INHIBITING ENZYMATIC FORMATION OF BLUE-GREEN PIGMENTS IN GARLIC
CLOVES

by

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Abstract

Blue-green pigments have the ability to form in crushed and whole garlic cloves following a rapid series of enzymatic and non-enzymatic reactions. Economic losses associated with a reduction in organoleptic quality of garlic (*Allium sativum* L.) containing blue-green pigments can be incurred. Preventing blue-green pigment formation can mitigate potential financial and brand equity damages. To prevent pigment formation, inactivation of the enzyme which yields pigment substrates is essential. Two methods to inactivate the enzyme associated with pigment formation were explored: blanch treatment of whole garlic cloves (100°C, 5 min); and soaking of whole, non-blanched garlic cloves in low pH (<3.0) acetic or citric acid pickling solutions for seven days.   The blanching treatment was effective in inactivating the enzyme as the pigment substrate decreased by approximately 99% as compared with pigment substrate concentrations in non-blanched garlic cloves. Soaking whole garlic cloves in low pH (<3.0) pickling solutions did not result in enzyme inactivation as pigments formed in cloves soaked in the acetic acid pickling solutions; however pigments did not form in cloves soaked in the citric acid pickling solution. This may be due to the different effect mono- and poly-carboxylic acids have on the permeability of garlic cell membranes.   Blanching garlic cloves can be implemented as a processing step to prevent pigment formation. Soaking garlic in a low pH pickling solution comprised of a poly-carboxylic acid does not inactivate the enzyme associated with pigment formation but pigment formation can be prevented. Other methods presented in the literature to prevent pigment formation include controlling growth, harvest and storage conditions, as well as treating garlic clove homogenates with chemical additives.

Table of Contents

List of Figures

List of Tables

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Chapter 1 - An Introduction to Garlic

History

Garlic is scientifically named as *Allium sativum* L. and belongs to the genus *Allium* which includes other crops such as onions, leeks, and chives. This genus is dispersed in mild climates throughout North America, Asia, and Europe, and contains several hundred species, most of which are edible and contain distinctly pungent odors and flavors (Block, 2010; Koch & Lawson, 1996). The earliest writings detailing the use of garlic as a food, spice, or herbal remedy date back as early as 2100 BC.   Hyams and Hehn (as cited in Koch & Lawson, 1996) consider Central Asia to be the most probable place of origin. From central Asia, garlic was introduced to the Middle East and Mediterranean regions by roaming hunters. Through trade routes, garlic continued its journey westward reaching sub-Saharan Africa and eventually the Americas by migrants and settlers (Block, 2010). Significant documentation exists in some of the worlds most storied civilizations describing how polarizing garlic and its relatives were considered. As has probably been the case throughout most of its history, Block (2010) described how garlic has a faithful following of alliophiles and alliophobes, he also described Moslem legends which recount garlic and onion plants sprouting from the ground in the wake of Satan's footsteps as he departed the Garden of Eden. In ancient, and some parts of modern Greece, garlic is considered a force to ward off evil spirits and is often hung in home entryways or placed under pillows for protection (Block, 2010). On the other hand, garlic was also known to be a powerful cure-all. Sanskrit medicinal textbooks dated approximately 500 AD classify garlic as a cure or therapy for "skin diseases, loss of appetite, dyspepsia, cough, anorexia, rheumatic conditions, abdominal diseases, spleen enlargement and hemorrhoids" (Weiss, 1983 as cited in Koch & Lawson, 1996).

Garlic Characterization and Cultivation

Growing Regions and Conditions

Garlic is second to onions as the most consumed *Allium* in the world, and is an herbaceous perennial most typically planted in autumn and harvested the following summer. However, garlic may also be planted biannually in early spring and early autumn. The plant grows best in sufficiently fertilized sandy soil, in areas of open, sparse vegetation and favors ample sunlight in a moderately dry environment with low wind. Garlic does not grow well in

low sunlight, rainy, or cool conditions. With the addition of nitrogen to the soil, garlic yield can be increased significantly (Block, 2010; Koch & Lawson, 1996). Like most plants, garlic is susceptible to disease, infestation, mold, virus, and infection during its lifecycle which can cause a significant reduction in yield and other issues including discoloration, rotting, and growth termination. Large growing regions of today include Egypt, China, India, Argentina, and the United States. California accounts for 90% of domestic production while the remaining balance is imported from China and Mexico (Koch & Lawson, 1996).

Description of the Plant and its Growth

An individual garlic clove is planted with the pointed end up. The plant will keep low to the ground while developing the bulbs underground. After planting, a hollow green stem protrudes from the ground encased by a set of long, flat, thin leaves on exact opposite sides of the stem. Approximately four to five sets of leaves will grow only halfway up the stem which can reach up to 35 inches in height. The stem will make interesting circular formations during its growth upward until reaching its maximum height at which point flowers begin to blossom during mid to late summer. From this flower, small bulbils will form and eventually drop to the ground. These bulbils can develop into a complete plant yielding garlic bulbs in its second growing season. Garlic plants require plenty of water during the early lifecycle with no water during the last few weeks before harvest. Garlic bulbs are protected by a parchment-like covering, which when peeled reveal individual, smaller, tightly packed bulbs called garlic cloves which are held in place by the basal plate (Block, 2010; Koch & Lawson, 1996).

Harvesting & Storage

The harvesting of garlic starts when the plants leaves turn brown and brittle and the bulb stems dry out. Following harvest, garlic should be stored at room temperature with low relative humidity and adequate ventilation for two to three weeks to reduce moisture by 20% in the process. Small scale harvests entail garlic bulbs being braided and hung to dry, while the post-harvest drying of garlic bulbs on large scale is most feasible on screens. Following the drying process, garlic will either enter the market as is and be sold by the bulb or be bought by specialized companies for further processing into granules, flakes, slices, pastes, purees, oils, and a host of other garlic-containing products and supplements. Long-term refrigeration may be required to prevent germination if the garlic is to be used commercially (Block, 2010; Koch &

Lawson, 1996). However, Lukes (1986) determined that garlic stored below 12°C may lead to increased concentrations of cysteine sulfoxides within the garlic thus increasing the propensity of garlic cloves to form blue-green pigments; this will be discussed at greater length in chapter 3. Hardneck and Softneck garlic, the two main garlic subspecies, yield roughly 6 to 11 and 24 cloves per bulb, respectively. Within the softneck subspecies, Silverskin and Artichoke garlic are the predominant varieties with Silverskin being the variety regularly found in supermarkets because of its exceptional long-term storability (Block, 2010).

Chapter 2 - Blue-Green Pigment Formation in Garlic Cloves

Composition of Garlic

General Composition

Water makes up approximately 65% of fresh garlic by weight which is unusually low when considering most other fruits and vegetables approach a water content of about 90%. Carbohydrates, mainly in the form of fructose polymers called fructans, make up approximately 28% of fresh garlic by weight. Protein accounts for approximately 3% (Koch & Lawson, 1996; Rejano, Sanchez, Castro, & Montano, 1997). Cysteine sulfoxides, which are sulfur containing amino acids derived from cysteine, make up as much as 1.9% of the fresh weight of garlic. These compounds are the precursors involved in a series of rapid and complex enzymatic and non-enzymatic reactions that cause garlic cloves to form blue-green pigments following tissue damage or disruption. Various lipids, fibers, vitamins, and minerals comprise the remaining 2% of fresh garlic (Aguilar & Rincon, 2007; Koch & Lawson, 1996; Kubec, Hrbacova, Musah, & Velisek, 2004; Kuettner, Hilgenfeld, & Weiss, 2002; Lee, Cho, & Lee, 2006a).

Sulfur Containing Compounds

While garlic contains close to forty unique sulfur compounds at unusually abundant concentrations (Koch & Lawson, 1996), a select few will be discussed in this report to explain the series of reactions that take place which cause garlic cloves to form blue-green pigments. The total sulfur content of garlic, which is roughly three times that of the amount contained in some of the more commonly consumed fruits and vegetables, is approximately 1.0% and 0.35% of garlics dry and fresh weight, respectively (Koch & Lawson, 1996). The vast majority of past

research conducted on the compounds found in garlic has focused on the sulfur-containing compounds. Koch and Lawson (1996) believe two main reasons exist for such focused research, the first being the effective "pharmacological activity of sulfur-containing drugs". Second, once a specific class of sulfur compounds found in garlic, thiosulfinates, are removed or otherwise absent, the positive health benefits associated with the consumption of garlic are no longer present. These benefits include antibacterial, antifungal, antithrombotic, and blood-lipid reducing effects. The earliest research attempting to identify the compounds which give garlic its distinguishing odor and flavor took place in the middle of the 19th century. While significant discoveries were made which pointed to clues that would ultimately aid in the characterization of garlics parent sulfur compounds, research after this point was unable to determine why whole, undamaged garlic cloves did not emit odor. This much was obvious in a statement made by two pioneering garlic researchers, Aurthur Stoll & Ewald Seebeck. They found a particularly "striking property of garlic is that, in the undamaged condition, it exhibits very little or no odor, but that, as soon as the bulbs are cut or crushed, an intense odor develops" (Stoll & Seebeck, 1951).

It wasn't until the middle of the 20th century when chemist Chester Cavallito was able to isolate and characterize allicin, one of the main sulfur compounds responsible for the odor and flavor of garlic. Through continued perplexing experiments, Cavallito soon hypothesized allicin could not be present in whole garlic cloves, but formed by the action of an enzyme on another compound found in whole garlic. Several years later, between 1947 and 1949, Aurthur Stoll & Ewald Seebeck were able to isolate this precursor to allicin and named it alliin (Block, 2010; Cavallito & Bailey, 1944; Koch & Lawson, 1996; Stoll & Seebeck, 1951). Follow-up research by Cavallito would later confirm his hypothesis. After freezing garlic cloves, he was able to isolate and purify the precursor alliin and the enzyme alliinase as white powders. When both are mixed together and combined with water, the reaction which produces allicin can take place and the pungent odor of garlic is produced. Proving why garlic has no odor in its whole clove form, Cavallito first boiled alliinase in ethyl alcohol, essentially deactivating the enzyme, and combined it with alliin in water which provided no pungent odor of allicin (Block, 2010).

The combined research of these scientists confirmed certain sulfur compounds are present in garlic when the cloves are in their whole, undamaged state. Other sulfur compounds are only formed through chemical reactions once garlic tissue is bruised or damaged. This

knowledge would lay the foundation for understanding the reactions which cause garlic cloves, typically cream in color, to form blue-green pigments, a phenomenon also known as "greening" (Lee et al., 2006a).

Cysteine Sulfoxides

Cysteine sulfoxides are the parent compounds involved in garlic greening, reacting enzymatically with alliinase to form thiosulfinates. Synthesis of cysteine sulfoxides in garlic cloves has been poorly explained. However, they are generally thought to be formed slowly during plant sprouting and bulb or clove development from sulfates and several amino acids including serine, cysteine, and valine. The cysteine sulfoxides serve little other purpose than to be converted into thiosulfinates upon damage to garlic tissue, a very rapid reaction which takes place in under ten seconds (Koch & Lawson, 1996).

Alliin

Alliin (Figure 1) is one of the main substrates involved in the complex reaction causing blue-green pigments to form in garlic cloves. It has only been found in large quantities in a handful of garlic types. Other nomenclature for alliin includes *S*-Allylcysteine sulfoxide, (+)-*S*-2-propenyl-L-cysteine sulfoxide or 2-PeCSO. Alliin makes up approximately 85% of the total cysteine sulfoxides found in garlic cloves (Aguilar & Rincon, 2007; Imai, Akita, Tomotake, & Sawada, 2006; Koch & Lawson, 1996). Alliin is a cysteine derived non-protein sulfur amino acid (Joslyn & Sano 1956; Kuettner et al., 2002; Lancaster & Collin, 1981). A colorless and odorless solid, alliin can be found at a concentration of approximately 0.24% in whole garlic cloves (Stoll & Seebeck, 1948 as cited in Yu & Wu, 1989). Concentration of alliin in garlic grown in different regions is fairly consistent and any variability would be caused by environmental conditions during growth, differences in soil composition, strain of garlic grown, and variations in analytical methods used for quantification. Stoll and Seebeck (1951) provided many contributions in characterizing and detailing alliin. They discovered alliin has high solubility in water, even at high temperatures. Alliin will only react with compounds which contain a free –SH group and it is a powerful oxidizing agent (Stoll & Seebeck, 1951).

