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Antioxidants and Beta-Carotene: A General Overview, A Research History, and Modern Scholarship

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Abstract

This paper seeks to provide a cursory overview of oxidative stress and the accompanying biomolecules which are used to combat it. From there, the paper will provide a somewhat comprehensive list of major historical discoveries regarding antioxidant molecules, in particular beta-carotene. After this, an overview of more modern scholarship on the issue of these molecule's antioxidant properties specific (from the 1970s onward) will be discussed up to modern times. The paper will conclude with an in depth look at the modern scholarship on beta-carotene that was performed here at Taylor University.

Antioxidant Overview

Antioxidants are biochemicals that have a long and controversial history in both research and popular culture. The purpose of antioxidant molecules in biological systems is complex strongly related to phenomenon commonly known as "oxidative stress" [1]. Concisely put, the phrase "oxidative stress" refers to the stress put upon biological systems as a result of their unavoidable interaction with diatomic oxygen. Diatomic oxygen does not have much destructive potential as O_2, but rather as closely related derivations from that molecule. These derivations are commonly known as "reactive oxidative species," or ROS. They include superoxide ions, peroxides, hydroxyl radicals, singlet oxygens, and many more [2]. A common trait to these compounds is the fact that they all have unpaired valence electrons. These compounds cause damage a variety of ways. For one thing, these compounds readily donate their unpaired electron to other, more important, cellular compounds and structures. A specific example of this includes the reaction of lipid peroxidation, whereby a reactive oxidative species passes its unpaired electron off to an unsaturated fatty acid. This can negatively affect plasma membrane rigidity as well as having other negative effects [3]. More generally, reactive oxidative species have the

potential to negative impact three major classes of biomolecules: both DNA and RNA polymers can be damaged via oxidative-assisted cleavage of the bonds making up the phosphate-sugar backbone, lipids are damaged by lipid peroxidation among other things, and proteins suffer adverse effects as several amino acid side chains are susceptible to oxidation [4]. Enzymatic activity is also negatively affected by the activities of reactive oxidative species as many coenzymes can be oxidized which deactivates them [4]. All of these effects can be mitigated, in theory, by reducing reactive oxidative species shortly after they form, before they can react with any other biomolecules [1]. Compounds that reduce reactive oxidative species for this purpose are referred to as "antioxidants." Several different specific compounds that fall under this categorical term have been identified, including the enzyme superoxide dismutase [5], the enzyme catalase [5], the enzyme glutathione peroxidase [5], the class of enzymes peroxiredoxins [5], glutathione [5], thioredoxin [5], ascorbate [5], alpha-tocopherol [5], uric acid [5], a group of fat-soluble compounds known colloquially as vitamin E [6], and beta-carotene. These antioxidants can be classified as either fat soluble (these are often found within phospholipid bilayers, to prevent them from suffering membrane rigidity from lipid peroxidation) or water soluble (these prevent free radical catalyzed destruction from occurring in the cytoplasm of cells) [1]. Suffice to say, the complete absence of antioxidants would result in severe difficulties for organisms as they currently exist; at the very least life would struggle with far more mutations relative to reality, unstable plasma membranes relative to reality, unstable amino acids relative to reality, and non-functional enzymes via oxidation of their coenzymes in a world devoid of these chemicals.

A History of Early Beta-Carotene Research

Many of the chemicals discussed as antioxidants have been known by chemists for a long time; in several cases, however, their antioxidant roles and properties were not determined until

