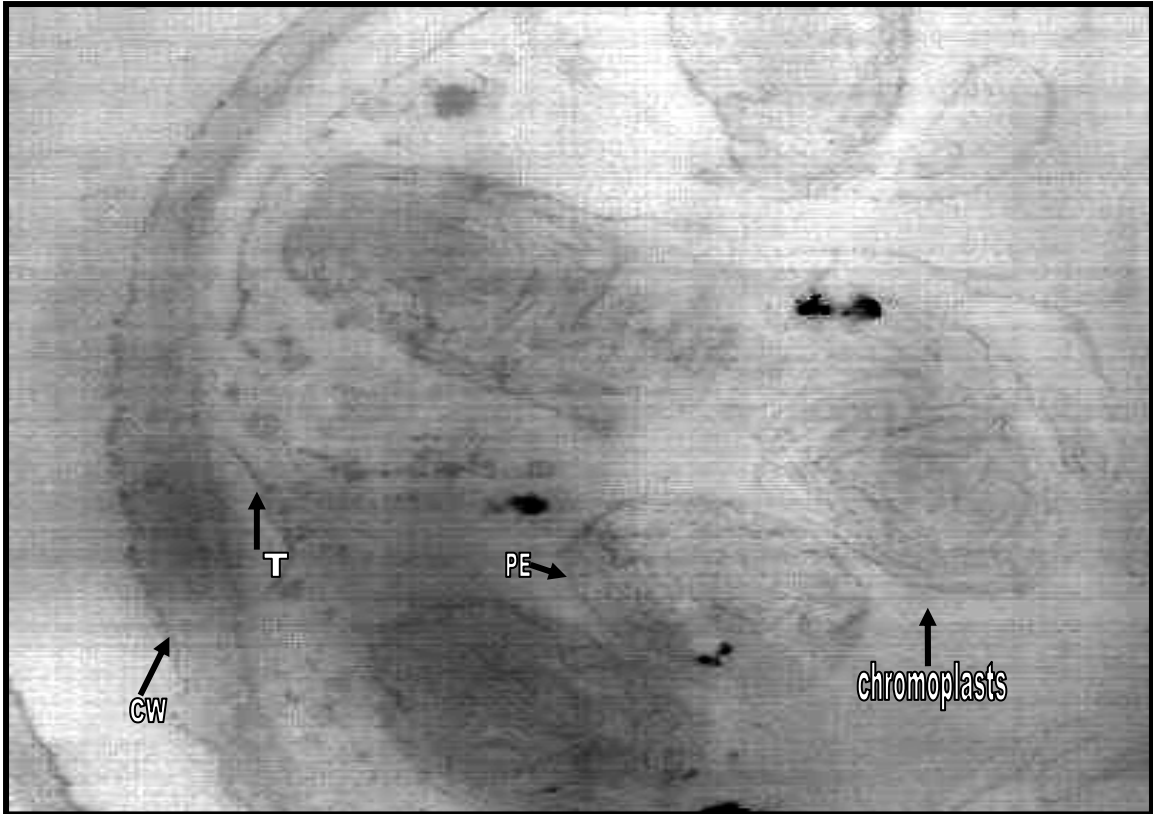
Figure 12. – 72X magnification micrograph of an immature watermelon showing numerous developing chromoplasts or plastids within the cell wall and tonoplast.

Structures are identified as follows:

PE):   Plastid Envelope
(cw):   Cell Wall
(T):   Tonoplast
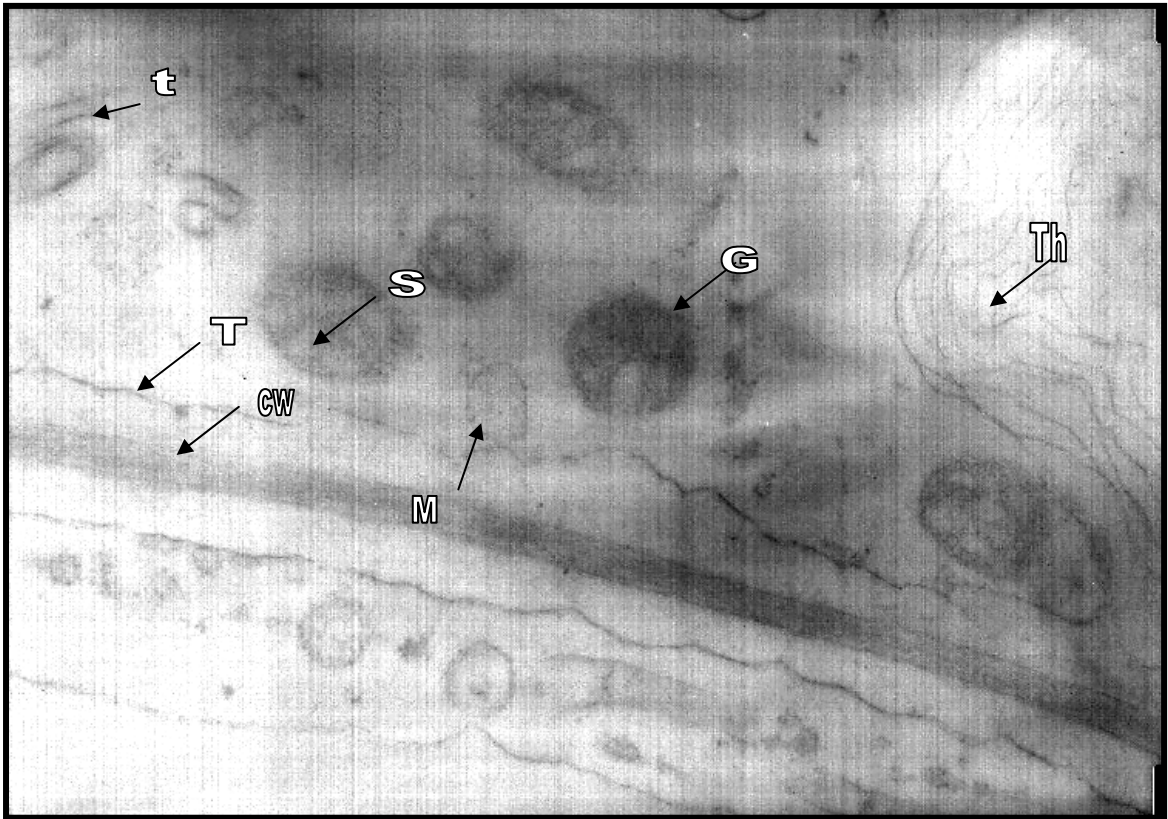
Figure 13. – 58X magnification micrograph of an immature watermelon showing partially developed lightly pigmented globules.

Structures are identified as follows:

(G):   Carotenoid-containing Osmiophilic Globule

(Th)   Dilated Thylakoid Granum

(t):   Individual Thylakoid Sac

(S):   Stroma

(cw):   Cell Wall

(T):   Tonoplast

(M):   Mitochondrion
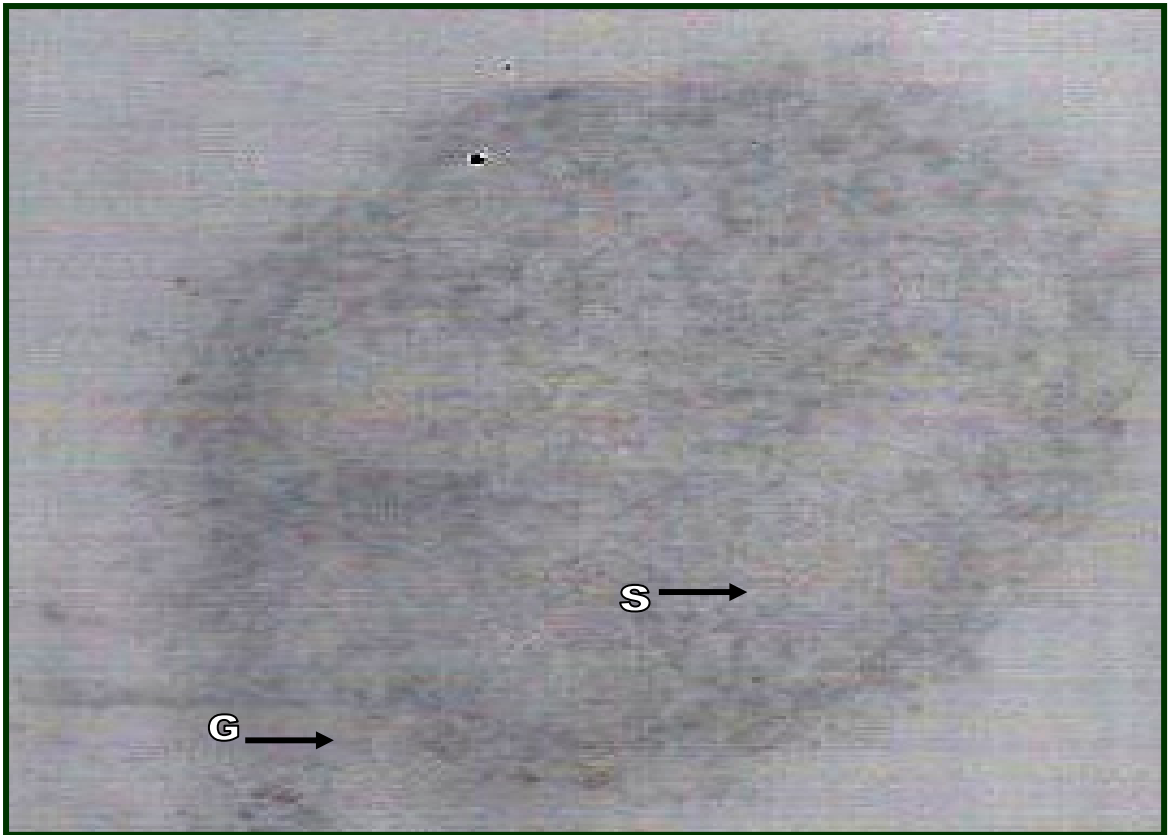
Figure 14. – 72X magnification micrograph of a immature watermelon showing rudimentary pigment containing globules with pigment accumulating into a definite network.