Figure 1: Alliin skeletal structure
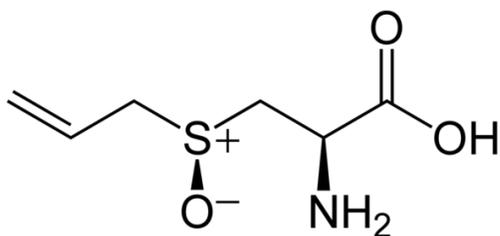
Isoalliin

Isoalliin (Figure 2), very similar in structure to alliin, is the second substrate involved in garlic greening. Nomenclature includes *S*-trans-1-Propenylcysteine sulfoxide, (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide or 1-PeCSO (Aguilar & Rincon, 2007; Koch & Lawson, 1996). While contributing a mere 5% of the total cysteine sulfoxide content of garlic, this compound is more prevalent in onions but essential in the reaction which causes blue-green pigment formation in garlic (Block, 2010; Imai et al., 2006; Koch & Lawson, 1996; Lukes, 1986). Methiin, the third cysteine sulfoxide found in garlic, comprises approximately 10% of the total cysteine sulfoxide content of garlic and is not involved in blue-green pigment formation (Koch & Lawson, 1996).

Figure 2: Isoalliin skeletal structure
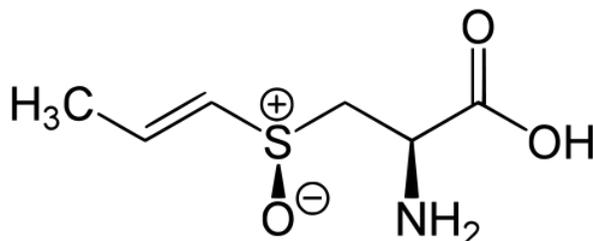
Alliinase

Alliinase is the enzyme which very rapidly acts on alliin and isoalliin to begin the reaction leading to garlic greening. Other nomenclature for alliinase includes cysteine sulfoxide lyase and alliin lyase. Alliinase is a "pyridoxal 5'-phosphate-dependent glycoprotein consisting of two subunits" (Goryachenkova, 1952 as cited in Koch & Lawson, 1996). When taking into consideration garlics total protein content, alliinase makes up approximately 10% which is an uncharacteristically high concentration for an enzyme. Stoll & Seebeck (1951) found alliinase able to lyse nearly 80% of alliin in approximately two minutes. Block (2010) contends this

reaction may be even faster than previously thought with alliinase lysing more than 97% of alliin in 30 seconds. A likely reason why Allinase reacts so rapidly with alliin is because their concentrations in garlic are nearly identical (Block, 2010; Koch & Lawson, 1996). Alliinase is also quite robust with the enzyme showing little loss in its ability to act on alliin in garlic powders after being stored for up to five years (Koch & Lawson, 1996). A pH between 5.0 and 8.0, and a temperature between 33°C and 37°C were found to be optimal conditions for alliinase activity (Stoll & Seebeck, 1951).

Thiosulfinates

Allicin

Allicin (Figure 3) is one of the main compounds in crushed garlic which provides its characteristic pungent odor and flavor (Brodnitz, Pascale & Van Derslice, 1971; Rejano et al., 1997). Allicin, or 2-propenethiosulfinate, is also the chief thiosulfinate produced when alliinase lyses alliin as it makes up nearly 70% of all thiosulfinates produced when garlic is crushed or damaged (Block, 2010; Lawson, Wood, & Hughes, 1991). Allicin makes up approximately 0.4% of garlic on a fresh weight basis. Allicin has also been found to have powerful antibacterial and antifungal activity, specifically against *Streptococcuspyogenes, Escherichia coli and Salmonella typhosa* (Koch & Lawson, 1996; Stoll & Seebeck, 1951). Allicin is quite unstable and highly reactive; it may also act as an anticancer agent and have exceptional antioxidant properties (Block, 2010). Allicin is ultimately formed after alliinase lyses alliin which forms an intermediate compound, 2-propenesulfenic acid. Two 2-propenesulfenic acid units instantaneously self-condense to form allicin while releasing a molecule of H_2O in the process. The rate at which 2-propenesulfenic acid condenses to form allicin is so fast that the compound has remained undetected for quite some time, even at temperature extremes. However, it has recently been detected and its half-life is postulated to be <1 second at room temperature (Block, Dane, Thomas, & Cody, 2010). To more clearly illustrate the reaction rate, "a burst of allicin is produced upon mixing precursor and enzyme" (Block, 2010).

Figure 3: Allicin skeletal structure

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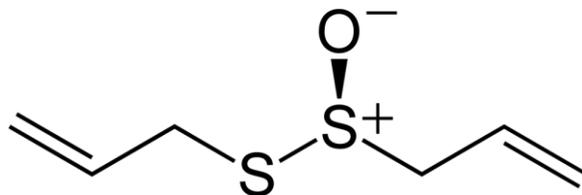
Compound Compartmentalization

Whole, intact garlic cloves are generally recognized to contain little to no odor. In other words, they are free of the thiosulfinate compounds that emit the familiar odor of freshly chopped garlic. This phenomenon led researchers in the mid-1950's to hypothesize that cysteine sulfoxides and alliinase must be physically separated or compartmentalized in whole garlic cloves (Koch & Lawson, 1996). Lancaster and Collin (1981) would eventually confirm early hypotheses of compound compartmentalization by locating the compounds physically separated from one another in different cell organelles in whole garlic cloves. They determined the substrate cysteine sulfoxides were stored in the cytosol of garlic cells while the enzyme alliinase was sequestered in the plants vacuoles. Upon the crushing or bruising of garlic tissue, vacuoles are ruptured which allows alliinase to mix with alliin and isoalliin to form allicin. Thus, the beginning of the garlic-greening reaction rapidly takes place, cysteine sulfoxides are lysed by alliinase giving rise to thiosulfinates (Aguilar & Rincon, 2007; Joslyn & Sano, 1956).

Pigment Formation

In general, garlic should possess a cream to light tan color (Ahmed & Shivhare, 2001). However, the phenomenon of garlic cloves forming blue-green pigments is recognized as a significant quality issue that "is still very poorly understood" regardless of having been investigated and discussed in scientific writings for the past half century (Ahmed et al., 2001; Joslyn, 1958 as cited in Block, 2010; Kubec et al., 2004). Overall, pigment formation consists of a rapid series of complex enzymatic and non-enzymatic reactions (Figure 4). When garlic tissue is crushed, damaged or bruised, the enzyme alliinase mixes together with and acts on the substrate cysteine sulfoxides alliin and isoallin. The action of alliinase on alliin gives rise to 2-propenesulfenic acid which very rapidly undergoes dimerization to form allicin while releasing one molecule of H_2O in the process. Concurrently with the formation of allicin, the action of

alliinase on isoalliin yields an intermediate thiosulfinate known as a color developer which then reacts non-enzymatically with naturally occurring amino acids present in garlic cloves. A pigment precursor, or pyrrole, is produced following the reaction of the color-developer and amino acid. In a final non-enzymatic reaction, allicin reacts with the colorless pigment precursor giving rise to blue-green conjugated pigments (Aguilar & Rincon, 2007; Block et al., 2010; Imai et al., 2006; Joslyn & Sano, 1956; Kubec & Velisek, 2007).

Figure 4: Proposed reaction mechanism leading to pigment formation (Imai et al., 2006)
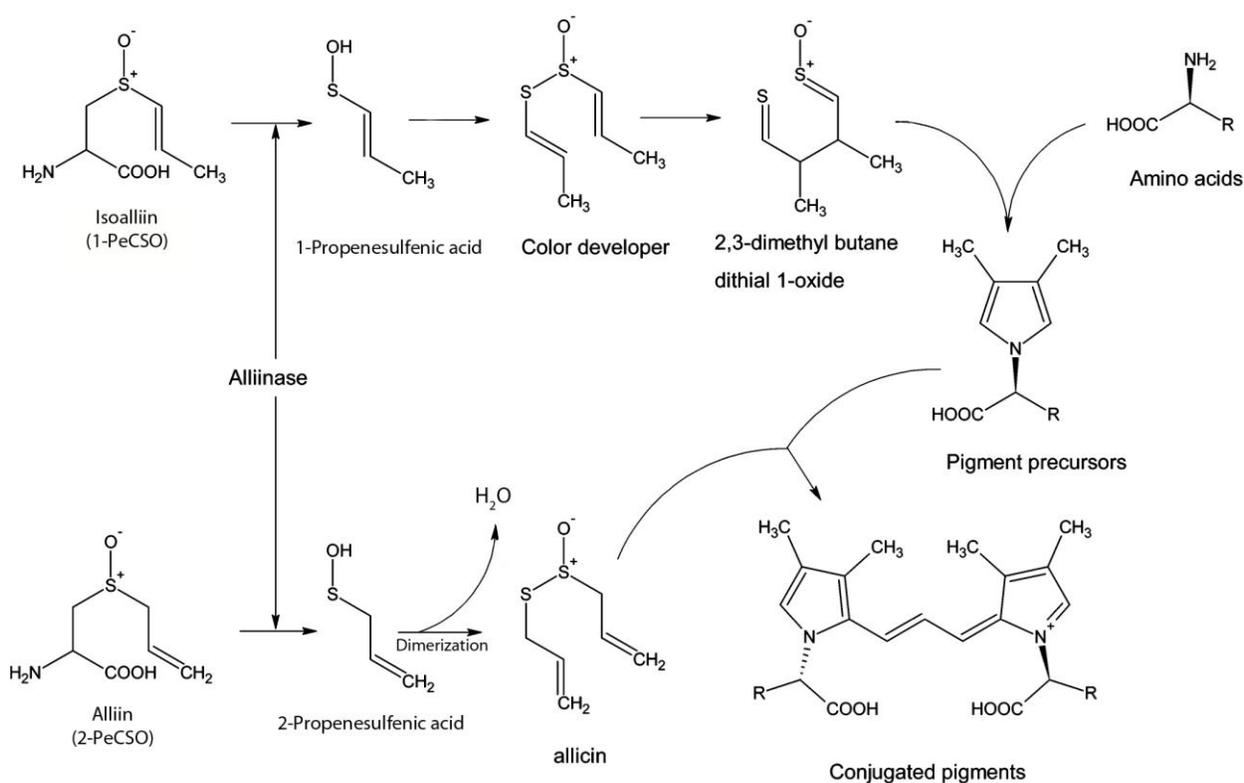
The proposed blue-green pigments are thought to consist of tri-, tetra-, or polypyrroles, compounds structurally similar to chlorophyll (Block, 2010; Imai et al., 2006; Joslyn & Sano, 1956). However further research in an effort to characterize these pigments show the pigments formed are new "nitrogenous water-soluble compounds differing significantly from all previously reported green pigments in plants" (Lee, Cho, Kim, & Lee, 2007).   Research by Lee et al. (2007) also demonstrated the pigments are somewhat viscous, contained the flavor of garlic, and when kept at room temperature for prolonged periods of time the pigments changed to yellow color followed by brown and eventually disappeared.

Conflicting research on the true color of the pigments formed has been documented. Upon isolating the pigment compounds formed, UV-vis absorption at 440 nm and 590 nm have indicated the pigments are most likely derived from separate blue and yellow pigments, which when mixed, form the visibly green color (Bai, Chen, Wang, Liao, & Zhao, 2005; Cho, Lee, Yoo, Lee, & Patil, 2009; Kubec & Velisek, 2007). Cho et al. (2009) contends the blue pigment, which is highly influenced by amino acid composition, may be the result of as many as eight individually formed pigments instead of one blue pigment.

