ANTHOCYANIN-ENRICHED PURPLE SWEET POTATO FOR COLON CANCER PREVENTION

by

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B.S., Yonsei University, Seoul, Korea, 2004

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Abstract

Anthocyanins are flavonoid pigments that account for the purple color in many plant foods. It has been investigated that anthocyanins' predominant occurrences in human diet and their health beneficial activities such as antioxidant, anti-inflammatory, and anti-carcinogenetic effects. Based on those scientific evidences, anthocyanins are now recognized as potential therapeutic compounds. Particularly, the chemopreventive effect of anthocyanins has been widely studied by many researchers in nutrition. However, their bioactivities are diverse due to different chemical structures of anthocyanins from different sources. In this study, we discuss the chemopreventive activity of anthocyanins from purple sweet potato. Previously, we selected a purple-fleshed sweetpotato clone, P40, crossbred seeds obtained from the International Potato Center in Lima, Peru. We hypothesized that anthocyanins enriched P40 may provide health beneficial activities in cancer prevention. For the first part of this study, we analyzed nutrient compositions, dietary fiber content, anthocyanins contents, total phenolics contents and total antioxidant activity. Even thought P40 presents similar composition and amount of nutrients with the control cultivars, white-fleshed O'Henry and yellow-fleshed NC Japanese, HPLC-MS analysis confirmed that it possesses much higher anthocyanin content even up to 7.5g/kg dry matter. Also, dietary fiber, particularly soluble dietary fiber content, total phenolics content, and total antioxidant capacity of P40 were significantly higher. For the second part of the study, we tested the potential anticancer characteristic of P40 cultivar in human colonic SW480 cancer cells and in azoxymethane-induced aberrant crypt foci in mice. Treatment with 0 – 40 μM of peonidin-3-glucoside or P40 extract containing corresponding amount of anthocyanins resulted in inhibition of cell growth in a dose-dependent manner. Interestingly, even though the patterns of growth inhibition were similar in the two treatment groups, the cells treated with P40 extract tend to survive significantly less than those treated with peonidin-3-glucoside. Cell cycle analysis confirmed that the growth inhibition was not due to cytotoxicity, but cytostatic mechanism with increased number at the G1 phase of the cell cycle. The cell cycle arrest was also significantly correlated with the anthocyanin contents in P40 cultivar when compared with the white-fleshed O'Henry and yellow-fleshed NC Japanese controls. After Azoxymethane (AOM) or saline injected mice were fed basal AIN-93M diet or diets containing 10~30% of P40,

20% O'Henry or 20% NC Japanese for 6 weeks, aberrant crypt foci (ACF) multiplicity was significantly inhibited by 10~30% P40 diet. Imunohistochemistry results of colonic mucosa showed that the expression level of apoptosis marker, caspase-3, was significantly induced in the mice treated with 10~20% P40 diet. Also, PCNA expression level, which is proliferation marker, was significantly inhibited by the 30% P40 diet. These findings indicated that consuming a purple sweet potato, P40, may prevent colon cancer by modulating antioxidant status, inducing apoptosis, and reducing cell proliferation.

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Dedicated to my parents, Wanki Lim and Soonyi Ahn, for your endless love, trust, and support.

Preface

Western diet is one of the main causes of chronic diseases including cancers and appropriate dietary modification can prevent many of these diseases. In particular, daily consumption of fruit and vegetables that contain phytochemicals is highly recommended in diet due to their health protection effects. Among these phytochemicals, phenolic compounds anthocyanins have been recognized for their anti-cancer potential.

Sweet potato is one of the most important crops in the world not only because of its considerable amount of nutrient, but also phytochemicals in its root and leaves. Also, it has its advantages of high yielding, drought tolerance, and wide adaptability to most of environment over the world. There are varieties of flesh color of sweet potatoes out there.

Among those varieties, purple-fleshed sweet potato is attracting lots of attention from people in nutrition. The strong color of purple sweet potato is contributed by phenolic pigment called anthocyanins. People have been trying to develop anthocyanin enriched purple sweetpotato. Also, it has been reported that purple sweetpotato presented excellent bioactivities such as antimutagenic, radical scavenging, antidiabetic, hepatoprotective, and chemopreventive activities.

Anthocyanins belong to flavonoids, a subgroup of dietary phenolics. They carry a positive charged ion in their flavylium ring, which makes them distinguished from other phenolic compounds. So far, more than 600 anthocyanins found in nature, however, 6 anthocyanidins - cyanidin, pelargonidin, delphinidin, petunidin, peonidin, and malvidin - are frequently found in human diet. They usually exist as glycosylated forms.

Anthocyanins are very reactive compounds and easily degradated due to the electronic deficiency of their flavylium ring. As pH condition changes, their colors are changed to red, blue, bown, or colorless. These properties of anthocyanin give limitations to quantify exact amount of anthocyanins because detecting techniques are based on their color, so there is high chance to overlook the colorless forms of anthocyanins during quantification analysis.

Anthocyanins are bioavailable and their biological efficienty of mainly depends on bioavailability such as absorption, metabolism, tissue distribution, and excretion. Most of the bioavailability studies are claiming their low bioavailability. They have been reported only nm to

uM range of anthocyanins detected in blood samples, which referring to low absorption, and .004 to 0.1 % of the intake amount of anthocyanins detected in urine, which also referring to low excretion levels.

Despite of their low bioavailabiliy, people have been reported cancer prevention effects of anthocyanins. In vitro studies showed that anthocyanins or anthocyandin rich extracts have exhibited growth inhibitory effects on various cancer cell lines, such as lung, breast, prostate, liver, and colon cancers. They also reported that anthocyanidins are more effective forms for inhibition of cancer cell growth than anthocyanins. Even very low dose of anthocyanidins (10-5M to 10-4 M) demonstrated their inhibitory effects on cancer cells. In vivo studies used animal cancer models fed with anthocyanin-rich diet showed that the diet inhibited tumor development, cell proliferation, inflammation, angiogenesis, ACF multiplicity, total tumor multiplicity, tumor burden, and adenocarcinoma multiplicity. Also the diet induced apoptosis in tumor tissues. Even though there are not many human studies about cancer prevention of anthocyanins, studies showed that anthocyanins intake improved oxidative damages, and decreased risk of certain type of cancer such as lung cancer.

In this study, we hypothesized that purple sweet potato diet may prevent colon cancer due to their high anthocyanin content. For this project, Dr. Carey and Dr. Griffin in horticulture department in Kansas State University bred special purple sweet potato cultivars. Among those new cultivars, we selected a purple-fleshed sweet potato clone, P40, from seeds obtained by crossbreeding in the International Potato Center in Lima, Peru. We quantified and qualified anthocyanins from P40 by HPLC-MS and compared them to those from two control cultivars, white fleshed O'Henry and yellow fleshed NC Japanese. Also, we analyzed nutrient composition and dietary fiber content of these sweet potato samples. Antioxidant activity of sweet potato samples was tested by FRAP assay and total phenolic content. To prove chemopreventive effect of anthocyanins from P40, we treated either the major anthocyanin in P40, peonidin 3-glusose or P40 extract on SW480 human colon cancer cells. Also, we used azoxymethan-induced aberrant crypt foci murine model to test the effect of P40 diet to investigate the potential mechanisms involved in this inhibition.

CHAPTER 1 – LITERATURE REVIEW

CHAPTER 1

Sweet potato

As the sixth largest food crop, sweet potato (*Ipomoea batatas* [L.] Lam.) is one of the most important foods in the world. In ancient Asia and Africa, it had been a great source of energy and nutrients during winter due to its excellent storability and reliability in case of other staple foods are failing from severe weather. In fact, sweet potato is very rich in nutrients such as carbohydrates(80-85%), vitamins, and minerals. It is also contains much higher levels of pro-vitamin A, vitamin C and minerals than rice or wheat (1). Among other root and tuber crops, the sweet potato is higher yielding and drought tolerant with wide adaptability to various climate and farming systems. Thus, it has been widely used for food and industrial application.

In addition to the nutritional values of sweet potatoes, it has been rediscovered as a functional food containing high levels of various phytochemicals which might have various health beneficial effects (2). Most studies on phytochemicals in roots or leaves of sweet potato mentioned their health promoting and/or disease preventing benefits related to the high level of polyphenols. In particularly, cancer preventive effects of polyohenols in sweet potato have been widely investigated. For example, Rabah et al. demonstrated cancer prevention activity of sweet potato (Cv. Koganesengan) extract and its correlation with its level of phenolic content (3).

It also has been noticed that the color of sweet potato may play a crucial role in their health beneficial effect. In some countries such as Kenya or sub-Saharan Africa, people have been suffering from severe vitamin A deficiency due to white sweet potato consumption as a staple food. Substitution of beta-carotene-rich orange-fleshed sweet potato helped to improve the deficiency (4-5). Also, purple-fleshed sweet potato cultivars have proved their excellent bioactivities such as antimutagenic (6-7), radical scavenging (8), antidiabetic (9), hepatoprotective (10), and chemopreventive activities (11-12). Those studies agreed that biological effects of purple sweet potato may be due to the phenolic pigment, "anthocyanin".

Sweet potato breeding

There are several goals of sweet potato breeding. Traditionally, yield maximization was one of the main goals in many countries where sweet potato is a food staple in their daily diet. Also, resistance against environmental stresses such as drought and flooding, or tolerance against pests and diseases can be a reason for breeding. Another goal has been improving nutritional qualities by controlling its nutrients production in their roots. Sweetness, moisture, texture, or root shape were also controlled to meet consumers' preference. For industrial uses, sweet potato breeding for producing specific pigments in their roots became a new area such as producing red, purple, or orange-fleshed cultivars (13).

Sweet potato breeding is focused on producing new varieties with highly nutritious characteristics. In fact, China, Korea, India, Peru, or US have been developing their national institutes or programs for new perspectives on sweet potato research. They particularly have been focused on developing new sweet potato cultivars with high content of phytochemicals such as anthocyanins in sweet potato. Even though traditional red-skinned sweet potato naturally contains high level of anthocyanins in its skin, it is usually removed before consumption. Thus, during the past few years, red-, purple-fleshed sweet potatoes have been developed and introduced mostly in Asian countries. At the same time, several genes for this trait in crops have been characterized (14). For instance, high anthocyanins content in crops can be achieved by overexpression of a single biosynthesis gene. In tomato, overexpression of the petunia CHI gene resulted in increased flavonoids including anthocyanins (15). Also, overexpression of the transcriptional factors such as R2R3 Myb, basic helix loop helix (bHLH), and WD40-type transcriptional factors is more commonly used to increase anthocyanin levels (16). The constitutive expression of the tomato ANT1 (a R2R3 Myb) gene is controlled for the effect (17). In sweet potato, IbMADS10 gene is involved to anthocyanin biosynthesis (18). However, so far, only a few purple-fleshed genotypes have been proven to be marketable (19).