well after their initial discovery. An example of this is the discovery of the group of chemicals known as vitamin E. These antioxidants were discovered in 1922 by Herbert McLean Evans and Katharine Scott Bishop [7]. However, the antioxidant properties of these compounds weren't revealed until years later when a different pair of researchers, Evans and Mattill, explored the exact biological action of this groups of chemicals [6]. A very similar story exists regarding the discovery of beta-carotene and its antioxidant properties. It was first isolated in 1831 in plant pigments by Heinrich Wilhelm Ferdinand Wackenroder [8]. Close to a century later, its empirical formula was discovered in 1907 by Richard Willstätter [9]. Beta-carotene's close metabolic relationship to vitamin A (beta-carotene is metabolized to vitamin A) wasn't proposed until 1919 by H Steenbock in at the University of Wisconsin-Madison [10]. Beta-carotene's structure was finally discovered in 1930 by Paul Karrer to much fanfare, as this was the first time the structure a vitamin of provitamin's structure had been deduced. Karrer was eventually awarded the 1937 Nobel prize in chemistry for this discovery, well over 100 years after its discovery in 1831. Antioxidants and beta-carotene are very well studied at this point in history, having been the subject of research for nearly two hundred years at this point.

Beta-carotene is in some ways the textbook example of an antioxidant as it has been studied extensively and know to the scientific literature for a relative long time. It has an isoprenoid structure with cyclization at either end of the molecule; the alternating system of double bond and single bond connecting the two rings are conjugated, implying electron delocalization [11]. It is almost certainly the conjugated nature of the double/single bond system which allows for the reduction of singlet oxygen and other reactive oxidative species without causing the immediate degradation of beta-carotene; the delocalized electrons of the conjugated system means that there is relatively little difference in magnitude between an excited state

electron and ground state electron and this energy difference can dissipated via small collisions with the solvent [11]. This means that radicalized beta-carotene poses little threat to the other biomolecules around it. Beta-carotene is a lipophilic molecule and therefore spends most of its time inside the plasma membrane.

A History of Modern Beta-Carotene Research

For a molecule whose existence has been known of for a comparatively long time and whose research has led to a Nobel prize, it took commercial markets until relatively recently to move to capitalize on it. It wasn't until 1954 that beta-carotene began to be produced commercially by Roche Inc, although at this point, it wasn't being marketed as a supplement with antioxidant properties [12]. In the 1970s, that changed, as the dangers of reactive oxidative species (most frequently called "free radicals" in popular imagination), already known for around one hundred years, captured the American public's attention when several studies published between 1972 and 1980 were published seeming to link reactive oxidative species with cancer incidence [13]. In the early 1980s, several scientific papers (epidemiological papers) were published which seemed to demonstrate that people who had diets high in fruits and vegetables also had relatively high beta-carotene blood concentration and experienced cancer at a lower rate than the general population [13]. The proposed link between beta-carotene consumption and decreased cancer risk was discovered accidently; the researchers did not design the study to discover a relationship between those variables. In several follow up studies performed later in the 1980s, rats treated with beta-carotene in both oral forms and intravenous forms seemed to form tumors at lower rate than control group rats [13]. These trials seemed to give strong evidence for beta-carotene's alleged anticancer properties, as rats in the control groups experienced tumor formation at up to twelve times the rate as rats in the highest control groups; this trend remained

constant across age and sex categories. These trials on rats led to the interventional studies involving humans in the 1990s [13]. Of course, it was expected that these trials demonstrate a similar relationship between cancer protection and beta-carotene intake as was observed in the rat trials only a few years prior [13]. While the first such trial seemed to show a modest trend in the expected direction, subsequent trials could not replicate its findings. In fact, one study even found that humans consuming beta-carotene from non-natural sources had an increased mortality rate relative to an untreated control group by as much as nine percent [13]. Other trials found similar worrying trends concerning artificial beta-carotene consumption in humans; many found the exact opposite trend hoped for, that is, cancer incidence increased with increased artificial beta-carotene consumption [13]. By the end of the 1990s, the scientific community had largely abandoned the idea that beta-carotene could be used to lower cancer incidence in human populations do to the interventional trials in humans [13]. While the scientific community largely moved on, the popular notion of beta-carotene being associated with anti-cancer properties endured in the public consciousness, a relic of the science community's hope from the 1870s and 1980s.