Structures are identified as follows:

(G):   Carotenoid-containing Osmiophilic Globule
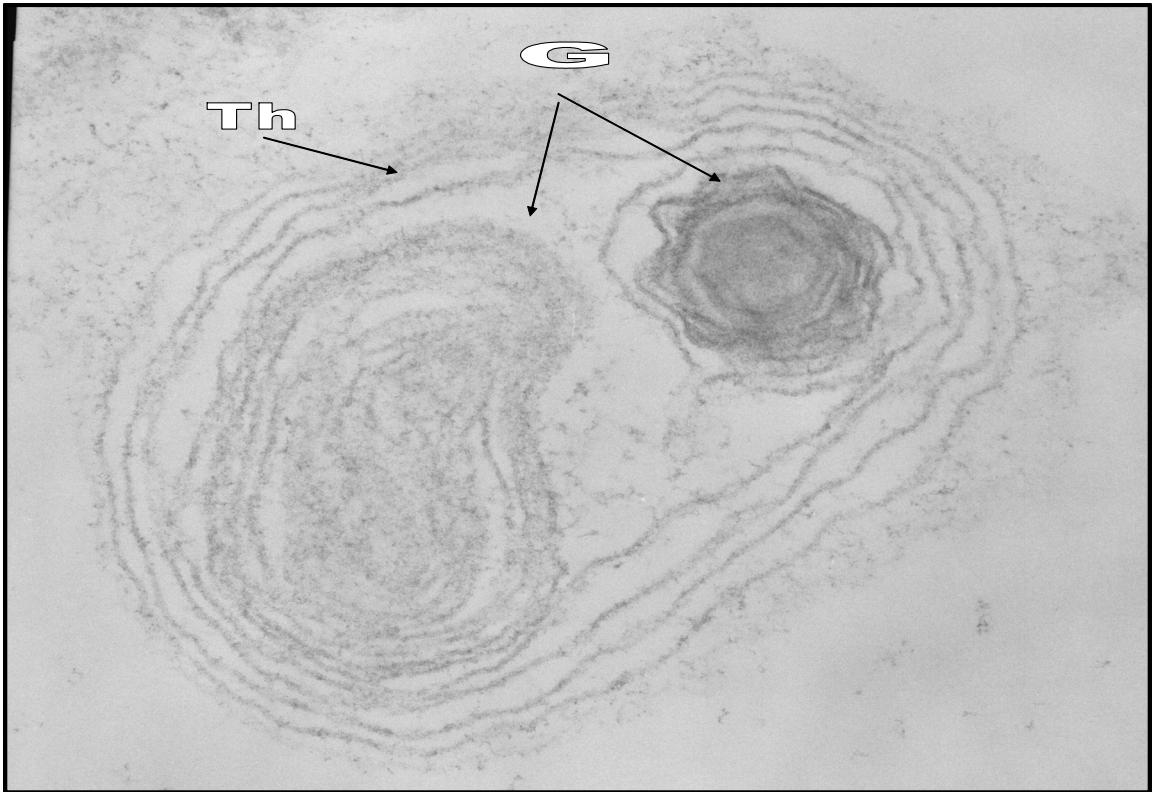
(S):   Stroma

MATURE

Figure 15. – 29X magnification micrograph of a mature watermelon showing globules engulfed within the thylakoid structures of a watermelon cell.