However, similar UV-vis measurements have been obtained in separate research after isolating the pigment formed. Lee et al. (2007) and Joslyn & Sano (1956) contend the 590 nm absorbance obtained is evidence enough to conclude the pigment formed was not a mixture of blue and yellow pigments but an individual green pigment. In any case, it is largely recognized that "the discoloration is an immensely complex process, yielding hard to separate mixtures of many colored compounds" (Kubec & Velisek, 2007).

Rate of Pigment Formation

The reaction which produces thiosulfinates from cysteine sulfoxides takes place very rapidly and is nearly complete after two minutes (Block, 2010; Koch & Lawson, 1996; Stoll & Seebeck, 1951). However, the rate at which pigments are formed from thiosulfinates can take place much slower (Yang, Zhang, Bai, Han & Zhao, 2010). Research conducted by Bai et al. (2005) in which garlic cloves were aged at 4°C for one month then pickled in a 5% acetic acid solution caused blue-green pigments to form in about two to four days. As incubation time in the acetic acid solution increased, total thiosulfinate content decreased. Bai et al. (2005) saw total thiosulfinate content diminish by 85% in 4 days and the remaining 15% decreased during days 4 to 14. It has been concluded that as thiosulfinate content of garlic decreases, pigmentation increases and complete formation of pigments can take up to two weeks to develop (Bai et al., 2005).

"Laba" Garlic

"Laba" garlic is a traditional Chinese homemade garlic product in which blue-green pigment formation is desirable. It is customary to serve "Laba" garlic for Chinese New Year. Garlic used to make "Laba" garlic is harvested fresh and must be aged for approximately four months in low temperatures, most typically from September through December. Once aged, the

whole, uncrushed garlic cloves are soaked in a 5% acetic acid pickling solution which causes blue-green pigments to form in the cloves over the course of approximately 7 days. Pigments eventually diffuse from the garlic cloves into the pickling solution (Bai et al., 2005). Acetic acid is required for pigments to develop in "Laba" garlic. This was demonstrated when blue-green pigments did not develop in garlic cloves soaked in two pickling solutions made with 5% citric acid and 5% malic acid (Bai et al., 2005). Further research conducted by Bai et al. (2005) showed alliinase to be essential for pigment formation in "Laba" garlic. When an alliinase inhibitor was added to pickling solutions at gradually increasing concentrations, UV-vis absorbance at 590 nm decreased. Limited information detailing the mechanism of pigment formation in "Laba" garlic is available (Bai et al., 2005). The fact that blue-green pigments can form in whole, uncrushed garlic cloves contradicts all previous research about the phenomenon which explain the need for garlic tissue to be damaged in order for alliin and isoalliin to mix with alliinase and form allicin.

Factors Affecting Pigment Formation

Effect of Monocarboxylic Acids on Pigment Formation in Whole Pickled Garlic Cloves

Blue-green pigments are able to form in intact garlic cloves when soaked in a 5% acetic acid pickling solution. It was demonstrated that both alliinase and acetic acid are essential for pigment formation in "Laba" garlic. However, blue-green pigments did not form in garlic cloves upon being soaked in malic acid or citric acid (Bai et al., 2005). This implies acetic acid has a unique function in the formation of blue-green pigments in whole pickled garlic cloves (Bai, Li, Hu, Wang, & Zhao, 2006). Research conducted by Bai et al. (2006) clarifies the mechanism for garlic greening in intact cloves and explains the role acetic acid plays in pigment formation.

Bai et al. (2006) recreated and expanded research conducted by Bai et al. (2005) incorporating several new mono-, di- and tri-carboxylic acids. Garlic cloves were soaked in nine separate pickling solutions containing 5% concentrations of acetic (Figure 5), propionic, butyric, valeric, caprioc, malic (Figure 6), oxalic, and citric (Figure 7) acids. The first five constituting the monocarboxylic acids, malic and oxalic are di-carboxylic acids and citric is a tri-carboxylic acid. Blue-green pigment formation only occurred in garlic cloves soaked in monocarboxylic acids. This led Bai et al. (2006) to hypothesize that monocarboxylic acids, specifically acetic acid which is widely used in food preservation and pickling, "increase the permeability of the

plasma membrane of garlic". To test this, the relative conductivity of the pickling solutions was measured; acetic acid was found to be at 90% conductivity with citric and malic at 60% conductivity. These results indicate higher levels of total dissolved solids in the acetic acid pickling solution. From this, the author hypothesized acetic acid could disrupt the cell organelles. To determine if organelle disruption was occurring, light micrograph pictures were taken of garlic tissue without treatment and garlic tissue soaked in acetic, malic, and citric acids (Figure 8). Tissue samples soaked in malic and citric acids showed little change and were similar to control, while the acetic acid sample showed distinct change and obvious organelle damage. Upon examining the organelle damage under a transmission electron micrograph (Figure 9), it became evident the garlic cells "underwent both morphological and microstructural changes" (Bai et al., 2006). Additional confirmation of garlic cell membrane disruption caused by treatment with acetic acid became apparent when the authors tested the thiosulfinate concentration of the pickling solutions containing acetic, citric, and malic acids. Test results show the thiosulfinate concentration in the acetic acid pickling solution contained 1.3 mg/mL, while malic and citric acid pickling solutions contained almost none (Bai et al., 2006).

From this research, Bai et al. (2006) inferred that whole garlic cloves soaked in an acetic acid pickling solution increases the permeability of the tonoplast, the membrane surrounding the vacuole. This allows the substrate cysteine sulfoxides, found in the cytosol, to move freely through the vacuolar membrane into the vacuole to mix with alliinase, forming thiosulfinates and eventually blue-green pigments (Bai et al., 2006).

From the conclusion that acetic acid breaks down the organelles inside garlic and malic and citric cannot, and the fact that malic and citric acids are naturally found in the large central vacuole, the authors hypothesized plant cells may have developed a way to transport or regulate malic and citric acid in a way that prevents them from damaging the vacuolar membrane. Conversely, the plant seemingly does not have a regulation system for mono-carboxylic acids which may be why acetic acid causes such damage to the organelles of garlic cells (Bai et al., 2006).

Figure 5: Acetic acid skeletal structure (mono-carboxylic acid)
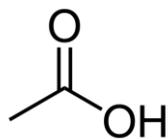
Figure 6: Malic acid skeletal structure (di-carboxylic acid)
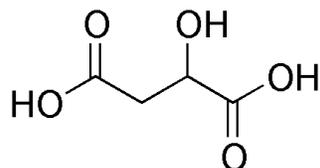
Figure 7: Citric acid skeletal structure (tri-carboxylic acid)
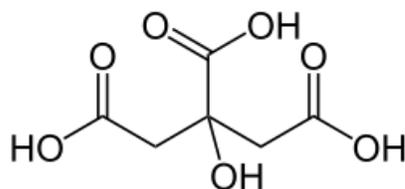
Figure 8: Light micrographs of 10-µm sections of garlic without treatment (a) and of garlic treated with 5% acetic acid (b), citric (c), and malic (d) acids. Samples are stained with safranin and fast green (Bai et al., 2006).

a

b

c

d
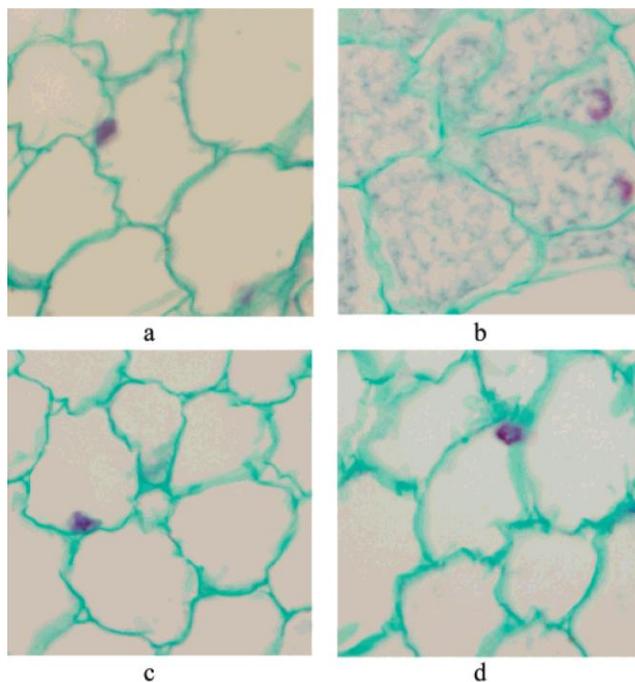
Figure 9: Transmission electron micrographs of sections of garlic without treatment (a) and of garlic treated with 5% acetic (b), citric (c), and malic (d) acids (Bai et al., 2006).
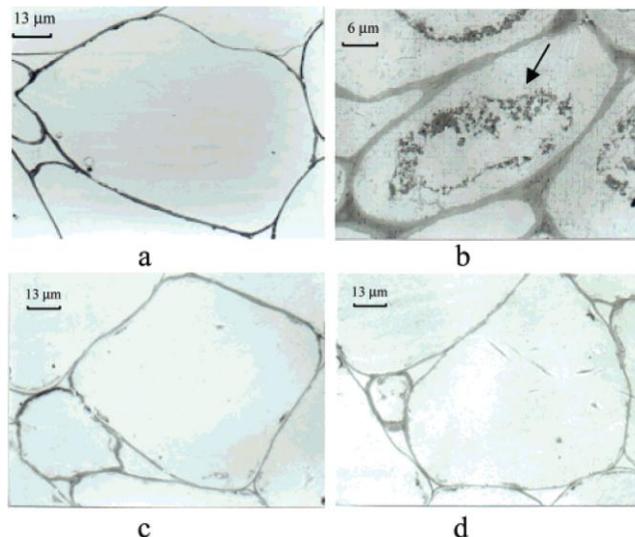
Amino Acids

It has been determined that alliin and isoalliin, reacting with alliinase to produce allicin, which reacts with amino acids is necessary for the formation of blue-green pigments in garlic cloves (Joslyn & Sano, 1956; Lukes, 1986; Stoll & Seebeck, 1951). Fresh garlic by weight contains approximately 1.3% amino acids (Koch & Lawson, 1996). Cho et al. (2009) identified optimum concentrations of cysteine sulfoxides needed to react with specific amino acids in order for blue-green pigments to form. Experiments conducted used inverse concentrations of alliin and isoalliin ranging from 0-100%. For example, one variable contained 40% alliin and 60% isoalliin while another contained 60% alliin and 40% isoalliin. Pigments ranging from pink to blue formed only in variables containing both alliin and isoalliin. The optimum ratio of alliin and isoalliin needed for generating blue pigment was found to be 70% and 30%, respectively.

All 22 amino acids were reacted with varying concentrations of the alliin and isoalliin mixture with alliinase. Pigments formed ranged from violet to blue to green when different amino acids reacted with the formed thiosulfinate mixture (Figure 10). While all 22 amino acids formed pigments, lysine, asparagine, and most notably glycine were most able to generate blue pigments (Cho et al., 2009). This supports other research which conclude glycine is not required for pigment formation but it "significantly enhanced the intensity of the color formed" (Kubec at al., 2004). Interestingly, the most predominant amino acids found in garlic are arginine,

glutamine, asparagine, (Lee et al., 2005 as cited in Cho et al., 2009). However, glycine, which showed the most potential to generate pigments, is found at very low levels in garlic cloves and in some varieties of garlic glycine is undetectable (Lee & Harnly, 2005 as cited in Cho et al., 2009).

Figure 10: Color variation by reaction with thiosulfinate and different amino acids. Each number corresponds to a different amino acid in Table 1 (Cho et al., 2009).

Table 1: List of amino acids used in the reaction with thiosulfinate solution and abbreviations (Cho et al., 2009).