In the John C. Pair Horticulture Research Center (Wichita, Kansas), we have developed a purple-fleshed sweet potato, P40. (Figure 2.1). The seeds were provided from the International Potato Center in Lima, Peru. Purple sweet potatoes were selected using seeds from controlled crosses of over 2000 seedlings from four full-sib progenies cultured, evaluated and selected in the field. Among them, one genotype, designated P40, with intense anthocyanin pigmentation and reasonable yield was the subject of this study.

Anthocyanins

As the name of anthocyanins is derived from Greek words, anthos (flower) and kyanos (blue), they are the largest group of water-soluble pigments widely distributed in the plant kingdom. They belong to a larger group of compounds known as flavonoids, a subgroup of dietary phenolics (Figure 1.1). They are responsible for the intense colors of many vegetables and fruits such as red grapes, berries, red cabbages and purple sweet potato (20-22). As one of the most abundant compounds among dietary polyphenols, they are widely present in human diets in the form of fresh fruits, vegetables, or beverages (23). The daily intake of anthocyanins in the human diet has been estimated at 180-215mg/d in the USA, which is about 9-fold higher than that of other dietary flavonoids such as genistein, quercetin and apigenin (20-25mg/d) (24).

In contrast to other flavonoids, anthocyanins carry a positive charge in the central ring (C-ring) structure. The aglycones or anthocyanidins exclusively found in nature are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. They are sharing the same 2-phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in 3, 5, and 7 positions, and differ by the number and position of hydroxyl and methoxyl groups in the B-ring (Figure 1.2). In plants, they are present mostly as forms of glycosidic compounds attached to many different natures of sugar moieties. D-glucose, D-galactose, L-rhamnose, D-xylose, and D-arabinose are the most predominant sugars. The sugar reisdues are usually acylated with cinnamic acids such as caffeic, p-coumaric, ferulic, or sinapic acid, and/or aliphatic acids such as acetic, malic, malonic, oxalic, or succinic acid. These acylated sugar components of anthocyanins are commonly conjugated to the C-3 hydroxy group in C-ring (25).

Anthocyanins are very reactive compounds and easily degradated due to the electronic deficiency of their flavylium ring. At acidic conditions (pH <2), they exist as a relatively stable form of favylium cation (red color). Increasing pH is accompanied by a rapid loss of a proton generating a blue quinoidal base. Hydration of the favylium cation results in yielding a colorless carbinol pseudo-base. Also, tautomerization through opening of the C ring generates a brown chalcone (Figure 1.3). The loss of pigmentation is also influenced by the presence of oxygen, enzymes, high temperature, and light. Therefore, it is very important to control those environments during analysis (20-23).

Bioavailability of anthocyanins

The biological efficiency of anthocyanins mainly depends on their absorption, metabolism, tissue distribution, and excretion. In general, anthocyanins are rapidly absorbed and eliminated. After ingestion, anthocyanin can be absorbed from the stomach and small intestine. After they break down into the aglycone and sugar molecules by microflora in the GI track and passing through the liver, they enter the blood circulation and urine (22). In a study that investigated rats fed with anthocyanin-enriched diet for 15 days, anthocyanins were found in the digestive area organs (stomach, jejunum and liver) and kidney, as well as brain. In the brain, total anthocyanin content reached 0.25 ± 0.05 nmol/g of tissue (26).

According to studies, anthocyanins appear to have low bioavailability. The limited amount of anthocyanins is absorbed from food, and only nM to low μM range of concentrations of anthocyanins is detected in blood (27-29). The excretion of anthocyanins has been reported as low range from 0.004% to 0.1% of the intake (30). However, the low bioavailability of anthocyanins is not conclusive due to limitations of these studies. For example, in human studies, recovery rate of anthocyanins in biological samples after volunteers consumed anthocyanin-rich foods or extracts was very low (31-34). Also, some colorless metabolites of anthocyanins such as carbinol and chalcone forms that are present in blood and urine may not be detected, and therefore may have been overlooked. A number of molecular structures of anthocyanins and their metabolites cause difficulties in determining accurate measurement. Currently, there are no selective and sensitive methods for determining the alternative molecular structures of anthocyanins (22).

Anthocyanins and cancer prevention studies

In vitro studies

A number of studies have examined the effects of anthocynins on cell growth or tumor-inducing cellular events. Anthocyanins or anthocyanin-rich extracts have exhibited growth inhibitory effects on a variety of cancer cells such as lung (35), breast (36), prostate (37), liver (38), and colon (39) cancers, etc. Most studies showed that aglycone anthocyanidins inhibited cancer cell growth more effectively than the glycosidic form. For example, anthocyanidins

significantly suppressed cell growth in lower concentration range (10^{-5}M) than anthocyanins (10^{-4}M). Among anthocyanidins, delphinidin showed the most effective inhibition on cancer cells (40).

In vivo studies

Anthocyanins or anthocyanins rich diet have been demonstrated to have cancer preventive properties in many type of cancer animal models. For example, in one study, after 2 weeks feeding with diets containing freeze-dried black raspberries (BRB) to tumor induced rats by N-nitrosomethylbenzylamine (NMBA), the diets suppressed tumor development, inhibited cell proliferation, inflammation, and angiogenesis, and induced apoptosis in tumor tissues (41). In another study, dietary purple corn color (anthocyanin-containing extract) and its major anthocyanin, cyanidin 3-O-beta-D-glucoside (C3-G) significantly inhibited DMBA-induced mammary carcinogenesis in human c-Ha-ras proto-oncogene transgenic (Hras128) rats and in their non-transgenic counterparts (42). Also, lyophilized black raspberries diet decreased aberrant crypt foci(ACF) multiplicity, total tumor multiplicity, tumor burden, and adenocarcinoma multiplicity on azoxymethane-induced colon tumors in male Fischer 344 rats (43). Results from animal studies are rather consistent and provide strong evidences of cancer preventive effect of anthocyanins.

Human studies

Unlike cell culture and animal studies, epidemiological studies are scarce, and do not provide much information about the anti-cancer mechanism of anthocyanin-rich diet. Still, some human studies are reporting possible cancer preventive effect of anthocyanin-rich fruits, vegetables or their food products. Consumption of tart cherry juice containing high levels of anthocyanins improves the capacity of older adults to resist oxidative damage during acute oxidative stress induced by forearm ischemia-reperfusion trial (44). In a prospective cohort study that consisted of 2,590 middle-aged eastern Finnish men, the relation between the intakes of 26 flavonoids including anthocyanidins, and the risk of lung, prostate and colorectal cancer was assessed. The data showed high intake of flavonoids is only associated with decreased risk of lung cancer in middle-aged Finnish smoking men, not with the risk of prostate or colorectal cancer (45). However, in a clinical study conducted with16 young cancer patients, the drug treatment containing 50 mg anthocyanin did not show an antitumor effect (46).

Relationship between chemical structure of anthocyanins and its cancer preventive effect

The chemical structure of anthocyanin is crucial to their biological activities. However, anthocyain structure-function relationships are not well established because of difficulties to assess from the studies conducted with so many different anthocyanin from different sources (47). Although the relationship could be different depending on experimental models, several possible relationships can be inferred by comparing the studies about cancer preventive effect of anthocyanins.

According to many study results, delphinidin presents the strongest inhibitory effect on cancer cell growth and survival among 6 anthocyanidins. It seems like the hydroxy groups on ring B of the anthocyanin molecule may have potency on anti-proliferation activity (40, 48). Also, Marko et al. compared the abilities of anthocyanidin to inhibit epidermal growth factor receptor(EGFR) tyrosin kinase which is associated with cancer development. They demonstrated that anthocyanidins decreased EGFR tyrosine kinase in the order delphinidin = cyaniding > pelagonidin > peonidin > malvidin (49). It suggests that potency might be related to the presence of hydroxy functions in positions 3' and 5' of the B ring of the anthocyanin molecule.

In contrast, the presence of methoxy groups in those positions may weaken the abilities. In a study on human leukemia cells, anthocyanidins possessing hydroxyl group in ring B also present higher pro-apoptotic activity including altering cell cycle than methoxy groups (50). However, another study reported that malvidin, which possesses only methoxy groups in 3' and 5' positions of ring B, exerted the greatest anti- proliferation activity among 6 anthocyanidins in stomach, colon, lung, breast, and central nerve system cancer cells (51).

Acylation and glycosidic patterns also effect on the biological activities of anthocyanins. In HT29 colon cancer cells, anthocyanins without acylation were more effective inhibitors than acylated anthocyanins (47). The growth inhibitory effects of delphinidin-3-galactoside and delphinidin-3- glucoside which were purified from bilberry extract were lower than those of the aglycon delphinidin on HL60 human leukemia cells and HCT116 human carcinoma cells (52). Anthocyanin triglycosides had less anti-proliferation ability than

diglycoside in HT29 colon cancer cells (47), which might imply the importance of glycoside residue of anthocyanins on their activities.

In terms of radical scavenging activity of anthocyanidins, it might be responsible for the presences of hydroxyl groups in position 3' of ring C and also in the 3', 4', and 5' positions in ring B of the molecules. Generally, the antioxidant activity of anthocyanidins is higher than that of anthocyanins, and it decreases as the number of sugar moieties increase (41).

Potential mechanisms of anthocyanins in cancer prevention

Anthocyanins also have demonstrated their strong antioxidant activity, which may be involved prevention of tumor development caused by excessive oxidative stress (41). The phenolic structure of anthocyanins may be related to their antioxidant and anti-carcinogenic effects. The phenolic structure appear to help scavenging reactive oxygen species (ROS) (53), increasing the oxygen-radical absorbing capacity of cells (54), stimulating the expression of Phase II detoxification enzymes (55), reducing the formation of oxidative adducts in DNA (56), and decreasing lipid peroxidation by modulating signal transduction pathways (57).

Inflammation may play a role in the promotion of some types of cancer. Abnormal up-regulation of inflammatory proteins such as nuclear factor-kappa B(NF-κB) and cyclooxygenase-2(COX-2) is commonly present in many cancers, and inhibitors of those proteins showed significant cancer preventive effect (58). Anthocyanins inhibit mRNA or protein expression levels of NF-κB, COX-2, and various interleukins (59,60).

Dysregulated apoptosis also plays major role in inducing cancers. However, the involved mechanisms are not conclusive and seem to depend on the cell lines and selected anthocyanin or anthocyanidins. For example, in a recent study, the anthocyanins isolated from Vitis coignetiae Pulliat induced apoptosis in colon cancer cells by activating p38-MAPK and suppressing Akt (61). Other researchers reported that the same anthocyanins induced apoptosis of human leukemia cells by being associated with modulation of expression of Bcl-2 and IAP family members and proteolytic activation of caspase-3, -8 and -9 (62). In another study, data showed that delphinidin induced apoptosis of human colon cancer cells via suppressing of NF-κB pathway (63).

Anthocyanins have proved their effect on cell-cycle regulation. By interrupting the cell cycle at G1 and G2/M, they may induce apoptosis and inhibit cancer cell proliferation. Anthocyain-rich blackberry extracts significantly reduced the G1 phase and increased proportion of cells in the sub G1 phase, indicating apoptosis (64). Delphinidin induced G2/M cell cycle arrest in human colon cancer cells (63). In human breast cancer cells, bilberry extract did cause an increase in the fraction of cells at the G2/M phase of the cell cycle (65).

