INHIBITION OF NON-ENZYMATIC PROTEIN GLYCATION BY POMEGRANATE

(*PUNICA GRANATUM*) POLYPHENOLICS

By

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(Under the Direction of Phillip Greenspan)

ABSTRACT

Protein glycation is the non-enzymatic reaction of a reducing sugar or sugar derivative with amino acids, peptides, or proteins resulting in advanced glycation endproducts (AGEs) and crosslinking of proteins. This process is accelerated in diabetes mellitus, and detrimental for proteins that are not readily recycled, such as collagen and lens crystallins. The current study investigates the effect of commercially available pomegranate juice, major phytochemicals found in pomegranate juice (ellagic acid, punicalagin), whole pomegranate fruit and various fractions of pomegranate fruit (peel, membrane, aril) on the *in vitro* fructose mediated glycation of bovine serum albumin (BSA). Total phenolic content and antioxidant capacity for all juices and pomegranate extracts were determined by the Folin-Ciocalteu method and the FRAP (ferric reducing antioxidant potential) assay, respectively. AGE formation was detected by a fluorescence spectroscopy assay. The effect of pomegranate juice on the inhibition of glycation for other proteins (gelatin, IgG, ribonuclease, lysozyme) was also investigated to determine whether pomegranate polyphenolics are generalized inhibitors of this process.

Pomegranate juice produced the greatest inhibition of BSA glycation on the basis of volume, phenolic content, and antioxidant capacity when compared to other juices. Punicalagin and ellagic acid produced similar results to that of pomegranate juice. Whole pomegranate had a much greater inhibitory activity than whole apple and the membrane extract of pomegranate fruit was the most potent inhibitor of BSA glycation compared to the aril and peel pomegranate extracts.

Pomegranate juice, incubated with gelatin, IgG, ribonuclease A (RNase), and lysozyme inhibited the glycation of these proteins in the presence of fructose, similar to that observed with BSA. SDS PAGE analysis of BSA and RNase revealed that pomegranate juice prevented chemical modifications to the native protein structure associated with glycation. This work suggests that the pomegranate is a robust inhibitor of protein glycation in various model proteins, owing the majority of its inhibitory activity to the antioxidant capacity of phytochemicals found within the membrane of the fruit.

INDEX WORDS: Protein glycation, Pomegranate, Fluorescence, SDS-PAGE, Bovine Serum Album, Ribonuclease A, Fruit, Juice

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DEDICATION

I lovingly dedicate this dissertation to my parents:

Edward and Wilhelmenia Garner

Jeremiah 29:10-14

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Epidemiological studies demonstrate a link between consumption of plant derived foods and various health benefits. These benefits have been linked to the phytochemcials found within these foodstuffs. Epidemiological studies also demonstrate a relationship between dietary habits and disease risk. Clinical trials investigating the metabolism, bioavailability, and tissue distribution of phytochemicals in humans are rare. Most tests involve animal models or *in vitro* assays. After stomach hydrolysis, polyphenolic compounds undergo extensive metabolism while they transit through the GI tract with few exceptions. Metabolism begins in the lumen of the small intestine where sugar moieties are cleaved, and the aglycone undergoes glucuronidation, sulfation, and/or methylation. The majority of polyphenols are not absorbed in the small intestine in their native form; they are however, structurally modified by colonic microflora (1). Secondary metabolites from primary compounds exist at low concentrations in the plasma; however, they can produce physiological effects (2). After circulation in the bloodstream, these secondary metabolites (low molecular weight phenolic acids) are excreted in the urine.

The mechanism by which a majority of phenolic compounds provides beneficial health benefits is scavenging free radicals. The bioactivity of phenolic compounds has been correlated to their antioxidant properties (ability to scavenge free radicals). Free radicals have been involved in the development of many chronic diseases such as LDL

oxidation in cardiovascular disease and DNA oxidation in cancer. Recently many bioactives from food have been marketed in the form of pharmaceutical products including but not limited to pills, capsules, powders, and aqueous solutions. This class of products is called nutraceuticals. Nutraceuticals are dietary supplements that provide a concentrated bioactive from a food source in a non-food matrix.

Many nutraceuticals currently available on the market are associated with health claims. Scientific evidence supporting their health benefits is lacking because supporting studies are performed *in vitro* or in animal model assays. The measurements of antioxidant capacity utilizing in vitro assays are used all the time to determine how "potent" an antioxidant is. FRAP, ABTS, DPPH, and lipid peroxidation evaluate the phytochemical's ability to scavenge artificially made radical species. Antioxidant capacity may not reflect its activity *in vivo*; however, *in vitro* antioxidant activity is used to characterize the compound. Phytochemicals are arranged in five different categories: carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds. Phenolics encompass: (a) phenolic acids, (b) flavonoids, (c) stilbenes, (d) coumarins, and (e) tannins.

Ellagic acid and ellagitannins are phenolics that belong to the phenolic acid and tannin category respectively. Ellagic acid was first studied in the 1960s for its effect on blood pressure (3). The dietary administration of ellagitannin containing foods such as strawberries and raspberries to rats has shown to inhibit events associated with the initiation and promotion/progression of chemically induced colon and esophageal cancers (4, 5). A more recent study showed that there was no effect on the number or size of adenomas in the small intestine of Apc-mutated Min mice after the administration of

ellagic acid and cloudberry (high ellagitannin content). This could suggest that the chemoprotective effect of ellagic acid is specific to the type of tumor and animal model.

Pomegranate juice is recognized as one of the most powerful *in vitro* antioxidant foods. Its activity is due to punicalagin, a very potent ellagitannin. In general, ellagitannins are not absorbed (6) however small amounts of punicalagin were detected in rats' plasma following long-term administration at high doses. Ellagitannins are hydrolyzed to ellagic acid in the small intestine (7). Reports show that ellagic acid is absorbed 30-90 minutes after ingestion suggesting absorption from the stomach (8). Various factors may affect the absorption of ellagic acid including the influence of the food matrix, the dose of free ellagic acid, and individual variability.

Ellagic acid can bind readily to the intestine epithelium also affecting its absorption. When ellagitannins or ellagic acid reach the distal part of the small intestine they are metabolized by gut microflora to render urolithin A and B, or hydroxyl-6H-dibenzo [b,d] pyran-6-one derivatives. Urolithin A and B are absorbed, conjugated, and detected in plasma at an approximate concentration of 10μM (9). These metabolites enter the enterohepatic circulation before becoming excreted in the urine. Ellagic acid methyl ether glucuronides have been detected in human plasma and urine. Extracts from red raspberry leaves and seeds and pomegranates are available in capsules, powders, tablets, and liquid form. These products have a GRAS status from the federal government; however, more toxicity studies need to be performed in humans. One study in humans shows an improvement in neutropenia after chemotherapy with patients ingesting 180mg of ellagic acid daily for 6 weeks (10). In the mid-1990s, ellagic acid and ellagic acid containing supplements were advertised as cancer preventing or cancer therapy products.

Based on initial studies, ellagic acid and ellagitannins are beneficial phytochemicals for use as supplements or within functional foods.

Pomegranate

Over 1000 cultivars exist of the pomegranate (Punica granatum) (11), which arose from the Middle East and extend throughout the Mediterranean, China, India, the American Southwest, and Mexico. References to pomegranates can be found in Christianity, Judaism, Islam, and Buddhism. Pomegranates are even displayed on the coat of arms of several British medical societies (12). Throughout history, the pomegranate has symbolized life, longevity, health, femininity, knowledge, morality, immorality, and spirituality (13). In Ayurvedic medicine, the pomegranate is considered a pharmacy contained within a fruit. Ancient medicine has utilized the pomegranate to cure diarrhea (14), and as a remedy for diabetes mellitus (15). Recent health benefits have been attributed to pomegranate's antioxidant, anti-inflammatory, and anti-cancer activity.

Pomegranate Fruit Composition

The pomegranate plant is considered a large tree or shrub, which bears a large berry as fruit. The fruit is surrounded by a tough, leathery exocarp, which contains numerous arils. Each aril is a translucent sac containing tart juice and a single seed. These arils are suspended within a matrix known as the membrane (Figure 1.1).

FIGURE 1.1 *Punica granatum*

Pomegranate seed is comprised of approximately 12-20% oil. It consists of 80% conjugated octadecatrienoic acid mainly comprised of cis 9, trans 11, and cis 13. Minor components of pomegranate seed oil include sterols, steroids, and cerebroside. On a phenolic basis pomegranate, seed oil also contains hydroxybenzoic acids (ellagic acid and 3, 3´-Di-O-methylellagic acid).

Pomegranate juice contains anthocyanins (cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, and pelargonidin 3-O-glucoside) which provide the juice and the fruit with an intense red color. The concentration of anthocyanins increased during ripening (16), however the concentration declined after the fruit was pressed for juice (17). Pomegranate juice that comes from squeezing the whole fruit contains several polyphenolics and sugars. These polyphenolics included: (a) punicalagin, a type of

hydrolyzable tannins (Figure 1.2), (b) gallic acid, (c) ellagic acid, (d) anthocyanins, and

(e) catechins.

FIGURE 1.2 Ellagic Acid and Punicalagin
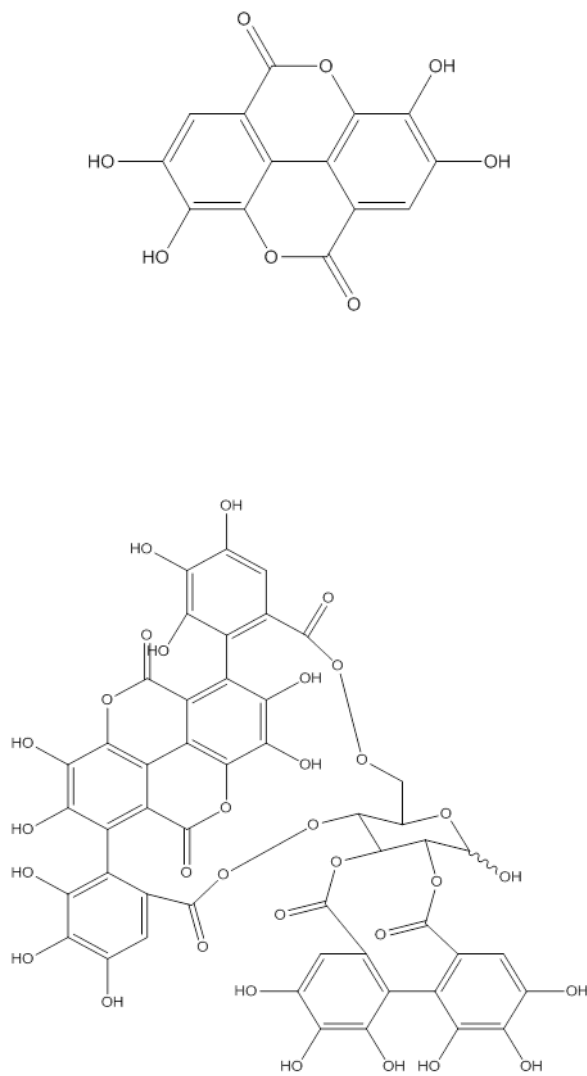
Pomegranate juice polyphenols can be placed in 4 major groups. The groups are anthocyanin pigments (i.e. cyanidin 3-glucoside), hydrolyzable tannins of the gallagyl type (i.e. punicalagin isomers and punicalin), ellagic acid and its glucosides, and complex hydrolyzable tannins (which degrade to ellagic acid after hydrolysis). Hydrostatic pressure from the juicing process crushes the entire fruit releasing juice from the arils and water-soluble ellagitannins from the rind (18). Despite their inherent size, pomegranate tannins can be absorbed by the intestines (19). It has been reported that ellagitannins of pomegranate are hydrolyzed extensively in mice, which excrete ellagic acid in their feces and urine (20).

The pericarp (peel and membrane) of pomegranate fruit contain abundant amounts of flavonoids and tannins. Pomegranate leaves contain hydroxybenzoic acids (gallic acid and ellagic acid), hydroxycinnamic acids (caffeic acid, chlorogenic acids, and ρ-coumaric acid), flavan-3-ols (catechin and epicatechin), flavonols (quercetin and kaempferol), flavones (luteolin and apigenin), and ellagitannins (punicalin, punicalagin).

Health Benefits of Pomegranate

Inflammation

Acute and chronic inflammation is the body's response to injury or tissue damage; if it has not been resolved in a timely matter chronic disorders of the immune system (rheumatoid arthritis and inflammatory bowel disease) and even cancer can arise (21, 22). Inhibition of cyclooxygenase by non-steroidal anti-inflammatory drugs is a conventional mode of therapy however, this treatment can provide an array of side effects. Whole pomegranate extract applied to mouse skin inhibited cyclooxygenase expression (23), and

compared to other commonly consumed juices it increased the anti-thrombotic prostanoid PGI_2 in human subjects.

Changes in phosphorylation of pro-inflammatory cytokines can prompt inflammatory cascades. Whole pomegranate fruit extract inhibited the phosphorylation of several cytokines in UV-B irradiated keratinocytes. Whole pomegranate fruit extract also inhibited the activation of NF-κB and MAPK, and cytokine formation in mouse skin exposed to 12-O-tetradecanoylphorbol-13-acetate (23). Patients with periodontitis that received intragingival chips containing pomegranate peel extract had reduced levels of inflammatory cytokines several months post treatment (24). Matrix metalloproteinases (MMP) are enzymes important in cancer progression (25). The activity of MMP in human chondrocytes was inhibited by whole pomegranate fruit extracts (26).

Heart Disease

There are several studies demonstrating the health benefits of pomegranate juice. Consumption of pomegranate juice by healthy individuals for two weeks significantly reduced oxidation of LDL and HDL and increased HDL associated paraoxonase1 (PON1) activity (27). Patients with carotid artery stenosis that consumed pomegranate juice for three years reduced serum oxidative stress, increased serum PON1 activity, and reduced the atherosclerotic lesion size (28). Fresh pomegranate juice can ameliorate the vasomotor effects of sheer stress in hypercholesterolemic mice (29).

Cancer

Cancer is an uncontrolled growth of malignant cells that can be propagated by inflammation. In female CD-1 mice with skin tumors induced by 7,12-dimethyl-benz[a]anthracene and promoted by 12-O-tetradecanoylphorbol 13-acetate, treatment

with 5% pomegranate seed oil produced significant decreases in tumor incidence and multiplicity. Human Burkitt's lymphoma cells exposed to pomegranate peel extract experienced cell cycle changes (30) due to modulation of the cell signaling molecules. Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide to bicarbonate, of which 14 isoforms are known in mammals. Carbonic anhydrase inhibitors strongly inhibit cancer cell growth *in vitro* and *in vivo*. Pomegranate peel extract inhibited the de-esterification of ρ-nitrophenyl acetate catalyzed by carbonic anhydrase, which establishes it as a carbonic anhydrase inhibitor. Aromatase catalyzes the formation of estrone and estradiol from androstenedione and testosterone, respectively (31). This enzyme can propagate hormone dependent cancers such as estrogen sensitive breast cancers. Both fermented pomegranate juice and pomegranate peel extract significantly inhibit aromatase. The growth of new blood vessels (angiogenesis) is necessary to supply oxygen and nutrients for tumor growth and metastasis. Angiogenesis in chicken chorioallantoic membrane *in vivo* was significantly suppressed by fermented pomegranate juice. Pro-angiogenic vascular endothelial growth factor (VEGF) was significantly downregulated in MCF-7 extrogen dependent cells by fermented pomegranate juice. Pomegranate peel extract led to apoptotic DNA fragmentation and suppression of growth in two human Burkitt's lymphoma cell lines, Raji and P3HR-1 (30). It has been demonstrated in many studies (32-34) that the anti-proliferative activity of polyphenolic compounds in pomegranate collectively is superior to singular compounds. There is a paucity of clinical work involving anti-cancer benefits from pomegranate; however, in a recent clinical trial, 46 men who consumed 8 oz. of

pomegranate juice daily experienced an increase in PSA (a clinical biomarker for prostate cancer mortality) doubling time from 15 to 37 months (35).

Glycation of Proteins

History

Protein glycation involves the non-enzymatic reaction of a reducing sugar or sugar derivative to amino acids, peptides, or proteins (36). This non-enzymatic reaction was first studied by L.C. Maillard in the early 1900s (37). Food chemists have studied the Maillard reaction as it relates to flavor, color, and texture in cooked, processed, and stored foods. In the 1970s and 1980s, researchers discovered that this process also occurred *in vivo*. Studies in the 1970s demonstrated that hemoglobin A1$_c$ (HbA1$_c$), naturally occurring minor human hemoglobin, is elevated in diabetics. Koenig and coworkers (38) found that the carbohydrate in HbA1$_c$ was attached as a 1-deoxy-1-fructosyl residue to the N-terminal valine nitrogen. They were also the first to propose using HbA1$_c$ as a means to monitor glycemic control in diabetic patients (39). In the 1980s, researchers began to understand the significance of Maillard reaction products in diabetic complications (40) and aging (41). These products were given the name glycated proteins to distinguish them from enzymatically glycosylated proteins. Complex pigments and crosslinks formed from glycated protein were termed advanced glycation end-products (AGEs).

Formation of Glycated Proteins

Reducing sugars (glucose, fructose, galactose, mannose, and ribose) are extremely reactive with nucleophillic nitrogen bases. Glucose is the least reactive of the common sugars, possibly leading to its selection as the principal free sugar *in vivo* (40). A

reducing sugar reacts with a free amino group forming a glycosylamine, which degrades to a Schiff base. In a Schiff base, the aldehydic carbon-oxygen double bond of the sugar is converted to a carbon-nitrogen double bond with the amine. The open-chain double bonded form of the Schiff base adducts of hexoses or pentoses are thermodynamically disfavored as opposed to the pyranose of furanose forms (glycosylamines) (42). Formation of the Schiff base from the reducing sugar and amine is fast and reversible. The Amadori rearrangement of a Schiff base to the Amadori product occurs via an open-chain end form, which tends to be slower than Schiff base formation. However, the reverse reaction (Amadori rearrangement) is much slower than the formation; therefore, Amadori products tend to accumulate on proteins. In the 1950s, it was realized that Amadori products (i.e. fructosamine) could form from aliphatic amines such as amino acids and not just aromatic amines (43). These adducts (Schiff base and Amadori products) are known as early stage glycation products. Despite continued research, these processes are still not completely understood. Adducts formed include fluorescent chromophores and browning pigments.

Recently it has become clear that α-dicarbonyl compounds are intermediates in the formation of AGEs. Free α-dicarbonyl glyoxal compounds such as 3-deoxyglucosone, methylgloxal, and glycoxal are formed from degraded glucose and Amadori products (44). Dicarbonyls can crosslink proteins forming AGEs directly and have been detected *in vivo* (45). Amadori products can also dehydrate at the 4-position to form 1-amino-4-deoxy-2,3 dione (Amadori dione) (46,47) and subsequently dehydrate at the 5-position to yield an unsaturated dione (Amadori ene-dione) (48). Amadori ene

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diones are also responsible for crosslinking proteins. This suggests that AGEs can form in the beginning or ending stages of the glycation process.