Order	Amino Acid	Abbreviation
1	cysteine	Cys
2	phenylalanine	Phe
3	glycine	Gly
4	methionine	Met
5	arginine	Arg
6	valine	Val
7	isoleucine	Ile
8	proline	Pro
9	lysine	Lys
10	serine	Ser
11	tryptophan	Trp
12	hydroxyl proline	h-Pro
13	alanine	Ala
14	aspartic acid	Asp
15	histidine	His
16	threonine	Thr
17	leucine	Leu
18	asparagine	Asn
19	glutamine	Gln
20	cystine	Cyt
21	glutamic acid	Glu
22	tyrosine	Tyr

A study carried out by Kubec and Velisek (2007) explored what effect the pH of garlic clove homogenates may have on pigment formation. Experiments entailed introducing thiosulfinates into amino acid solutions between the pH of 2.0 and 9.0. Results showed pH significantly effects pigment formation, intensity of the color produced, and shade of color produced. Among all pH levels, pigment formation was generally greatest at around pH 5.5 which is peculiarly close to the natural pH range of garlic cloves which is $6.1 - 6.3$. All amino acids tested were able to form pigments except cysteine and proline. Kubec and Velisek (2007) obtained similar results to Cho et al. (2009) when they determined alanine, glycine, and glutamic acid were the amino acids which most readily formed pigments (Kubek & Velisek, 2007). pH is an influential factor governing garlic greening. Non-enzymatic reactions most readily occur at low pH (2.0-3.0) while enzymatic reactions most readily occur at higher pH (6.0 or above). Thus, for the entire reaction of pigment formation to occur, a pH in between 4.0-5.0 is ideal (Bai et al., 2005; Joslyn & Sano, 1956; Kubec et al., 2004).

Factors Not Affecting Pigment Formation

Copper and other Metals

In non-scientific literature, pigment formation has been popularly attributed to the sulfur containing compounds of garlic and their tendency to bind with trace metals or copper ions to form blue-green pigments or copper sulfate (Kubec et al., 2004; Kubec & Velisek, 2007). Trace metals have an approximate concentration of 0.12% in garlic pigments. From this, it can be assumed that the blue-green pigments formed are not metal complexes and the trace metals are impurities (Kubec et al., 2004). However, to further disprove the notion that metals impact pigment formation, Kubec et al. (2004) mixed three of the more common metal sulfates, iron sulfate, copper sulfate, and zinc sulfate, with a pigment precursor solution. Results showed no significant difference in the rate or intensity of pigment formation between solutions with and without added metal sulfates. Based on these results, it can be concluded that metals are not involved with pigment formation (Kubec et al., 2004).

Pesticides

For some time, those familiar with blue-green pigment formation attributed the cause to pesticides used during the growth of the plant. However, there is no research to support the theory that pesticides are the cause or are involved with pigment formation in garlic cloves (Lee et al., 2006b).

Plant Defense Mechanism

Based on the previous information, it is obvious that an elaborate series of chemical reactions take place when garlic tissue is crushed or damaged. It has already been mentioned that allicin is a thiosulfinate, an organic sulfur containing compound. Thiols are another class of organosulfur compounds; they are one of the major products of microbial degradation or the putrefaction process, which is often followed by the creation of harmful toxins. Knowing this, it may not be unrealistic to conclude that "primates should be highly sensitive to such compounds in order to avoid intoxication" (Laska, 2007 as cited in Block, 2010). Furthermore, as is common with some spicy foods, when raw garlic is consumed, a burning or tingling sensation in the mouth may follow. The perceived burning is what takes place after pain receptors in the mouth are triggered by allicin, "an electrophilic defense compound" (Block, Cody, Dane, Sheridan, Vattekkatte, & Wang, 2010) similar in nature to that of other irritants such as car exhaust and tear gas (Block, 2010).

Koch and Lawson (1996) suggest that nature may have generated an elaborate system for garlic to produce and store sulfur compounds, along with alliinase, in order to rapidly generate allicin upon disruption or infestation of the plant. There are also several instances of prior research hypothesizing that this chemical reaction is a primeval defense mechanism to fend off attack from pests, bacteria, and fungus (Block, 2010; Block et al., 2010; Fujisawa, Suma, Origuchi, Kumagai, Seki, & Ariga, 2008; Koch & Lawson, 1996; Kuettner et al., 2002). Conclusive proof of this theory does not exist, however, it is interesting that insects such as ticks and mosquitoes are readily deterred by garlic (Macpherson, 2005 as cited in Block, 2010).

Chapter 3 - Preventing Pigment Formation

Challenges of Pigment Formation

Undesirable color change of food products during their processing and shelf life is a major challenge in the food industry (Aguilar & Rincon, 2007). When blue-green pigments are formed in garlic cloves, significant financial losses in products containing affected cloves may be realized due to a decrease in organoleptic quality (Kubec & Velisek, 2007). Often times, affected cloves are unsalable (Lukes, 1986). However, consumption of garlic cloves containing blue-green pigments at low concentrations poses no food safety risk (Block, 2010).

Changes in Garlic Consumption

Throughout most of history, garlic has typically been stored, shipped, and sold in its whole bulb form, after which, the bulb is broken down and individual cloves are peeled, prepared, and consumed. Today's garlic is processed in numerous forms, including whole, purees, pickled, juice, powder, and oleoresin (Lee et al., 2006b). Over the past several decades, the continued increase in consumer demand for fresh, ready-to-eat vegetables has led to a variety of "minimally processed, pre-peeled and chopped garlic products" (Cho et al., 2009; Hong & Kim, 2001; Lee et al., 2006b). From this, it is reasonable to infer that during the peeling or processing of ready-to-eat garlic products, the bruising or damaging of garlic tissue can result thus leading to an increased occurrence of garlic-greening.

Prevention through Plant Growth, Harvest, and Storage

Research carried out by Joslyn and Sano (1956) determined garlic planted in the fall and harvested in late spring or early summer did not form green pigments nearly as readily as garlic planted in the spring and harvested in late summer. The phenomenon of early garlic having increased propensity to cause garlic greening than late garlic was also observed by Lukes (1986). Selecting early summer garlic may be one of several ways to reduce the propensity of green pigment formation in garlic cloves. This observation made by Joslyn and Sano (1956) may be explained by research which shows a variation in the concentrations of cysteine sulfoxides in different parts of the plant leading up to harvest.

Cysteine Sulfoxide Concentration in Garlic at Harvest

Analytical tests to determine the amount of cysteine sulfoxides distributed in garlics roots, bulbs, leaves and stem in the weeks leading up to the plants full maturity and harvest are very interesting. Results show that alliin and isoalliin concentrations in garlic bulbs increase by approximately 250% and 40%, respectively up to seven weeks before the plant is ready for harvest. During the same seven week time-span, results show alliin and isoalliin concentrations in the above ground leaves and stem decrease by approximately 85% and 63%, respectively (Koch & Lawson, 1996). These results indicate an apparent migration of cysteine sulfoxides from the above-ground plant to the below-ground bulbs in the weeks leading up to full plant maturity. Seven weeks before full maturity, garlic bulbs contain approximately 36% of the total plant cysteine sulfoxides. However, by full maturity at harvest time "90% of the total plant cysteine sulfoxides are found in the bulb", an increase of 150% (Lawson & Wang, 1994 as cited in Koch & Lawson, 1996). To further reduce the potential for garlic greening, harvesting garlic seven weeks premature seems to limit the total amount of cysteine sulfoxides found in the bulb. It is reasonable to conclude if garlic bulbs contain 150% less substrate cysteine sulfoxides, greening intensity or incidence may be significantly reduced.

Effect of Storage Conditions on Cysteine Sulfoxide Concentrations in Garlic

As discussed in chapter 1, after garlic is harvested it must go through a curing process for several weeks so that its total moisture content is reduced. During the preparation of garlic for commercial use, long term storage of garlic bulbs may be required. Commercial garlic stored for extended periods of time is kept at refrigeration to sub-refrigeration temperatures to prevent the sprouting of cloves (Block, 2010). There have been several conclusive studies examining what effect certain storage conditions has on garlic cysteine sulfoxide concentrations and the occurrence of blue-green pigment formation.

Research conducted by Lukes (1986) confirmed garlic greening is directly affected by the temperature at which garlic is stored. Incidence of garlic greening is increased when garlic is stored at or below 12°C but does not occur when stored at or above 23°C. Lukes (1986) was able to eliminate and produce green pigments in garlic cloves by transferring samples to and from warm and cold storage conditions several times. Each time garlic was transferred, the rate of color change took longer to complete. Upon analyzing the differences in the greening and

non-greening garlic, Lukes (1986) found significant differences in their isoalliin concentrations, 1.62 mg/g garlic and 0.2 mg/g garlic, respectively. Thus, isoalliin content of garlic in cold storage increases while isoalliin content of garlic stored at room temperature decreases (Lukes, 1986). Lukes (1986) was able to prevent green pigment formation by storing garlic at or above 23°C at least 32 days before processing.

Yamazaki, Yamamoto, and Okuno (2012) built upon research first observed by Lukes (1986) when they found a significant correlation between the isoalliin content of garlic bulbs stored at 3°C and the degree of green discoloration in garlic. Their research shows what seems to be a synergistic relationship between certain compounds in garlic upon being stored at low temperatures. It was determined that activity of the enzyme gamma-glutamyl transpeptidase is increased when garlic is stored at around 3°C. This enzyme is responsible for catalyzing a reaction which synthesizes isoalliin from γ-Glutamyl-*S-trans*-1-propenylcysteine (Glu-PEC). As gamma-glutamyl transpeptidase activity increases, metabolism rate of isoalliin from Glu-PEC increases and is highly correlated to degree of discoloration (Figure 11). Enzyme activity was subsequently slowed once garlic was stored at or above 35°C. Garlic cloves which showed greening in cold storage reportedly "reverted to their original cream color during warm storage, with an accompanying decrease in isoalliin" (Yamazaki et al., 2012).

Secondly, Yamazaki et al. (2012) showed no change in isoalliin content of garlic bulbs when stored at 25°C for 4.5 months. However, there was shown to be a 1225% increase in isoalliin per 100 g garlic on a dry weight basis when this garlic was stored at 3°C for 4.5 months. Additionally, isoalliin concentrations in garlic initially stored at 25°C for three months increased upon being transferred to storage at 3°C. A similar effect was seen when garlic bulbs were stored at 3°C for three months and then transferred to storage conditions ranging from 25°C - 40°C for two weeks, the occurrence of greening diminished as temperature and storage duration increased. It has been postulated that while alliin and isoalliin are both required for garlic greening, the severity of greening is dependent mainly on isoalliin because it is found in garlic at significantly lesser quantities than alliin (Yamazaki et al., 2012).

Figure 11: Effects of cold storage of garlic bulbs on processed puree color (A), and clove isoalliin (B) and Glu-PEC (C) contents (Yamazaki et al., 2012).
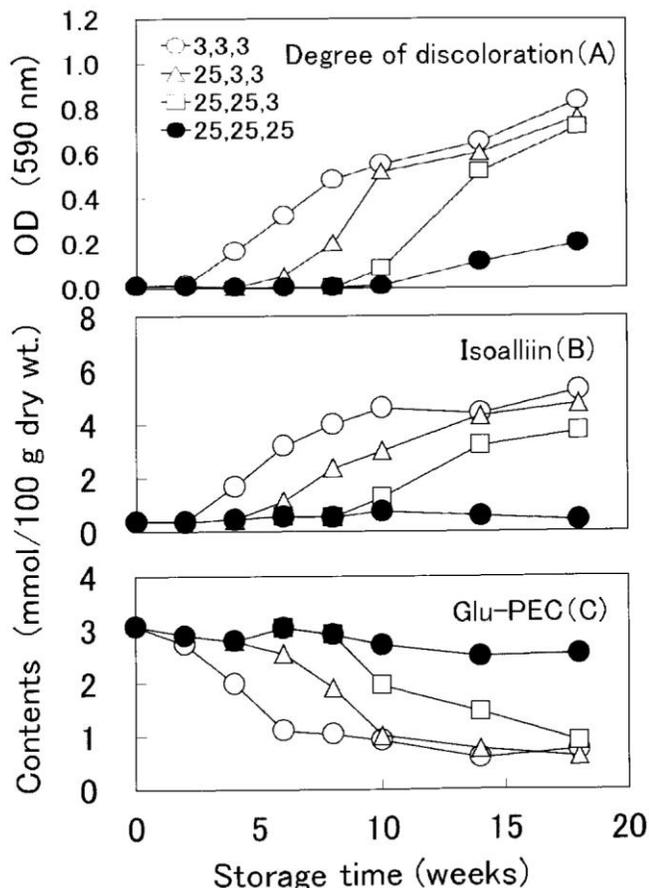
Additional research conducted by Lee et al. (2006b) showed similar findings to Lukes (1986) in that garlic stored at 0 and 10°C for 10 and 6 months, respectively developed blue-green pigments. Garlic stored at 20°C did not develop pigments. However, the sprouting of garlic cloves only occurred when garlic was stored at 10°C and 20°C. Lee et al. (2006b) concluded from these observations that sprouting does not prompt pigment formation. While greening was only observed in garlic stored at 0°C and 10°C, the total cysteine sulfoxide content of garlic stored at all temperatures increased (Lee et al., 2006b).