Today, pharmaceutical companies exploit the public's antiquated perception of beta-carotene as cancer preventative to sell beta-carotene supplements. There is considerable opposition to the claims made by the industry in the science community [5 14 15 16]. Some researchers believe that all antioxidants aimed reducing reactive oxidative species post-formation are ineffective because in even the most conservative estimates of biological reactive oxidative species concentration, they are significantly higher than the concentration of antioxidants (including beta-carotene) [5]. Other researchers point to the fact that beta-carotene supplements have been shown to have virtually no effect on the growth of skin keratoses (which are an

indicator of future skin cancer) whereas sunscreen has been shown to have a positive measurable impact of the development of skin keratoses [14]. Beta-carotene has actually been shown to cause reactive oxidative species formation in biomolecules *in vitro* under certain conditions [5], and other researchers doubt its potency and safety as a supplement for that reason. Beta-carotene supplements have also not conclusively been shown to have positive health benefits outside of cancer prevention. For example, beta-carotene has not been shown to prevent macular degeneration [15]. And in regards to the biggest myth in the American psyche about beta-carotene that is perpetuated by companies, a meta-analysis of 13 large studies all performed after 1999 regarding the possible effects of beta-carotene supplementation on human cancer rates failed to find any evidence in connection to the anticancer claim [16]. In light of sustained research and at least somewhat conclusive results, it is probably somewhat safe to say that beta-carotene supplements at best have no measurable effect on human health and may even be detrimental in some cases.

That is not to say that beta-carotene is completely worthless as object of study or that consumption of beta-carotene in all cases is at best worthless and a scam or at worst detrimental to one's health. Beta carotene is found naturally in many easily available fruits and vegetables, and there is some evidence that consumption of beta-carotene in these forms could lead to some modest health benefits (Pakistan et al., 2007). Food high in beta-carotene include lettuce, spinach, and carrots. People who eat higher than average amounts of lettuce have been found to have significantly lower mortality rates than their non-lettuce-eating peers as well as many more specific health benefits; this at least partially attributed to lettuce's high beta-carotene content [18]. A similar trend regarding mortality rates concerning carrots has also been demonstrated by numerous studies. Specific health benefits of carrot consumption include immune system boost

and lowered risk for certain types of cancer (lung cancer). Again, at least some of these benefits to high carrot consumption can be attributed to the beta-carotene content of carrots [19]. It has been demonstrated that consumption of carrots does lead to measurable increases in blood plasma concentration of beta-carotene [20]. Spinach has also been shown to have many health benefits including lowering blood pressure and decreasing the risk for some types of cancers [21]; it is very unlikely that none of the health benefits inherent to spinach consumption come from its beta-carotene content. The difference between consumption of beta-carotene supplements and beta-carotene in vegetables is that beta-carotene in supplements are being consumed alone. It is very possible that beta-carotene requires other compounds found in natural foods that assist in its uptake by the body or enhance its potency within the body [11,13,20]. This synergistic interaction between beta-carotene and the other chemicals indigenous to vegetables to give measurable health benefits to humans is hard to quantitatively measure and difficult to prove conclusively, but it is currently the best explanation for the observed phenomenon.

The current research trend in this area has been towards quantification of beta-carotene and other antioxidants in foods. This reflects the prior research discussed earlier in the paper, as it has been shown that beta-carotene supplements (that is, beta-carotene consumed in a concentrated form in the absence of other biomolecules occurring naturally in food) don't have any real, profound effects on human health, but that consuming antioxidant molecules (including beta-carotene) within food may have some health benefits, including prevention of some cancers. It makes sense to identify which foods have higher quantities of beta-carotene and other antioxidant molecules so that the potential health benefits of consuming those foods may be exploited.