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FIGURE LEGENDS

FIGURE 1.1. Dietary phenolics.

FIGURE 1.2. Structures of common anthocyanidins and anthocyanins.

FIGURE 1.3. Stuructural changes in the anthocyanin chromophore and their pH-dependent color changes in aqueous solution

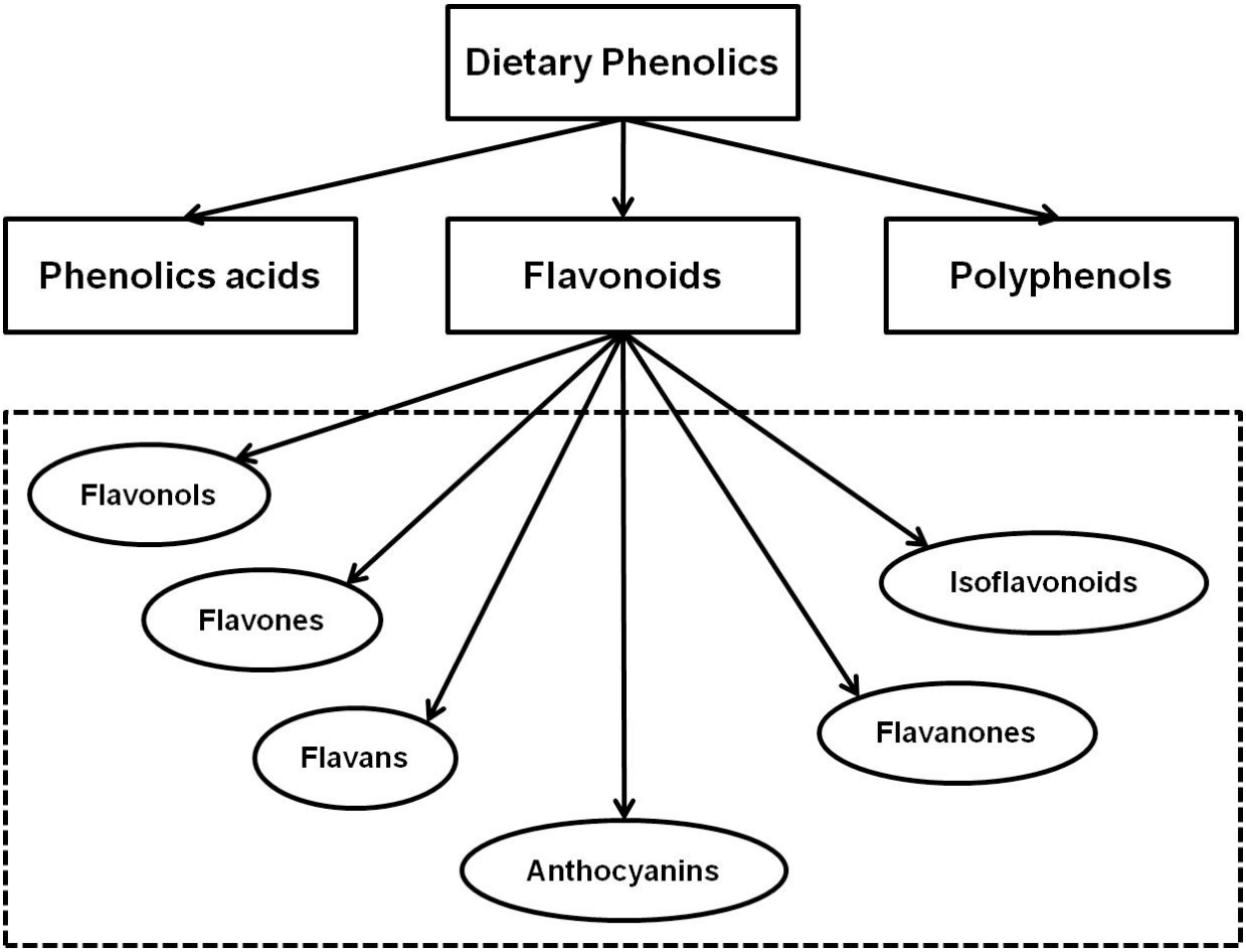
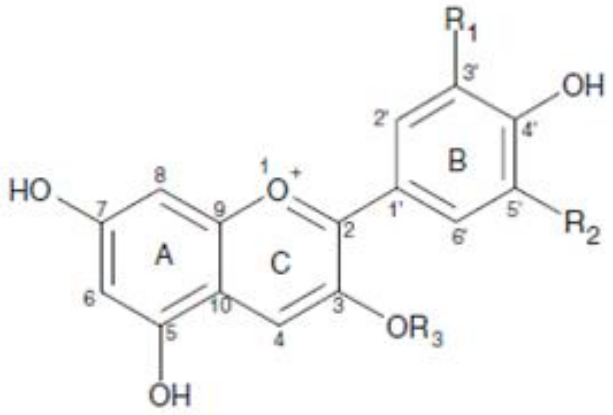

Dietary Phenolics
Phenolics acids
Flavonoids
Polyphenols
Flavonols
Flavones
Flavans
Anthocyanins
Isoflavonoids
Flavanones

FIGURE 1.1



Anthocyanidins	Substitutes	
	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH3	H
Petunidin	OCH3	OH
Malvidin	OCH3	OCH3

R_3 = Glucose, galactose, rhamnose, xylose, or arabinose

FIGURE 1.2

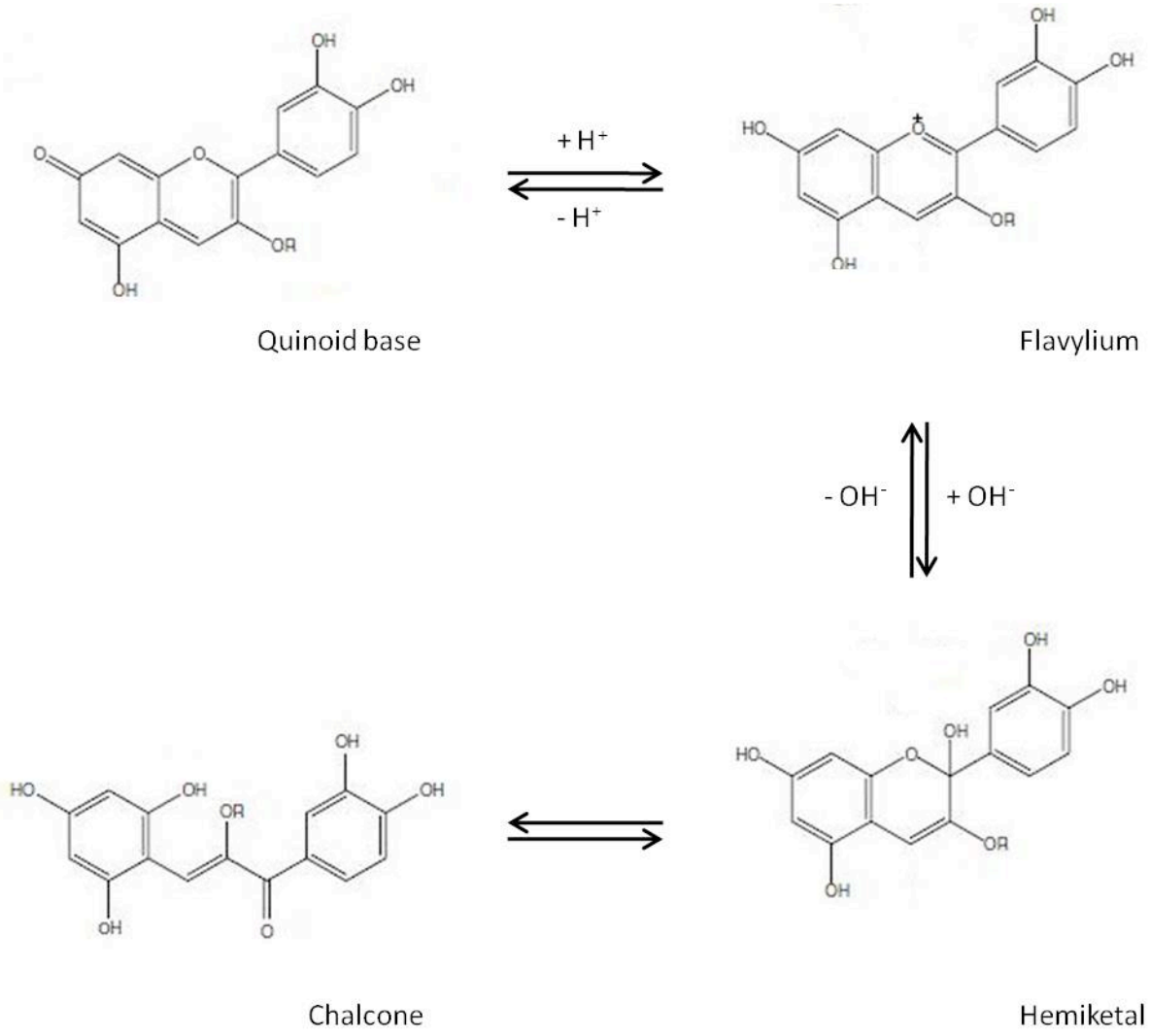


FIGURE 1.3

CHAPTER 2 - CHEMICAL PROPERTIES OF ANTHOCYANIN-ENRICHED PURPLE-FLESHED SWEET POTATO BRED IN KANSAS

Chemical Properties of Anthocyanin- Enriched Purple-Fleshed Sweet Potato Bred in Kansas[1]

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[3]Abbreviations used : HPLC-MS/ESI, high performance liquid chromatography-mass spectrometry / electron spray ionization ; TDF, Total dietary fiber ; IDF, Insoluble dietary fiber ; SDF, Soluble dietary fiber ; PN3G, Peonidin 3-glucoside ; PN3GE, peonidin 3-glucoside equivalent ; GAE, Gallic acid equivalent ; TE, Trolox equivalent.

ABSTRACT

Previously, we selected a purple-fleshed sweet potato clone, P40, from seeds obtained by crossbreeding. We analyzed nutrient composition, dietary fiber content, anthocyanins quantification, total phenolics content, and also measured their total antioxidant activity in P40 in comparison with white-fleshed O'Henry and yellow-fleshed NC Japanese controls. Despite differences in flesh color, P40 had similar composition and amount of nutrient compared to control cultivars. However, HPLC-MS analysis confirmed that it possesses much higher anthocyanins content than others, even up to 7.5g/kg dry matter. Dietary fiber, particularly soluble dietary fiber content of P40 was also significantly higher than controls. The high content of total phenolics likely was responsible for significantly higher antioxidant activity than controls. Taken together, these findings indicate novel characteristics of P40 on its potential health benefits.