Research by Lee et al. (2006b), Lukes (1986), and Yamazaki et al. (2012) showed an apparent correlation between the refrigerated storage of garlic and an increase in isoalliin content of garlic, which ultimately increases the formation of blue-green pigments. When the garlic bulb senses low temperatures, as is the case during planting in autumn, sprouting occurs and coincides

22

with an increase in isoalliin synthesis (Yamazaki et al., 2012). As discussed in chapter 2, this could be an example of the plants natural defense mechanism in action.

Inactivation of Alliinase

The enzyme alliinase is the catalyst which converts alliin into allicin, a thiosulfinate which reacts with amino acids to form blue-green pigments in garlic cloves. Nearly all prior researchers attempting to elucidate the reaction that takes place leading up to and during blue-green pigment formation consider alliinase essential for the reaction to occur (Aguilar & Rincon, 2007; Bai et al., 2005; Block et al., 2010; Imai et al., 2006; Joslyn & Sano, 1956; Kubec & Velisek, 2007). Aside from harvesting premature garlic and controlling the postharvest storage conditions of garlic bulbs, the inactivation of alliinase is fundamental in preventing blue-green pigment formation in garlic cloves (Rejano et al., 1997).   Stoll and Seebeck (1951) determined the optimum temperature of alliinase to be 37°C; they were able to show its activity decline rapidly above this temperature with its inactivation beginning at temperatures exceeding 50°C (Stoll & Seebeck, 1951).

Blanching

Rejano et al. (1997) explored what effect blanching garlic may have on the ability of pigments to form in pickled garlic cloves. Garlic was blanched in a 90°C water bath for 15 minutes. Blanched cloves were packed in two sets of glass jars with standard pickling brine; one set was pasteurized for 5.5 minutes at 90°C while the other set was unpasteurized. Both sets of jars were stored for 4 months at 27°C and neither set displayed pigment formation. In an effort to understand the specific blanch temperature that will eliminate pigment formation, Rejano, de Castro, Sanchez, Casado, and Montano (2004) blanched garlic slices from 60°C through 80°C at 5° increments for 15 minutes. Temperatures below 75°C were not adequate in completely eliminating pigment formation. However, no blue-green pigment formation was detected when a blanching treatment at 80°C for 15 minutes was implemented which is the recommended blanching treatment to prevent greening in garlic slices (Rejano et al., 2004).

pH

Research shows alliinase activity is unaffected in weak acidic and weak alkaline conditions. A pH less than 5.0 and greater than 8.0 will begin to reduce alliinase activity (Stoll & Seebeck, 1951). Alliinase activity should rapidly decrease under acidic conditions with a pH of 3.5 or less (Koch & Lawson, 1996) and should be irreversibly inactivated at pH of less than 3.0 (Aguilar & Rincon, 2007). This will prevent the production of allicin and thus the formation of blue-green pigments. Additionally, if initial pH is 3.0 or less, neutralization of this pH has been shown to have no impact on the restoration of alliinase activity (Koch & Lawson, 1996).

Pickling

Research conducted by Rejano et al. (1997) showed the ability for blue-green pigments to form in pickled garlic. Garlic was packed in two sets of glass jars in pickling brine containing a mix of lactic acid and acetic acid which provided a final equilibrium pH of 4.0. A non-pasteurized set of jars and a pasteurized set of jars, heated to 90°C for 8 minutes, were stored at room temperature while observations were recorded. Green pigments formed in the pasteurized jars only a few hours after pasteurization while greening became clear in the non-pasteurized jars after two to three days of storage. Blue-green pigments were most intense after nine days and were strongest in the non-pasteurized jar (Rejano et al., 1997).

A separate study investigating allicin content of pickled garlic aging in a 2% acetic acid solution over 60 days showed marked results. There was a 70% decrease in allicin content over the first 10 days and allicin was undetectable at day 60. Using sensory test results over the 60 day study, researchers were able to conclude a direct correlation between reduced allicin content and diminished garlic pungency (Kim, Yun, & Sok, 1994).

Freezing

During the freezing and thawing processes, enzymatic activity can usually be reduced or permanently lost (Deutscher, 1990 as cited in Wäefler, Shaw, & Lancaster, 1994). Research shows freezing alliinase contributes to some extent in its complete inactivation. Work carried out by Wäfler et al. (1994) demonstrated a rapid freeze cycle of a homogenate containing alliinase did not change its activity. However, the slow freezing and thawing of onion tissue containing alliinase resulted in a total loss of its activity. From this, the hypothesis was formed that during slow freezing or thawing, ice crystal formation ruptures cell organelles and allows for

proteolytic destruction of alliinase normally sequestered in vacuoles. In testing this hypothesis, onion homogenate was made without the use of protease inhibitors and a reduction in alliinase activity was insignificant. Thus, proteases do not have an effect in inhibiting alliinase activity. Results proved to be inconclusive and the authors could not fully attribute the total inactivation of alliinase to the slow freeze-thaw process alone but rather other unknown chemical or physical dynamics that occur during freezing and disruption of cell organelles (Wäefler et al., 1994).

Additives

Hydroxylamine

As previously discussed, alliinase is a pyridoxal phosphate-dependent enzyme and its activity may be reduced with a pyridoxal phosphate antagonist such as Hydroxylamine. As increasing concentrations of hydroxylamine were added to garlic homogenates (Figure 12), alliinase activity was completely inactivated and green pigment formation did not occur (Lee et al., 2006b). The use of Hydroxylamine as an alliinase inhibitor is further confirmed through research conducted by Bai et al. (2005) when the authors demonstrated a decrease in UV-vis absorption at 590nm which corresponded with increasing concentrations of hydroxylamine. However, hydroxylamine may not be used in food products since it is not listed on the FDA GRAS list under CFR 21 Part 184.

Figure 12: Effects of hydroxylamine on GCI and alliinase activity in garlic homogenates (Lee et al., 2006b).
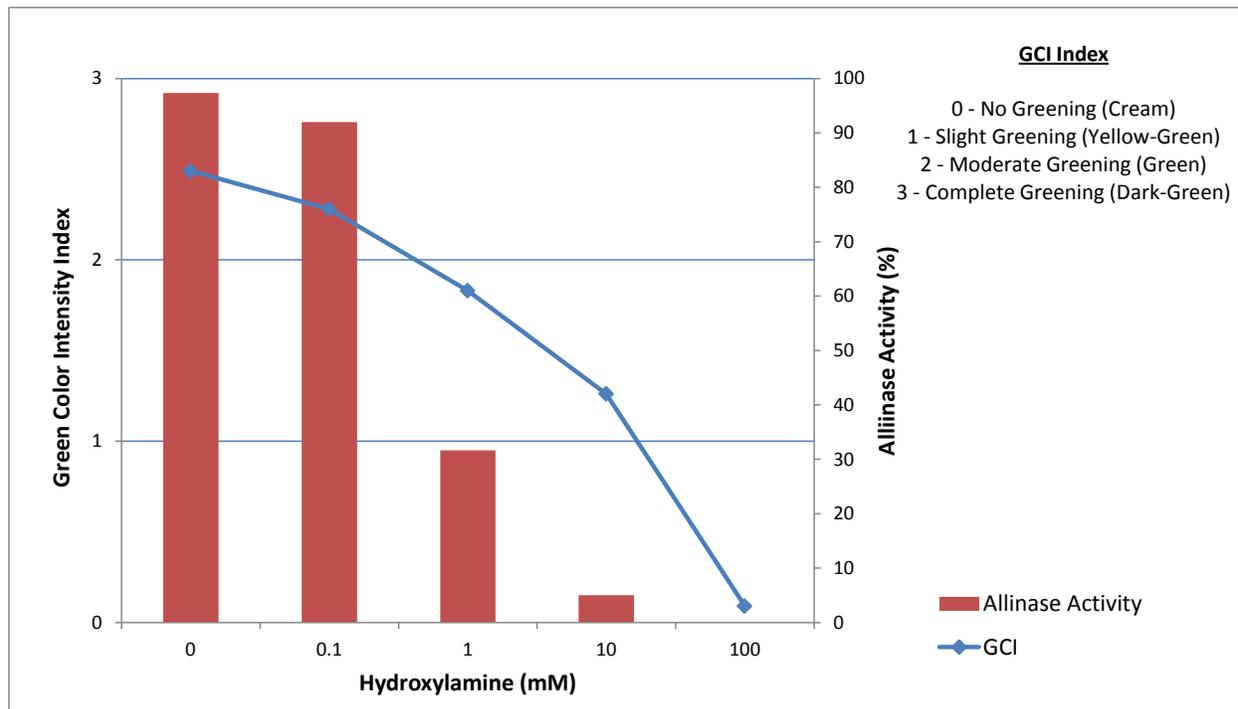
Alcohols

In general, alcohols are known to denature proteins and have had some success in inhibiting alliinase. It has been determined after using a combination of methanol and ethanol at 40%, alliinase was still very active. Concentrating these alcohols up to 99% causes a precipitous decrease in alliinase activity, however, approximately 1% alliinase still remains active based on a very small amount of allicin produced (Lawson & Wang, 1994 as cited in Koch & Lawson, 1996). Research conducted by Bai et al. (2005) showed ethanol as able to decrease alliinase activity at concentrations from 5% to 80% (v/v) by demonstrating a decrease in UV-vis absorption at 590 nm.

Cysteine

As described in chapter 2, alliin will only react with compounds which contain a free –SH group (Stoll & Seebeck, 1951). Since cysteine contains a free –SH group, it will react with alliin thus reducing the amount of available alliin able to be converted into allicin by alliinase (Aguilar & Rincon, 2007). It is for this reason why adding the amino acid cysteine to garlic homogenates may play a role in preventing blue-green pigment formation. The average free

cysteine content in garlic cloves is somewhat variable and ranges from approximately 50-400 mg/100 g garlic (Lee & Harnly, 2005). It has been demonstrated that adding approximately 1% cysteine to garlic paste prevented pigment formation. However, levels of cysteine exceeding 1% concentration seemed to have a "negative effect on greening prevention" (Aguilar & Rincon, 2007). Research also shows a decrease in UV-vis absorption at 590 nm with increasing concentrations of L-cysteine (Bai et al., 2005). The specific amount of cysteine that should be used to prevent garlic greening will be dependent on garlic variety, growing conditions, post-harvest storage conditions, and processing conditions since the concentrations of all compounds involved in the pigment formation reaction can be impacted by the aforementioned variables (Aguilar & Rincon, 2007).

Ineffective Preventative Methods

High Hydrostatic Pressure

High hydrostatic pressure treatment has been shown to affect enzyme activity in food products by "altering intermolecular structures" (Hoover D.G. et al., 1989 and Barbosa-Canovas et al., 1998 as cited in Hong & Kim, 2001). Its use can also lead to the activation or inactivation of enzymes. Activity of polyphenol oxidase in pears increased by as much as five times upon being subjected to a high hydrostatic pressure treatment (Asaka & Hayashi, 1991 as cited in Hong & Kim, 2001). However, polyphenol oxidase activity in apples and avocados decreased gradually with increasing hydrostatic pressure (Weemaes et al., 1998 as cited in Hong & Kim, 2001). The objective of a recent study was to determine what effect a high hydrostatic pressure treatment would have on inactivation of alliinase and thus prevention of blue-green pigment formation in garlic cloves. Results show high hydrostatic pressure treatment did not have an impact on the ability for blue-green pigments to form in garlic cloves (Hong & Kim, 2001).