Fluorescent AGEs

The characteristic brown color and fluorescence are properties used to estimate AGE formation. Fluorescent AGE crosslinks include: (a) pentosidine, (b) crossline, (c) AGE-XI,(d) pentodilysine, (e) vesperlysine A, B,&C, and (f) FPPC. Pentosidine was first identified in dura matter collagen by Sell and coworkers (49). Pentosidine can be formed from the reaction of lysine or arginine with glucose, ribose, ascorbic acid, or 3-deoxyglucosone. Pentosidine has been found in human tissues not limited to the skin, tracheal cartilage, cortical bone, aorta, cardiac muscle, lung, liver, kidney, and eye lens. Studies have shown that there is a direct correlation between skin pentosidine levels and the severity of diabetic complications in patients with diabetes mellitus (49). Crosslines (50) and vesperlysines (51) have been detected *in vivo*.

Non-Fluorescent AGEs

Fluorescent AGE crosslinks are an easy marker for AGE formation due to their detection but they only account for one percent of the total amount of crosslinking structures *in vivo* (52). AGE structures mostly responsible for protein-protein crosslinking *in vivo* are non-fluorescent and have not been fully identified yet. Non-fluorescent AGE crosslinks include pyrraline imine, AFGP (alky formyl glycosyl pyrrole) imine, amadori dione, α-amino acid amides, imidazolium dilysine, aminoimidazoline imine, glucosepan, and ALI (arginine-lysine-imidazole).

Imidazolium dilysine, also known as GOLD/MOLD crosslinks, have been isolated from the reaction of two glyoxal derivative molecules with two lysine residues *in vitro*, and they have been detected *in vivo* (53). Imidazole dilysine are present at levels 10 to 50 fold higher than pentosidine in tissue (54).

AFGP imines form from two sugar molecules with one alkylamine molecule (56). The α positions of the side chains attached to the pyrrole ring carbons in AFGP imines are susceptible to nucleophilic attack by thiols (57) and lysine amine groups (56). This indicates that AFGPs are cross-linked proteins.

The pyrraline crosslink (N-alkyl-5-hydroxymethyl-2-pyrrolaldehyde) is a monomeric AGE that forms on lysine residues *in vivo*. The aldehyde of pyrraline can form a Schiff base with another amino group, which has the possibility to form lysine-lysine crosslinking *in vivo*. Sugar and Amadori compounds react with primary amines to form N,N'-dialkyl-alanine or α amino acid amide crosslinks. Gloxal derivatives from Amadori product breakdown are possible intermediates for their formation (58). They are difficult to isolate because they are cleaved under protein hydrolysis to yield carboxymethylysine.

Aminoimidazoline imine crosslinks are derived from the reaction between arginine and an α-oxoaldimine Schiff base of a lysine residue or glyoxal derivatives (i.e. methylgloxal, 3-deoxyglucosone) (59).

ALI (arginine-lysine-imidazole) crosslink is derived from the reaction between an Amadori dione and an arginine residue (60). ALI is immunochemically close to AGE structures on glucose modified bovine serum albumin (61). Amadori dione crosslink is a

conjugate addition of a nucleophilic protein side chain to a protein bound Amadori ene-dione, which results in a protein-protein crosslink containing an α-diketone structure in the linker.

A number of non-crosslinking AGE structures have a profound effect on protein structure and function *in vivo*. They serve as precursors to crosslinks or biological receptor ligands, which induce adverse cellular and tissue changes. These non-crosslink structures include: (a) pyrraline, (b) 1-carboxy-alkyllysine, and (c) imidazolone A and B. Pyrraline is a pyrrole aldehyde AGE found *in vivo* (62). It is derived from a reaction between 3-deoxyglucosone and lysine residues, and is known to form crosslinks between proteins. 1-carboxyalkyllysines involves a 1-carboxyalkyl group attached to a free amino group of an amino acid residue (i.e. N^ϵ-(carboxymethyl) lysine and N^ϵ-(1-carboxyethyl) lysine). These compounds have been found *in vivo*. They may form from reactions of lysine residues with glyoxal derivatives (63) or from autoxidation of early stage AGEs (i.e. Amadori products) (53). Imidazolones form from the reaction of glyoxal, methylglyoxal, or 3-deoxyglucosone with the guanidino group of arginine.

AGE Formation in Various Human Tissues

Collagen is a prime target for AGE formation due to its low turnover rate. AGEs damage vascular collagen, which contributes to atherosclerosis, coronary disease, kidney damage, retinal pathology, and poor peripheral circulation. Lens crystallins are also a prime target for AGE formation, which can lead to cataracts. Collagen lysine residues and hydroxylysine residues are oxidized by lysl oxidase, which converts Є-amino groups to aldehydes, which crosslinks with lysine or hydroxylysine residues in adjacent collagen

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molecules. AGEs mediate crosslinking in collagen, which causes loss of bulk elasticity, flexibility, and increased brittleness. Collagen can also react with exogenous molecules such as albumin, immunoglobulin, and LDL (64), resulting in the thickening of the basement membrane and the development of atherosclerotic lesions

Human lens crystalline can last for the human lifetime. Yellow brown pigments that have the spectral and fluorescent properties of AGEs form in the lens as a function of age (65). Glycation can modify amino groups in crystallins resulting in conformational changes. These changes expose sulfhydryl groups which autoxidize to form intermolecular disulfide bonds (66). These aggregates are substantial enough in size to scatter light and produce a cataract.

Protein Glycation in DNA

The presence of AGEs on DNA can cause unusual transpositional rearrangements (67). Like protein, DNA contains amino groups. The 2-amino group of guanosine is the most reactive. In mammalian cells, AGE formation on DNA may be responsible for insertions containing repetitive sequences of the Alu family that disrupt human genes (68). Protein glycation in DNA can cause congenital malformations in infants of poorly controlled, insulin dependent diabetic mothers.

Implications of Glycated Protein in Cardiovascular Disease

The Amadori product and later stage AGEs undergo autoxidation and have pro-oxidant effects on other molecules as well (69). The AGE radical may extract a hydrogen atom from a biomolecule nearby converting it to a radical, leading to its autoxidation. This effect is demonstrated in the glycation of lipoproteins like LDL (70). The glycation

of hemoglobin increases its oxygen affinity and makes it more susceptible to oxidation (71). AGE modification of LDL can occur on amino groups on the apoprotein (72) and the aminolipid (i.e. phosphatidylethanolamine) (73). AGE formation on the apoprotein can cause crosslinking of LDL to the collagen layer of the blood vessel wall (64) increasing the half-life of LDL in serum by impeding the recognition site for its receptor mediated uptake (72). This will increase the probability of autoxidation of the lipid component (73). Oxidation of LDL can lead to loss of recognition by cellular LDL receptors and induce uptake by macrophage scavenger receptors (74). Macrophages attracted to AGEs on vessel wall collagen may accumulate modified LDL, resulting in their conversion to foam cells, which are thought to be key in the atherosclerotic process. Inactivated oxygen does not normally react with most organic compounds; however, redox chemistry can allow iron or copper ions to induce the addition of oxygen to olefinic bonds of unsaturated fatty acids to form hydroperoxides. Further reactions of these hydroperoxides with metals can form hydroxyl and peroxy radicals that will cleave fatty acids to form aldehydes. Free metals are usually not present in significant amounts *in vivo*; therefore, the ability of AGEs to initiate oxidative reactions in the absence of metals offers a mechanism for lipoprotein lipid peroxidation *in vivo* (70).

Implications of Glycated Protein in Alzheimer's disease

There is evidence that AGEs play a role in abnormal amyloid aggregation in Alzheimer's disease. Analysis of the plaque revealed that Alzheimer's disease patients had three times the amount of AGE content per mg of amyloid in comparison to control subjects (75). The amyloid plaque continues to increase as the disease progresses, its AGE content increasing crosslinking, and resisting proteolytic degradation and removal.

Implications of Glycated Protein in Diabetes Mellitus

Bookchin and Gallop (76) first characterized glycated hemoglobin ($HbA1_c$), and its increase in persons with diabetes was reported by Rahbar (77). Long term monitoring of diabetes mellitus is currently performed by self-monitoring blood glucose (SMBG) and $HbA1_c$ levels every 3-6 months. A 2007 Freemantle study of 1286 type 2 diabetes patients over 5 years found that neither SMBG testing nor its frequency was associated with glycemic benefit in type 2 diabetes patients . A 2006 study of 3000 type 2 diabetes patients on oral antidiabetic drugs or a restricted diet found no benefit from SMBG in glycemic control for either group (78). Hemoglobin resides in the red blood cell, which has a half-life of 120 days, therefore the amount of $HbA1_c$ in a patient's blood becomes a record of glycemic control over a 3-6 month period. $HbA1_c$ is a verified standard for being able to predict the risk of having diabetic complications. Another short-term marker is apolipoprotein B (a component to LDL) that becomes glycated, and is involved in atherogenesis. LDL is recycled every three to five days; representing glycemic control over the preceding few days. Glycated albumin (GA) has also been used as a method to assess glycemic control.

Albumin is the largest component of plasma proteins, representing more than 60% of total plasma protein concentration. Albumin is responsible for maintenance of oncotic pressure. The structure of albumin is divided into three domains, and each domain is divided into two subdomains, which are held together by disulfide bonds. In a healthy human the amount of glycated albumin is roughly 1-10%, however with diabetes mellitus this amount can increase by threefold (79).

Lysine, arginine, and cysteine are predisposed to non-enzymatic glycation in serum albumin. Of the 29-glycation sites found, 18 of them are lysine residues. Lysine-525 is main site for non-enzymatic glycation in serum albumin; and it accounts for 30% of the overall glycation to albumin (80). Arginine-410 is predominantly responsible for albumin glycation mediated by methylglyoxal (81). Kisugi and coworkers (82) have demonstrated that there is a strong correlation between the amount of glycated albumin and the number of glycation sites. In a diabetic patient with no glycemic control there were 10 glycated sites, however after insulin therapy there were only three glycation sites.

Glycation of albumin is measured by: (a) colorimetric assay, (b) enzymatic assay, (c) HPLC and affinity chromatography, (d)immunoassay, and (e) ELISA. The thiobarbituric acid assay (TBA) and the fructosamine assay are common colorimetric methods used to measure glycation. TBA measures the amount of ketoamine bound to albumin based on the release of glucose from albumin in the form of 5-hydroxymethylfurfural (5-HMF). However, the 5-HMF is heat sensitive and free glucose in the assay can interfere with results (83). The fructosamine assay involves the reduction of nitro blue tetrazolium with ketoamines to form Formazan (chromophore). Thiol groups, uric acid, and lipemia can interfere with this assay (84). An enzymatic assay has been developed to measure the amount of glycated albumin using albumin-specific proteinase, keto amine oxidase, and bromocresolpurple reagent. This method provides an easier system to measure the amount of glycated albumin. (85).

Glycation of albumin is greater in people with coronary artery disease and unlike HbA1$_c$ is a predictor of coronary artery disease in type 2 diabetes. A recent study (86)

found that glycation of albumin decreased with improved glycemic control in comparison to HbA1$_c$. The reaction rate of non-enzymatic glycation of albumin is nine times greater than human hemoglobin. There is evidence to use glycated albumin to detect short-term glycemic control, which is highly recommended in gestational diabetes.

Receptors for Advanced Glycation Endproducts

Scavenger receptors with an affinity for AGEs have been found on many cells including macrophages, lymphocytes, and barrier cells (i.e. endothelial and mesangial cells) (87,88). Phagocytic cells that express these recpetors can endocytose old, AGE modified proteins, releasing AGE modified peptides similar to those absorbable from food and excreted by the kidneys. The cellular uptake of AGE proteins signals synthesis and release of certain cytokines and growth factors that stimulate the re-synthesis of proteins that have been removed (89).

RAGE is the most widely characterized receptor, which is distributed among endothelial cells, smooth muscle cells, and macrophages. Diabetic erythrocytes can induce oxidative stress in endothelial cells, however the effects are dampened by RAGE (90). Interaction between AGEs and RAGE can up-regulate the expression of adhesion molecules, including VCAM-1, which is responsible for atherosclerotic lesion formation (91). There are other receptors for AGEs besides RAGE, however they are less selective. The activity and function of RAGE and other AGE receptors *in vivo* is still unclear.

Tissue overgrowth can occur if this process is not regulated. For example, high levels of AGEs on matrix proteins in the diabetic kidney signals mesangial cells to

produce large amounts of matrix proteins, resulting in a thickened basement membrane incapable of normal kidney filtration (92).

Controversy over Diabetic Complications

Diabetic complications are hypothesized to originate from the following sources collectively: AGES, aldose reductase (93), oxidative stress (94), pseudohypoxia (95), true hypoxia (96), carbonyl stress (97), altered lipoprotein metabolism (97), increased protein kinase C activity (98), and altered growth factor expression (99). There has been controversy as to whether oxidative stress occurs in early stages of diabetes or is it a consequence of tissue damage. Increase in reactive carbonyls derived from oxidative and nonoxidative (carbonyl stress) leads to increased chemical modification of proteins, oxidative stress, and eventually tissue damage.

Carbonyl stress is usually caused by an increase in the concentration of reactive carbonyl precursors of AGEs (glycoxidation and lipoxidation products), or an increase in substrate stress. Baynes and coworkers (69) propose that carbonyl stress in diabetes is the result of a deficient or overloaded detox pathway. Carbonyl trapping may be a more efficient means to inhibit the progression of diabetes in comparison to antioxidants. This is evident in certain vitamins or coenzymes such as glutathione, carotene and vitamin E.

Controversy also surrounds the role AGEs play in the formation of diabetic complications (69). Primarily, AGEs are detectable at trace amounts in tissue proteins therefore discounting the idea that they have a quantitative effect in the development of complications. These moieties tend to become very unstable in conditions required for isolation and analysis making characterization impossible. Secondly, the concentration

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of AGEs in older adults is similar to that of younger diabetic patients with severe complications, which would suggest there is little direct correlation. However, older adults with high concentrations of AGEs are at increased risk for cardiovascular and Alzheimer's disease. Thirdly, AGE concentrations are low in tissues from diabetic animal models compared to those in humans, which could suggest AGEs are not a common cause of diabetic complications in certain mammalian species.

Development of AGE Inhibitors

The development of AGE inhibitors involves two different approaches. First inhibition by carbonyl blocking agents such as aminoguanidine, and second the cleavage of already formed AGE protein-protein crosslinks (i.e. DPTC). The ketone group of the 1-amino-1-deoxyfructose residue in the Amadori product is the key in AGE forming reactions. Aminoguanidine, a low molecular weight compound, chemically deactivates the ketone group making it inert. Aminoguanidine is highly nucleophilic, since it reacts with ketones and aldehydes. Research has demonstrated the effectiveness of aminoguanidine at inhibiting AGE formation *in vivo* in a wide array of systems and tissues (100-102). The limitation to aminoguanidine is that it cannot repair or undue previous AGE damage or crosslinking. Aminoguanidine is an Amadorin, which can only inhibit the formation of Amadori products.

A new class of anti-AGE agents that contain a thiazolium structure can chemically break α-dicarbonyl compounds (i.e. glyoxals, α-diketones) by cleaving the carbon-carbon bond between the carbonyls. For example, 4, 5 dimethyl-3-phenacylthiazolium chloride (DPTC) is a promising compound that reversed large artery

stiffness and crosslinking of tail collagen in streptozocin diabetic rats (1 mg/kg/day) after 1 to 3 weeks (103).

Inhibition of AGE formation with polyphenols

There are three mechanisms by which phenolics may inhibit glycation. They are antioxidants (blocking glucose autooxidation), metal chealation, or trapping reactive dicarbonyl compounds (Figure 1.3).

FIGURE 1.3 Schematic of Advanced Glycation Endproduct Formation

Glucose
(Reducing Sugar)

O_2^-, H_2O_2, HO

Glyoxal, Methylgloxal, Glucosone
(Reactive Dicarbonyl Products)

Protein Amino Group

Protein Amino Group

Glucosepane
Pentosidine
Carboxymethylysine
(AGEs)

N——Protein
‖
CH
|
CHOH
|
(CHOH)$_3$
|
CH$_2$OH

HN ——Protein
|
CH$_2$
|
CHOH
|
(CHOH)$_3$
|
CH$_2$OH

Oxidative Fragmentation
O_2^-, H_2O_2, HO
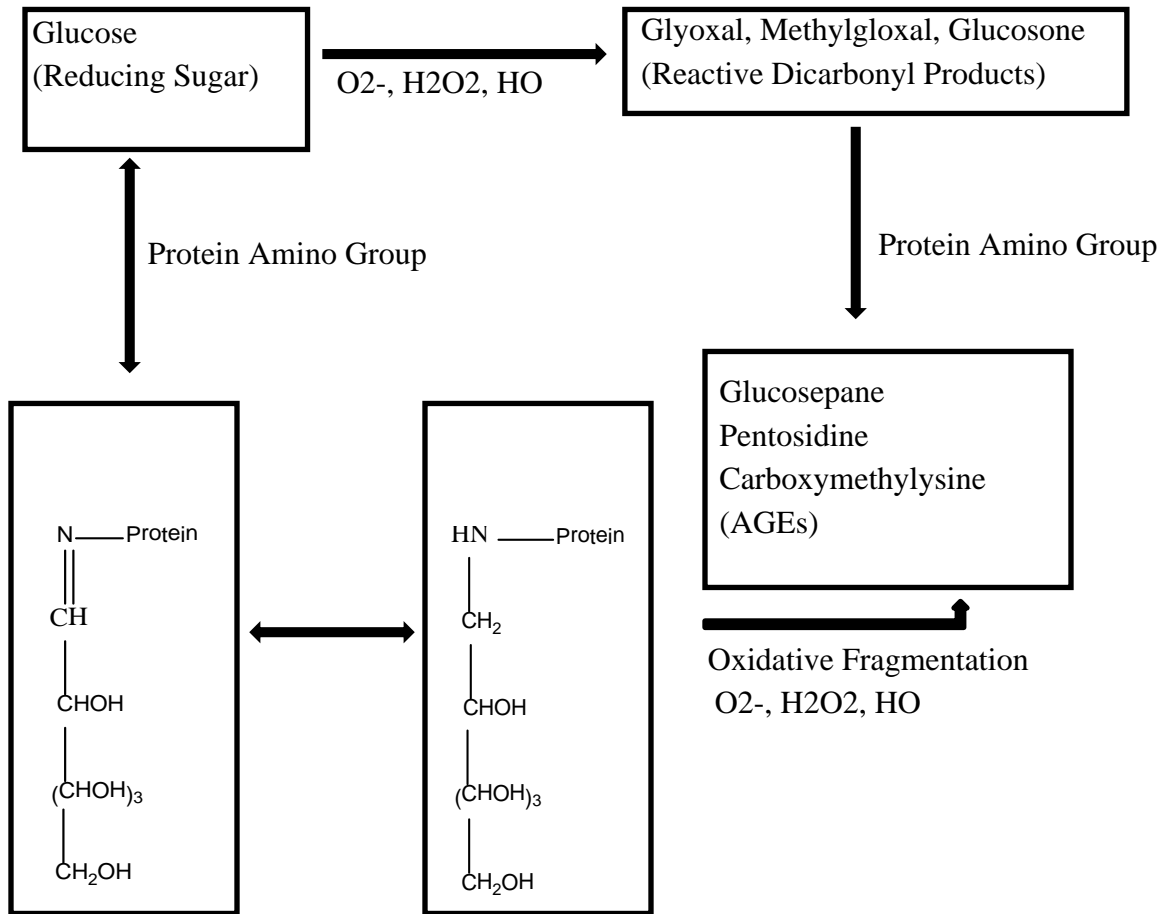
AGES tend to accumulate in proteins with a long half-life such as the proteins found in the neurological system. Epidemiological observations have linked flavonoid intake to a reduced risk of neurodegenerative disease (104).