INTRODUCTION

Anthocyanins, a class of natural polyphenol compounds, are widely distributed in the human diet such as fruits, beans, cereals, and vegetables. They are responsible for intense colors of plants. In recent years, they have been studied for their various biological activities including antioxidant (1-6), anti-inflammatory (7-9), antimicrobial (10), antihyperglycemic(11), and anti-carcinogenic activities(12-19).

Purple-fleshed sweet potatoes (*Ipomoea batatas* L.) exhibit an intense blue or purple color in both skin and flesh of the storage root due to a high level of anthocyanins accumulation. The anthocyanins from sweet potato belong either to the cyanidin or peonidin and are linked with sophorose and glucose. According to Oki et al (20). and Yoshinaga et al (21), they categorized anthocyanin composition of sweet potato into two groups based on the shade of color and peonidin/cyanidin(pn/cy) ratio: blue domain cyanidin types(pn/cy<1.0) and red domain peonidin types(pn/cy>1.0). They are usually mono- and diacylated with ferulic, caffeic, and ρ-hydroxybenzoic acid, which make them more stable than those from strawberry, red cabbage, perilla and other plants (22). Therefore, purple sweet potato has been regarded as an excellent source of stable anthocyanins. As well as the unique color they present, they are superior in

nutrition and health promoting benefits (23). They contain higher contents of various vitamins, minerals, and protein than other vegetables (24). Physiological functions of purple sweet potato have been widely reported such as antimutagenic (25), antihyperglycemic (26), hepatoprotective (27), anticancer (28,29), and antioxidant activities (30-33).

Reactive oxygen molecules generated during oxidative stress play an important role in development of various diseases such as cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, artherosclerosis, cararacts, inflammation, and aging (34-36). By scavenging reactive oxygen species (ROS), ending radical chain reactions, or chelating transition metals, the antioxidant nutrients constitute a vital endogenous defense against free radical cellular and tissue damage (37,38).

According to Suda et al. (39) and Harada et al. (40), anthocyanins of purple sweet potato (PSP) are rapidly absorbed into the body and have a higher antioxidant activity than anthocyanins from other sources. Also, anthocyanins from PSP showed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity in PSP anthocyanin-injected rats and PSP beverage-administered volunteers (30). A study of hepatoprotective effect of purple sweet potato color (PSPC) suggested that PSPC may protect the liver by attenuating lipid peroxidation, renewing the activities of antioxidant enzymes and suppressing inflammatory response (41).

In this part of study, we selected a purple-fleshed sweet potato clone, P40, from seeds obtained by crossbreeding. To identify novel characteristics of P40, we quantified and qualified anthocyanins from P40 by HPLC-MS and compared them to those from two control cultivars, white fleshed O'Henry and yellow fleshed NC Japanese. Also, we analyzed nutrient composition and dietary fiber content. Antioxidant activity of sweet potato samples was tested by FRAP assay and total phenolic content.

MATERIALS AND METHODS

Reagents

All organic solvents were HPLC grade, and purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Total dietary fiber assay kit was purchased from Megazyme international (Wicklow, Ireland). Folin-Ciocalteu reagent, gallic acid, 2,4,6-tri[2-pyridyl]-s-triatine (TPTZ),

$FeCl_3$. trolox, Celite, Peonidin-3-glucoside chloride was obtained from Chromadex (Irvine, CA, USA).

Purple-(P40), white-(O' Henry) and yellow-(NC Japanese) fleshed sweet potatoes (Figure 2.1) were provided from the John C. Pair Horticulture Research Center, Wichita, Kansas. Purple-fleshed sweet potato was selected using seeds from controlled crosses provided by the International Potato Center in Lima, Peru. Over 2000 seedlings from four full-sib progenies were cultured, evaluated and selected in the field. One genotype, designated P40, with intense anthocyanin pigmentation and reasonable yield was the subject of this study.

Sample preparation and extraction

For each sweet potato sample, tubers were randomly taken, washed with tap water, diced into approximately 0.5cm cubes, freeze-dried (general purpose freeze dryer VirTis GPFD 36DX66, SP Scientific, Gardiner, NY) and ground by cutting mills (Retsch, Newtown, PA) into flour. Prepared flour was stored at -80˚C until use. For preparation of anthocyanin extracts, 1g of flour was extracted with 8ml of acidified MeOH (1N HCL, 85:15, v/v) to obtain a sample-to solvent ratio of 1:8. The flasks containing flour/solvent mixture were covered with aluminum foil to avoid exposure to light, stirred on magnetic stirrer for overnight. After 12hrs extraction, extracts were centrifuged (1,800rpm, 30min) and supernatant was taken. Supernatant was filtered through Whatman no. 1 and syringe filter.

Nutrient composition analysis

Nutrient compositions of sweet potato powder were used for analysis according to the official AOAC methods (42): Dry Matter (#930.15), Crude Fat (# 920.39), Crude Protein (# 990.03), Ash (# 942.05), Starch (# 920.40) and Minerals (# 968.08). This analysis was conducted by Dr. Dave Trumble in department of animal sciences and industry in Kansas State University.

Dietary fiber content

Total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) in sweet potato were measured using total dietary fiber assay kit which is based on the official methods

AOAC # 985.29, AOAC # 991.43, AACC # 32-07, AACC #32-05, AACC #32-21, and AACC #32-06 (43-45). Sweet potato flour (1:40, w/v) was homogenized in MES/Tris buffer (1:40, w/v) by stirring. Samples treated with heat-stable α-amylase were placed in shaking water bath at 95−100°C for 35 min with continuous agitation. After cooling to 60°C, the samples were digested with protease for 30 min in a water bath at 60°C with continuous agitation. After adjusting the mixture to pH 4.1-4.8, an amyloglucosidase solution was added and incubated for 30 min in a shaking water bath at 60 °C under continuous agitation. To determine the TDF content, 95% ethanol preheated to 60°C was added to the digested sample and allowed to precipitate to form at room temperature for 1 h. The precipitated enzyme digest was filtered through crucible containing Celite, and washed with 78% ethanol, 95% ethanol, and absolute acetone. Crucible containing residue was dried overnight by placing in 103°C oven, and weighed to the nearest 0.1mg. To determine IDF and SDF contents, the enzyme digest was filtered through a crucible into a filtration flask. Insoluble residue was washed twice with preheated distilled water to 70°C. The filtrate and water washings were kept for SDF determination. After washing insoluble residue in crucible with 95% ethanol and acetone, the crucible with IDF was dried and weighed the same way as described for TDF. On the other hand, the filtrate and water washings were precipitated by adding 95% ethanol preheated to 60°C followed by filtration and washing with 78% ethanol, 95% ethanol, and absolute acetone. SDF was also dried and weighed by the same as above.

HPLC-MS/ESI analysis of anthocyanins

The quantification of anthocyanins in sweet potato extracts was conducted based on previous methods (46, 47). Analysis was carried out on an Esquire 3000+ electrospray ion-trap mass spectrometer, ESI/MS, (Bruker Daltonics, GmH, Billerica, MA) connected to an Agilent 1100 series high performance liquid chromotography system, HPLC, (Agilent Waldbronn, Germany) equipped with a photodiode array detector (Waters 996, Waters Corp., Milford, MA). Reverse phase liquid chromatography, RPLC, was preformed with a Gemini C18 stationary phase column (2.0mm x 150mm i.d.,, 3µm, 110Å). An optimum column temperature of 25 ˚C was set. The mobile phase A consisted of 1% formic acid in double deionized water (ddiH_2O), and the mobile

phase B consisted of 90:10 mixture of acetonitrile and 1% formic acid in ddiH2O. At a flow rate of 0.2mL/min the gradient conditions were as follows : solvent B: 0min, 2%; 15min, 20%; 25min, 95%; 26min, 98%. For HPLC-UV/VIS analysis, peaks were spiked with 5μM peonidin3-glucoside as an internal standard and detected by monitoring absorbance at 520nm wavelength for anthocyanins. Peak identification of each anthocyanin was based on comparison of relative retention time (RT), percentage peak area, and spectral data with anthocyanin standard, peonidin 3-glucoside (0-500μM). Right after the HPLC analysis, the ESI/MS spectra were acquired in positive ionization mode from m/z 100 to 1000. The ESI/MS data was used to confirm the mass of the anthocyanin HPLC peaks. The mass spectrometry instrument was controlled by the esquire control 5.3 software (Bruker Daltonics, GmbH, Billerica, MA). The data were processed with Data analysis 3.3 software (Bruker Daltonics, GmbH, Billerica, MA).

Total phenolic content

The total phenolics in sweet potato extracts were measured by Folin-Ciocalteu method (48) with slight modification. Each extract (10μl) was mixed with 600μl of H_2O in a microcentrifuge tube. In the same tube, 50μl of Folin-Ciocalteu reagent was added. The mixture was allowed to stand for 1-8min in room temperature, then, mixed with 150 μl of 20% Na_2CO_3 solution. It was incubated at 23℃ for 2hrs followed by dilution with 190μl of H_2O. The reaction solution from the tube was applied into 96-well plate and detected its absorbance at 760nm. Gallic acid was used as a standard reagent.

Total antioxidant capacity

Total antioxidant capacity of extracts from three sweet potato varieties was determined by measuring their ability to reduce Fe^{3+} to Fe^{2+}as established by the Ferric-reducing ability of plasma (FRAP) test (49). Working FRAP reagent was prepared as required by mixing 20 ml of 300mM acetate buffer (pH 3.6), 2.0 ml of 10mM TPTZ solution, 2.0 ml of $FeCl_3$ solution and 2.4 ml of distilled water. Each extract (30ul) was diluted with water and mixed with 270ul of freshly prepared working FRAP reagent pre-warmed at 37˚C. The absorbance was measured in a microplate reader of 96 well plates at 593 nm. Trolox was used as standard.

Statistical analysis

Data were analyzed by using SAS statistical system, version 9.2. Results were evaluated by the analysis of variance (ANOVA). Each experiment was conducted in multiplication (n=3~7), and the results were expressed as means±SDs. Means were separated using Turkey's studentized range test. A probability $p < 0.05$ was considered significantly.

RESULTS

Nutrient composition and dietary fiber content

The nutrient content of purple fleshed sweet potato, P40 appeared to be similar with control sweet potato, white fleshed O' Henry or yellow fleshed NC Japanese in nutrient we analyzed (Table 2.1). Starch (9.84-17.54%) is the most predominant nutrient component of three sweet potato samples followed by protein (1.50-2.19%), small amounts of fat and minerals (<0.53% or <18.93ppm). However, dietary fiber, particularly soluble dietary fiber content of P40 was significantly higher than controls ($p < 0.05$) (Table 2.1).

HPLC-MS/ESI analysis of anthocyanins from sweet potato samples

By HPLC analysis, anthocyanin peaks were detected from 12min to 17min at 520 nm. We detected some minor peaks of anthocyanins from NC Japanese extract and 9 major peaks from P40 extract, whereas peaks were undetectable for extract from O' Henry (Figure 2.2). Commercially available peonidin-3 glucoside (5μM) was added in the extracts as an internal standard (Figure 2.2, peak 5), and was also used as a standard for quantification. The Anthocyanin content of sweet potato was calculated as peonidin 3-glucoside equivalent (PN3GE) value. Total anthocyanin content of P40 was 7.5334g PN3GE/kg dw, and it was significantly higher than that of controls ($p < 0.05$). The amount of anthocyanins in NC Japanese was 1.8707g PN3GE/kg dw, which presumably was extracted from its purple-colored cortex (Table 2.1).