Factors to Consider when Preventing Pigment Formation

Organoleptic Changes to Garlic upon Blanching

The blanching of garlic cloves has been shown to inactivate the enzyme responsible for converting alliin to allicin (Rejano et al., 2004; Rejano et al., 1997) which reacts with amino acids to form blue-green pigments (Imai et al., 2006). As previously stated, allicin is one of the

main thiosulfinates in garlic responsible for its pungent flavor and odor (Brodnitz et al., 1971; Rejano et al., 1997). Upon inactivating alliinase, a loss of pungency in garlic may result due to a lack of allicin production (Kim et al., 1994). Blanched garlic may also exhibit a color that is "less white than raw garlic" (Rejano et al., 1997).

Blanching garlic cloves in a hot water bath for extended periods of time may lead to reduced firmness and loss of nutrients (Fante & Zapata, 2012; Rejano et al., 2004; Rejano et al., 1997). As blanch time and temperature increase, garlic cloves begin to soften and garlic pungency is diminished (Rejano, Sanchez, Casado, Montano, & de Castro, 2007; Rejano et al., 2004). A combination of steam and hot water blanching may help garlic maintain its texture while still inactivating enzymes (Fante et al., 2012).

Diminished garlic pungency and turgor upon blanching or pickling garlic cloves should be considered when food manufacturers incorporate garlic cloves into food products with the expectation of the cloves providing characteristic garlic flavor. Optimal blanching parameters should be determined for specific applications.

Chapter 4 - Experiments & Data

Effect of Blanching on "Laba" Garlic in 5% Acetic Acid Pickling Solution

Introduction

Bai et al. (2005) and Bai et al. (2006) demonstrated the ability for blue-green pigments to form in intact garlic cloves soaked in a 5% acetic acid pickling solution. The ability of acetic acid to increase the permeability of the vacuolar membrane and allow alliin to diffuse into the vacuole and react with alliinase to form allicin is the main mechanism for pigment formation in intact garlic cloves (Bai et al., 2006). Rejano et al. (1997) showed an ability to inactivate alliinase via the blanching of garlic cloves at 90°C for 5.5 minutes. The objective of this experiment was to determine if blanching garlic cloves at 100°C for 5 minutes inactivates alliinase prior to soaking in a 5% acetic acid pickling solution, thus preventing blue-green pigment formation. A short blanch time of 5 minutes at a high blanch temperature of 100°C was chosen to thoroughly inactivate alliinase. A non-blanched sample of garlic cloves soaked in 5% acetic acid pickling solution represents control while a blanched sample of garlic cloves soaked

in 5% acetic acid pickling solution represents test. Bai et al. (2006) demonstrated the ability to detect high concentrations of thiosulfinates diffused from garlic cloves in acetic acid pickling solutions. If blanching garlic cloves inactivates alliinase, then allicin would not be produced. To determine if allinase was inactivated, color change will be monitored over a seven day period and equilibrated pickling solution will be tested for allicin content.

Materials & Methods

Equipment

A stainless steel kitchen pot was used to boil water and blanch garlic cloves. A 710 ml glass pickling jar was used to store pickled garlic cloves and 5% acetic acid solution. A calibrated Fisher Scientific (300 Industry Drive, Pittsburgh, PA, 15275) Accumet AB15 pH meter was used to measure pH of equilibrated homogenized garlic clove and acetic acid solution. A 118 ml polypropylene Tyco specimen cup (VWR catalog number 13915-772) was used to submit garlic for allicin quantitation. A "Magic Bullet" food processor with "short cup" was used to chop and homogenize equal amounts of garlic cloves and pickling solution before testing pH. A calibrated AcuRite (965 Wells Street, Lake Geneva, WI, 53147) stainless steel instant read thermometer was used to determine the temperature of the water used for blanching.

Ingredients

Garlic cloves used were grown in California and purchased from a Whole Foods Market, 222 Main Street, Madison, NJ 07940. Distilled white vinegar with 5% acetic acid concentration was used and purchased from a local grocery store. Distilled water was purchased from CVS Pharmacy, 410 Rt. 10, Whippany, NJ 07936.

Sample & Equipment Preparation

Garlic cloves were stored at 4°C for 20 days in their bulb form. Once 20 days elapsed, they were removed from refrigeration and hand peeled to avoid bruising or damage and double rinsed with distilled water. Glass jars, caps and kitchen pot were machine washed and then double rinsed with distilled water.

Ingredient Storage

Based on research conducted by Lukes (1986), storage at 5°C should increase alliin and isoalliin concentrations in whole garlic cloves which should increase the tendency for blue-green pigments to form in garlic cloves. For the sake of this experiment, it was necessary to increase the propensity for pigment formation in garlic cloves in order to determine if blanching prevents its occurrence. Consequently, garlic cloves were stored at 5°C for 20 days prior to blanching and being added to pickling solution.

Allicin Quantitation

Quantitation of allicin was carried out by Eurofins Scientific of Petaluma, California. The method was adapted from USP, Garlic Monograph; and INA Method 110.001, *Allicin by High-Performance Liquid Chromatography* which uses reversed phase high-performance liquid chromatography separation with UV detection. The method is applicable for the analysis of allicin content in raw material and finished products. Quantitation of allicin was performed by comparison to an external reference material of a known concentration. An aliquot of sample was accurately weighed and diluted with refrigerated Milli-Q water. The sample solution was then sonicated and filtered through polytetrafluoroethylene into an amber auto-sampler vial and analyzed under an isocratic instrument condition, with menthol, water and phenomenex. A Prodigy 5u ODS3 (4.6 x 250 mm, 5.0 micron) column from Phenomenex (411 Madrid Ave, Torrance, CA, 90501) was used in separation at 240 nm UV. All samples were run in duplicate.

Procedure

Peeled, whole, non-blanched garlic cloves were accurately weighed to 150 grams and added to a 710 ml glass pickling jar. One hundred and fifty grams of distilled white vinegar (5% acetic acid content) were poured over the non-blanched garlic cloves and the jar was then hand capped and left to sit on a lab bench under ambient conditions.

Approximately six cups of distilled water was poured into kitchen pot, put on stove and set to high flame to reach target blanch temperature of 100°C. One hundred and fifty grams of whole peeled garlic cloves were weighed and added to water after temperature reached 100°C. Garlic was blanched for five minutes, then cloves were removed from blanch water and added directly to a bowl of ice water to quickly cool for 5 minutes. After five minutes, blanched cloves were added to a glass pickling jar. One hundred and fifty grams of distilled white vinegar (5%

acetic acid content) was poured over the blanched garlic. Jars were hand capped and allowed to sit on a bench top for seven days. Observed changes in both jars were recorded on a daily basis for seven days.

Final equilibrated pH of blanched and non-blanched garlic cloves soaked in 5% acetic acid pickling solution for seven days was measured after homogenizing equal parts of soaked garlic cloves with pickling solution.

Results & Discussion

Observations

Upon adding blanched and non-blanched cloves to the jars, the blanched cloves sank to the bottom while the majority of the non-blanched cloves floated near the surface of the vinegar. On day 1 (24 hours after adding cloves to vinegar), the blanched cloves showed no signs of pigment formation while almost every non-blanched clove showed a small amount blue-green pigment development on the side of the clove that would have been connected to the basal plate of the bulb. Upon opening both jars, the jar containing non-blanched cloves smelled like pungent garlic and vinegar while the blanched cloves only smelled like vinegar. On day 2, pigment formation in non-blanched cloves could be seen in all cloves. Blue-green pigments nearly fully enveloped smaller cloves while pigments in larger cloves covered approximately 25%-50% of the clove migrating upwards from the bottom end. Smaller non-blanched cloves were completely enveloped by pigments by day 3 while larger cloves were approximately 50% covered. Blanched cloves exhibited no blue-green pigment formation, however, a light yellow to tan color was observed in the cross section of the cloves. On day four, the pickling solution used to soak the blanched cloves first exhibited a very slight yellow hue and the product smelled like pungent vinegar with a slight mild garlic aroma, nothing like the pungent fresh garlic aroma apparent in the non-blanched cloves. Blanched cloves still displayed no greening, while the pigments now covered more than half of the flesh in the non-blanched cloves with the blue-pigments in the vinegar now moderately apparent. On day 5, the majority of the non-blanched cloves began to sink and the pigments now enveloped approximately 75% of the larger cloves. Blue-green pigments leaching into the brine became apparent. For the blanched cloves, vinegar now exhibited an apparent light yellow hue similar to the color observed in the cross section of the garlic clove, no greening was apparent. On day 6, no noticeable changes occurred in the

31

blanched cloves while the larger non-blanched cloves were approximately 90% enveloped with blue-green pigments. By day 7, blue-green pigments had completely enveloped non-blanched cloves (Figures 14 & 16) and its pickling solution (Figure 17) displayed a noticeable blue-green color. No blue-green pigments formed in the blanched cloves (Figures 13 & 15), and its pickling solution (Figure 17) displayed a noticeable light yellow color.

From a texture standpoint, the non-blanched cloves were firm and crunchy as compared to the blanched cloves which were perceived to be softer upon cutting to examine cross-section. The softening of blanched cloves is a known phenomenon previously observed by Rejano et al. (2007).

Cross sections of blanched garlic cloves at the start were cream colored, characteristic of fresh garlic, and turned light yellow to tan in color as time elapsed, consistent with observations made by Rejano et al. (1997).

Figure 13: Cross-section of blanched garlic cloves soaked in 5% acetic acid pickling solution over seven day period

Figure 14: Cross-section of non-blanched garlic cloves soaked in 5% acetic acid pickling solution of seven day period
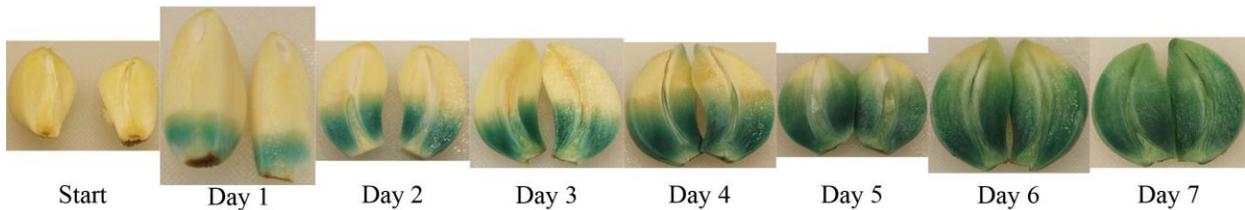
Figure 15: Jars containing blanched garlic cloves soaked in 5% acetic acid pickling solution over seven day period
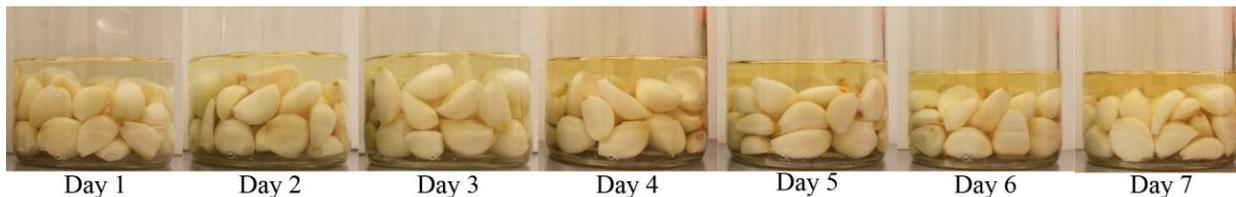
Figure 16: Jars containing non-blanched garlic cloves soaked in 5% acetic acid pickling solution over seven day period
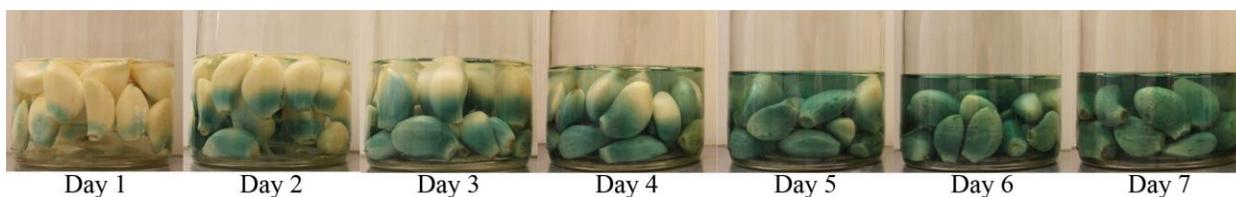
Figure 17: Blanched and non-blanched equilibrated 5% acetic acid pickling solutions after seven day period
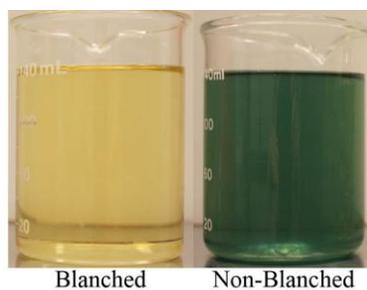
Data