Inhibition of AGE formation with pomegranate polyphenols

Verzelloni and coworkers (1) investigated the inhibitory activity of select colonic microbiota derived polyphenol catobolites against advanced glycation endproducts formation *in vitro* and their ability (at physiological concentrations) to counteract mild oxidative stress in cultured human neuronal cells. Three groups were tested for their inhibition against protein glycation: ellagitanin group (pyrogallol, urolithin A, urolithin B), coffee group (dihydro caffeic acid, dihydroferulic acid, and feruloylglycine), berry/red wine anthocyanin group (3-hydroxyphenylacetic acid, 3,4-dihydroxyphenyl acetic acid, 3-methoxy-4hydroxyphenylacetic acid). The ellagitannin group was extremely effective in protecting albumin from glycation at a concentration of 2μmol/L; reducing AGE formation by almost 50% compared to untreated albumin. Rout and Banerjee (105) explored the effect of a polysaccharide fraction from pomegranate rind on the glycation of BSA (10 mg/ml) in the presence of fructose and glucose (25 mM). The polysaccharide fraction from pomegranate rind inhibited AGE formation by 28% at a concentration of 10μg/ml.

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CHAPTER 2

INHIBITION OF NON-ENZYMATIC PROTEIN GLYCATION BY POMEGRANATE

(*PUNICA GRANATUM*) JUICE

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Abstract

Diabetes affects nearly eight percent of the United States population, and is the eighth leading cause of death according to the Centers for Disease Control and Prevention. Complications from this chronic disease encompass kidney failure, non-traumatic lower limb amputations, blindness, heart disease, and stroke. Elevated glucose levels generate non-enzymatic glycation of proteins and the formation of advanced glycation endproducts; this ultimately leads to the crosslinking of proteins which are thought to be responsible for diabetic complications. Since many antioxidants and polyphenolic compounds have been shown to inhibit protein glycation both in vitro and in vivo, our study sought to demonstrate the effect of commonly consumed ready to drink (RTD) juices (pomegranate, cranberry, black cherry, pineapple, apple, and Concord grape) on the fructose mediated glycation of albumin. Pomegranate juice exhibited the highest total phenolic content and antioxidant potential compared to the other RTD juices. The extent of albumin glycation after a 72 hour incubation of albumin (10 mg/ml) and fructose (250 mM) was ascertained by measuring fluorescence intensity at the wavelength pair of 370/440 nm. Albumin glycation decreased by 98% in the presence of 10 μl of pomegranate juice/ml while other RTD juices (cranberry, black cherry, pineapple, and concord grape) inhibited glycation by only 25%. Pomegranate juice produced the greatest inhibition of protein glycation when compared to other RTD juices incubated at the same phenolic concentration and the same antioxidant potential (FRAP units). Major phenolic constituents of pomegranate fruit, punicalagin and ellagic acid, significantly inhibited the glycation of albumin by 92 and 94%, respectively, at 5μg GAE/ml. These results demonstrate that pomegranate juice and two of its major phenolic constituents are potent inhibitors of protein glycation mediated by fructose.

Introduction

Protein glycation, also known as the Maillard reaction, is a complex series of sequential and parallel steps that begin with the non-enzymatic binding of a reducing sugar or sugar derivative to an amine group of a protein (1). Molecular rearrangements (Schiff base formation and Amadori rearrangements) lead to the formation of advanced glycation endproducts (AGEs) and ultimately the crosslinking of proteins. This is clearly observed in proteins that are not readily recycled in the body such as collagen and crystallins in the lens of the eye. AGEs, extremely reactive compounds, increase in proteins with time; however this process is accelerated in diabetes (2). AGEs formation has now been recognized to participate in the pathogenesis of retinopathy (3), nephropathy (4), neuropathy (5), and atherosclerosis (6).

Long term consumption of foods that are rich in polyphenols can ameliorate or provide prevention against certain disease states (7, 8). One such fruit, the pomegranate, has been used for medicinal purposes since ancient times (9). The high phenolic content of the pomegranate is a natural defense against environmental stressors in the very arid regions of the world which include Iran, Afghanistan, and northern India. The antioxidant potential of pomegranate juice has been reported to exceed that of red wine and tea (two phenolically rich beverages), and phenolically rich ready to drink (RTD) juices (Concord grape, blueberry, black cherry, acaí, and cranberry) (10). Pomegranate juice contains significant amounts of hydrolyzable ellagitannins, gallotannins, ellagic acid and various flavonoids (11). Hydrolyzable tannins account for more than 90% of the antioxidant potential of pomegranate fruit; the major phytochemical contributor is punicalagin (12). In recent years, studies have documented that punicalagin has significant anti-inflammatory, antiproliforative, and apoptotic properties (13). Punicalagin and other

hydrolyzable tannins originate from the peel of pomegranate fruit, and are found with ellagic acid, in commercially available juices (14). This would suggest that the juicing of pomegranates with hydrostatic pressure leaches hydrolyzable tannins from the pomegranate peel.

Numerous polyphenolic compounds have been shown to inhibit non-enzymatic glycation of proteins (15-19) both *in vitro* and *in vivo*. In this study, we investigated the effect of pomegranate juice and its major phenolics (punicalagin and ellagic acid) on the formation of AGEs after incubation of bovine serum albumin with fructose. The inhibition observed by pomegranate juice was compared to other commonly consumed ready to drink (RTD) juices.

Materials and Methods

Materials

Bovine serum albumin (essentially fatty acid free), D-(-) fructose, Chelex 100 (sodium form), Folin-Ciocalteu reagent, TPTZ (2, 4, 6-tri[2-pyridyl]-s-triazine, ferrous sulfate heptahydrate, ellagic acid, anhydrous ferric chloride, and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO). Punicalagin was obtained from Chengdu Biopurify Phytochemicals Ltd (Chengdu, Sichuan China). The RTD juices, Organic Apple (R.W. Knudsen), Black Cherry (R.W. Knudsen), Concord Grape (R.W. Knudsen), Organic Cranberry (Lakewood), Organic Pineapple (Lakewood), and 100% Pomegranate Juice (POM Wonderful), were purchased locally from an Earth Fare Supermarket. Laemmli sample buffer, Bio-Safe Coomassie blue, and Criterion precast gels (4-15% in Tris HCl, pH 8.6) were purchased from Bio-Rad (Hercules, CA).

Total Phenolic Content

The total phenolic content for all RTD juices was determined by the Folin-Ciocalteu method as described by Slinkard and coworkers (20), utilizing gallic acid as a standard. Briefly, gallic acid standards (20 µl) and each RTD juice (20 µl) was combined with distilled water (1580 µl), Folin Ciocalteu reagent (100 µl), and 300µl of sodium carbonate (1.6M). Distilled water (20 µl) was used as a blank. This mixture was allowed to incubate for 45 minutes at room temperature, after which absorbance was read at 765 nm on a Beckman DU 600 series spectrophotometer. The results are expressed as gallic acid equivalents (GAE/ml).

Ferric Reducing Antioxidant Potential (FRAP) Assay

The Ferric Reducing Antioxidant Potential of all RTD juices was determined by a modified method described by Benzie and coworkers (21), where iron (II) sulfate heptahydrate was the standard. Briefly iron (II) sulfate heptahydrate standards (10 µl) and each RTD juice (10 µl) were combined with distilled water (30 µl), and 300 µl of reagent (25 ml acetate buffer (300 mM, pH 3.6), 25 ml of 10 mM TPTZ solution, and 2.5 ml of 20 mM ferric chloride solution). Distilled water (10 µl) was used as a blank. The mixture was allowed to incubate for six minutes after which each sample was combined with distilled water (340 µl). Absorbance was read at 593nm on a Beckman DU 600 series spectrophotometer. The results are expressed as mM $FeSO_4$ equivalents/ml.

Modification of Albumin by Fructose

The glycation of bovine serum albumin was determined by a method described by Farrar and coworkers (22). Bovine serum albumin (10 mg/ml) was incubated in the presence of D-(-) fructose (250 mM) and various concentrations of RTD juices, ellagic

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acid, and punicalagin in 200 mM potassium phosphate buffer (pH 7.4) at 37ºC for 72 hours. Potassium phosphate buffer was treated with Chelex 100 prior to use. Ellagic acid and punicalagin were dissolved in 99.5% H_2O/0.5% 1N NaOH and 50% EtOH respectively. All samples were corrected for the native fluorescence of incubated albumin with RTD juices, ellagic acid or punicalagin. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using a Perkin-Elmer LS 55 Luminescence Spectrometer with slit widths set at 3nm.

The fluorescence emission spectra of the albumin/fructose solution incubated for 72 hours in the absence and presence of pomegranate juice was determined by setting the excitation wavelength at 370nm. The emission spectra was scanned from 380 to 550 nm with the slit width set at 3nm.

Modification of Albumin by Methylglyoxal

The glycation of bovine serum albumin by methylglyoxal was performed using a method described by Lee and coworkers (23). Bovine serum albumin (100 μM) was incubated in the presence of methylglyoxal (1mM) in Chelex 100 treated 0.1 M sodium phosphate, pH 7.0. After 96 hours, the fluorescence was measured at a wavelength pair of 350/409 nm. All samples were corrected for the native fluorescence of albumin incubated with pomegranate juice.

Analysis of Protein Modifications

Bovine serum albumin (10 mg/ml) was incubated in the presence of D-(-) fructose (250 mM), in the presence and absence of pomegranate and apple juice (2.5 and 5 μg GAE/ml) at 37ºC for 14 days. In this experiment 10μl of apple and pomegranate juice were added to the incubations every 3 days. Protein modifications induced by protein

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glycation were assessed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) (24). Aliquots for each sample were diluted 1:1 with Laemmli sample buffer containing 62.5mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (v/v) Bromophenol Blue, and 5% (v/v) 2-mercaptoethanol. Samples were boiled at 100ºC for five minutes after dilution and then centrifuged. Centrifuged samples were loaded onto a 15% resolving polyacrylamide gel with a 4 % stacking polyacrylamide gel and subjected to electrophoresis for 45 minutes. Gels were stained with Bio-safe Coomassie Blue for one hour and de-stained with distilled water. All gels were scanned on a FluorChem HD2 system with Alpha View software.

Statistical Analysis

Experiments were performed in triplicate and expressed as mean ± SEM. Data was analyzed utilizing a one-way analysis of variance (ANOVA) and multiple comparisons were performed employing Tukey's test. Statistical significance was set at $p < 0.05$.

<u>Results</u>

Locally purchased RTD fruit juices were initially tested for their phenolic content employing the Folin Ciocalteu assay (20). Pomegranate juice contained the highest concentration of total phenols (Table 1), nearly 4 mg GAE/ml, which is in agreement with the results of Aviram and coworkers (25). Concord grape and black cherry juice contained the second and third highest concentration of total phenols. The RTD juices were also analyzed for their antioxidant capacity as determined by the FRAP assay (Table 1). Pomegranate juice had the highest antioxidant capacity of all the RTD juices examined, approximately 3 times greater than the next highest RTD juice. Similar to that

observed with the content of phenolic compounds, Concord grape and black cherry juice possessed the second and third highest antioxidant capacity. These results are in agreement with Seeram and coworkers (10). It is interesting to note that while pomegranate juice contained approximately five times the phenolic content of apple juice, the FRAP values of pomegranate juice were nearly twenty times greater in comparison to apple juice.

To examine the effect of the RTD juices on protein glycation, each juice (10 μl/ml) was incubated with a solution containing bovine serum albumin (10 mg/ml) and fructose (250 mM). After 72 hours, the control incubation of BSA and fructose in the absence of any RTD juice resulted in a significant increase in fluorescence (Figure 1). When BSA and fructose were incubated in the presence of the RTD juices (10 μl of juice/ml), pomegranate juice produced the greatest inhibition; the fluorescence intensity observed in the presence of POM juice was 2% of control. Black cherry, concord grape, cranberry, and pineapple juice all resulted in a decrease in fluorescence of approximately 20%. The least effective inhibitor of protein glycation was apple juice.

The emission spectrum of the albumin/fructose solution after 72 hour incubation in the absence of any RTD juice is shown in Figure 2. With the excitation set at 370 nm, a broad fluorescence spectra is observed having an emission maximum at 440 nm. When pomegranate juice was present at a concentration of 10 μl/ml during the course of the 72 hour incubation, the resulting emission spectrum resembles a plateau with no major emission maximum observed at 440 nm, the emission spectrum is quite similar to spectrum observed when albumin is incubated with pomegranate juice (Figure 2). These

results demonstrate the near absence of fluorescent AGE products in the presence of pomegranate juice.

The superior inhibition observed with pomegranate juice on protein glycation when performed on the basis of volume was not unexpected due to the fact it contains the highest concentration of phenolic compounds (Table 1). To further examine the relative inhibition of RTD juices on protein glycation, the effect of RTD juices was studied when the juices were present at the same phenolic concentration. In this experiment, the phenolic content for each juice was normalized to 5 μg GAE/ml of the incubation mixture. When pomegranate juice was incubated at this concentration, it still provided the greatest inhibition of protein glycation; the decrease in fluorescence intensity was approximately 90% (Figure 3). Pineapple yielded the next greatest decrease in fluorescence intensity (66%). The other RTD juices produced lesser levels of inhibition. The resulting inhibition pattern among RTD juices in this *in vitro* assay varies greatly, and demonstrates that not all phenolic compounds inhibit glycation equally. The phenols present in pomegranate juice are clearly the most potent inhibitors when compared to the other RTD juices (apple, cherry, concord grape, cranberry, pineapple).

To further characterize the relative uniqueness of pomegranate juice inhibition of protein glycation, an experiment was performed in which the different RTD juices were added to the albumin/fructose mixture at a single antioxidant capacity value based on the FRAP assay. In this experiment, the RTD juices were added at a concentration of 0.20 mmolFeSO$_4$ equivalents/ml (Figure 4). Under these conditions, pomegranate juice still produced the greatest decrease in fluorescence intensity (98%); black cherry juice was the second best inhibitor with an approximately 60% decrease in fluorescence intensity.

These results demonstrate that the degree of inhibition of protein glycation among these juices can vary significantly even when they are present at the same antioxidant capacity.

Pomegranate juice, prepared by squeezing the whole fruit, is rich in hydrolyzable tannins, namely punicalagin, the major ellagitannin found in the commercial pomegranate fruit juices (14). The concentration dependent inhibition of protein glycation by punicalagin is shown in Figure 5. Similar to that seen with pomegranate juice, punicalagin was an extremely effective inhibitor at a concentration of 5μg/ml. Both at 5μg/ml and at 2.5μg/ml, the punicalagin inhibition of protein glycation was greater than 90%. At 1μg/ml, inhibition of protein glycation was not observed. Ellagic acid, a metabolite of ellagitannins (i.e. punicalagin), is formed after digestion (26); the data in Figure 6 demonstrates that ellagic acid inhibits protein glycation to the same extent as punicalagin.

Methylglyoxal is a reactive α-dicarbonyl which is formed from the auto-oxidation of glucose. Reactive carbonyls have the ability to bind with amino groups of proteins to form AGEs directly. When methylglyoxal was incubated with bovine serum albumin for 96 hours, a dramatic inhibition of fluorescence intensity was not observed by pomegranate juice at phenolic concentrations of 5 and 10 μg GAE/ml (Figure 7). These results suggest that pomegranate juice does not appear to be an effective inhibitor of glycation mediated by reactive α-dicarbonyls such as methylgloxal.

The effect of pomegranate juice was also examined (Figure 8) on protein modifications. Albumin (10mg/ml) incubated in potassium phosphate buffer for 14 days (column A) resulted in a distinct protein band at 65KDa. Albumin (10mg/ml) incubated

in the presence of fructose (250mM) for 14 days (column B) had a noticeable widening of the albumin band. These results are similar to those of Yamagishi and coworkers (27) who observed a widening of the BSA band incubated in the presence of glucose. Albumin incubated in the presence of fructose and apple juice (5µg GAE/ml) (column C) resulted in the same band widening as column B. Interestingly, albumin incubated in the presence of fructose and 5µg GAE/ml pomegranate juice (column D) resulted in a sharp 65 KDa band, similar to that of albumin incubated in the absence of fructose (column A). Thus, pomegranate juice prevented the band widening of albumin resulting from incubation with fructose. SDS-PAGE analysis revealed that pomegranate juice, and not apple juice, prevented protein modifications associated with albumin glycation.

Discussion

Glucose or fructose can react with amino groups of proteins to initiate the Maillard reaction forming a glycosylamine which degrades to a Schiff base. The Schiff base undergoes an Amadori rearrangement to form fructosamine (28), after which the degradation of fructosamine forms stable products known as AGEs. However, α-oxoaldehydes (carbonyls) formed from degraded glucose, fructose, or Schiff bases can react with amino groups of proteins to form AGEs directly; therefore AGEs can be formed in early or late stages of glycation. The mechanism of action of phenolic compounds on glycation is thought to be related to their antioxidant properties (29); they prevent the oxidation of Amadori products and the subsequent formation of AGEs, sometimes referred to as glycooxidation. Some polyphenols may inhibit the later stages of glycation by preventing the binding of dicarbonyls to protein amino groups (30). Verzelloni and coworkers (31) suggest that phenolic compounds may inhibit the

formation of Amadori products because of their specific binding to albumin. The data in this paper suggests pomegranate juice is an effective natural inhibitor of protein glycation independent of trapping dicarbonyl species for there was no effect on AGEs formed from α-oxoaldehydes (methylgloxal) (Figure 7). In addition, the inhibitory effects of pomegranate on glycation appears independent of protein binding of phenolic compounds; a similar pattern of inhibition by pomegranate juices was found in proteins other than albumin, such as gelatin and immunoglobulin G (data not shown).