Right after HLPC quantification, the MS/ESI data were collected by monitoring the molecular iron characteristic for each of 9 peaks. These compounds identified by their respective m/z

values of molecular iron peaks (Figure 2.2, Figure 2.4); cyanidin 3-sophoroside-5-glucoside (m/z 773), peonidin 3-sophoroside-5-glucoside(m/z 787), cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside(m/z 893), peonidin 3-p-hydroxybenzoylso -phoroside-5-glucoside (m/z 907), peonidin 3-glucoside (m/z 498), cyanidin 3-caffeoyl-p-hydroxybenzoylsophoroside-5-glucoside (m/z 1055), pelargonidin 3-sophoroside-5-glucoside (m/z 757), cyanidin 3-(6"-feruloylsophoroside)-5-glucoside (m/z 949), and peonidin 3-caffeoyl-p-hydroxybenzoyl-sophoroside-5-glucoside (m/z 1069). Most of these anthocyanins are exclusively peonidin or cyanidin 3-sophoroside-5-glucoside and their acylated derivatives with caffeic, ferulic, and/or hydroxybenzoic acids except peak5 and peak7.

Total phenolics and total antioxidant activity

The total phenolic content of extracts from three sweet potato samples is shown in Table 2.1. The rank order of total phenolic content was P40 (74.6170 mg GAE/g dw) > NC Japanese (25.5901 mg GAE/g dw) > O' Henry (16.1491 mg GAE/g dw), corresponding fairly closely to the anthocyanin content. NC Japanese extract was significantly higher than O' Henry. The antioxidant capacity of extracts was determined by FRAP assay and also shown in Table 2.1. P40 (84.3522 mg TE/g dw) had the highest antioxidant capacity values followed by O' Henry (49.1195 mg TE/g dw) and NC Japanese (47.9120 mg TE/g dw). Although O' Henry had the lowest phenolic content, it showed significantly higher antioxidant capacity than that of NC Japanese ($p < 0.05$).

DISCUSSION

This study was designed to show that the antioxidant effect of purple sweet potato was due to its high level of anthocyanins content. For this study, we used a purple sweet potato cultivar, P40 which we previously bred in Kansas. One of our objectives was to prove the higher level of anthocyanin content compared to other tested sweet potatoes samples, O' Henry, and NC Japanese. Our new purple sweet potato cultivar, P40 shows its prominent level of anthocyanins content (7.5 g of peonidine 3-glucoside equivalent/kg dw) compared to controls (Table 2.1). Among control sweet potato samples, yellow-fleshed NC Japanese also showed small amount of anthocyanins, which we came from the purple color skin of NC Japanese may have been the

source of anthocyanins. We also found out that peonidin and cyanidin are the most predominant anthocyanidins in P40 extract, and they are likely to exist as peonidin or cyanidin 3-sophoroside-5-glucoside forms. They are mostly acylated with caffeic, ferulic, and/or hydroxybenzoic acids (Figure 2.2). In this matter, our results agree with others (47, 50, 51). In 1995 a new cultivar, Ayamurasaki, with high anthocyanin content was released from the sweet potato research group in Kyushu National Agricultural Experiment Station (KNAES) in Japan (52). The Ayamurasaki cultivar contains anthocyanins of 0.59 mg of peonidin 3-caffeoylsophoroside-5-glucoside (Pn 3-Caf·sop-5-glc) equivalent/g (53). However, our result showed that P40 was even higher than Ayamurasaki. In this study, we could not include other purple sweet potato cultivars as controls since we did not grow any of them in our farm. In order to control the environmental factors which might affect their nutrients or anthocyanin content, we selected white-fleshed O' Henry and yellow-fleshed NC Japanese as control sweet potato samples to compare with purple-fleshed P40 because they were grown in the same farm under the same environment.

P40 was also significantly high in phenolic content (74.6170 mg GAE/g dw) as well as in antioxidant capacity (84.3522 mg TE/g dw) (Table 2.1). As we assumed, antioxidant capacity determined by FRAP assay showed that P40 had the highest antioxidant capacity among sweet potato samples we tested. Interestingly, O' Henry showed significantly higher antioxidant capacity than that of NC Japanese ($p < 0.05$) despite its low phenolic content. It might contain more antioxidant phytochemicals besides anthocyanins. Numerous studies suggested that the phenolic structure of anthocyanins might act as an antioxidant. Also, the presumed antioxidant capacity of anthocyanins might be related to their anticancer activity by scavenging reactive oxygen species (ROS) (54), increasing the oxygen-radical absorbing capacity of cells (55), stimulating the expression of Phase II detoxification enzymes (1), reducing the formation of oxidative adducts in DNA (56), decreasing lipid peroxidation (57) and finally inhibit tumor development caused by excessive oxidative stress (58).

Our data suggested that P40 was also high in soluble dietary fiber compared to controls (Table 2.1). Even though we did not evaluate the chemical composition of dietary fibers in our sweet potato samples, according to Mei et al. (59), dietary fibers from sweet potato is mostly composed of soluble dietary fiber, pectin and insoluble dietary fibers such as hemicellulose, cellulose, lignin. There also have been studies documenting the benefits of the soluble dietary fiber, pectin on intestinal health. Fiber also provides fecal bulk and act as a laxative (60). Pectin has been

reported to alleviate the symptoms associated with intestinal inflammation (61), and a role preventing colon cancer (62).

In conclusion, we demonstrated the superior anthocyanin content in purple sweet potato, P40 and its excellent antioxidant activity. Anthocyanin content of P40 was extremely higher than controls as well as other cultivars reported. These findings suggest that new purple-fleshed sweet potato, P40 may be the excellent therapeutic agent for various diseases possibly caused and developed by oxidative stress including cancers.

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Table 2.1. Nutrient composition, dietary fiber content, anthocyanins content, total phenolic content, and antioxidant capacity of sweet potato samples

	O'Henry	NC Japanese	P40
Dry matter (%)*	19.9	32.0	29.0
Protein (%)*‡	1.7	2.2	1.5
Fat (%)*	0.2	0.2	0.2
Ash (%)*	1.1	1.4	1.3
Starch (%)*	9.8	17.5	14.4
Dietary fiber (%) §			
IDF	9.3 ± 0.2	8.4 ± 1.9	8.4 ± 1.3
SDF	4.5 ± 1.0[b]	3.6 ± 1.7[b]	7.4 ± 1.4[a]
TDF	13.8 ± 0.9	12.0 ± 0.5	15.8 ± 0.5
Anthocyanins content ¥ (g PN3GE/kg dw)	UD	1.9 ± 0.0[b]	7.5 ± 0.4[a]
Total phenolic content ¥ (mg GAE/g dw)	16.2 ± 0.5[c]	25.6 ± 1.4[b]	74.6 ± 0.3[a]
Antioxidant capacity ¥ (mg TE/g dw)	49.1 ± 11.8[b]	47.9 ± 1.1[c]	84.4 ± 1.4[a]

* Data are reported on a 100% fresh weight basis.

‡ Protein is calculated using a 6.25 conversion factor.

§ Abbreviations are as follows : IDF, Insoluble dietary fiber; SDF, Soluble dietary fiber; TDF, total dietary fiber. Data are reported as percent of dry matter and expressed as Means ± SD, n=4

¥ Abbreviations are as follows : UD, Undetectable value; dw, dry weight; PN3GE, Peonidin 3-glucoside equivalent value; GAE, Gallic acid equivalent value; TE, Trolox equivalent value. Data were expressed as Means ± SD, n=4

[abc]Means in the same row without a common letter are significantly different, $p < 0.05$

FIGURE LEGENDS

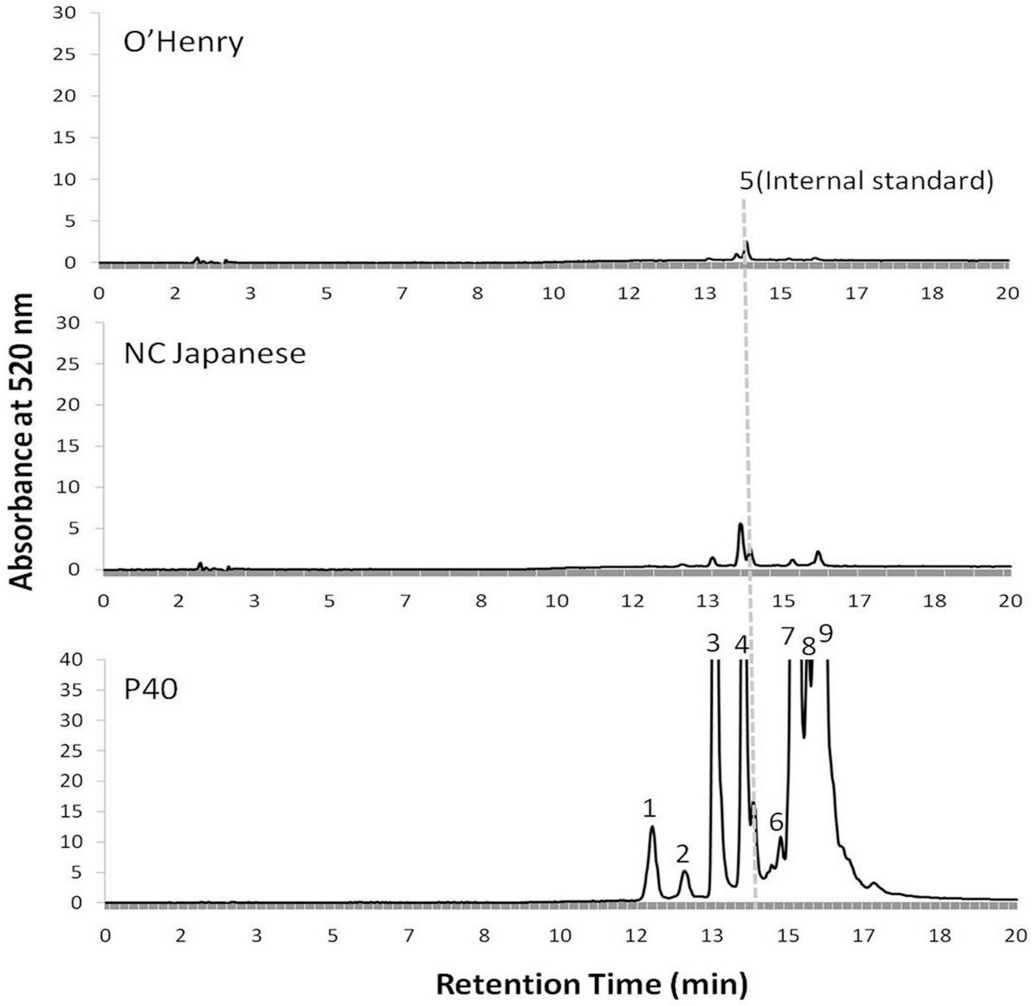
FIGURE 2.1. Selected sweet potato samples. White-fleshed O'Henry, yellow-fleshed NC Japanese, and Purple-fleshed P40 were grown in the John C. Pair Horticulture Research Center, Wichita, Kansas.