Table 2: pH and allicin content from equilibrated blanched and non-blanched garlic cloves soaked in 5% acetic acid pickling solution

	Equilibrated pH	Allicin Content of Pickling Solution %(w/w)
Blanched Cloves	3.72	<0.0001
Non-Blanched Cloves	3.62	0.0327

Discussion & Conclusion

Blue-green pigments leaching from the non-blanched cloves into the vinegar occurred on day 3 and seemed to coincide with the pigments completely enveloping the smaller cloves. When the cap was removed from the jar of non-blanched garlic cloves, a pungent garlic smell was apparent and seemed to increase as soak time elapsed. Since allicin is the main sulfur compound contributing to garlics characteristic flavor and aroma (Brodnitz et al., 1971; Rejano et al., 1997), the increase in pungent garlic odor over the seven day period can most likely be attributed to increasing amounts of allicin or other thiosulfinates diffusing from the garlic cloves into the vinegar pickling solution. The diffusion of allicin from the garlic clove into the pickling solution became clear upon testing the acetic acid pickling solution itself for allicin content (Table 2). While the pickling solution containing the blanched garlic cloves contained negligible amounts of allicin, the non-blanched pickling solution contained 0.0327%(w/w) of allicin, a clear indication diffusion of allicin from the garlic has taken place.

With the lack of pigment formation in the blanched garlic cloves, and the absence of diffused allicin in the blanched clove pickling solution, blanching garlic cloves at 100°C for 5 minutes is an effective method of alliinase inactivation thus preventing blue-green pigment formation. Since the equilibrated pH of garlic cloves was above 3.0, this experiment does not show if low pH pickling solutions inactivate allinase as described by Aguilar and Rincon (2007).

Effect of low pH Acetic Acid Pickling Solutions on Pigment Formation in "Laba" Garlic

Introduction

As shown above, and demonstrated by Bai et al. (2005), blue-green pigments are able to form in intact garlic cloves soaked in a mild acetic acid pickling solution. Stoll and Seebeck (1951) determined alliinase to have an optimal pH range of 5.0 to 8.0 with its inactivation at a pH of 3.0 or less (Aguilar & Rincon, 2007). The objective of this experiment was to confirm if the enzyme alliinase is inactivated in whole garlic cloves soaked in low pH acetic acid pickling solutions, thus preventing blue-green pigment formation.

Materials & Methods

Equipment

As described in the initial experiment.

Ingredients

As described in the initial experiment. Distilled white vinegar with 20% and 30% acetic acid concentration was used and obtained from Fleischmann's Vinegar, 12604 Hiddencreek Way Suite #A, Cerritos, CA 90703.

Sample & Equipment Preparation

As described in the initial experiment.

Ingredient Storage

As described in the initial experiment.

Allicin Quantitation

As described in the initial experiment.

Procedure

Two sets of peeled, whole garlic cloves were accurately weighed to 150 grams and added to two glass pickling jars. One hundred and fifty grams of distilled white vinegar at 20% acetic acid content was poured over the first jar of peeled garlic cloves and 150 grams of distilled white vinegar at 30% acetic acid content was poured over the second jar of peeled garlic cloves. Jars were capped and allowed to sit on bench top for seven days. Observed changes in both jars were recorded on a daily basis for seven days.

Final equilibrated pH of garlic cloves soaked in 20% and 30% acetic acid pickling solutions for seven days was measured by taking approximately 40 grams of soaked garlic cloves and 40 grams of pickling solution, macerating together in the "Magic Bullet" blender to form a homogenous mixture and measuring pH.

Results & Discussion

Observations

Pigment formation ultimately occurred in both 20% and 30% acetic acid pickling solutions. Pigment formation in garlic cloves soaked in the 20% acetic acid pickling solution appeared to be more intense and more rapid than formation in the 30% acetic acid pickling solution of the course of the first three days. By day four, the pigment formation appeared to be similar in intensity and progress in both samples, and pigments could be seen in approximately 90% of the clove. By day five, all cloves in both jars were completely enveloped in pigment. Soaking of garlic cloves took place until day seven with minimal changes occurring in the final two days of the experiment. A pungent garlic aroma was observed from day one through seven when the jars were opened. When comparing pigment formation in the 20% and 30% acetic acid pickling solutions to the 5% acetic acid pickling solution, pigments formed in the 20% and 30% pickling solutions were more of a light green color as opposed to a deep blue-green seen in the 5% pickling solution sample. Pigments leached from the brine were a yellow-brown-green color (Figure 22).

Figure 18: Cross-section of non-blanched garlic cloves soaked in 20% acetic acid pickling solution over seven day period

Day 1   Day 2   Day 3   Day 4   Day 5   Day 6   Day 7

Figure 19: Cross-section of non-blanched garlic cloves soaked in 30% acetic acid pickling solution over seven day period

Day 1   Day 2   Day 3   Day 4   Day 5   Day 6   Day 7

36

Figure 20: Jars containing non-blanched garlic cloves soaked in 20% acetic acid pickling solution over seven day period
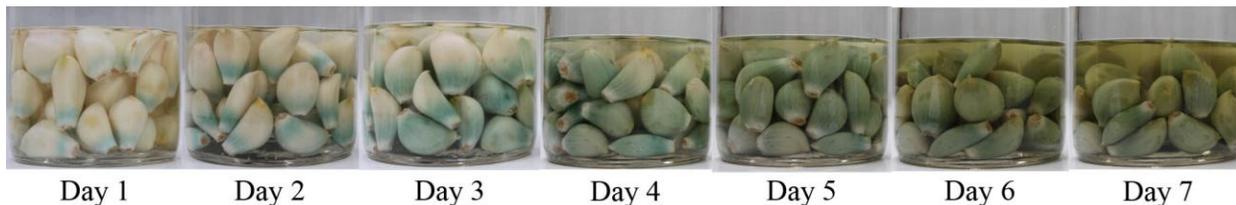
Figure 21: Jars containing non-blanched garlic cloves soaked in 30% acetic acid pickling solution over seven day period

Figure 22: Equilibrated 20% and 30% acetic acid pickling solutions after seven day period
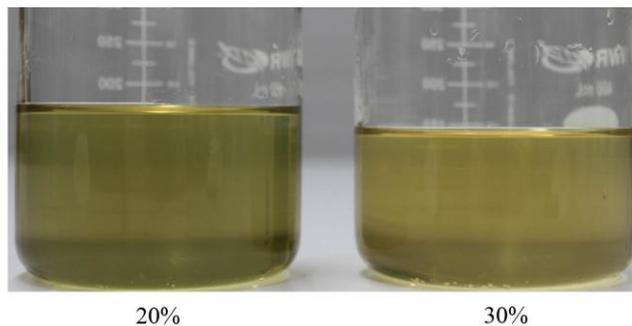
Data

Table 3: pH and allicin content from equilibrated garlic cloves soaked in 20% and 30% acetic acid pickling solutions

	Equilibrated pH	Allicin Content of Pickling Solution %(w/w)
20% Acetic Acid Solution	3.10	0.0260
30% Acetic Acid Solution	2.81	0.0271

Discussion & Conclusion

Blue pigments formed in garlic cloves soaked in pickling solutions containing 20% and 30% acetic acid concentrations. In most other research demonstrating alliinase inactivation by low pH (<3.0), garlic was in the form of a paste or homogenate instead of whole cloves. In intact garlic cloves, Bai et al. (2006) hypothesized acetic acid pickling solutions have the ability to increase the permeability of the the tonoplast, the cell membrane surrounding the vacuole which is the organelle where alliinase is sequestered. By increasing the permeability of the cell membrane and facilitating diffusion, either the cysteine sulfoxides traverse the tonoplast to mix with alliinase in the vacuole, or alliinase traverses the tonoplast to mix with cysteine sulfoxides in the cytosol (Bai et al., 2006). Since blue-green pigments were able to form in whole garlic cloves soaked in low pH (3.10 & 2.81) pickling solutions (Figures 20 & 21), it is reasonable to conclude when acetic acid increases the permeability of the tonoplast, cysteine sulfoxides traverse the tonoplast diffusing into the vacuole, reacting with alliinase to form allicin before the low pH conditions of the pickling solution have an opportunity to inactivate alliinase.

Blue-green pigments formed faster in cloves soaked in 20% and 30% acetic acid pickling solutions than pigments formed in cloves soaked in 5% acetic acid solution. However, the pigments formed in the higher acetic acid concentrations were less intense than the pigments formed in the 5% acetic acid pickling solution. This may be attributed to the low pH pickling solution inactivating a higher amount of alliinase than the 5% acetic acid pickling solution. Additionally, approximately 19% less allicin diffused from the garlic cloves into the 20% and 30% acetic acid pickling solutions than the 5% acetic acid pickling solution. This would support the hypothesis that low pH pickling solutions inactivate more alliinase in intact garlic cloves than the 5% acetic acid pickling solution.

Effect of Citric Acid Pickling Solution on Pigment Formation in Whole Garlic Cloves

Introduction

Research conducted by Bai et al. (2005) and Bai et al. (2006) show the inability for blue-green pigments to form in whole garlic cloves soaked in a citric acid pickling solution. Since citric acid is a tri-carboxylic acid, it does not increase the permeability of the tonoplast in whole garlic like acetic acid does. The diffusion of cysteine sulfoxides into the vacuole or diffusion of

alliinase into the cytosol does not take place and pigment formation does not occur in garlic cloves soaked in a citric acid pickling solution. The objective of this experiment was to confirm the findings of Bai et al. (2005) and Bai et al. (2006) and to provide an alternate acidulant for pickling whole garlic cloves by demonstrating the viability of citric acid pickling solution through the absence of blue-green pigment formation.

Materials & Methods

Equipment
As described in the initial experiment.

Ingredients
As described in the initial experiment. Citric Acid FCC, USP, Anhydrous Powder was obtained from Tate & Lyle 2200 East Eldorado St. Decatur, IL, 62525.

Sample & Equipment Preparation
As described in the initial experiment.

Ingredient Storage
As described in the initial experiment.

Allicin Quantitation
As described in the initial experiment.

Procedure
A 10% citric acid solution was made from the powdered citric acid and distilled water. Approximately 150 grams of peeled garlic cloves were accurately weighed and added to the glass pickling jar. One hundred and fifty grams of the citric acid pickling solution was accurately weighed and poured over the garlic cloves in the pickling jar. Jars were capped and allowed to sit on bench top for seven days. Observed changes in both jars were recorded on a daily basis for seven days.

Final equilibrated pH of garlic cloves soaked in citric acid pickling solution for seven days was measured by taking approximately 40 grams of soaked garlic cloves and 40 grams of

pickling solution, macerating together in the "Magic Bullet" blender to form a homogenous mixture and measuring pH.