In this study pomegranate juice had a higher phenolic content and antioxidant capacity when compared to other commonly consumed RTD juices (apple, cherry, Concord grape, cranberry, pineapple) (Table 1). However, when pomegranate juice and the other RTD juices were normalized on the basis of phenolic content (5 μg GAE/ml of mixture) (Figure 3) and antioxidant capacity (0.20 mmol $FeSO_4$/100ml) (Figure 4), pomegranate juice was superior to other RTD juices in inhibiting albumin glycation. This would suggest that pomegranate juice contains phenolic compounds that are more potent inhibitors of protein glycation than phenolic compounds found in other RTD juices. Yokozawa and cowokers (16) demonstrated that different phenols inhibit protein glcyation to varying degrees and similar studies have been performed to illustrate this concept (18). The major polyphenols present in each RTD juice examined in this study are listed in Table 2. Concord grape, black cherry and cranberry juice contain anthocyanins, while Concord grape, cranberry, and apple contain proanthocyanidins.

Matsuda and coworkers (29) examined the relationship between flavonoid structure, the inhibition of protein glycation, and radical scavenging properties of sixty-two flavonoids. Flavonoids with a strong scavenging activity (i.e. DPPH radical

scavenging) tended to be, with a few exceptions, robust inhibitors of AGE formation. In the current study this was observed when the RTD juices were added at the same volume (Figure 1).

A comparison of the anti-glycative activity of the fruit juices was also performed at the same antioxidative capacity (Figure 4). While significant inhibition was observed for all juices, pomegranate juice was the best inhibitor of glycation. These results agree with those of Kim and coworkers (32), who also correlated the inhibition of glycation and DPPH scavenging activity and found that compounds which scavenge free radicals to the same extent could have widely different capacity to inhibit glycation. Comparing the major polyphenolic groups listed in Table 2, ellagitannins are unique to pomegranate juice. The phenolic profile of pomegranate juice can be attributed to ellagic acid and hydrolyzable tannins mainly found in high concentrations in the peel of the fruit (33). A study conducted by Seeram and coworkers demonstrated that the antioxidant properties of punicalagin and ellagic acid are enhanced or provide a synergistic effect in combination with other polyphenols (i.e. pomegranate juice) (13). In this study we tested the effect of punicalagin and ellagic acid (Figure 5 and 6) on the inhibition of albumin glycation. Punicalagin and ellagic acid were found to drastically inhibit protein glycation at 2.5 μg/ml (corresponding to 8 μM for ellagic acid). This would suggest that ellagic acid and hydrolyzable tannins such as punicalagin are key factors in pomegranate juice's ability to significantly inhibit protein glycation. Muthenna and coworkers (34) have recently demonstrated that ellagic acid is an excellent inhibitor of protein glycation. Verzelloni and coworkers (31) demonstrated that ellagic acid-derived catabolites

(urolithins A and B) are also effective in protecting albumin from glycation at a concentration of 10 μM.

At the present time, there are no FDA approved medications that directly inhibit the glycation of proteins in diabetes, with the subsequent slowing in the progression of diabetic complications. The only way to pharmacologically arrest protein glycation is to lower plasma glucose levels. Unfortunately, even with many drug therapy options, too many diabetics are unable to maintain plasma glucose concentrations at near normal levels; this leads to an elevated rate of glycation. For this reason, phenolic compounds appear to offer another approach to slow the glycation process. In 2003, Nagasawa and coworkers (35) illustrated this concept in a diabetic rat study. They studied the effect of G-rutin (0.2% of the diet) on the protein glycation in plasma and kidney in these animals. After one month, G-rutin treated rats had a much lower content of glycation products in the serum and kidney. However, in this experimental model, G-rutin did not significantly lower the elevated blood glucose levels found in the diabetic rats. Therefore, at least in this animal model of diabetes, addition of a phenolic compound to the diet suppressed the extent of glycation of tissue proteins independently of blood glucose concentration.

There have been two studies that determined the levels of hemoglobin A1c after pomegranate juice consumption in humans. Sumner and coworkers (36) studied the effect of three month pomegranate juice consumption on myocardial perfusion in twenty-six patients with coronary heart disease with normal levels of hemoglobin A1c. Rosenblat and coworkers (37) studied the effect of three month pomegranate juice consumption on the oxidative status in ten diabetic patients. In both studies, no effect of pomegranate juice consumption was found on serum blood glucose levels or hemoglobin A1c

concentrations. Unfortunately, based on the number of subjects, neither study was designed to determine if pomegranate juice would significantly lower serum glucose or hemoglobin A1c. It is interesting to note that consumption of pomegranate juice was not associated with significant increases in plasma blood glucose concentrations in either study.

In summary, pomegranate juice and its constituents were found to be effective inhibitors of protein glycation. Unlike many studies examining the effects of various phenolic compounds on protein glycation, pomegranate juice employed in these experiments is commercially available. Therefore, proper studies, employing a sufficient number of subjects, can be readily performed to ascertain whether the anti-glycative properties described in this communication is also observed in experimental animals with diabetes and in diabetic patients.

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Tables and Figures

Table 2.1: The total phenolic content and ferric reducing antioxidant potential of commonly consumed ready to drink juices.

Ready To Drink Juices	Total Phenolic Content (mg Gallic Acid/ml)	Ferric Reducing Antioxidant Potential (mmol $FeSO_4$/ml)
Apple (R. W. Knudsen)	0.78 ±.01	2.70 ±.01
Black Cherry (R.W. Kndusen)	1.58 ±.01	10.5 ±.1
Concord Grape (R. W. Knudsen)	2.13 ±.01	16.8 ±.1
Cranberry (Lakewood)	1.13 ±.01	8.74 ±.01
Pineapple (Lakewood)	0.66 ±.02	3.46 ±.01
Pomegranate (POM)	3.96 ±.01	45.9 ±.1

Table 2.2: Primary phenols present in each RTD juice.

| Ready To Drink Juices	Primary Phenols
Pomegranate	Ellagitannins and anthocyanins (14)
Concord Grape	Proanthocyanidins, anthocyanins (38)
Black Cherry	Anthocyanins, flavan-3-ols (39)
Cranberry	Proanthocyanidins, anthocyanins (40)
Apple	Polyphenolic acid, proanthocyanidins (41)
Pineapple	p-Coumaric acid (42)
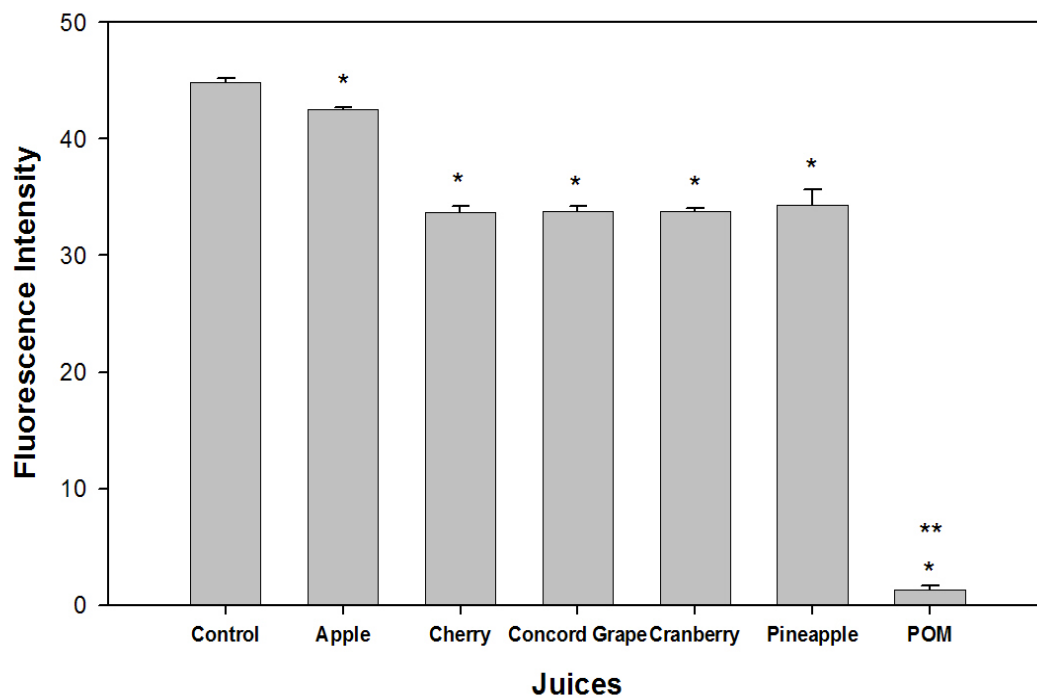
Figure 2.1: Inhibition of albumin glycation by RTD juices on the basis of volume. Albumin (10 mg/ml) and fructose (250 mM) were incubated with 10µl of RTD juices/ml for 72 hr at 37ºC. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *p< 0.05 when compared to control; **p< 0.05 when compared to other RTD juices
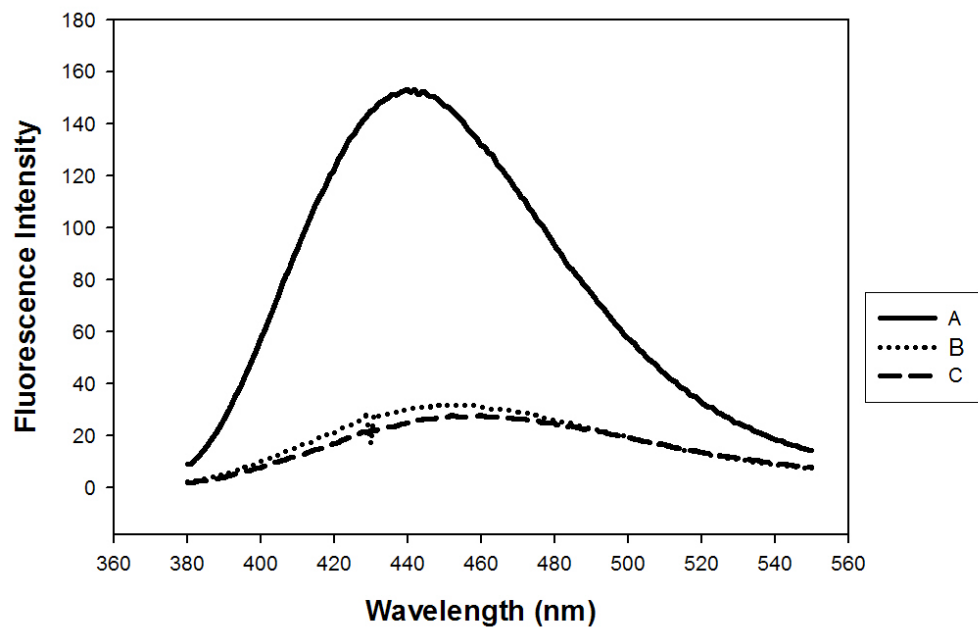
Figure 2.2: Emission spectra of 10 mg/ml albumin incubated with fructose (250 mM) for 72h (A), albumin incubated with fructose in the presence of POM juice (5 µg GAE/ml) (B), and albumin incubated in the presence of POM juice (C). The excitation wavelength was set at 370 nm.
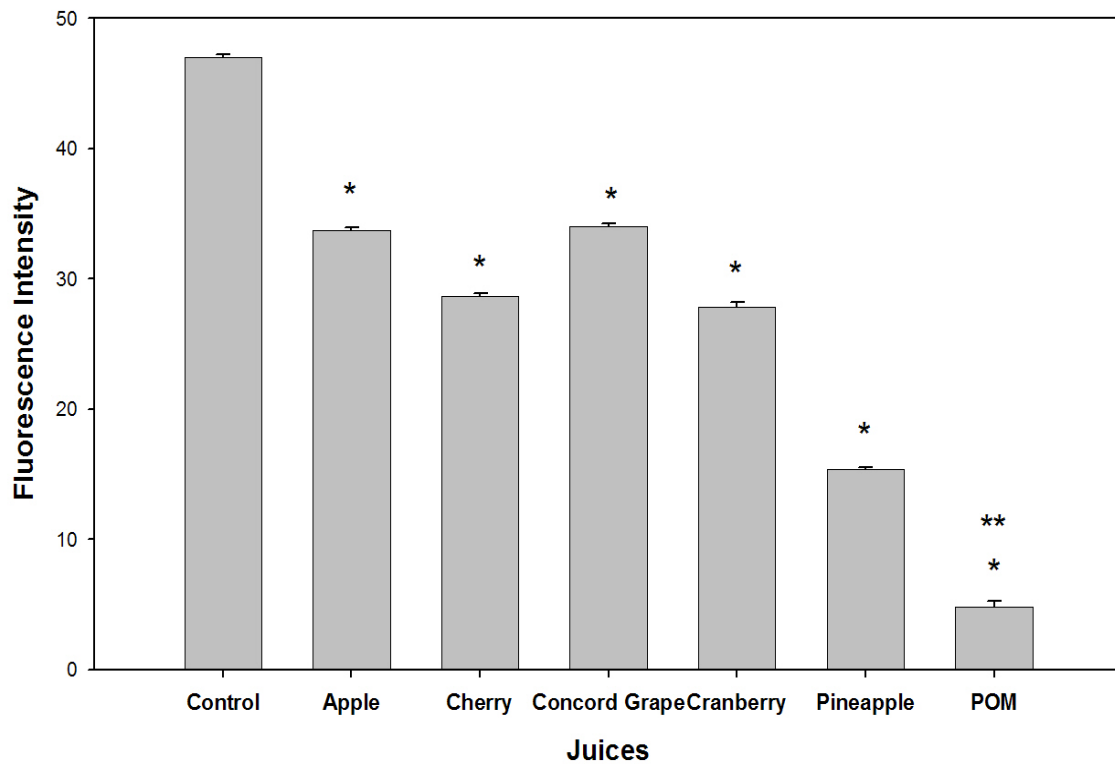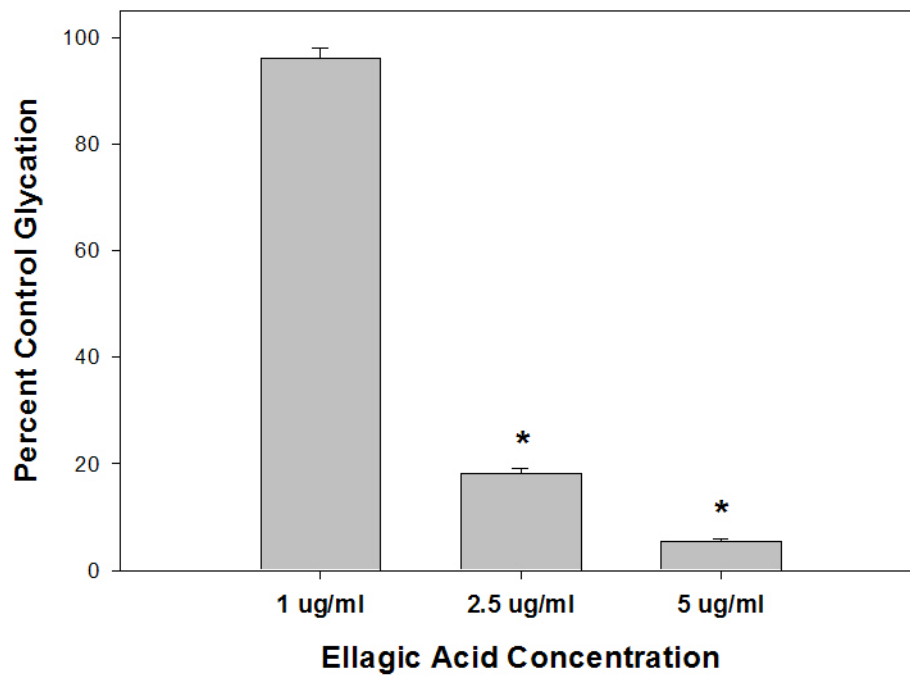
Figure 2.3: Inhibition of albumin glycation by RTD juices on the basis of phenolic content. Albumin (10 mg/ml) and fructose (250 mM) were incubated with RTD juices (5µg GAE/ml) for 72 h at 37ºC. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations.* p< 0.05 when compared to control; p< 0.05 when compared to other RTD juices**
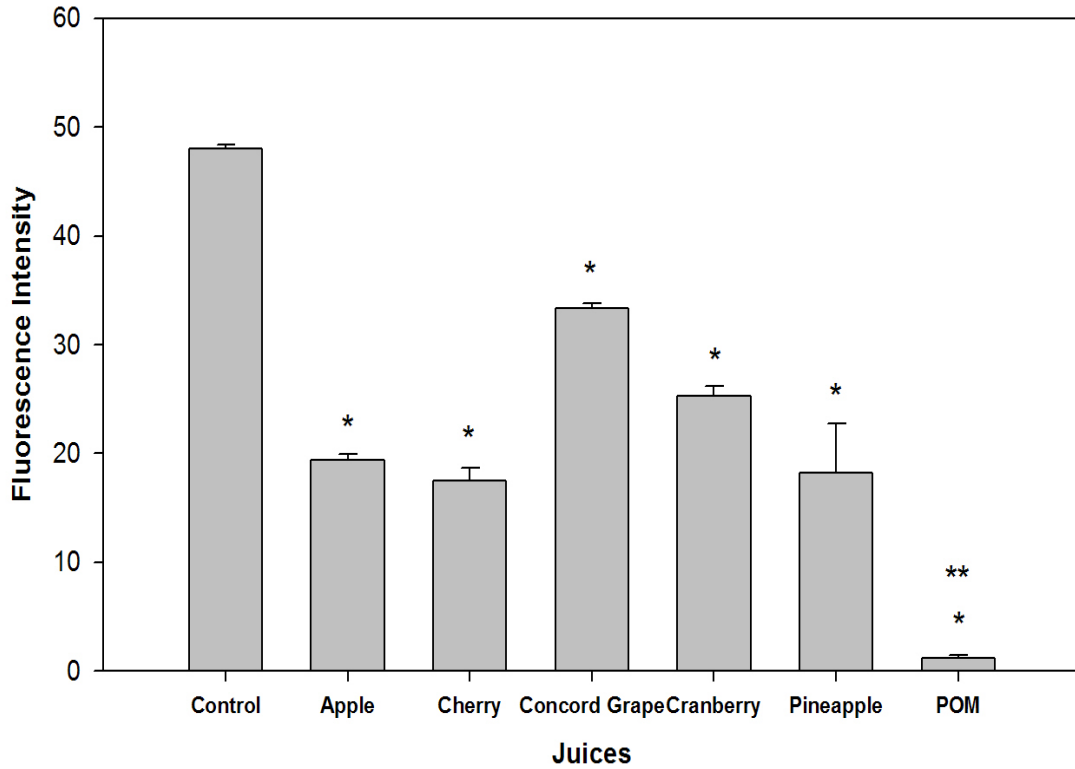
Figure 2.4: Inhibition of albumin glycation by RTD juices on the basis of FRAP values. Albumin (10 mg/ml) and fructose (250 mM) were incubated with RTD juices (0.20 mmol FeSO$_4$ equivalents/100 ml) for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *p< 0.05 when compared to control; **p<0.05 when compared to other RTD juices
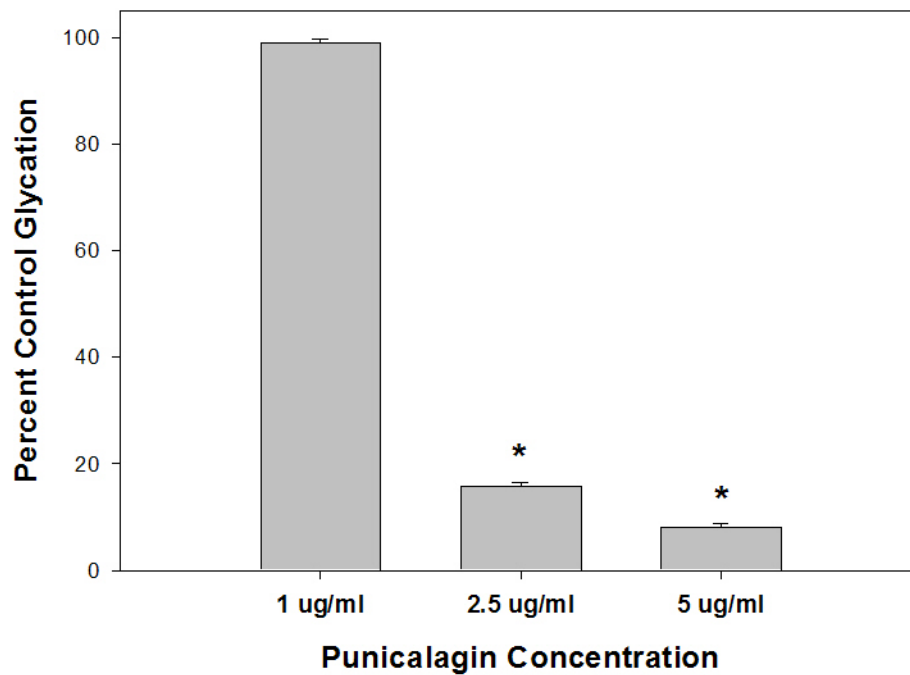
Figure 2.5: Effect of punicalagin on albumin glycation. Albumin (10 mg/ml) and fructose (250 mM) were incubated with punicalagin for 72h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *p< 0.05 when compared to control value