FIGURE 2.2. Representative HPLC chromatogram, UV/vis spectra and mass spectrometric data of anthocyanins detected in extracts from three sweet potato samples. Peaks were dectcted at 520nm. Peonidin 3-glucoside was used as an internal standard(peak no.5). Each number of peak identities were confirmed by mass and listed in table below.

FIGURE 2.3. Mass spectrometric data of peonidin 3-glucose detected in extracts from sweet potato samples.

FIGURE 2.4. Mass spectrometric data of anthocyanins detected in purple sweet potato P40 extract.

FIGURE 2.1



Peak no.*	MW (m/z)	Anthocyanins	Precursor of Anthocyanidins (m/z)
1	773	cyanidin 3-sophoroside-5-glucoside	cyanidin (287)
2	787	peonidin 3-sophoroside-5-glucoside	peonidin (301)
3	893	cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside	cyanidin (287)
4	907	peonidin 3-p-hydroxybenzoylsophoroside-5-glucoside	peonidin (301)
5	498	peonidin 3-glucoside	peonidin (301)
6	1055	cyanidin 3-caffeoyl-p-hydroxybenzoylsophoroside-5-glucoside	cyanidin (287)
7	757	pelargonidin 3-sophoroside-5-glucoside	pelargonidin (271)
8	949	cyanidin 3-(6"-feruloylsophoroside)-5-glucoside	cyanidin (287)
9	1069	peonidin 3-caffeoyl-p-hydroxybenzoyl-sophoroside-5-glucoside	peonidin (301)

FIGURE 2.2

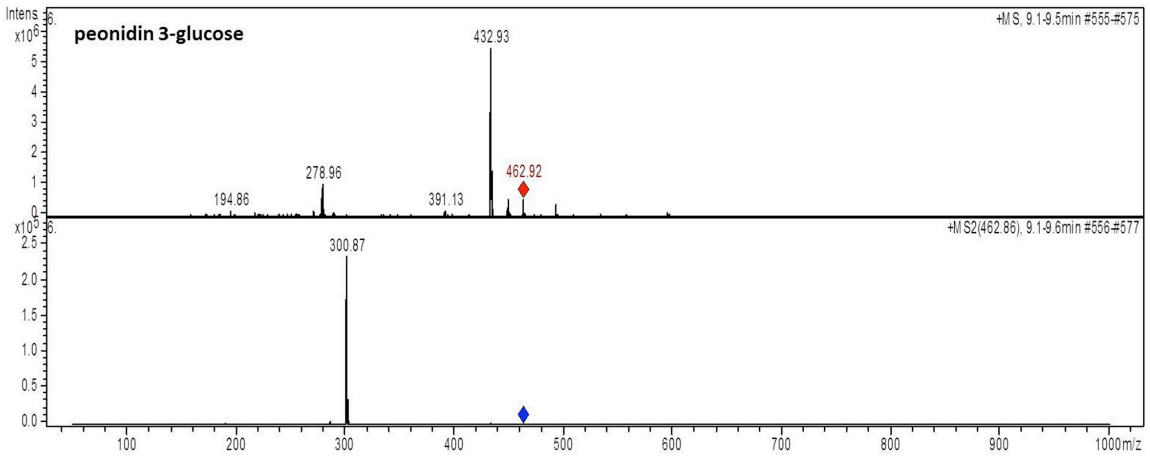

peonidin 3-glucose
+MS, 9.1-9.5min #555-#575
432.93
278.96
462.92
194.86
391.13
+MS2(462.86), 9.1-9.6min #556-#577
300.87
100
200
300
400
500
600
700
800
900
1000m/z

FIGURE 2.3

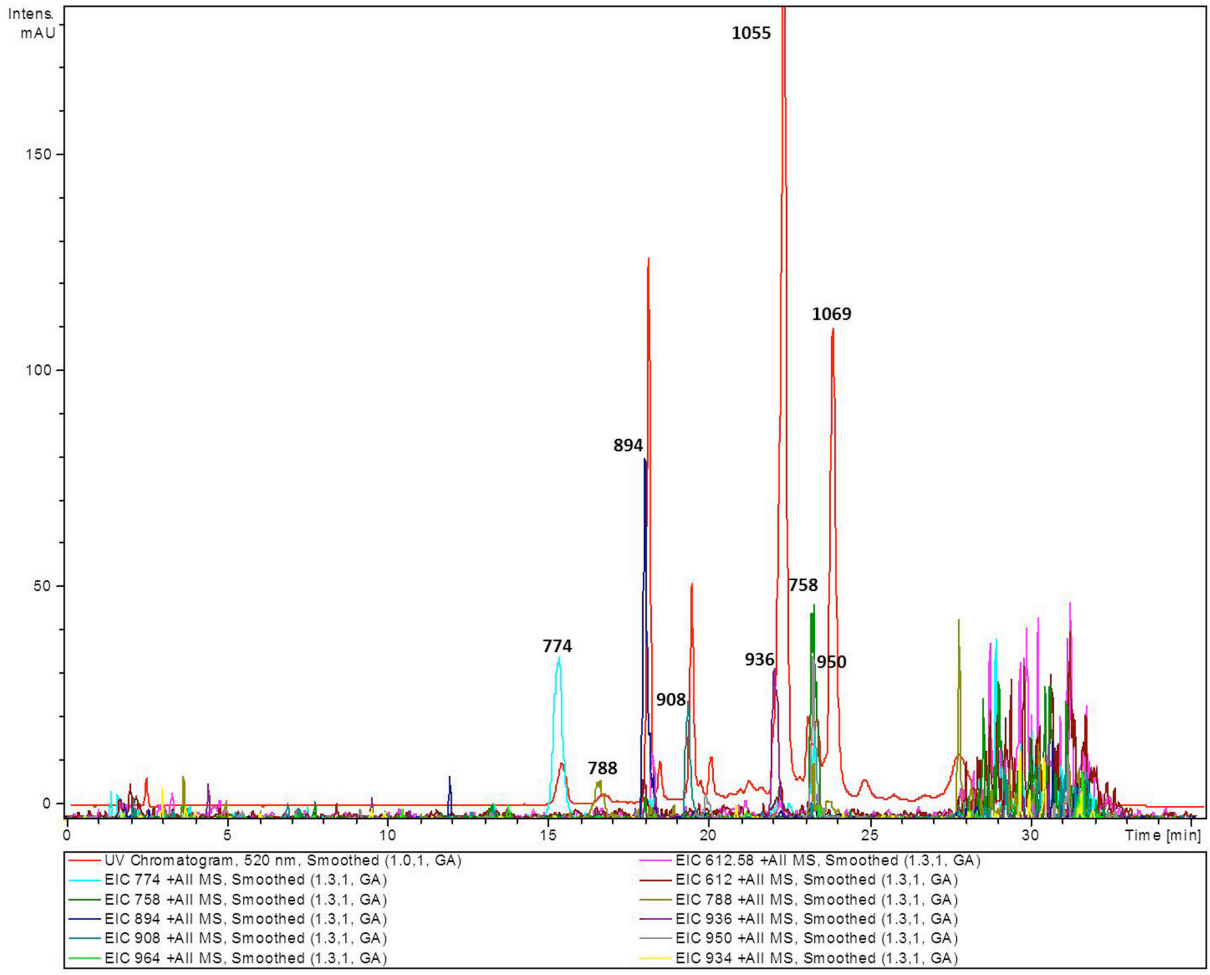

Intens.
mAU
150
100
50
0
1055
1069
894
758
774
936
950
908
788
0
5
10
15
20
25
30
Time [min]
UV Chromatogram, 520 nm, Smoothed (1.0,1, GA)
EIC 774 +All MS, Smoothed (1.3,1, GA)
EIC 758 +All MS, Smoothed (1.3,1, GA)
EIC 894 +All MS, Smoothed (1.3,1, GA)
EIC 908 +All MS, Smoothed (1.3,1, GA)
EIC 964 +All MS, Smoothed (1.3,1, GA)
EIC 612.58 +All MS, Smoothed (1.3,1, GA)
EIC 612 +All MS, Smoothed (1.3,1, GA)
EIC 788 +All MS, Smoothed (1.3,1, GA)
EIC 936 +All MS, Smoothed (1.3,1, GA)
EIC 950 +All MS, Smoothed (1.3,1, GA)
EIC 934 +All MS, Smoothed (1.3,1, GA)

FIGURE 2.4

CHAPTER 3 – ANTHOCYANIN ENRICHED PURPLE-FLESHED SWEET POTATO FOR POTENTIAL CANCER PREVENTION

Anthocyanin- Enriched Purple-Fleshed Sweet Potato For Potential Colon Cancer Prevention[1]

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[3]Abbreviations used : AOM, azoxymethane; ACF, Aberrant crypt foci; PCNA, Proliferating Cell Nuclear Antigen;

ABSTRACT

Previously, we selected a purple-fleshed sweet potato clone, P40, from seeds obtained by crossbreeding. This study is to identify the chemopreventive effect of anthocyanins from purple sweet potato, P40. We treated SW480 human colon cancer cells with 0 - 40μM of peonidin-3-glucoside or P40 extract containing corresponding amount of anthocyanins. Both of the treatments inhibited cell growth in a dose-dependent manner, however, cells treated with P40 extract tends to survive significantly less than those treated with peonidin-3-glucoside. However, there was no cytotoxicity occurrence during/after treatment. By checking the cell cycle changes, we found the growth inhibition was not due to cytotoxicity, but due to cytostatic mechanism with increased number of cells arrested at G1 phase. We also assessed cancer preventive effect of purple sweet potato diet by using azoxymethane (AOM)-induced aberrant crypt foci (ACF) in mice. AOM or saline injected mice were fed basal AIN-93M diet or diets containing 10~30% of P40, 20% O' Henry or 20% NC Japanese for 6 weeks. After the dietary treatment, ACF multiplicity was significantly inhibited by 10~30% of P40 diet. Results of imunohistochemistry in colonic mucosa showed that the expression level of apoptosis marker, caspase-3, was significantly induced in the mice fed 20% of NC Japanese or 10~20% of P40 diet. Also, PCNA expression level, which is proliferation marker, was significantly inhibited by 30% of P40 diet compared to basal diet fed mice. Both in vitro and in vivo results suggest a promising chemopreventive effect of P40 in cancers.

INTRODUCTION

Studies on the biological and nutraceutical properties of sweet potatoes tend towards focusing on purple sweet potato. Studies have shown the free radical scavenging (1), antidiabetic (2), and chemopreventive activity of purple sweet potato roots and leaves (3,4). These biological effects of purple sweet potato may be due to the phenolic pigment "anthocyanin".