Local Research on Beta-Carotene

The research of beta-carotene at Taylor University (in the spirit of most modern research on this topic) has been directed mostly at issues regarding quantification of beta-carotene and determining the relative amounts of antioxidant properties various pieces of fruit have. The fruit under examination was papaya, as other fruit and vegetables had already been studied quite extensively in regards to beta carotene content, specifically bananas [22], spinach [23–28], kale [28–30], carrots [26,31–33], lettuce [17,30,34,35], and brussel sprouts [36,37] among many others. The local research was not concerned with quantifying the absolute amount of beta-carotene in papaya, but with demonstrating the relative amount of antioxidant potential that beta-carotene extract from differently processed papaya possess. So, while a concentration of beta-carotene in papaya was not determined at any point in the research, the local research was able to distinguish which type of papaya processing allowed for greater antioxidant property in the extract.

In order to study beta-carotene *in vitro* or to measure the amount of beta-carotene in a food, extraction of beta carotene must occur. This is either to obtain a pure sample on which to perform experiments *in vitro* or to quantify the concentration of beta-carotene in a given food source. One commonly used method to quantify the amount beta carotene in food is to use either HPLC or column chromatography on homogenized food extracts [38]. Another very common way of isolating beta-carotene is to use beta-carotene rich algae and isolate it from there [39]. The method used in the research performed here at Taylor University was slightly more crude. First the fruit from which beta-carotene was being extracted (papaya) was homogenized with acetone and diethyl ether and then the organic liquid was separated via filtration from the solid. The liquid was then subject to rotary evaporation until orange crystals or sludge was left. This extract was then used for the procedure of the projects. Equal amounts of papaya (in mass) were used at

the beginning of the extraction process, implying that equal amounts of extract ought to be able to be meaningfully compared in the concentration category, as only the type of papaya processing differed between papaya extracts.

While the antioxidant properties of foods are often determined via the absolute quantification of the various antioxidant molecules in different fruits and vegetables using high performance liquid chromatography [17,20,24,28,36,37], the local research group took a different method that included measuring the antioxidant properties of the various extracts by carefully measuring the reaction between the presumed antioxidants in the extracts with a known concentration of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a known reactive oxidative species. DPPH as a free radical appears black to the eye and absorbs strongly 450 nanometer wavelength light but as after reaction with an antioxidant, becomes clear; this property was exploited by the local research group to measure the progress of reaction, a metric which could be translated roughly into relative antioxidant capacity of the various papaya extracts.