Figure 2.6: Inhibition of albumin glycation by ellagic acid. Albumin (10 mg/ml) and fructose (250 mM) were incubated with ellagic acid for 72h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *p< 0.05 when compared to control value
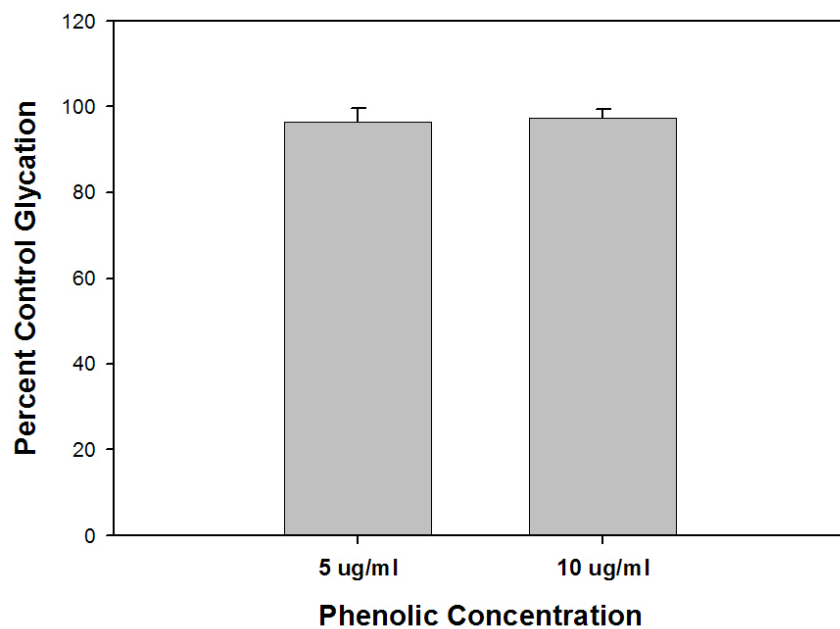
Figure 2.7: Effect of pomegranate juice on albumin glycation mediated by methylglyoxal. Albumin (100 μM) was incubated with methylglyoxal (1mM) and various concentrations of pomegranate juice for 96h at 37°C. The fluorescence intensity was measured at 350/409 nm. Results represent the mean ± SEM for triplicate determinations.
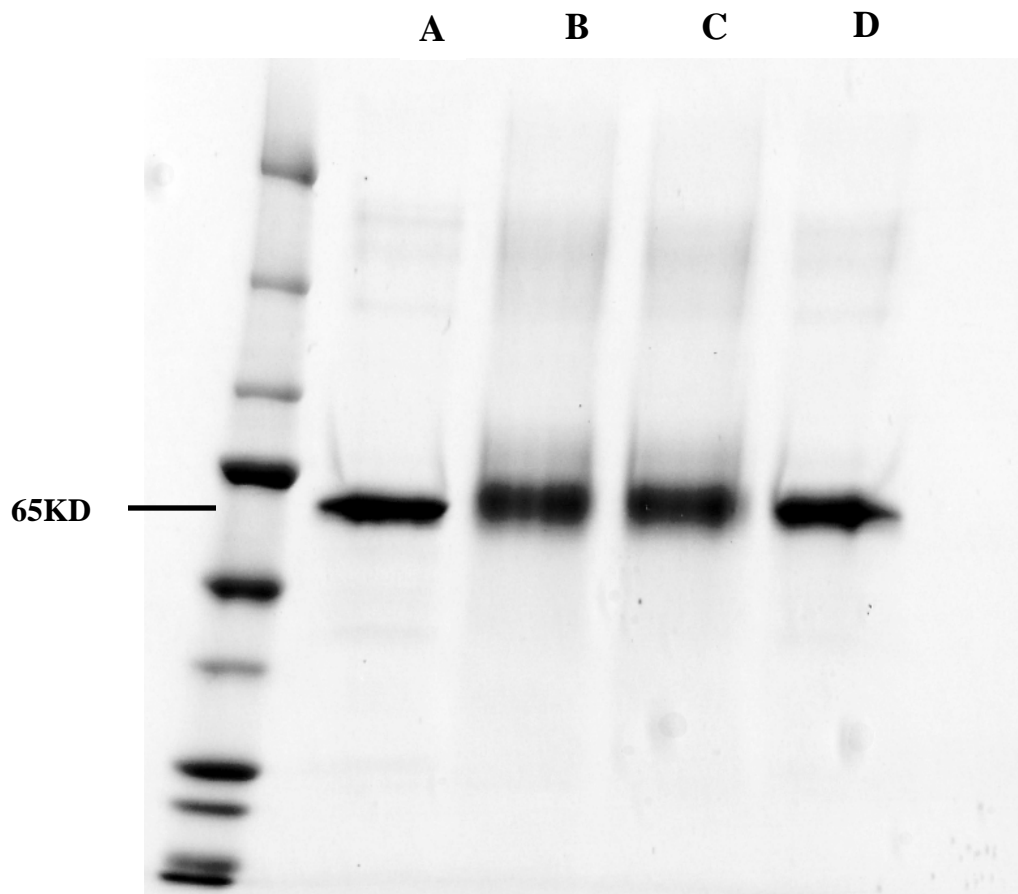
Figure 2.8: SDS PAGE profile of (A) 2µg BSA, (B) 2µg BSA incubated with fructose (250mM), (C) 2µg BSA incubated with fructose (250mM) and apple juice (5µg GAE/ml), and (D) 2µg of BSA incubated with fructose (250mM) and pomegranate juice (5 µg GAE/ml) for 14 days. Samples were loaded onto a 15% resolving polyacrylamide gel with a 4% stacking polyacrylamide gel. Staining was performed with Coomassie brilliant blue. Size markers are shown to the left.

CHAPTER 3

INHIBITION OF NON-ENZYMATIC PROTEIN GLYCATION BY WHOLE

POMEGRANATE (*PUNICA GRANATUM*) AND COMPONENTS OF THE PERICARP

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Abstract

The non-enzymatic glycation of proteins, an oxidative dependent process, initiates the formation of advanced glycation endproducts (AGEs) which eventually leads to the crosslinking of proteins. Over the past decade many studies have focused on the antioxidant potential of pomegranate fruit; however little work has been performed on the relationship between its antioxidant activity and ameliorating the diabetic state. This study investigates the effect of a phenolic whole pomegranate fruit extract and various fractions (aril, peel, and membrane) of pomegranate fruit on the *in vitro* fructose mediated glycation of albumin. Compared to apple, whole pomegranate fruit exhibited a much higher total phenolic content and antioxidant potential. Pomegranate fruit decreased glycation by 80% and 90% when incubated at a phenolic concentration of 2.5 and 5 μg GAE/ml respectively; apple, at these phenolic concentrations, inhibited protein glycation by only 20% and 40% respectively. Pomegranate membrane exhibited the highest total phenolic content and antioxidant potential compared to the pomegranate aril and peel. At 2.5 μg GAE/ml, the membrane fraction decreased glycation by 85% compared to the aril (42%), and peel (75%). Pomegranate membrane also produced the greatest decrease in glycation when these fractions were incubated at the same antioxidant capacities (FRAP values). These results demonstrate that pomegranate fruit is a potent inhibitor of fructose mediated albumin glycation when compared to whole apple. The inhibition observed is attributed to the presence of ellagitannins, which are not found in whole apple.

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<u>**Introduction**</u>

Complications stemming from diabetes mellitus such as neuropathy (1), nephropathy (2), and retinopathy (3) are initiated by the accumulation of advanced glycation endproducts (AGEs) in various tissues. AGEs are formed from the non-enzymatic binding of a protein, nucleic acid, or lipid to a reducing carbohydrate (4). Hyperglycemia is known to cause oxidative damage and an imbalance between reactive oxygen species and antioxidant detoxification pathways. Diabetic individuals are more susceptible to oxidative processes due to an increased production of reactive oxygen species (5), and a lower concentration of inherent antioxidants (vitamins C and E) (6). Many mechanisms are involved in hyperglycemia mediated oxidative stress including the oxidation of glucose, protein glycation, and the formation of AGEs; free radicals and oxidation reactions are involved in the glucose-mediated modification of proteins (7).

Diets rich in fruits and vegetables reduce the risk of cancer and other chronic diseases (8). Fruits and vegetables are also an important dietary source of polyphenols (9), and the typical human intake of flavonoids (a major group of polyphenols) is reported to be 1g per day (8). Diets rich in fruits and vegetables (5 or more servings a week), as demonstrated by Sargeant and coworkers (10), significantly reduced HbA1$_C$ levels in a cohort of over 5,000 adults. In the United States, apples (33.1%) are the largest contributors of polyphenols to the American diet followed by oranges (14.0%), grapes (12.8%), and strawberries (9.8%) (11). Polyphenols found in various botanical extracts (12-14) have been shown to inhibit the formation of AGEs both *in vitro* and *in vivo*; flavonoids have the greatest inhibitory effect among phenolics examined (15).

Pomegranate fruit is rich in polyphenols and found in the arid regions of the world. Ancient medicine has utilized the pomegranate to treat many maladies (16, 17),

however the health benefits of pomegranate stem from its anti-oxidative and anti-inflammatory properties (18,19). Previously we have shown that polyphenols found in commercially available pomegranate juice are superior inhibitors of fructose mediated protein glycation when compared to commonly consumed juices at the same phenolic content and antioxidant capacity (20). This suggests that the major polyphenols contained within the pomegranate are unique inhibitors of the glycation process. The major group of polyphenols, ellagitannins, is found in the peel and membrane of the fruit (21). During the juicing process, pomegranate is squeezed whole, leaching ellagitannins from the peel and membrane into the resultant juice. The current study examined the effect of extracts from whole pomegranate fruit and its various components (peel, membrane, arils) on the glycation of bovine serum albumin mediated by fructose. Apple was employed as a control fruit, for we previously demonstrated that pomegranate juice was superior to apple juice in inhibiting protein glycation (20).

Materials and Methods

Materials

Bovine serum albumin (essentially fatty acid free), D-(-) fructose, Chelex 100 (sodium form), Folin-Ciocalteu reagent, TPTZ (2, 4, 6-tri[2-pyridyl]-s-triazine, ferrous sulfate heptahydrate, anhydrous ferric chloride, and Amberlite XAD-16 were purchased from Sigma Chemical Company (St. Louis, MO). Pomegranates (POM Wonderful variety) and apples (Red Delicious) were purchased locally from Publix Supermarket.

Extraction of Pomegranate and its Pericarp Components

Pomegranates are characterized as large berries covered by a leathery exocarp (peel). A white, spongy, and very bitter tissue connected to the peel extends into the interior of the fruit (membrane). The membrane provides a matrix for edible seeds surrounded by a sack of juice (arils) (22). POM Wonderful pomegranates were cut into quarters (exposing the pericarp of the fruit), and placed in distilled water to facilitate the separation of the arils, membrane, and peel. Arils, membrane, peel, and the fruit as a whole were macerated in a Super 5000 Vitamix (Cleveland,OH) until a slurry was formed. The resultant slurries were subjected to a hot water extraction at 100ºC for two hours, after which they were vacuumed and filtered twice. A burette (20ml) packed with Amberlite XAD-16 was washed four times with deionized water, and the eluent was disposed of. The fruit slurries were loaded onto the burette and washed several times with deionized water, to achieve a Brix level of zero. The Brix level was measured with a pocket PAL-1 Atago pocket refractometer (Tokyo, Japan). Phenolic compounds were eluted from the Amberlite XAD-16 using methanol. The samples were placed on a A-210 Buchi rotovapor (Switzerland) at 200 MPa for one hour, after which they were freeze dried with a FreeZone 2.5 Labconco freeze dry system (Kansas City,MO). All sample extracts were reconstituted in 50% EtOH at a concentration of 1mg/ml.

Total Phenolic Content

The total phenolic content for all fruit extracts was determined by the Folin-Ciocalteu method as described by Slinkard and coworkers (23), utilizing gallic acid as a standard. Briefly, gallic acid standards (20 μl) and each fruit extract (20 μl) were combined with distilled water (1580 μl), Folin Ciocalteu reagent (100 μl), and 300 μl of

1.6 M sodium carbonate. Distilled water (20 µl) was used as a blank. The mixtures were incubated for 45 minutes at room temperature, after which absorbance was read at 765 nm on a Beckman DU 600 series spectrophotometer.

Ferric Reducing Antioxidant Potential (FRAP) Assay

The Ferric Reducing Antioxidant Potential of all fruit extracts was determined by a modified method described by Benzie and coworkers (24), where iron (II) sulfate heptahydrate was the standard. Briefly iron (II) sulfate heptahydrate standards (10 µl) and each fruit extract (10 µl) were combined with distilled water (30 µl), and 300 µl of reagent (25 ml of 300 mM acetate buffer (pH 3.6), 25 ml of TPTZ solution, and 2.5 ml of 20 mM ferric chloride solution). Distilled water (10 µl) was used as a blank. The mixtures were allowed to incubate for six minutes after which each sample was combined with distilled water (340 µl). Absorbance was read at 593nm on a Beckman DU 600 series spectrophotometer.

Modification of Albumin by Fructose

The glycation of bovine serum albumin was determined by a method described by Farrar and coworkers (25). Bovine serum albumin (10 mg/ml) was incubated in the presence of D-(-) fructose (250 mM) and various concentrations of the fruit extracts in 200 mM potassium phosphate buffer (pH 7.4) at 37℃ for 72 hours. Potassium phosphate buffer was treated with Chelex 100 prior to use. All samples were corrected for the fluorescence of albumin incubated with each fruit extract. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using a Perkin-Elmer LS 55 luminescence spectrometer with slit widths set at 3nm.

Modification of Albumin by Methylgloxal

The glycation of bovine serum albumin by methylgloxal was performed using a method described by Lee and coworkers (26). Bovine serum albumin (100 μM) was incubated in the presence of methylgloxal (1mM) in Chelex 100 treated 0.1 M sodium phosphate buffer, pH 7.0. After 96 hours, the fluorescence was measured at an excitation/emission wavelength pair of 350/409 nm using a Perkin-Elmer LS 55 luminescence spectrometer with slit widths set at 3 nm. All samples were corrected for the fluorescence of albumin incubated with each extract.

Statistical Analysis

Experiments were performed in triplicate and expressed as mean ± SEM. Data was analyzed utilizing a one-way analysis of variance (ANOVA) and multiple comparisons were performed employing Tukey's test. Statistical significance was set at $p < 0.05$.

<u>Results</u>

The phenolic fractions of pomegranates, the various components of pomegranates and apples were freeze dried and resuspended in 50% ethanol at a concentration of 1 mg/ml. Whole pomegranate fruit had a higher content of total phenols than whole apple (Table 1), which is in agreement with a study by Martin and coworkers (27). Whole apple and pomegranate fruit were also analyzed for their antioxidant capacity as determined by the FRAP assay (Table 1); pomegranate exhibited an antioxidant capacity that was approximately four times greater than that of apple.

Total phenolic content and antioxidant capacity for components of pomegranate's pericarp were analyzed. Peel and membrane extracts exhibited total phenolic content of

0.79 and 0.81 mg GAE/ml respectively, slightly greater than the phenolic content of whole fruit (Table 2). The aril extract had a much lower phenolic content, which is in agreement with work by Tzulker and coworkers (28).   It is noteworthy to mention that peel and membrane extracts exhibited an antioxidant capacity that was approximately three times greater than the aril extract (Table 2).

To examine the effect of pomegranates and apples on protein glycation, pomegranate and apple extracts (2.5 and 5 µg GAE/ml) were incubated with a solution containing bovine serum albumin (10 mg/ml) and fructose (250 mM). When the BSA/fructose mixture was incubated in the presence of whole pomegranate extract (2.5 µg GAE/ml), glycation was inhibited by approximately 80% (Figure 1), however the same concentration of apple phenolic compounds resulted in only a 20% decrease in control glycation. Increasing the concentration of whole pomegranate extract to 5 µg GAE/ml resulted in a further decrease in glycation to approximately 10% of control values. However, apple phenolics, at 5 µg GAE/ml did not produce the degree of inhibition observed with pomegranate phenolics at 2.5 µg GAE/ml. These results are in agreement with our previous findings on the effect of pomegranate and apple juices on protein glycation (20).

The effect of extracts from various components of the pomegranate pericarp (arils, peel, membrane) on protein glycation was also examined. In this study (Figure 2), the phenolic content for each component was normalized to 5 µg GAE/ml in the incubation mixture. At this concentration, all components inhibited protein glycation by over 80%; similar to that observed with the whole pomegranate extract. The membrane extract produced the greatest decrease in fluorescence intensity to approximately 5% of

control fluorescence. When the extracts from the various components were incubated at both 2.5 and 5 µg GAE/ml, the membrane extract again produced the greatest decrease in glycation followed by the peel extract (Figure 3).