Structures are identified as follows:

(Th)   Dilated Thylakoid Granum
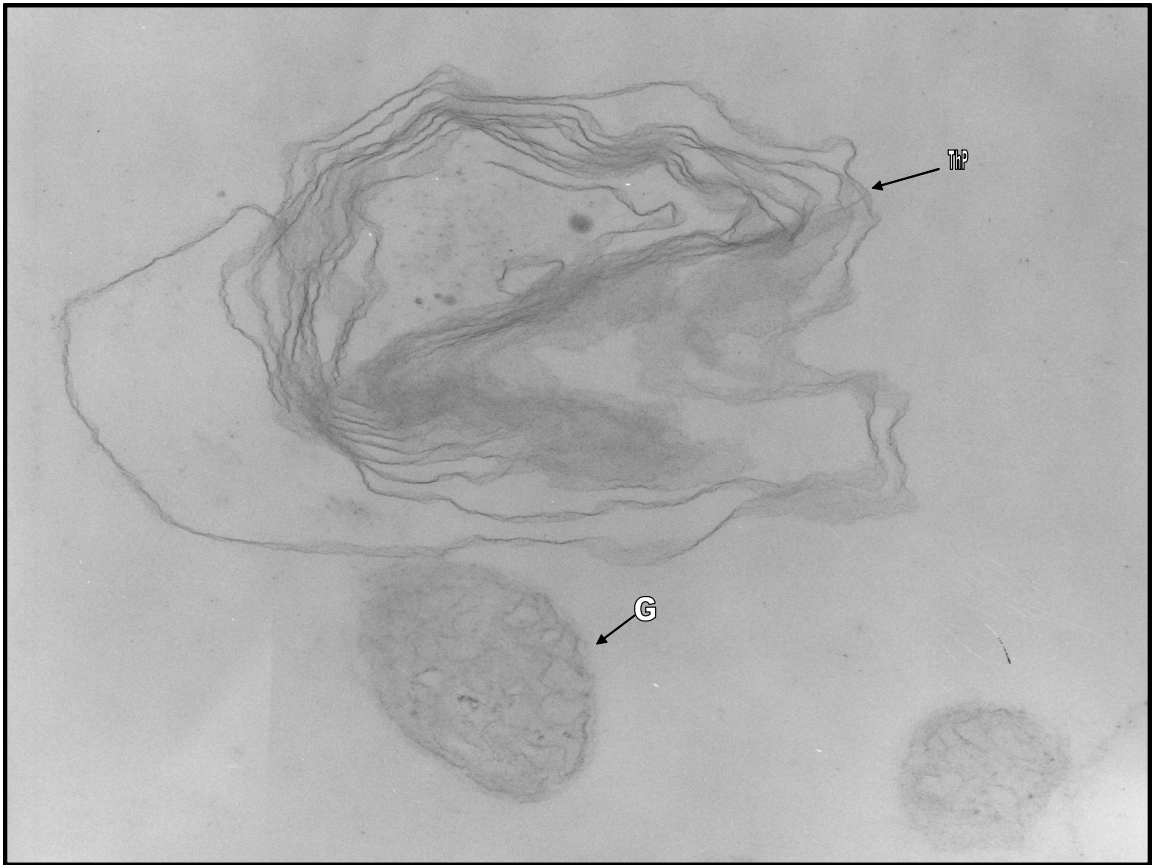
(G):   Carotenoid-containing Osmiophilic Globule

Figure 16. – 19X magnification micrograph of a mature watermelon showing a thylakoid plexus along with other chromoplasts.

Structures are identified as follows:

(Th)   Dilated Thylakoid Granum
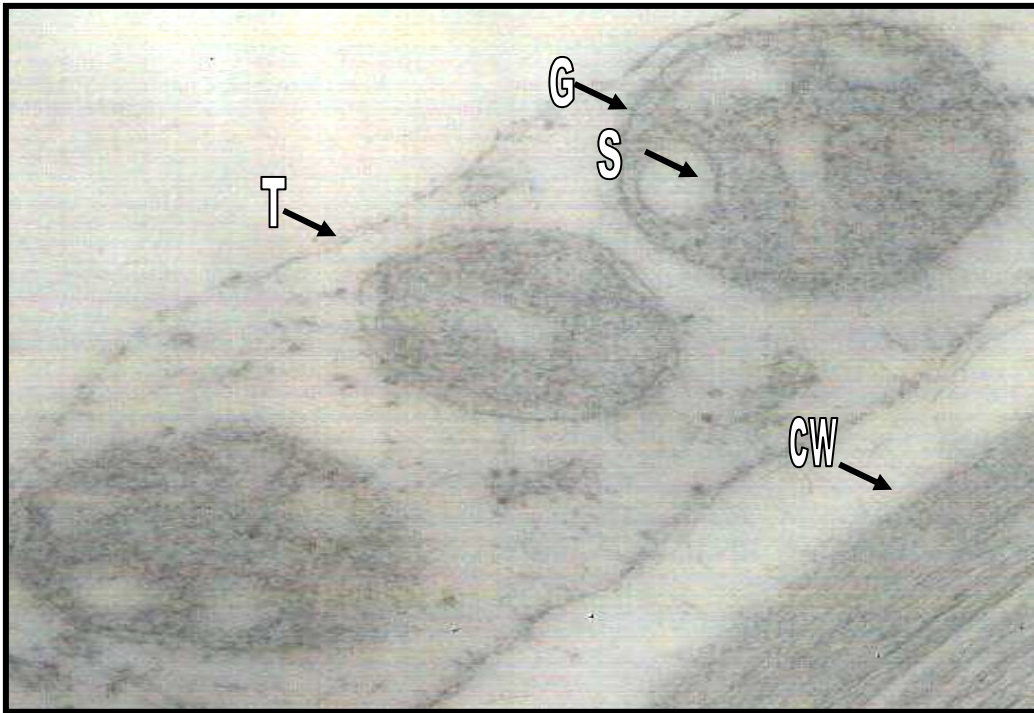(G):   Carotenoid-containing Osmiophilic Globule

Figure 17. – 36X magnification micrograph of a mature watermelon showing pigment containing globules intact within tonoplast and inside the cell wall.

Structures are identified as follow:

(G):   Carotenoid-containing Osmiophilic Globule
(S):   Stroma
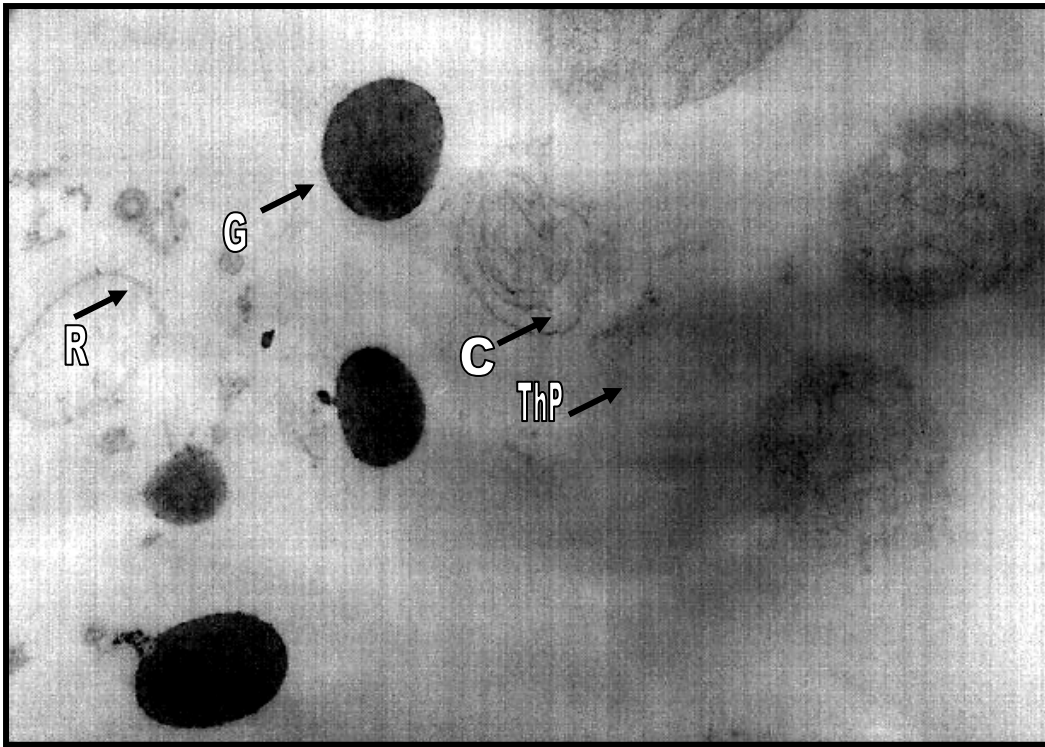(CW): Cell Wall
(T):   Tonoplast

Figure 18. – 19X magnification micrograph of a mature watermelon showing formation of thylakoid plexus (ThP).