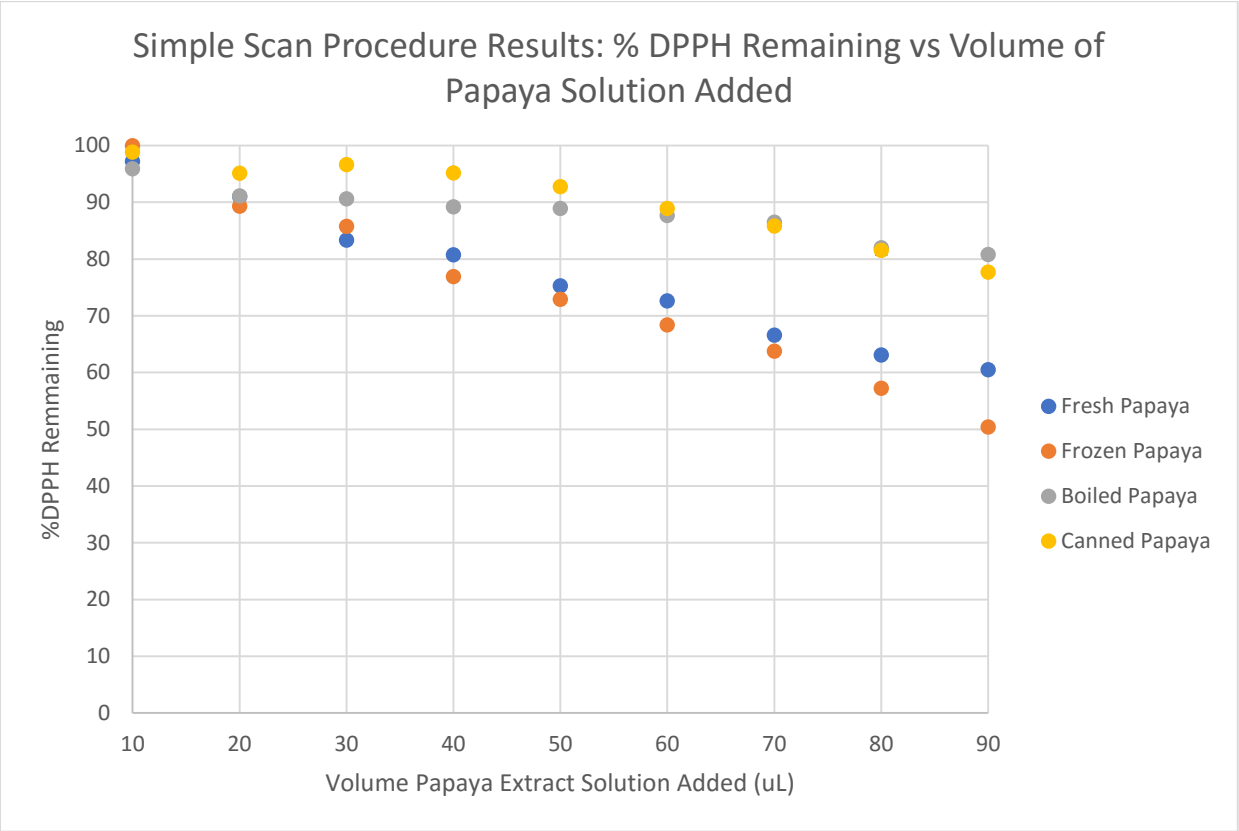
One way the color change associated with DPPH was exploited to determine the relative amounts of antioxidant property in the various extracts was simply using a single beam spectrophotometer to make simple reads. One scan was taken of a DPPH solution prior to the addition of extract solution. After the extract solution was added, the reaction was allowed to proceed unhindered for 90 seconds, at which another simple scan was taken. The difference in absorbance was used as evidence of the reaction proceeding and the magnitude of that procedure.

Another way that DPPH's color change can be exploited is by using a spectrophotometer to perform kinetic runs to obtain a rate of absorbance change. In the case of the local research group, a single beam spectrophotometer was used again as with the simple runs. The idea was

that extracts with more antioxidant property would demonstrate higher reaction rates, something that was easily calculated from the rate of absorbance change.

The results of both procedures designed to infer relative antioxidant activity in the various extracts coincided very well. The results of the simple scan procedure showed that the extract derived from frozen papaya was able to react the most with the DPPH standard solution relative to the other extract types, indicating the greatest amount of antioxidant capacity. The extract that was able to react the second most with the DPPH solution was the extract derived from fresh papaya, indicating the second most amount of antioxidant property. The canned and boiled papaya extract were very similar in how much DPPH they were able to produce. They both were the extract types that reacted the least with the DPPH solution, indicating that they both contained the least amount of antioxidant property. These trends were mirrored almost perfectly by the kinetic procedure results. The frozen papaya extract reaction was found to occur at the fastest initial rate, co-indicating with the simple scan data that the frozen papaya extract contained the greatest amount of antioxidant property of the various papaya extracts tested. The trend found via the results of the simple scan procedure was also found to be in alignment with the kinetic data, as the fresh papaya extract was found to have the second fastest rate of all the papaya extracts. The boiled and canned papaya extract behavior in the kinetic scan procedure deviated from the results found in the simple scan procedure in a small way by not being quite as nicely juxtaposed; the canned papaya extract reacted with DPPH at a significantly higher rate than the boiled papaya extract did. This contradicted the earlier results obtained by the simple run procedure. However, both were still found to be much lower than the rate of reaction induced by the fresh and frozen papaya extracts, so the data agrees on that point. The local group also tested an additional extract type using the kinetic scan procedure but not the simple scan