The effect of each pomegranate component extract, set at two different antioxidant capacities, on protein glycation was then examined (Figure 4). At an antioxidant capacity of 0.04 mmol $FeSO_4$/100 ml, the membrane extract produced the greatest inhibition in glycation followed by the peel extract, with arils still showing significant inhibitory activity. At 0.05 mmol $FeSO_4$/ 100 ml all extracts inhibited glycation by over 90%, with the membrane extract still producing the greatest inhibition among the components. Combined with data presented in Tables 2 and 3, it is apparent that the type of phenols found in the peel and membrane of the fruit is the determining factor for the inhibition of protein glycation. Ellagitannins (the major phenolic group in pomegranate fruit) are mainly located in the peel and outer pericarp of pomegranate fruit (28) which includes the pomegranate membrane.

Reactive dicarbonyls, such as methylglyoxal, bind to proteins to form AGEs directly bypassing earlier stages of protein glycation (Schiff bases and Amadori products) (4). When pomegranate and apples extracts (2.5 and 5 µg GAE/ml) were incubated with albumin (100 µM) in the presence of methylgloxal (1mM), there was only a slight decrease in glycation observed (Figure 5). Pomegranate aril, peel, and membrane extracts were also examined for their effect on methylglyoxal mediated glycation (Figure 6). Only the membrane extract, at 5µg GAE/ml, significantly inhibited glycation. The extent of inhibition (approximately 20%) at 5 µg GAE/ml was quite weak compared to that observed with fructose mediated albumin glycation (Figure 2). These results demonstrate

that the dramatic inhibition of fructose mediated protein glycation by pomegranates is not observed in other pathways of protein glycation.

Discussion

There are numerous studies centered on the antiglycating activity of natural products; however there are few focused on the inhibition of this process by functional foods. The antioxidant and anti-inflammatory effects of pomegranate fruit are well documented but its effect on protein glycation has not been investigated. Previously we explored the effect of pomegranate juice and other commonly consumed juices (black cherry, concord grape, pineapple, apple, and cranberry) on the formation of AGEs (20). While all fruit juices inhibited protein glycation at a concentration of 1% (v/v), pomegranate juice was the best inhibitor of the group. Based on these findings, we explored the effect of the pomegranate fruit and the various components of its pericarp on the inhibition of protein glycation. The extract of whole pomegranate produced a greater inhibition of protein glycation when compared to whole apple extract (Figure 1). These results agree with our previous work (20); apple juice was much less effective in inhibiting protein glycation when compared to pomegranate juice. This is not the first report on the effect of the apple on protein glycation. Lavelli and coworkers (29) reported that dehydrated apple extracts were effective inhibitors of protein glycation. Based on molar concentration, procyanidin B2 was found to be the most effective phenolic inhibitor, followed by catechin and epicatechin.

In contrast, pomegranates and other berries (strawberries, blackberries, and raspberries) are phenolically rich in ellagitannins and ellagic acid (hydrolytic product of ellagitannins) (30). The highest concentrations of ellagitannins are found in the

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membrane and peel of the pomegranate (21) and these phenolic compounds provide the majority of the anitoxidative properties of the fruit. The peels of many different fruits contain a high concentration of antioxidants which provide protection against environmental stressors (31,32). In a similar matter, Yamaguchi and coworkers (33) incubated 10μM of garcinol (purified product from Garcina indica fruit rind) with BSA (20 mg/ml) and D-fructose (500 mM); glycation was inhibited by 50%. The findings in the current study in regards to the pomegranate peel and membrane agree with previous results where the major phenolics found in pomegranate, punicalagin (ellagitannin) and ellagic acid, inhibited protein glycation by over 90% at 2.5 μg GAE/ml (20). Thus, the rather high content of ellagitannins in pomegranate fruit appears to be responsible for the dramatic effect on protein glycation.

Shao and coworkers (32) documented that apple phenolics, phloretin and phloridzin were capable of trapping reactive dicarbonyl species such as methylglyoxal and glyoxal. Based on the results presented in Figure 5, the phenolics present in both pomegranates and apples did not appreciably inhibit methylgloxal mediated protein glycation at a concentration of 5 μg GAE/ml. Phloretin and phloridzin represent approximately 22% of the total major apple phenolics (33). The concentration of methylglyoxal in the current study mixture was 1mM, which is approximately 100 times the concentration of phenolic compounds. In contrast, Shao and coworkers (32) used nearly equivalent concentrations of dicarbonyl and phenolic species to demonstrate the carbonyl trapping properties of apple phenolics.

Hyperglycemia has been associated with the overproduction of the superoxide anion radical from the mitochondrial electron transport chain (34). These free radicals

damage many biomolecules (lipids, proteins, DNA), that can lead to many chronic diseases and diabetic complications. Flavonoids with a strong scavenging activity (antioxidant capacity) tend to have a greater inhibitory effect on AGE formation than weaker scavenging phenolics (35). However, as shown in Figure 4, different pomegranate fractions inhibit protein glycation to varying degrees even at the same antioxidant capacity. This would suggest that antioxidant capacity is not the sole factor in determining inhibition of AGE formation.

Pomegranate has been extolled for many years due to its medicinal properties. However, there are few studies that highlight the inhibitory properties of pomegranate fruit on protein glycation. A recent study involving a water soluble extract of pomegranate rind (10μg/ml) inhibited glucose and fructose mediated glycation of BSA by 28%; however vitamin C (10μg/ml) inhibited protein glycation by 41% (36). The extracts from the whole fruit and various components of the whole fruit (arils, peel, membrane) in the current study demonstrated inhibition of protein glycation at over 90% at 5 ug GAE/ml (Figure 3). The current study is the first to demonstrate the inhibitory properties of whole pomegranate fruit and various components of its pericarp on protein glycation.

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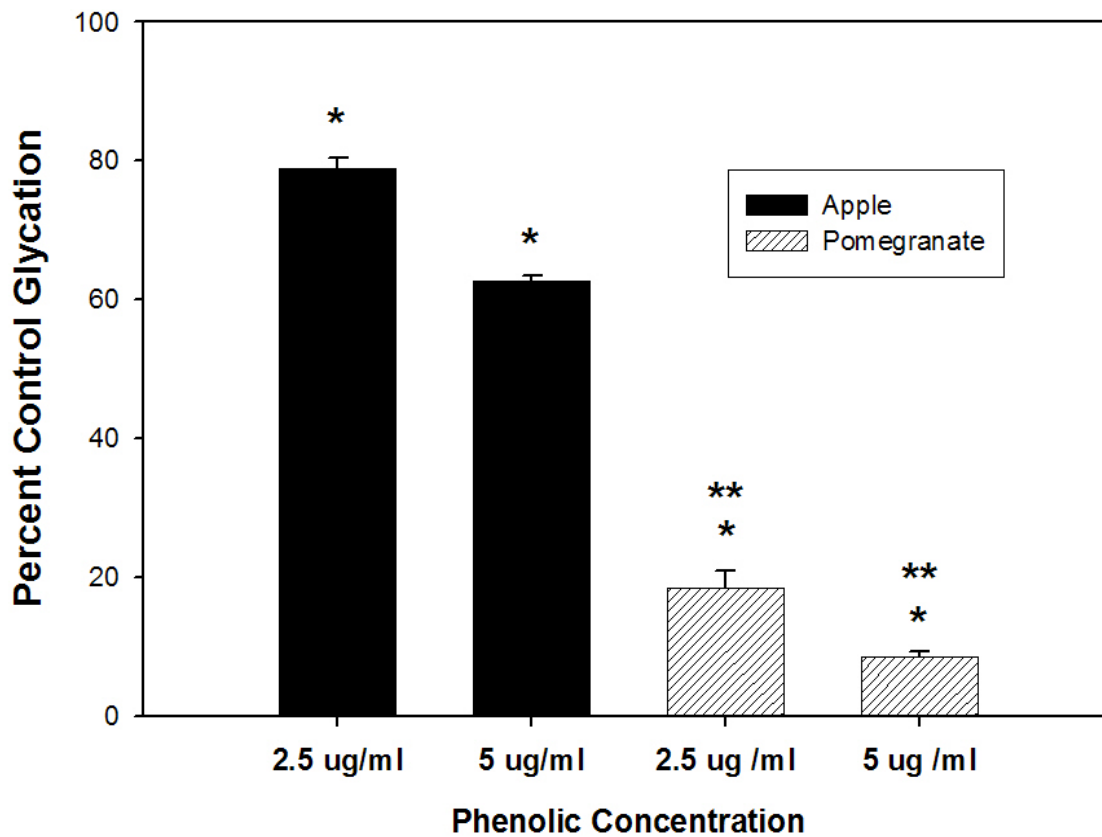
Tables and Figures

Table 3.2: The total phenolic content and ferric reducing antioxidant potential of whole pomegranate and apple fruit extracts.

Fruit	Total Phenolic Content (mg Gallic Acid/ml)	Ferric Reducing Antioxidant Potential (mmol FeSO$_4$/ml)
Pomegranate	0.60 ±0.01	13.8 ±0.1
Apple	0.39 ±0.01	3.6 ±0.1

Table 3.2: The total phenolic content and ferric reducing antioxidant potential of extracts of the components of the pericarp of the pomegranate.

| Pomegranate Components	Total Phenolic Content (mg Gallic Acid/ml)	Ferric Reducing Antioxidant Potential (mmol FeSO$_4$/ml)
Arils	0.39 ±0.01	5.0 ±0.1
Peel	0.79 ±0.02	14.9 ±0.1
Membrane	0.81 ±0.02	16.0 ±0.1

Figure 3.1: Inhibition of albumin glycation by whole pomegranate and apple. Albumin (10 mg/ml) and fructose (250 mM) were incubated with whole pomegranate and apple extracts at a concentration of 2.5 and 5 μg GAE/ml for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *P< 0.05 when compared to control values; **P< 0.05 when compared to the corresponding apple extract
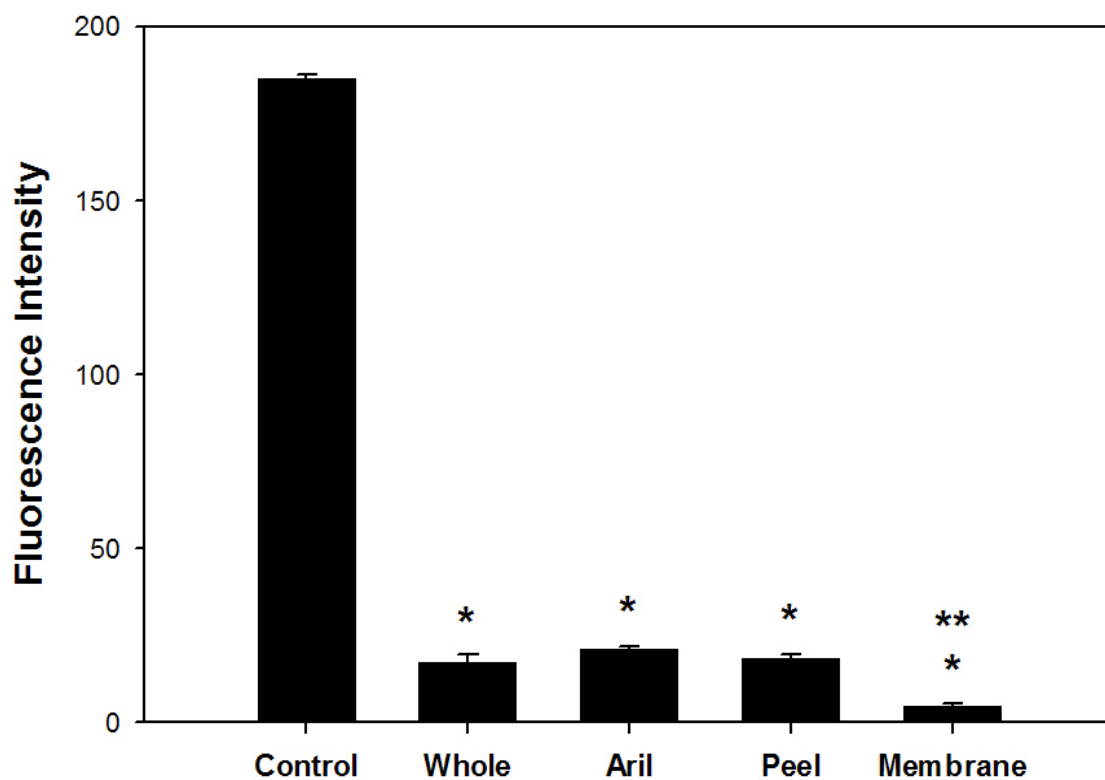
Figure 3.2: Inhibition of albumin glycation by whole pomegranate fruit and various components of the pericarp (aril, peel, membrane). Albumin (10 mg/ml and fructose (250 mM) were incubated with aril, peel, and membrane extracts at a concentration of 5 µg GAE/ml for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *P<0.05 when compared to control values; **P<0.05 when compared to other extracts
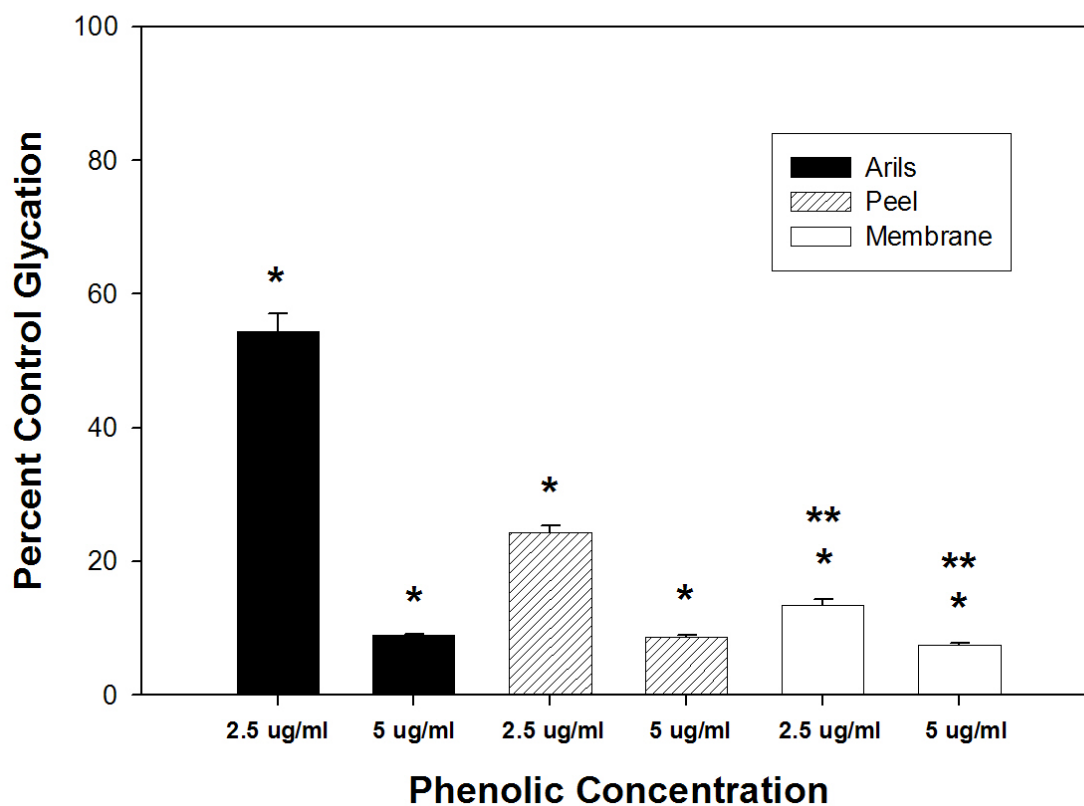
Figure 3.3: Inhibition of albumin glycation by components of the pomegranate pericarp (aril, peel, membrane). Albumin (10 mg/ml) and fructose (250 mM) were incubated with arils, peel, and membrane extracts at a concentration of 2.5 and 5 μg GAE/ml for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *P<0.05 when compared to control values; **P<0.05 when compared to corresponding aril and peel extracts
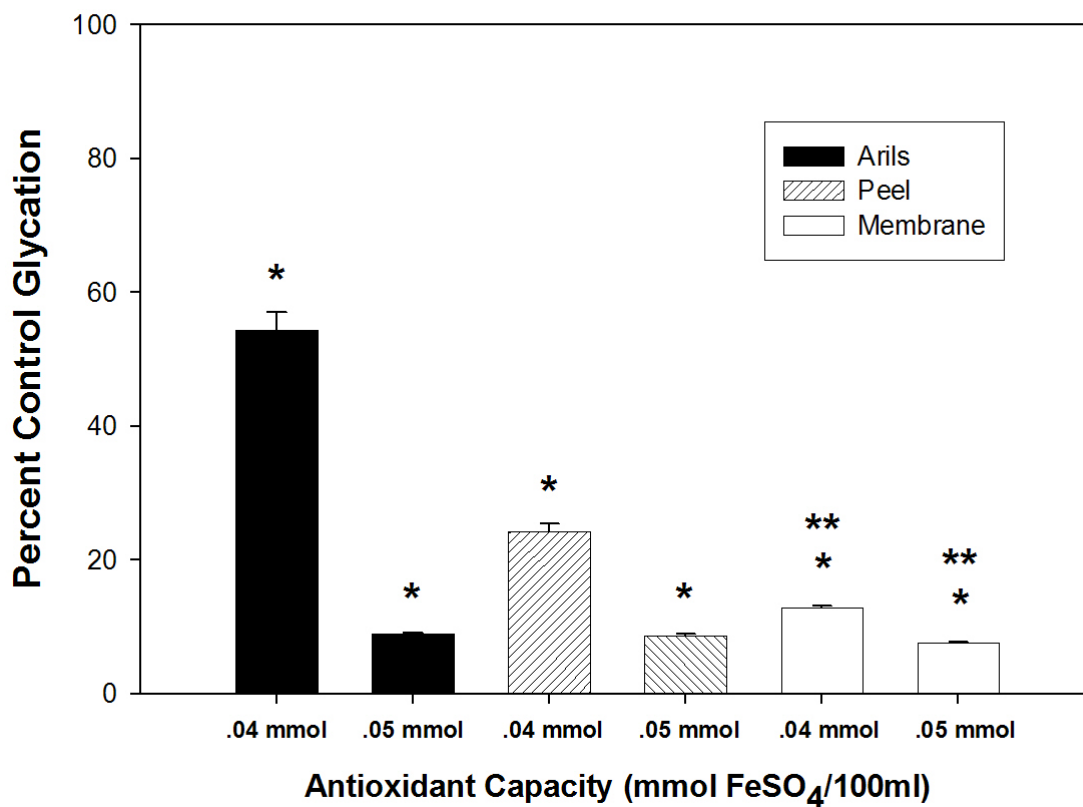
Figure 3.4: Inhibition of albumin glycation by components of the pomegranate pericarp (aril, peel, membrane). Albumin (10 mg/ml) and fructose (250 mM) were incubated with arils, peel, and membrane extracts at antioxidant capacities of 0.4 and 0.5 mmol FeSO$_4$/100 ml for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *P<0.05 when compared to control values; **P<0.05 when compared to corresponding aril and peel extracts
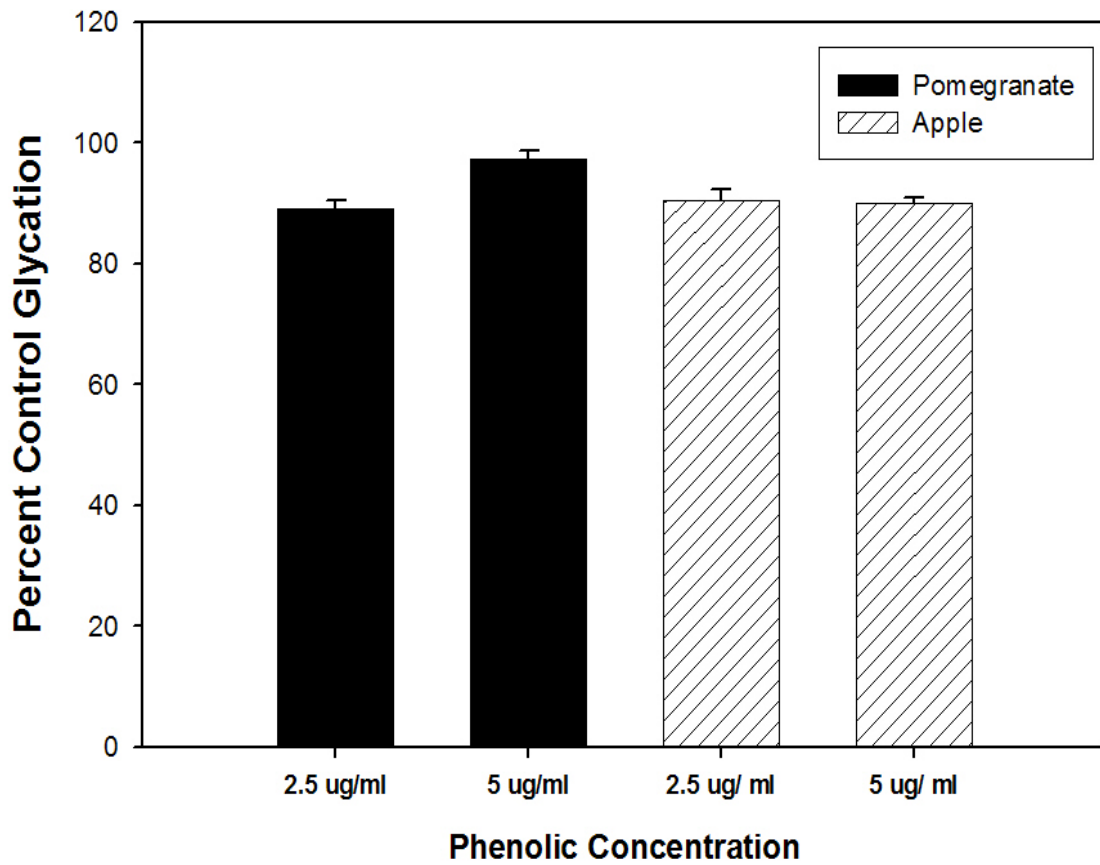
Figure 3.5: Effect of whole pomegranate and apple extract on methylglyoxal mediated albumin glycation. Albumin (100 µM) and methylgloxal (1mM) were incubated with pomegranate and apple extracts at a concentration of 2.5 and 5 µg GAE/ml for 96 h at 37°C. The fluorescence intensity was measured at 350/409 nm. Results represent the mean ± SEM for triplicate determinations.
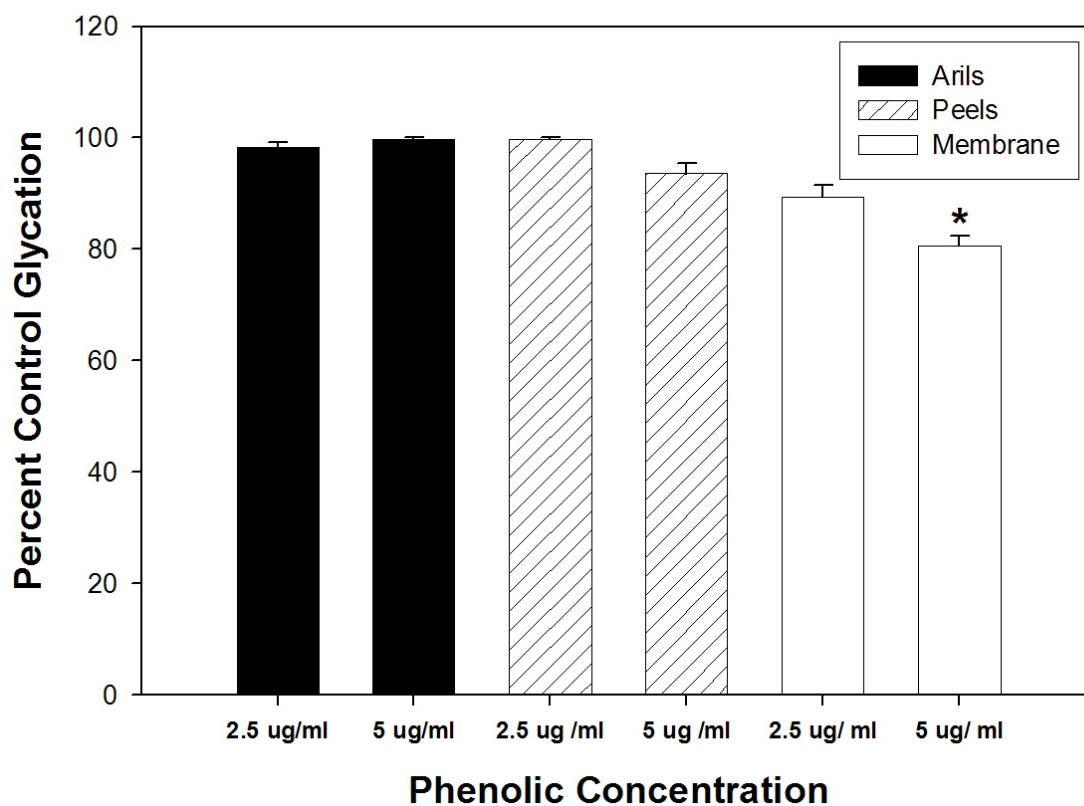
Figure 3.6: Effect of various components of the pomegranate pericarp on methylglyoxal mediated albumin glycation. Albumin (100 µM) and methylgloxal (1mM) were incubated with aril, peel, and membrane extracts at a concentration of 2.5 and 5 µg GAE/ml for 96 h at 37°C. The fluorescence intensity was measured at 350/409 nm. Results represent the mean ± SEM for triplicate determinations. *p<0.05 when compared to control values