Anthocyanins are polyphenolic compounds, which are responsible for the intense colors of many fruits and vegetables such as red grapes, berries, red cabbages and purple sweet potato (5,6). Anthocyanins not only plays important role in industry as a natural food colorant, but also provides various health benefits including antioxidant and anti-inflammatory effects (7-9). They may also reduce the risk of cardiovascular disease (10), diabetes (11), and age-related neurodegenerative diseases (12).

Anthocyanins or anthocyanin-rich extracts have exhibited inhibitory effect on cancer cell growth or tumor-inducing cellular events in variety of cancer cells such as lung (13), breast (14), prostate (15), liver (16), and colon (17) cancers, etc. Also, animal studies have been conducted to prove their anti-cancer activities by using carcinogen-treated animal models. Those studies have shown that a anthocyanin-rich diet induced apoptosis and inhibited cell proliferation, inflammation, and angiogenesis, aberrant crypt foci (ACF) multiplicity, total tumor multiplicity, tumor burden, and adenocarcinoma multiplicity in tumor tissues of cancer induced animals (18,19).

Studies have suggested that several anti-cancer mechanisms of anthocyanins may be involved such as their strong antioxidant, anti-inflammatory properties, and apoptosis induction by regulating cell cycle in cancers. Phenolic structure of anthocyanins may act as an antioxidant and inhibit tumor development caused by excessive oxidative stress (20-25). Also, anti-inflammatory effect of anthocyanins may play an important role in cancer prevention. Abnormal up-regulation of inflammatory proteins such as nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 (COX-2) is commonly present in many cancers, and inhibitors of those proteins showed significant cancer preventive effect (26). Inhibitory effects of anthocyanins on mRNA or protein expression levels of NF-κB, COX-2, and various inflammatory interleukins have been reported (27,28). Studies showed that anthocyanin treatments may inhibit cell growth and induce apoptosis in cancer cells by interrupting cell cycle at G1 and G2/M phase (29,30). However, the involved mechanisms are still not conclusive and results differ depending on tested anthocyanins from different sources.

In the present study, we bred a new variety of purple-fleshed sweet potato clone, P40, from seeds obtained by crossbreeding from the International Potato Center in Lima, Peru. We hypothesized that P40 may have high anthocyanin content and have health beneficial activities compared to other sweet potato cultivars. To prove this, we included two cultivars as controls,

which are white- (O' Henry) and yellow-fleshed (NC Japanese) sweet potato. Chemopreventive effect of anthocyanin from purple sweet potato was tested on SW480 human colon cancer cells and azoxymethan-induced aberrant crypt foci in mice. Finally, we investigated the potential mechanisms involved in this inhibition.

MATERIALS AND METHODS

Reagents

All organic solvents were HPLC grade, and purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's Modified Eagle medium, fetal bovine serum, penicillin/streptomycin, trypsin-EDTA, trypan blue, propidium iodide (PI), RNase, azoxymethan (AOM, 98% pure), 10% neutral buffered formalin, methylene blue, and rabbit anti-caspase 3 were obtained from Sigma-Aldrich (St. Louis, MO, USA). PCNA staining kit was purchased from Invitrogen (Carlsbad, CA, USA). Secondary label donkey anti-rabbit HRP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peonidin-3-glucoside chloride was obtained from Chromadex (Irvine, CA, USA). Human colon cancer line, SW480 was obtained from ATCC (Manassas, VA, USA). Female CF-1 mice (n=52) were purchased from Charles River (Wilmington, MA, USA). Powder ingredients for AIN-93M diet formulation were purchased from Harlan (Indianapolice, IN, USA).

Purple (P40), white (O' Henry) and yellow (NC Japanese) fleshed sweet potatoes (Figure 2.1) were provided from the John C. Pair Horticulture Research Center, Wichita, Kansas. Purple-fleshed sweet potato was selected using seeds from controlled crosses provided by the International Potato Center in Lima, Peru. Over 2000 seedlings from four full-sib progenies were cultured, evaluated, and selected in the field. One genotype, designated P40, with intense anthocyanin pigmentation and reasonable yield was the subject of this study.

Sample preparation and extraction

For each sweet potato sample, tubers were randomly taken, washed with tap water, diced into approximately 0.5cm cubes, freeze-dried (general purpose freeze dryer VirTis GPFD 36DX66,

SP Scientific, Gardiner, NY) and ground by cutting mills (Retsch, Newtown, PA) into flour. Prepared flour was stored at -80˚C until use. For preparation of anthocyanin extracts, 1g of flour was extracted with 8ml of acidified MeOH (1N HCL, 85:15, v/v) to obtain a sample-to solvent ratio of 1:8. The flasks containing flour/solvent mixture were covered with aluminum foil to avoid exposure to light and stirred on magnetic stirrer overnight. After 12hrs extraction, extracts were centrifuged (1,800rpm, 30min) and supernatant was taken. Supernatant was filtered through Whatman paper no. 1 and syringe filtered.

Cell viability and cytotoxicity assay

Human colon cancer cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded at a density of 6×10^5 cells in 6-well plates at 37˚C in a 5% CO_2 atmosphere for cell attachment and spreading. After cells were washed twice with PBS, the medium was changed to DMEM medium containing peonidin 3-glucoside at 0-40μM. Cells are also treated with P40 extract at 0-40 μM of peonidin-3-glucoside equivalent doses based on anthocyanin content measured in Chapter 2 (Table 2.1). After 48hrs incubation with treatment, the survival cells were detached by trypsin-EDTA, stained by trypan blue, and counted by hemocytometer. The survival cell numbers in treated cells were compared with that in medium controls. Cytotoxicity was also checked in adherent cells by trypan blue staining as described by our previous publication (31).

Cell cycle analysis

SW480 cells were seeded at a density of 6×10^5 cells per well in 6-well plates, and after 48h treatment with medium control or peonidin 3-glucoside (10-40μM), cells were trypsinized and fixed in ethanol for 24h at -20˚C. The centrifuged pellet was resuspended with phosphate buffer saline solution (pH7.4), containing 20g/L propidium iodide (PI) and 5000 U/L of RNase for 30min at 37˚C. PI stained cells were analyzed using fluorescence-activated cell sorter (FACS) Calibur flow cytometer (FACSCaliber, Becton Dickinson, New york, NJ) with an excitation at 488 nm and an emission at 630 nm. Results were reported as percent cells in each phase of the cell cycle (31).

Preparation of animal diet

We mixed animal diet ingredients based on AIN-93M diet formulation and used for control diet. Experimental diets containing 10-30% sweet potato powder were balanced for protein, fat, energy, and fiber as similar as AIN-93M control diet. Freeze-dried sweet potato powder was mixed into diet to achieve the 20% O' Henry, 20% NC Japanese, and 10-30% P40 concentration and stored at 4˚C in the dark (Table 3.1). All diets were adjusted to be iso-caloric.

Animals and treatments

Female CF-1 mice (n=52), aged 6-7 wk, 22.6 ±1.1 g body weight, were housed 3-5 mice per cage containing paper bedding. They were maintained under standard conditions (23 ± 0.5˚C, 22 ± 5 % humidity) on a 12:12h light-dark cycle for the duration of the study. Feed and water were available ad libitum throughout the course of the study. For 1- wk of acclimatization period, all animals were fed AIN-93M diet. Mice were monitored daily. Body weight was recorded weekly, and food intake was recorded daily. On the second week, mice were randomized into 2 control groups (Group 1 and Group2) and 5 experimental diet groups (Group 3-7). The animals in groups 2-7 received an injection of azoxymethan (AOM) in a saline vehicle at 10mg/kg body wt intraperitonially (i.p) once per week for 2 wks. At the same time, animals in Group 1 received equal volumes of saline. After the first injection, animals were switched to the experimental diets of 0% sweet potato (Group 1 and Group 2), 20% O' Henry (Group 3), 20% NC Japanese (Group 4), and 10-30% P40 (Group 5-7).

Aberrant crypt foci

At 5½ wk after the final AOM injection, all mice were sacrificed by diethyl ether and decapitation. Organ weight for liver, spleen or kidneys was measured in order to monitor toxicity of sweet potato high diet. Colons were removed from the end of the cecum to the end of the rectum, opened longitudinally, rinsed in PBS, and fixed in 10% neutral buffered formalin. Fixed colon tissues were stained with 0.2% methylene blue solution, and the aberrant crypt foci (ACF) were counted using a light microscope at ×40 magnification. ACF were classified on the basis of

the number of crypts per focus (i.e., small: 2-3, Medium:4-5, or Large: >5 crypts) (32). The same colon tissues were subsequently examined for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry procedures followed those in our previous publication (32) with slight modification. Briefly, five pieces of lower intestine were dissected from each of the seven samples groups and embedded in paraffin so that the absorption loops were at the sagittal plane. The samples were de-paraffinized in xylene, rehydrated through alcohol to TBST (Tris buffered saline / 1% Tween -20). Antigen bearing was achieved through 95˚C steam bath in citrate buffer (pH 6) containing 1% Tween 20 for 30 min. Tissues were blocked with the serum of the secondary antibody species. For caspase 3 labeling, sections were incubated overnight at 95˚C rabbit anti-caspase 3 at 1:500. The samples were rinsed three times in TBST for 3 min. The secondary label donkey anti-rabbit HRP was used at 1:5000 for 20 min at room temperature, and rinsed with TBST. Substrate 3,3'-Diaminobenzidine (DAB) exposure (10 min) was followed by counterstain Harris hematoxylin (1 min). Both were rinsed with distilled water. Stained slides were dehydrated and cover slipped. Each group of samples was evaluated at 400x with a light microscope and given a score 0-40 based on stain intensity and percent of area stained using computer standards by a pathologist blinded to slide identity. For PCNA labeling, a PCNA staining kit was used though the same process as above. Quantification of staining was performed at 400x by counting total cells and total PCNA-stained cells in every 5th absorption loop of the colon. Stained cells were further graded as light stain or dark stain. Data were summarized as percent of all stained cells/total cells.

Statistical analysis

Data were analyzed by using SAS statistical system, version 9.2. Results were evaluated by analysis of variance (ANOVA). Each experiment was conducted in multiplication(n=3~7), and the results were expressed as means±SDs. Means were separated using Turkey's studentized range test. A probability $p < 0.05$ was considered significantly.

RESULTS

Cell viability and cytotoxicity

Based on HPLC-MS/ESI data, we learned that peonidin is the most predominant anthocyanidin in P40. Therefore, peonidin-3-glucoside was chosen for treatment of SW480 human colon cancer cells in order to evaluate the effect of anthocyanins on cell growth. After 48h treatment of peonidin-3-glucoside (0-40μM), cell growth was significantly inhibited in dose-dependent manner ($p < 0.01$). When the cells were treated with P40 extract at 0-40 μM peonidin 3-glucoside equivalent doses, cell growth was also significantly inhibited with the same pattern as a result of peonidin 3-glucoside treatment, but even stronger ($p < 0.01$) (Figure 3.1). Cytotoxicity was also checked by the tryphan blue staining, and there was no significant difference (data not shown).