procedure; the dried papaya extract exhibited a rate of reaction that was between that of the canned papaya extract and the fresh papaya extract, indicating an antioxidant property intermediate that of canned papaya and fresh papaya. And finally, the local research group also performed an additional kinetic run using a stock beta-carotene solution and the DPPH solution, in order to help give a rough estimate of the absolute amount of beta-carotene in each extract solution. The rate of reaction obtained from this kinetic run was intermediate the rates obtained from the fresh and frozen papaya, indicating that the frozen papaya extract had relatively greater antioxidant property than the stock solution of beta-carotene while the fresh papaya extract had relatively less antioxidant property. The ranking of the relative antioxidant property from the simple scans from greatest to least amount of antioxidant property is as follows: frozen papaya extract, fresh papaya extract, and a tie between canned and boiled papaya extract. The ranking from greatest antioxidant property to least amount of antioxidant property according to the kinetic scan data including addition of dried papaya extract and beta-carotene were as follows: frozen papaya extract, beta-carotene stock solution, fresh papaya extract, boiled papaya extract, dried papaya extract, and canned papaya extract. It was in alignment with the local research group's expectations that both the canned and boiled papaya had less antioxidant capacity than the fresh papaya; it is very reasonable to assume the addition heat would oxidize biomolecules (which would deactivate antioxidant molecules) and that this is what happened here. The only real contradiction between these results is the relative lack of congruity between the boiled papaya extract's antioxidant property and the canned papaya extract's antioxidant property, and this was not significant to the research. Otherwise, the data from both procedures are in excellent agreement.



KINETIC RUN PROCDURE RESULTS: AVERAGE RATES OF REACTION	
Papaya Type:	**Absolute Value of Rate (given in absorbance/seconds):**
Fresh Papaya	0.0009375
Frozen Papaya	0.001060833
Boiled Papaya	0.000640833
Canned Papaya	0.000195833
Dried Papaya	0.000440833
Beta-Carotene Stock Solution	0.002748333

The local research group fully expected that the fresh papaya would have the most antioxidant capacity. The most startling result of the experimental procedures was that this expectation was not met, as the frozen papaya extract edging out the fresh papaya extract for the top position. There are several reasons for why this result might have been obtained. If the mechanism for determining why the bioavailability of beta-carotene (or other antioxidant

molecules) becomes greater when a food is consumed can be elucidated, that discovery might lead to different processing methods for things like fruit.

One of the first theories posed by the local research team regarding the experimental result of frozen papaya extract possessing the most antioxidant property relative to the other extracts, particularly the fresh extract, is that freezing the fruit in preparation somehow removed water from the fruit. This would have had the effect of concentrating any beta-carotene (and other lipophilic antioxidant molecules) relative to the mass of the fruit. Of course, this change would have been reflected all the way up through the procedures of the project. The amount of papaya measured for extraction would have relatively more beta-carotene (and other lipophilic antioxidant molecules) in it relative untreated papaya from the same fruit. After the extraction procedure was completed, the frozen papaya extract would still have a relatively greater concentration of beta-carotene in it relative to fresh papaya extract. And this greater concentration would manifest itself in both of the experimental procedures, the simple scan procedures and the kinetic scan procedures. If this explanation is correct, there are no reasons to necessarily freeze papaya prior to consumption, as the beta-carotene (and potentially other lipophilic antioxidant molecules) bioavailability is not necessarily increasing with freezing, but rather water is leaving the fruit, concentrating the existing biologically available beta-carotene. This has no effect on the actual quantity of biologically available beta-carotene though. Rather, the same amount of beta-carotene will be biologically available upon consumption regardless of whether the fruit is frozen or not. As far as real world consideration is concerned, if this explanation is correct, the only difference between a papaya that's been frozen and a papaya that hasn't been is that the frozen papaya has less water weight than the fresh papaya. A completely satisfactory mechanism regarding exactly how the freezing procedure could result in loss of

water from papaya has not been found or tested for, but it is possible that the dry nature of Taylor University's stock room refrigerator contributed to the evaporation of water from the papaya; loss of water via desiccation is possible.