CHAPTER 4

POMEGRANATE (*PUNICA GRANATUM*) JUICE: A NON-SPECIFIC INHIBITOR OF

PROTEIN GLYCATION

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<u>Abstract</u>

Oxygen drives the Maillard reaction between a reducing sugar and protein at physiological pH and temperature. This reaction yields fluorescent and non-fluorescent glycoxidation products that contribute to the chemical modification and crosslinking of proteins. The Maillard reaction is a key component in the development of diabetic complications. In previous studies we found that bovine serum albumin incubated in the presence of fructose yielded fluorescent glycoxidation products and pomegranate juice was highly effective in arresting the progression of this reaction. In the current study we sought to examine the effect of pomegranate juice on the *in-vitro* fructose mediated glycation of four different proteins (gelatin, IgG, lysozyme, and ribonuclease A). Pomegranate juice, at 2.5 and 5 µg GAE/ml, reduced the formation of fluorescent glycoxidation products by 87 and 92% respectively when gelatin was incubated in the presence of fructose. Pomegranate juice also produced a significant reduction in AGE formation when IgG was incubated in the presence of fructose. Lysozyme incubated in the presence of fructose did not yield an appreciable amount of fluorescent glycoxidation products, possibly due to lysozyme's lysine residue content. SDS-PAGE analysis revealed that ribonuclease A incubated in the presence of fructose resulted in the formation of many oligomeric products visible at different molecular weights. Pomegranate juice (5µg GAE/ml), when incubated with ribonuclease A and fructose, prevented the formation of oligomeric products. In contrast, apple juice at the same phenolic concentration did not alter the modifications to ribonuclease A observed in the presence of fructose. This work demonstrates that pomegranate juice is a natural and effective *in vitro* inhibitor of glycation of various mammalian proteins.

Introduction

Proteins in the body are exposed to many factors which can result in their modification; oxidation and glycation are two major non-enzymatic alterations that affect a protein's native structure (1). The alterations to these proteins are often cleared by receptors on neighboring cells; one important receptor is RAGE (receptor for advanced glycation endproduct). However, in people who suffer from chronic diseases (who are more susceptible to oxidation), these altered proteins may accumulate, leading to pathological conditions. In diabetes mellitus, these alterations are a hallmark for many diabetic complications such as retinopathy.

Albumin is the most abundant protein found in blood plasma with a concentration of 35 to 50g/l (2). Albumin's tertiary structure allows it to bind to small molecules such as metal ions, fatty acids, bilirubin, and various pharmaceutical agents (3). Due to its long half-life (21 days) and its high concentration in blood plasma, albumin is sensitive to non-enzymatic modification by carbohydrates. Many structural modifications occur as a result of the glycation of albumin including an increase in molecular weight. This increase in molecular weight can be attributed to one or several glucose units attached to certain amino acid residues such as arginine, lysine and cysteine and the eventual crosslinking of albumin molecules.

Modifications to the native protein structure are also correlated to changes in the binding properties of albumin (1). Nonetheless, there are many contradictory studies involving the binding affinity of modified albumin. One study indicated that the alteration of certain albumin binding sites by glycation causes a decrease in binding affinity for certain fatty acids (4). Conversely, in another study lengthy incubations (60

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days) of albumin in the presence of glucose (9 mol glucose/mol albumin) enhanced the binding of warfarin (5); however Okabe and coworkers (6) reported no effect on warfarin binding by human serum albumin glycated by glucose at a lower glucose concentration (2 mol glucose/mol albumin).

In previous studies (data not shown), we have demonstrated that pomegranate juice and certain parts of pomegranate fruit are effective inhibitors of the glycation of bovine serum albumin mediated by fructose. These inhibitory properties are attributed to ellagitannins and ellagic acid found within the membrane and peel of the fruit. Based on these data, we wanted to demonstrate that the inhibitory effect of pomegranate's major polyphenolics on albumin glycation is not unique to albumin, but also is observed with other model proteins. Glycation occurs to many proteins *in-vivo*, specifically those that have a long half-life such as collagen and laminin (7). Bovine serum albumin is commonly used to study *in vitro* protein glycation and it has an 80% structural similarity to human albumin (8). Other investigators (9,10) have employed other proteins to study glycation. In this study, gelatin, IgG, lysozyme, and ribonuclease A were incubated in the presence of fructose and the effect of pomegranate juice on glycoxidation was determined. Ribonuclease A was subjected to SDS-PAGE analysis to demonstrate the inhibition of glycative modifications to the native protein structure by pomegranate juice.

Materials and Methods

Materials

Bovine serum album (Fraction V, essentially fatty acid free), gelatin (bovine skin), ribonuclease A (bovine pancreas), lysozyme (chicken egg white), Chelex 100 (sodium form), and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO). Bovine IgG was purchased from Equitech-Bio (Kerville, TX). Laemmli

sample buffer, Bio-Safe Coomassie blue and Criterion precast gels (8-16% Tris HCl) were purchased from Bio-Rad (Hercules, CA). Apple juice (R.W. Knudsen) and pomegranate juice (POM Wonderful) were purchased locally from an Earthfare Supermarket.

Modification of Albumin by Fructose

A modified method described by Farrar and coworkers (11) was used to determine the degree of glycation for gelatin, IgG, and lysozyme. Gelatin, IgG, and lysozyme (2 mg/ml) were incubated in the presence of D-(-) fructose (125 mM) and pomegranate juice (2.5 and 5 μg GAE/ml) in 200 mM potassium phosphate buffer at 37ºC for 72 hours. The potassium phosphate buffer was treated with Chelex 100 prior to use. All incubation mixtures were corrected for the native fluorescence of incubated gelatin, IgG, and lysozyme, with pomegranate juice. The fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using a Perkin-Elmer LS 55 Luminescence Spectrophotometer with slit widths set at 3nm.

Analysis of Protein Modifications

Ribonuclease A (10mg/ml) and D-(-) fructose (250mM) were incubated in the presence and absence of pomegranate juice (5μg GAE/ml) and apple juice (5 μg GAE/ml) in 200 mM potassium phosphate buffer at 37ºC for 14 days. In this experiment 10μl of apple and pomegranate juice were added to the 3ml incubations every 3 days. Modifications induced by protein glycation were assessed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) (12). Aliquots for each sample were diluted 1:1 with Laemmli sample buffer containing 62.5mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (v/v) Bromophenol Blue, and 5% (v/v) 2-

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mercaptoethanol. Samples were boiled at 100ºC for five minutes after dilution and then centrifuged. Centrifuged samples were loaded onto a 16% resolving polyacrylamide gel with an 8 % stacking polyacrylamide gel and subjected to electrophoresis for 45 minutes. Gels were stained with Bio-safe Coomassie Blue for one hour and de-stained with distilled water. All gels were scanned on a FluorChem HD2 system with Alpha View software.

Statistical Analysis

Experiments were performed in triplicate and expressed as mean ± SEM. Data was analyzed utilizing a one-way analysis of variance (ANOVA) and multiple comparisons were performed employing Tukey's test. Statistical significance was set at $p < 0.05$.

<u>**Results**</u>

In previous studies, the Folin Ciocalteu method was used to determine the phenolic content of pomegranate juice (data not shown). The concentration of total phenols was approximately 4mg GAE/ml. On the basis of phenolic content we were able to determine that a normalized concentration of 5µg GAE/ml inhibited the formation of fluorescent glycoxidation products in albumin incubated in the presence of fructose by 90%.

Gelatin is a denatured protein that originates from the processing of collagen. *In vivo*, collagen is extremely sensitive to glycooxidation due to its long half-life, and is involved in the development of diabetic complications. In this study gelatin, (2mg/ml) was incubated in the presence of fructose (125 mM) for 72 hours at 37°C. Pomegranate juice was added to the reaction mixture at a normalized phenolic content of 2.5 and 5µg

GAE/ml (Figure 1). Pomegranate juice, at both 2.5 and 5µg GAE/ml, produced a dramatic inhibition of glycation, similar to that observed with bovine serum albumin (data not shown). These results demonstrate that pomegranate juice is a generalized inhibitor of glycation and this inhibition is not unique to bovine serum albumin.

IgG is the smallest antibody present in the human body, and it is soluble in aqueous solutions. Glycated IgG has been implicated in the pathogenesis of rheumatoid arthritis (13) and the progression of nephropathy (14). Figure 2 illustrates the concentration dependent inhibition of IgG glycation by pomegranate juice. Pomegranate juice, at 5µg GAE/ml, decreased the fluorescence intensity by 72%. The inhibition of glycation was approximately 50% when pomegranate juice was present at a concentration of 2.5 µg GAE/ml. These data illustrate that pomegranate juice is also an effective inhibitor of IgG glycation mediated by fructose.

Lysozyme, also known as muramidase, is an enzyme that can cleave the cell walls of bacteria and viruses (15). Ahmad and coworkers (10) demonstrated that lysozyme can be used to investigate glycation induced crosslinking due to the fact that it produces oligomers which are easily detected with SDS-PAGE analysis. In our study lysozyme incubated in the presence of fructose (125 mM) for 3 days did not yield the same increase in fluorescence intensity observed with albumin, gelatin or IgG (Figure 3). While the degree of glycation in lysozyme was approximately one-fifth of the other proteins observed, pomegranate juice, at 2.5 and 5µg GAE/ml, still produced a decrease of fluorescence intensity in comparison to the control incubations.

According to Eble and coworkers (16), ribonuclease is an excellent model protein for studying hexose induced crosslinking because it possesses many reactive lysl residues which form oligomers. The occurrence of crosslinked RNase oligomers can easily be determined by SDS-PAGE analysis (17). The current study examined the effect of pomegranate juice on fructose mediated protein modifications of RNase utilizing SDS-PAGE analysis (Figure 4). The effect of apple juice on the modification of RNAase by fructose was also examined; we previously demonstrated that apple juice, at the same phenolic concentration as pomegranate juice, was a less effective inhibitor of glycation than pomegranate juice. RNase (10mg/ml) incubated in potassium phosphate buffer for 14 days (column A) yielded a distinct protein band at 14 KDa. RNase (10 mg/ml) incubated in the presence of fructose (250 mM) for 14 days (column B) resulted in the broading of the protein band at 14 KDa. In contrast to the native protein, two lightly stained bands at 32 and 45 KDa respectively were apparent after RNase was incubated with fructose. When RNase (10 mg/ml) was incubated in the presence of fructose (250 mM) and pomegranate juice (5 μg GAE/ml) (column C), SDS Page profile was quite similar to native RNAase; there was no broading of the 14 KDa band and no evidence of bands at 32 and 45KDa. In contrast, RNase incubated in the presence of fructose and apple juice (5 μg GAE/ml) resulted in a broadening of the protein band at 14 KDa and two lightly stained bands at 32 and 45KDa. The SDS-PAGE analysis demonstrates that pomegranate juice, and not apple juice, is an effective inhibitor of modifications to RNase mediated by fructose.

Discussion

Albumin is the model protein to illustrate *in vitro* protein glycation because it contains reactive lysine residues which are instrumental in producing vesperlysine (18), a type of fluorescent AGE. In previous studies we have measured the formation of fluorescent AGEs, such as vesperlysine, by incubating albumin in the presence of fructose and measuring fluorescence at the excitation/emission wavelength pair of 370/440 nm. The glycation of albumin is also associated with tertiary structure modifications, that involve unfolding of the protein at tryptophan residues 134 and 214 (19,20). Conformational changes resulting from glycation can yield new binding sites for certain ligands (1), and can impair the affinity of diazepam, sulfisoxazole, phenytoin, cyclosporine, and valproic acid binding to albumin (21). The resulting conformational changes in proteins with a long half-life (i.e. collagen) and eventual cross-linking of these proteins can result in altered protein function and free radical formation.

Our past studies have demonstrated that pomegranate juice can prevent the non-enzymatic glycation of bovine serum albumin (data not shown). We illustrated that major phenolic compounds present in pomegranate juice (punicalagin and ellagic acid) were effective inhibitors of albumin glycation. However, the purpose of this current study is to demonstrate that AGE inhibition by pomegranate and pomegranate juice was not unique to albumin, perhaps because of a critical and unique binding site of these phenolics to the protein. Rather ellagitannins and ellagic acid are effective generalized inhibitors of glycation and that this inhibition would be observed in a wide variety of proteins, with the subsequent prevention of modifications to the native protein structure.