Other structures are identified as follow:

(G):   Carotenoid-containing Osmiophilic Globule

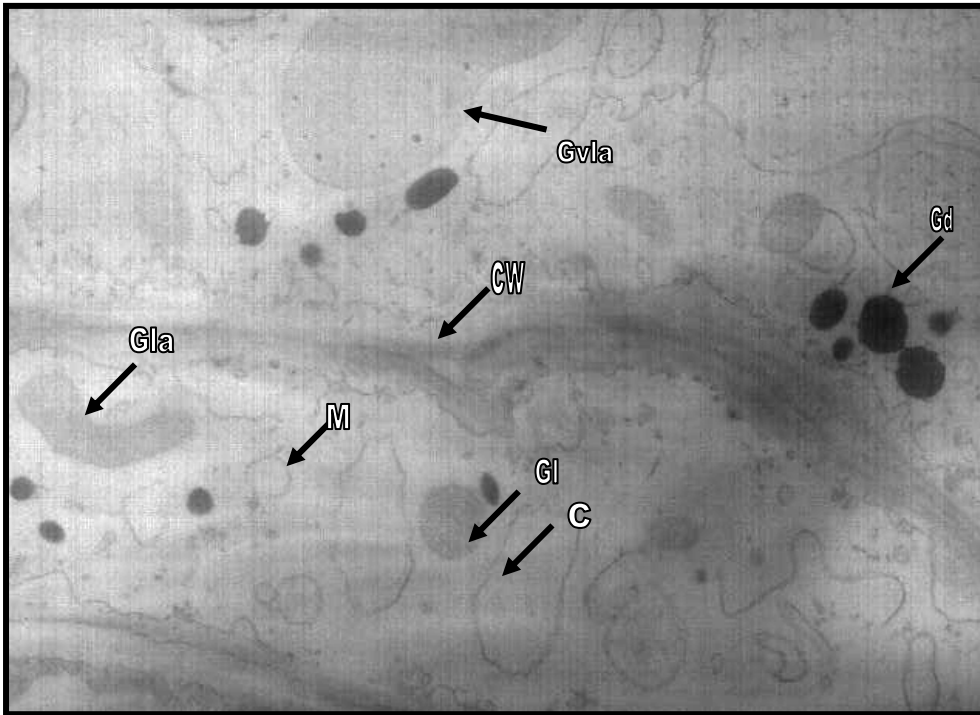(C):   Undulating Thread-like Structure

(R):   Ribosome

Figure 19. – 72X magnification micrograph of a mature watermelon showing a number of coiled thread like structures (C).

Other features include globules ranging from large (Gla) to very large asymmetrical globular structures (Gvla) and light underdeveloped (Gl) to dark completely developed globules (Gd).

Other structures are identified as follow:

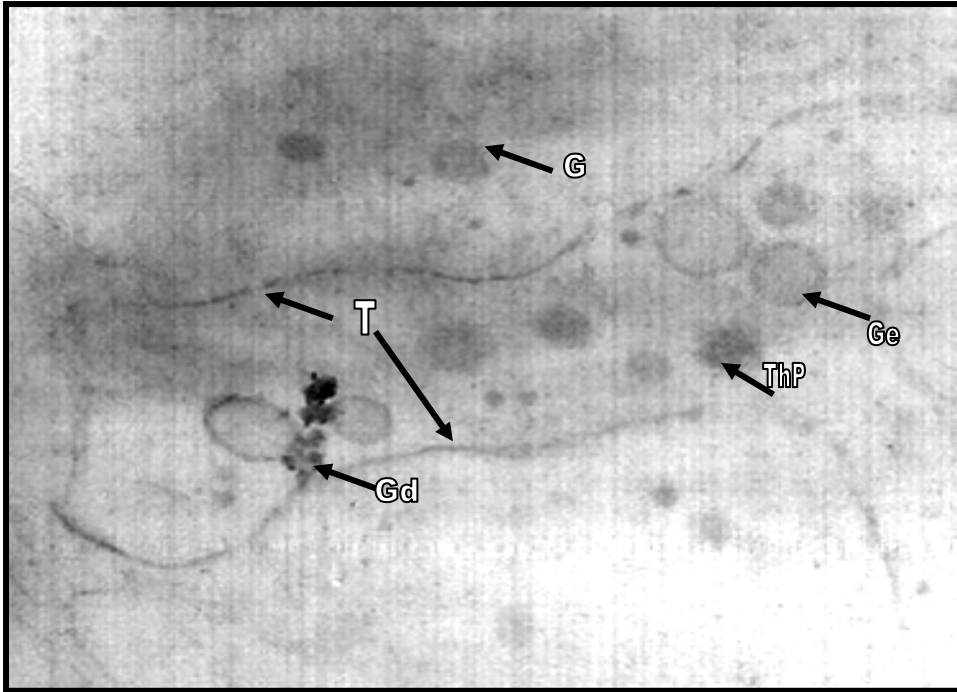(cw):   Cell Wall
(M):   Mitochondrion

OVERMATURE

Figure 20. – 10X magnification micrograph of a over mature melon showing diffusion of pigments from globules into the cell stream (Gd), light, mostly empty globules (Ge) and regions of plexus (ThP) formation.

Other structures are identified as follows:

(G): Carotenoid-containing Osmiophilic Globule
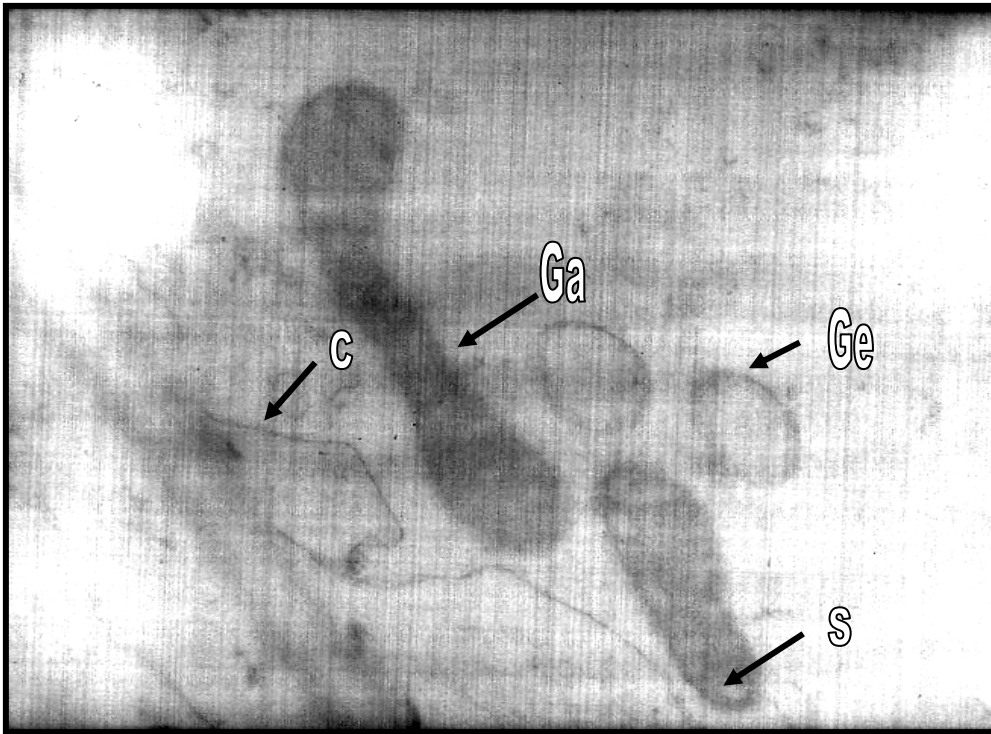
(T): Tonoplast

Figure 21. – 19X magnification micrograph of an over mature melon showing both empty globules (Ge), and asymmetric globules (Ga) with retained pigmentation.

Other structures are identified as follows:

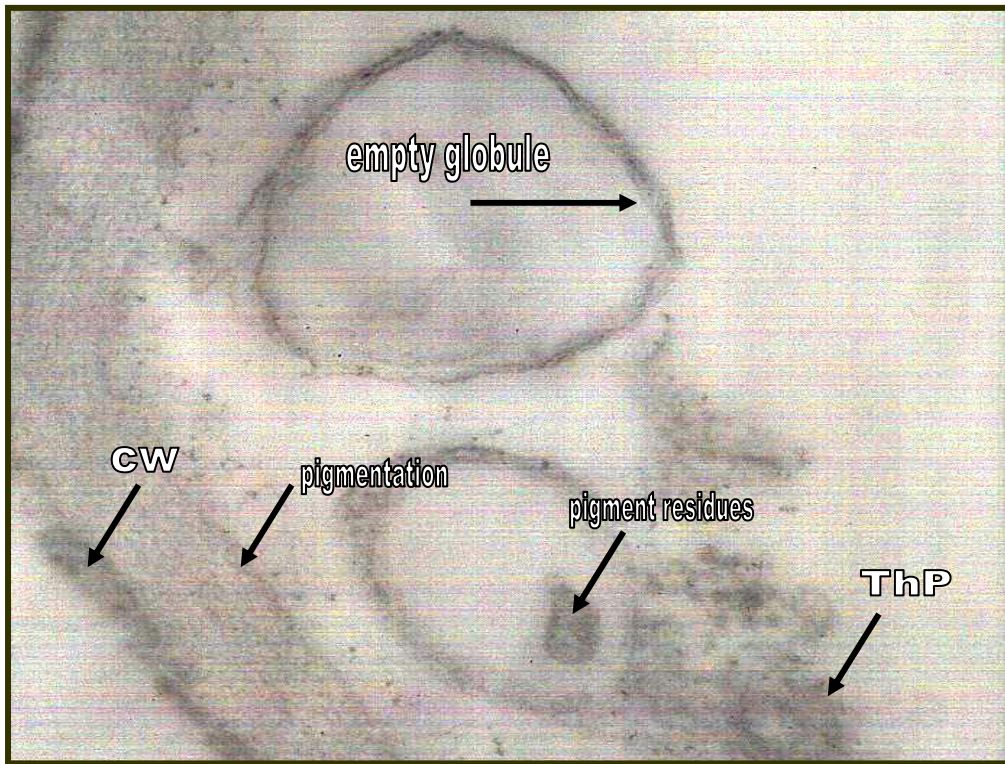(C):   Undulating Thread-like Structure

(S):   Stroma

Figure 22. – 58X magnification micrograph of a over mature watermelon showing empty globules, aglobule with residual and diffusing pigmentation into the cell stream, and plexus regions (ThP).

Other structures are identified as follows:

(cw):   Cell Wall

VITA

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Doctor of Philosophy

Thesis: Influence of watermelon maturity on tissue ultrastructure, lycopene distribution, and antioxidant activity

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Pages in Study: 78

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Scope and Method of study: Lycopene, a lipophilic antioxidant, plays a crucial role in biological systems. Watermelon, a natural source of lycopene (~48.7µg/g), could be a useful ingredient in functional foods. Correlating lycopene concentration in a food product with its antioxidant activity would hence be critical in determining effective and economical rates of lycopene addition. Also, it is necessary to understand the association of lycopene with other structural organelles during melon maturation, while designing processes to maximize lycopene retention in watermelon-based extracts and/or functional foods. The objectives outlined in this study include: (i & ii) correlating the antioxidant activity of lycopene with lycopene concentration using the a modified $ORAC_{FL}$ assay involving the use of β-cyclodextrin (β-CD) and randomly methylated β-cyclodextrin (RMCD) separately as solubility enhancers (iii) studying watermelon ultrastructure and relating melon maturity to the distribution and organization of lycopene within structural organelles in watermelon mesocarp. Lycopene was extracted (from Lyc-O-Mato^{TM} 6 % extract) by a modified spectrophotometric assay developed by inclusion of β-CD and RMCD separately (at concentrations of 0, 0.4, 0.8 & 1.6 %) and the antioxidant activity of lycopene measured with $ORAC_{FL}$ assay. Experiments were conducted in quadruplicate and statistical pooled correlations analyzed. Also for the third objective study employed Transmission Electron Microscopy (TEM) to conduct research on the ultrastructure of watermelon mesocarp for watermelon obtained at different maturities.

Findings and Conclusions: Lycopene concentration correlated poorly with $ORAC_{FL}$ (R^2=0.33) in the absence of either β-CD or RMCD. Correlations improved both with increasing levels of β-CD (R^2=0.58 and 0.91 for 0.4 and 0.8 % β-CD respectively) and RMCD (R^2=0.9284 and 0.9959 for 0.4 and 0.8 % RMCD respectively). A very high β-CD concentration (1.6%) decreased the correlation between ORAC and lycopene concentration. Inclusion of cyclodextrins in $ORAC_{FL}$ assay improves correlation between ORAC and lycopene concentration, thus expanding the scope of the $ORAC_{FL}$ assay for similar other fat soluble antioxidants. Electron micrographs from TEM studies showed chromoplasts changing from less organized globular form in immature to a definite, symmetrical form in mature and finally to an asymmetrical structure in over-mature melons.

ADVISOR'S APPROVAL: Dr. William McGlynn