It is also possible that the lower temperature of freezer could have changed the activity of some lytic enzymes within the cells of the papaya. If this is the case, and lower temperatures could be shown to make these lytic enzymes more active, than those "extra active" enzymes could contribute to cellular lysis. If cellular lysis is occurring, it could lead to more beta-carotene being bioavailable. Obviously cellular lysis doesn't create any more beta-carotene, but instead frees up more to react. It does this by allowing some of the beta-carotene that is caught up in the plasma membrane of cells and within the cells themselves to react with free radicals (and in the case of the local research, with DPPH). So, to summarize this theory, lower temperatures could lead to a higher activity of certain lytic enzymes, which could lead to a greater rate of cellular lysis which would free up extra beta-carotene within the plasma membrane of the papaya cells and the cells themselves to react. If this is the case, freezing might represent a practical way to make beta-carotene more bioavailable in a human diet as only one cycle of freezing would be necessary to activate the necessary enzymes to lyse the cell and free up the beta-carotene for use in a human. This would be a relatively cheap and effective method of increasing bioavailability of beta-carotene in humans. It is however, a remote possibility that this is the case. Enzyme activity nearly always goes down when the temperature drops, which would mean that its more likely that lytic enzymes get less active rather than more active in freezing temperatures similar to the temperatures the local research group exposed the frozen papaya to. There is, however, another alternative explanation that isn't predicated on the dubious idea that some lytic enzymes get more active as the temperature get colder.

A final theory to explain the apparent greater ability of frozen papaya extract to exhibit antioxidant capacity than even fresh papaya could be the fact that the process of freezing and thawing lyses cells. The lysis of cells would free up beta-carotene to react exactly as described in the preceding paragraph. This theory would be somewhat easy to confirm experimentally as freezing and thawing a papaya several times over should free up proportionally more beta-carotene for reaction than papaya that had only undergone one cycle of freezing and thawing. Should this prove to be the case, it might be possible to apply a series of cycles of freezing and thawing to make beta-carotene more bioavailable in papaya for human consumption. The implications of this theory are very similar to the implications of the lytic enzyme theory; if true, a relatively cheap method of increasing the bioavailability of beta-carotene could exist. It would merely entail freezing and thawing papaya several times before sale to the customer. This is more likely the cause of why the frozen papaya experimental group outperformed the fresh papaya experimental group in both procedures than the lytic enzyme explanation. Lysis of cells via freezing and thawing is well documented whereas enzymes that become more active in colder temperatures would be the exception to the rule.

Conclusion

The history of research on antioxidants in general and beta-carotene in particular is fascinating and rewarding. While the scientific community may be long past researching the effects of beta-carotene on humans in isolation (that is, beta-carotene without the rest of the hundreds of chemicals that are found in food) As important as beta-carotene and antioxidants in general are, there is still a lot of research to be done on this topic; a lot current research is focused on quantifying the amount of beta-carotene in different foods. And it is very possible that future research will be focused on coming up with new ways to enrich food with beta-

carotene or on increasing the bioavailability of the beta-carotene that's already within foods. This is an obvious next step as several studies have shown the benefits (mainly anticancer benefits) of consuming beta-carotene "naturally" within a diet. The local research group did good relevant work in this area, and while further research is warranted on some on their findings, they already have a significant finding; frozen papaya seems to have more antioxidant property that fresh papaya. While the days of winning Nobel prizes off of research related to beta-carotene may be over (Karrer won the 1937 Nobel prize for his work on beta-carotene), there is still useful work to be done concerning it and other antioxidant molecules that could have the potential to save many more lives.

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