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In the current study, pomegranate juice produced a dose dependent inhibition of fluorescent AGE formation in three differents proteins (Figures 1-3) and the results are similar to that observed previously with bovine serum albumin (data not shown). The data suggests that the phenolic compounds within pomegranate juice are effective inhibitors of Schiff base formation (early stage glycation). In previous studies pomegranate juice provided very little protection against Amadori product formation (late stage glycation) mediated by an α-dicarbonyl (methylglyoxal). While pomegranate juice consumption has recently been shown to be beneficial for cardiovascular health (22), these findings suggest that pomegranate juice may also lessen the pathogenesis of complications resulting from non-enzymatic glycation.

While pomegranate juice did inhibit the production of fluorescent AGEs, lysozyme incubated in the presence of fructose did not form an appreciable amount of fluorescent AGEs when incubated for 72 hours (Figure 3). These results may be due to the fact that lysozyme lacks a considerable amount of reactive lysine residues needed for rapid glycation in comparison to BSA (23,24) and the length of incubation was not sufficient (>3 days). Other studies have indicated proteins rich in lysine and arginine residues undergo rapid structural changes in the presence of a reducing sugar (25). Ahmad and coworkers (10) incubated lysozyme with glucose (500 mM) for 35 days and found significant protein modification.

In previous work, we examined the effect of pomegranate juice on the protein modification of fructosylated bovine serum albumin (data not shown). BSA incubated in the presence of fructose produced only a widening of the native BSA band; no larger weight bands, indicative of significant protein modification (crosslinking) was observed.

We also demonstrated that pomegranate juice, at a given phenolic content, inhibited the widening of the native albumin band. In this study, RNase was chosen based on the work by Litchfield and coworkers (9). In their study, RNase A (10mg/ml) was incubated in the presence of glucose or arabinose (250 mM) for 21 days. SDS-PAGE analysis revealed the formation of multiple larger molecular weight bands. Amino acid analysis demonstrated that lysine and arginine residues were the only amino acids to undergo significant chemical modifications. In our study, we also observed the formation of larger molecular bands when RNase was incubated with fructose; these bands were not produced when pomegranate juice was present in the incubation mixture, but did appear when apple juice, a poor inhibitor of glycation, was present.

The mechanism of action for many natural product glycation inhibitors is still unknown; however studies have indicated that compounds with a high antioxidant capacity provide protection against glycation mediated by reducing sugars (26). Pomegranate juice is commercially available and an effective inhibitor of protein glycation. We have shown that pomegranate juice provides protection against glycation in four different model proteins as evidenced by fluorescence spectroscopy. Pomegranate juice also prevents the structural modification of two proteins, albumin and ribonuclease A, as described by SDS-PAGE analysis. This present work supports the theory that the inhibition of bovine serum albumin glycation by pomegranate juice is not unique to that protein, and is not a result of the documented binding of polyphenolic compounds to albumin (27). In summary, pomegranate juice is rich in ellagitannins (punicalagin and ellagic acid) and possesses high antioxidant capacity which lends itself to being an exceptional inhibitor of protein glycation.

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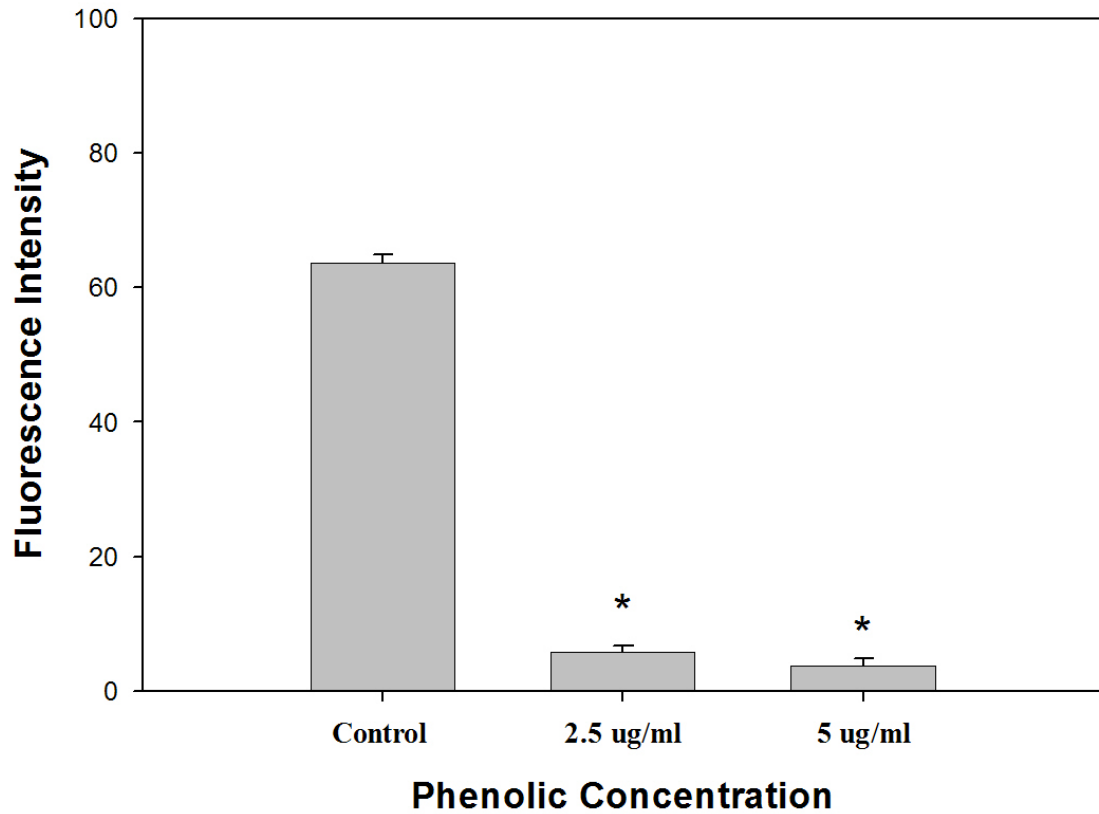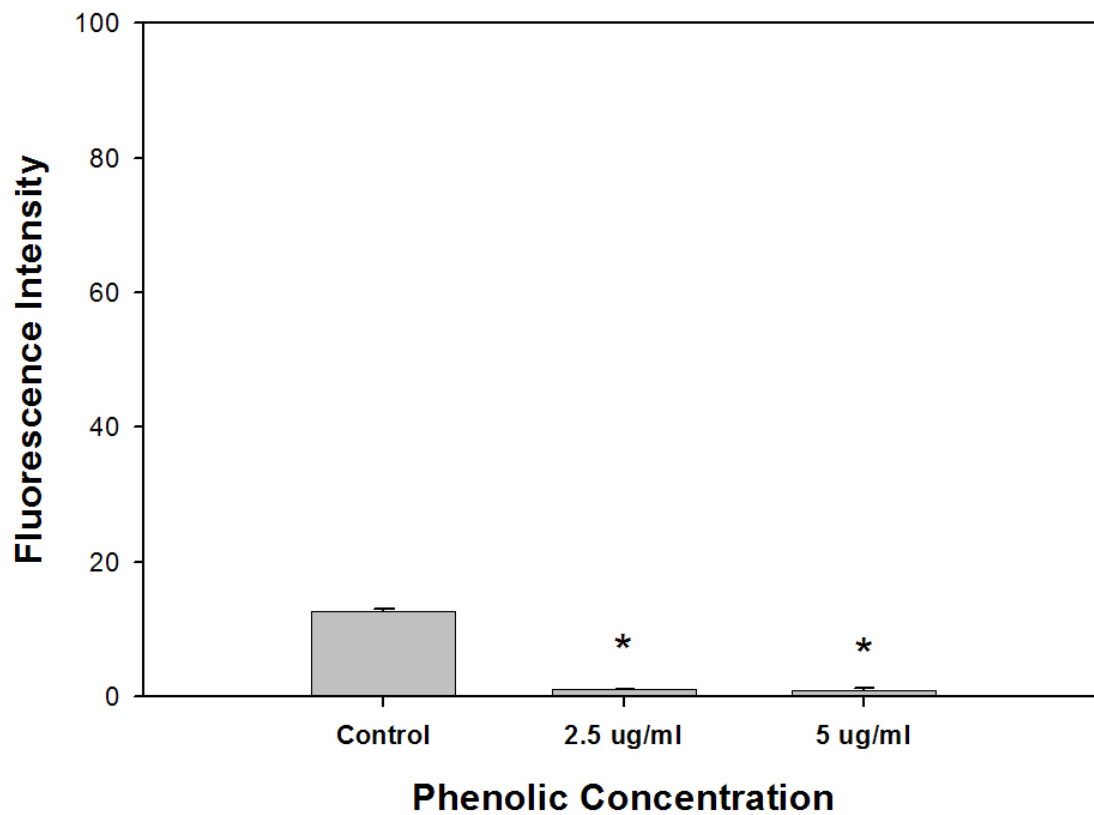
Figure 4.2: Inhibition of the glycation of gelatin by pomegranate juice. Gelatin (2mg/ml) and fructose (125 mM) were incubated with pomegranate juice for 72 h at 37°C. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *p< 0.05 when compared to control
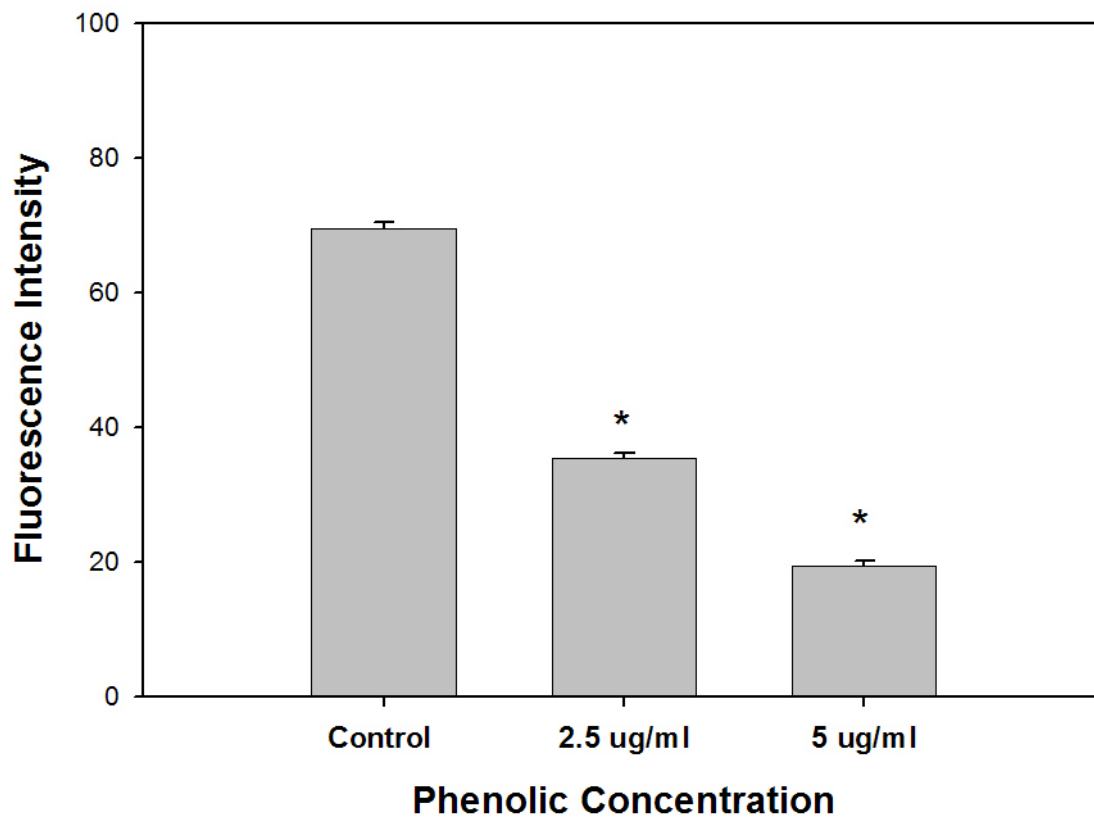
Figure 4.2: Inhibition of the glycation of IgG by pomegranate juice. IgG (2mg/ml) and fructose (125 mM) were incubated with pomegranate juice for 72 h at 37ºC. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *$p < 0.05$ when compared to control

Figure 4.3: Inhibition of the glycation of lysozyme by pomegranate juice. Lysozyme (2mg/ml) and fructose (125 mM) were incubated with pomegranate juice for 72 h at 37ºC. The fluorescence intensity was measured at 370/440 nm. Results represent the mean ± SEM for triplicate determinations. *p<0.05 when compared to the control
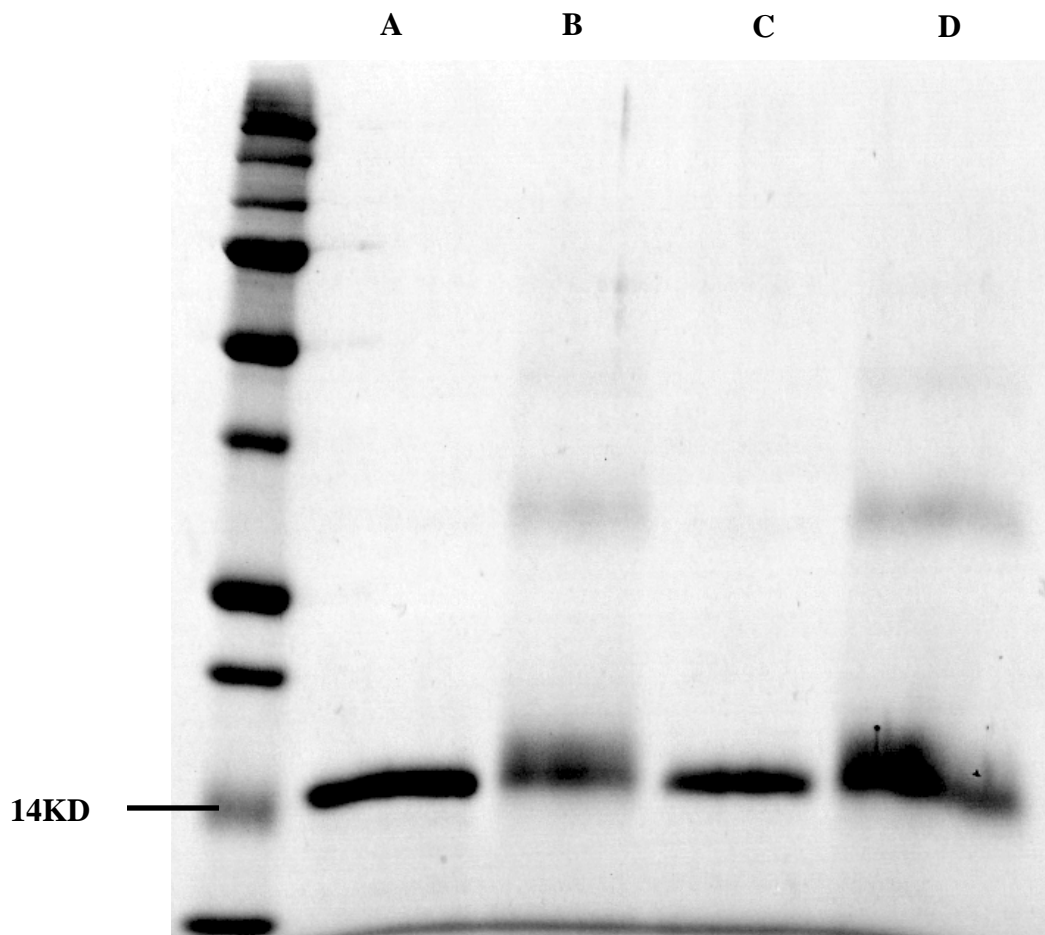
Figure 4.4: SDS PAGE profile of (A) 2µg Ribonuclease A, (B) 2µg of Ribonulcease A incubated with fructose, (C) 2 µg of Ribonuclease A incubated with fructose and pomegranate juice, and (D) 2 µg of Ribonuclease A incubated with fructose and apple juice for 14 days. Samples were loaded onto a 16% resolving polyacrylamide gel with a 8% stacking polyacrylamide gel. Staining was performed with Coomassie brilliant blue. The size marker is shown to the left.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

There are many FDA approved drugs designed to lower blood glucose levels; however no drug currently exists to combat diabetic complications stemming from protein glycation. Non-enzymatic protein glycation involves the binding of a protein amine group to the carbonyl group of a reducing sugar. Resultant Schiff base compounds are usually removed by various receptors; however in proteins that are not readily recycled, irreversible AGEs adducts form.

Within the past decade there has been a large paradigm shift to increase the consumption of phytochemicals (antioxidants) in the human diet. Rich in phytochemicals, pomegranate has been extolled for its many medicinal qualities. In the current study pomegranate juice, ellagic acid and punicalagin (major phenolics found in pomegranate), extracts of pomegranate membrane and peel all demonstrated robust inhibitory activity of albumin glycation mediated by fructose when compared to commonly consumed fruit and fruit juices. Similar results were observed in other model proteins (gelatin, IgG, ribonuclease A) demonstrating that pomegranate polyphenolics are non-specific inhibitors of protein glycation.

To continue this research, the effect of pomegranate on the formation of AGES using *in vivo* models of diabetes should be performed. One possible experimental system is the streptozotocin induced diabetic rats and determining the rate of AGE formation in tail collagen. To expand this project further, studies can be performed to determine

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whether pomegranate juice or whole extracts of pomegranate fruit decrease the concentration of glycated albumin in diabetic human subjects. Finally, chemical analogs of punicalagin or ellagic acid could be synthesized in an attempt to find even more potent anti-AGE compounds.

CHAPTER 6

APPENDIX
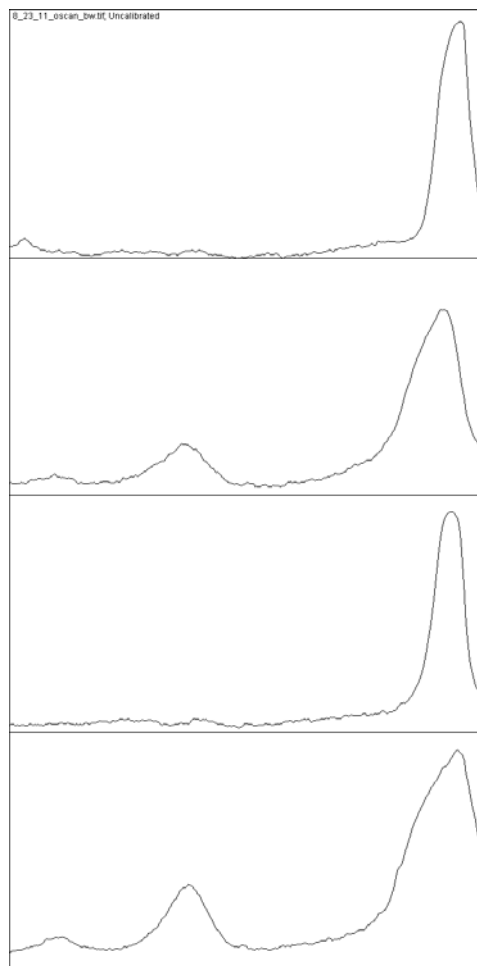
Figure 6.1: Densitometry scan of Figure 4.4 using ImageJ software