Results & Discussion

Observations

Over the course of the seven days in which whole garlic cloves were soaked in the citric acid pickling solution, no blue-green pigment formation was observed. Garlic cloves maintained their original cream to light tan color (Figures 23 & 24). Over the course of the seven days, a mild to moderate aroma characteristic of fresh garlic was observed upon opening the jars. The citric pickling solution used to soak the garlic cloves for seven days did not change color and remained clear.

Figure 23: Cross-section of non-blanched garlic cloves soaked in 10% citric acid pickling solution over seven day period
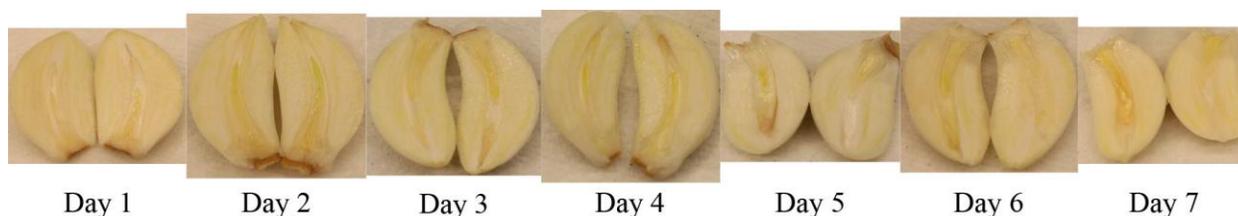
Figure 24: Jars containing non-blanched garlic cloves soaked in 10% citric acid pickling solution over seven day period

Data

Table 4: pH and allicin content from equilibrated garlic cloves soaked in 10% citric acid pickling solution

	Equilibrated pH	Allicin Content of Pickling Solution %(w/w)
10% Citric Acid Solution	2.42	<0.0001

Discussion & Conclusion

Consistent with research conducted by Bai et al. (2005) and Bai et al. (2006), whole garlic cloves soaked in a citric acid pickling solution did not form blue-green pigments and nearly no allicin (Table 4) was detected in the citric acid pickling solution. This can be attributed to the inability of citric acid to increase the permeability of cell membrane of the organelle which sequesters alliinase (Bai et al., 2006). These results show citric acid pickling solutions can be used as an alternative acidulant to acetic acid for the purpose of pickling or preserving whole garlic cloves for future use. The ideal concentration of citric acid would need to be determined in order to ensure stability and desired shelf life of whole garlic cloves soaked in a citric acid pickling solution.

Effect of Refrigerated Storage on Allicin Content in Blanched and Non-blanched Garlic Cloves

Introduction

Garlic cloves intended for commercial use are often stored under refrigerated conditions for prolonged periods of time to prevent the cloves from sprouting which increases year round availability (Block, 2010). It has been well documented that storing garlic in refrigerated conditions increases cysteine sulfoxide concentrations in whole garlic cloves leading to an increased incidence and intensity of pigment formation (Lukes, 1986; Yamazaki et al., 2012). To prevent the potential increased occurrence of pigment formation, blanching cloves to inactivate alliinase may be implemented. Commercial food manufacturers looking to use garlic in a product formulation will most likely have their garlic stored under refrigeration to prevent sprouting. Once the garlic bulb is ready for use, it is be peeled and a blanch treatment of the cloves may be applied to prevent pigment formation. The objective of this experiment was to explore the effect a blanch treatment may have on allicin content of garlic cloves after having been stored under refrigerated conditions. The intent is to investigate the effect blanching garlic cloves has on the inactivation of alliinase as substrate cysteine sulfoxide concentrations increase over time as a result of refrigerated storage.

Materials & Methods

Equipment

As described in the initial experiment.

Ingredients

As described in the initial experiment.

Sample & Equipment Preparation

As described in the initial experiment.

Ingredient Storage

Garlic cloves were stored at 5°C until being removed for blanching and allicin quantitation.

Allicin Quantitation

As described in the initial experiment.

Procedure

In order to establish a baseline allicin content of garlic cloves, initial allicin content of blanched and non-blanched cloves were measured prior to the garlic cloves being stored at 5°C. All cloves were then stored at 5°C. Two sets of 3-4 garlic cloves were peeled from the bulbs and removed from refrigeration on a bi-weekly basis with the first set being removed after two weeks under refrigeration. One set was blanched in distilled water at 100°C for 5 minutes and the second set was not blanched. Both sets were submitted for allicin quantitation. The process was repeated every two weeks for eight weeks total.

Results & Discussion

Data

Table 5: Allicin content of non-blanched and blanched garlic cloves stored at 5°C for eight weeks

	Allicin Content of Cloves - %(w/w)	
Weeks stored at 5°C	Non-Blanched Cloves	Blanched Cloves
0 (Baseline)	0.547	0.0033
2	0.641	0.0009
4	0.676	0.0174
6	0.616	0.0011
8	0.454	0.00235

Discussion & Conclusion

Non-blanched garlic cloves held at refrigeration temperatures (5°C) for extended periods of time seemed to increase slightly in allicin content. These results may be consistent with Lukes (1986) and Yamazaki et al., (2012) who determined storing garlic cloves at or below 12°C for extended period's increases total clove content of isoalliin, the cysteine sulfoxide which governs the total potential amount of allicin produced. An approximate 97% decrease in allicin content was apparent after blanching the garlic cloves stored in refrigeration. However, concentrations of allicin in blanched cloves stored under refrigeration for the same period of time fluctuated. It would be premature to draw a conclusion attributing the increase in allicin content in garlic cloves stored under refrigeration to a potential change in allicin content after blanching garlic cloves. From this, a larger data set would be needed to visualize potential trends and accurately determine what effect storing garlic cloves in refrigerated conditions for extended periods would have on the allicin content of garlic cloves after they are blanched.

Chapter 5 - Discussion & Conclusions

Blanching garlic cloves to inactivate the enzyme which causes blue-green pigment formation has shown its effectiveness. It may be necessary to establish an optimal blanch time

and temperature for specific applications using a larger sample size to determine optimal parameters for preventing blue-green pigment formation and minimizing loss of firmness associated with blanching.

Results suggest that low pH pickling solutions are able to reduce alliinase activity in whole garlic cloves. However, full inactivation of alliinase may not be viable via soaking whole garlic cloves in low pH pickling solutions as pigment formation still occurs.

It was confirmed that a citric acid pickling solution can be used as an alternative to acetic acid since citric acid does not increase the permeability of the tonoplast, thus preventing blue-green pigment formation in whole garlic cloves.

Areas for Further Research

Aguilar and Rincon (2007) demonstrated that adding 1% cysteine to garlic paste inhibited blue-green pigment formation. However, when cysteine was added to garlic paste at concentrations exceeding 1% there was a "negative effect on greening". Additional research could be conducted to determine why levels of cysteine exceeding 1% has a negative effect on garlic greening.

Research conducted by Rejano et al., (2004) and Rejano et al., (1997) explored blanching garlic cloves and slices using time and temperature treatments ranging from 60°C to 90°C for as long as 60 minutes. More research, using a larger sample size needs to be conducted to determine the optimal blanch time and temperature required to effectively inactivate alliinase. Also, research could be conducted to determine what concentration of alliinase is needed to bring out blue-green pigment formation.

Garlic intended for commercial use is stored at refrigerated temperatures to prevent sprouting of the cloves (Block, 2010). Lukes (1986) has shown that garlic stored at refrigerated temperatures has a significantly higher tendency to form blue-green pigments than garlic stored above 23°C. If garlic is stored at refrigerated temperatures to prevent sprouting and then stored above 23°C for a month to prevent greening, further research could be conducted to determine what effect storage of the garlic at 23°C for one month will have on sprouting of the cloves. It would be interesting to see if sprouting would still occur.

Wäfler et al. (1993) showed the slow freezing of tissue containing alliinase led to its complete inactivation but a mechanism for its inactivation was not elucidated. More research

could be completed to characterize the chemical or physical process taking place that leads to the inactivation of alliianse following a slow freeze/thaw cycle.

Stoll and Seebeck (1951) determined alliinase to have an optimal pH range of 5.0 to 8.0 with its inactivation at a pH of 3.0 or less (Aguilar & Rincon, 2007). As demonstrated above, blue-green pigments were able to form in garlic cloves soaked in a pickling solution whose pH was less than 3.0. Further research needs to be conducted to determine the ability, efficacy and rate of alliinase inactivation in whole garlic cloves soaked in low pH pickling solutions.

Some of the suggested areas for additional research may be challenging to determine and form an accurate conclusion that can be used to prevent pigment formation as concentrations of cysteine sulfoxides are dependent on a wide variety of variables from growing conditions to storage conditions.

Ideal Pigment Formation Preventative Measures

Solutions discussed to inhibit blue-green pigment formation should be implemented on a case by case basis after determining the best solution or solutions for a specific application.

Harvesting garlic seven weeks premature can reduce cysteine sulfoxide concentrations by as much as 150% which may diminish the occurrence or intensity of pigment formation. However, further investigation is needed to determine what effect the premature harvesting of garlic would have on yield.

Storing garlic above refrigerated temperatures can be implemented as a preventative method to reduce garlic greening, though it does not prevent the sprouting of garlic cloves. Garlic greening and sprouting are two factors to consider when selecting a storage temperature for garlic cloves. It may be necessary to refrigerate garlic for long term storage purposes to prevent sprouting and then store garlic under temperatures at or above 23°C one month prior to processing to prevent greening as demonstrated by Lukes (1986).

Blanching garlic may be the most favorable preventative measure as it has proven successful in inactivating alliianse. However, blanching will cause garlic cloves to lose firmness and their characteristic pungent flavor.

As shown above, and discussed by Bai et al. (2005) and Bai et al. (2006), whole garlic cloves soaked in acetic acid pickling solutions contribute significantly to pigment formation. Pickling whole garlic in low pH acetic acid solutions have been shown to be ineffective at

inactivating alliinase. Therefore, pickling whole garlic cloves in solutions not containing acetic acid or mono-carboxylic acids is recommended.

Freezing garlic cloves has been shown to inactivate alliinase to some degree but results are inconclusive. Freezing garlic cloves to inactivate alliinase would have to be studied in detail using the intended final application to determine efficacy.

While incorporating additives such as cysteine and hydroxylamine to garlic products has been shown to reduce greening, implementing preventative measures during the growth, storage, or processing of garlic bulbs and cloves is preferred (Yamazaki et al., 2012).

Conflicting and Inconsistent Prior Research

Pigment development is an "immensely complex process" (Kubec & Velisek, 2007) and can be dependent on many factors including weather conditions during growth, growing region, garlic variety, maturity at harvest, time of harvest, storage conditions, and processing conditions (Aguilar & Rincon, 2007; Block, 2010; Kubec et al., 2004; Kubec & Velisek, 2007; Lawson and Wang, 1994 as cited in Koch & Lawson, 1996; Lukes, 1986; Yamazaki et al., 2012). It is acknowledged that research conducted to further elucidate the mechanism of and factors contributing to blue-green pigment formation in garlic are at times conflicting and inconsistent (Aguilar & Rincon, 2007). Research on this phenomenon has been taking place for the past several decades and has mostly been "very poorly understood" (Joslyn & Sano, 1956; Kubec et al., 2004). For example, Kubec and Velisek (2007) state that research investigating the relationship between certain amino acids and their ability to form pigments is "fragmentary and, in many cases, quite controversial, being obtained under different experimental conditions". Part of the reason why the greening phenomenon has been so poorly understood and elucidated may be because green pigment development is sporadic and inconsistent, "even when the same cultivar is harvested simultaneously from the same field" (Yamazaki et al., 2012). However, advances have been made in recent years. Factors previously thought to contribute to pigment formation such as copper ions and pesticides have been dismissed (Kubec et al., 2004; Kubec & Velisek, 2007; Lee et al., 2006b). Clearer elucidations of the mechanism of pigment formation and methods to prevent or diminish its occurrence have been determined (Aguilar & Rincon, 2007; Bai et al., 2005; Bai et al., 2006; Imai et al., 2006).

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