Cell cycle arrest

After 48h treatment with anthocyanins, the percent distribution of cells in the G0/G1, S, and G2/M phases was determined by FACS analysis after 48h treatment with peonidin-3-glucoside (Figure 3.2). The treatments significantly increased the percentage of cells in G0/G1, and decreased the percentage in S ($p < 0.05$). It suggested that cells were arrested at G1 phase by the treatment.

Diet consumption and mice body weight

Diet consumption of groups of mice fed sweet potato diet (Table 3.2) was significantly lower than control groups fed AIN93M diet. However, final body weight was significantly less in 20% O' Henry, 20% NC Japanese, and 10% P40 diet groups than control, not in 20~30% P40 diet groups ($p < 0.05$). AOM injection did not affect either diet intake or weight gain. Organ to body weight ratio of liver, spleen, or kidney did not reveal any significant differences between any diet groups (Table 3.2).

Aberrant crypt foci

ACF were induced in groups of animals injected with AOM (Figure 3.3.B and Table 3.3), and most of ACF were observed in the distal portion of the colon. Total number of ACF, large (≥ 5 multiplicity) and medium (4-5 multiplicity) ACF were significantly decreased in colons of mice fed 10-30% P40 diet when compared with mice fed the AIN93M control diet ($p < 0.05$) (Table 3.3). 20% NC Japanese diet also significantly inhibited large ACF formation in mice colons ($p < 0.01$). There were no significant differences among small ACF formation in any diet groups.

Caspase 3 and PCNA expression detected by immunohistochemistry

To evaluate mechanisms involved in the chemopreventive activity of sweet potato diet, we further analyzed the protein levels of caspase 3 and PCNA in colon tissues by immunohistochemistry. Caspase 3 positive staining showed brownish yellow color (Figure 3.4. B). Percent of cells stained for caspase 3 both in 20% NC Japanese (14.8±4.9 5) ($p < 0.05$) and 20% P40 diet group (21.3±3.8%) ($p < 0.01$) were significantly more than those in control diet group (2.3 ±1.3 %) (Figure 3.4.C). For PCNA expression, the number of stained cells was significantly less in mouse fed the 30% P40 diet than control diet group (Figure 3.5) ($p < 0.01$).

DISCUSSION

Colon cancer is the one of the most common cancers and the third leading cause of cancer death for both men and women in the United States (33). The need for new chemopreventive agents without adverse effects have led researchers' interest to identifying phytochemicals for their potential use. Studies have constantly shown that preventive effects of phytochemical anthocyanins and their aglycons on colon cancer (3, 17, 19, 23, 29, 32, 34-36). As part of those efforts, we evaluated SW480 colon cancer cell growth and cell cycle changes by treating anthocyanin-rich purple sweet potato, P40, extract or its major anthocyanin, peonidin 3-glucoside. After 48h incubation, both of the treatments significantly inhibited cancer cell growth without cytotoxitity (Figure 3.1). Interestingly, despite the fact we treated the cells with the same level of anthocyanins in both treatments, the inhibitory effect was much higher in the cells

treated with P40 extract than those with peonidin 3-glucoside itself. P40 extract may contain various chemopreventive phytochemicals other than anthocyanins such as phenolics. We also learned that 48h treatment with peonidin 3-glucoside significantly arrested cancer cells increased at G1 phase (Figure 3.2). These findings suggest that P40 may inhibit cancer cell growth by inducing apoptosis, not by cytotoxicity.

Azoxymethane (AOM) is a classic carcinogen that has been used to initiate and promote tumor development in animal models that has characteristics similar to human colonic tumors (37). Aberrant crypt foci (ACF) system has been used extensively to identify modulators of colon carcinogenesis as the earliest identifiable putative premalignant precursors of animal or human colon cancers (32, 38-40). In agreement with others, we used AOM-induced mice model to test the chemopreventive effect of purple sweet potato diet. We fed the mice with formulated diet high in sweet potato (10~30% P40, 20% O' Henry, or 20% NC Japanese based on AIN93M diet (Table 3.1), and then evaluated the changes in ACF formation. 10-30% P40 diets effectively inhibited ACF growth indicating preventive effect of anthocyanin diet on tumor development in colon during the initiation phase by blocking ACF development. The result also showed 20% NC Japanese diet significantly inhibited large ACF formation in mice colons ($p < 0.01$) (Table 3.3). It could be because of small amount of anthocyanins which came from its purple cortex. It is also possible that the inhibition effect might be associated with beta-carotenoids, which usually exist in yellow-or orange-fleshed sweet potatoes (41). In this study, we only evaluated inhibitory effects of P40 on ACF formation. However, we are planning to do an extended study for observing the effect of anthocyanin on tumor endpoint.

Subsequently, we analyzed the mice colon tissues for protein expressions by immunohistochemistry. Studies have used various immunohistochemical markers as prognostic indices of tumors, for the most part carcinoma. Among those markers, we used caspase-3 and PCNA. As critical mediators of mitochondrial events of apoptosis, caspase 3 staining is an important method for indicating apoptosis level induced by a wide variety of apoptotic signals (42). PCNA is associated with most of the carcinogenesis, which develops proliferative abnormalities, thus it is often used as a cell proliferation marker (43). Our results showed that 20% NC Japanese diet and 20% P40 diet significantly induced caspase 3 expression in mice colon compared to control diet (Figure 3.4.C.). However, there was no induction of caspase 3 expression in 30% P40 diet group. We assume it might be experimental error since

immunohistochemistry only gives semi-quantitative data. We will confirm the data with further studies. In the colon of mouse fed 30% P40 diet, immunohistochemical staining level of PCNA decreased statistically significantly compared to control diet group (Figure 3.5.C). These findings suggest that 20% P40 diet may effectively upregulate Caspase 3 expression in mice colon tissues, thus, promote cell apoptosis, and 30% P40 diet showed inhibitory effect on cell proliferation in mice colon crypts.

In conclusion, we demonstrated the anticancer activity of anthocyanin-rich purple sweet potato, P40 in vitro and in vivo. In vitro, we showed cell growth inhibition effect of P40 due to its high level of anthocyanins content and suggested potential mechanism involved; high antioxidant capacity of P40 and ability of arresting cancer cells at G1phase. In vivo, P40 diet significantly inhibited ACF multiplicity by inducing apoptosis and inhibiting cell proliferation in AOM-induced mice crypts. These findings suggest that new purple-fleshed sweet potato, P40 may be an excellent therapeutic agent for preventing colon cancer in human.

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Table 3.1. AIN93M-based diet formulations

	g/kg total diet					
	Control	**20% O'Henry***	**20% NC Japanese €**	**10% P40 ℓ**	**20%P40**	**30% P40**
Sweetpotato powder	**0**	**200.0**	**200.0**	**100.0**	**200.0**	**300.0**
Casein	**140.0**	**122.8**	**126.3**	**134.9**	**129.7**	**124.6**
L-cystine	**1.8**	**1.8**	**1.8**	**1.8**	**1.8**	**1.8**
Corn starch	**465.7**	**366.7**	**356.0**	**416.4**	**367.0**	**317.7**
Maltodextrin	**155.0**	**80.8**	**85.7**	**113.3**	**71.7**	**30.0**
Sucrose	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
Soybean Oil	**40.0**	**38.4**	**38.7**	**39.4**	**38.9**	**38.3**
Cellulose	**50.0**	**42.1**	**43.9**	**46.7**	**43.4**	**40.1**
Mineral Mix ¥	**35.0**	**35.0**	**35.0**	**35.0**	**35.0**	**35.0**
Vitamin Mix ¶	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**
Choline Bitartrate	**2.5**	**2.5**	**2.5**	**2.5**	**2.5**	**2.5**
TBHQ, antioxidant	**0.008**	**0.008**	**0.008**	**0.008**	**0.008**	**0.008**

* Each gram of O'Henry powder contains 86.2mg of protein, 8.0mg of fat, 495.1mg of starch, and 39.5mg of fiber

€ Each gram of NC Japanese powder contains 68.5mg of protein, 6.4mg of fat, 548.3mg of starch, and 30.3mg of fiber

ℓ Each gram of P40 powder contains 51.4mg of protein, 5.7mg of fat, 493.4mg of starch, and 32.9mg of fiber

¥ AIN93M-mineral mix formulation

¶ AIN93M-mineral mix formulation

Table 3.2. Food intake, weight gain, and organ to body weight ratio in rats fed AIN93M-based sweet potato diet*

Diet groups[¥]	AOM injection	Food intake (g/day/mouse)	Final body weight (g)	Organ to body weight ratio		
				Liver(%)	Spleen(%)	Kidneys(%)
AIN93M	+	9.3 ± 1.3[a]	34.0 ± 3.2[a]	4.8 ± 0.5	0.4 ± 0.2	1.1 ± 0.2
AIN93M	−	9.6 ± 1.0[a]	38.3 ± 4.3[a]	4.1 ± 0.6	0.3 ± 0.1	1.1 ± 0.1
20% O'Henry	+	6.3 ± 0.8[b]	28.1 ± 2.4[c]	4.9 ± 0.5	0.5 ± 0.2	1.1 ± 0.1
20%NCJapanese	+	5.7 ± 0.8[b]	29.1 ± 3.2[c]	4.9 ± 0.6	0.5 ± 0.2	1.2 ± 0.4
10% P40	+	5.8 ± 0.5[b]	31.3 ± 3.7[b]	4.6 ± 0.5	0.5 ± 0.2	1.2 ± 0.3
20% P40	+	6.1 ± 1.2[b]	32.9 ± 3.2[a]	3.9 ± 0.4	0.3 ± 0.0	1.1 ± 0.2
30% P40	+	6.3 ± 1.0[b]	32.9 ± 3.5[a]	4.2 ± 0.7	0.5 ± 0.2	1.2 ± 0.4

* Data were expressed as Means ± SD

¥ Number of mice examined for diet groups: AIN93M, n=5 ; 20% O' Henry, n=6 ; 20% NC Japanese, n=6; 10% P40, n=7; 20% P40, n=7; 30% P40, n=5

[abc]Means in the same column without a common letter are significantly different, $p < 0.05$

Table 3.3. Effect of sweet potato diet on aberrant crypt foci (ACF) multiplicity in Azoxymethane-induced mice[¥]

Diet groups[€]	AOM injection	Total ACF/colon	Small (2-3)	Middle (4-5)	Large (≥5)
AIN93M	+	53.4 ± 11.4	22.0 ± 7.1	18.0 ± 3.2	13.4 ± 3.4
AIN93M	−	3.0 ± 1.9**	1.0 ± 1.0**	1.4 ± 0.9**	0.6 ± 0.6**
20% O' Henry	+	41.2 ± 1.3	17.3 ± 2.2	14.7 ± 1.8	9.2 ± 0.8
20% NC Japanese	+	35.8 ± 13.6	17.2 ± 6.6	12.2 ± 4.8	6.5 ± 2.7**
10% P40	+	32.6 ± 10.7*	15.7 ± 5.9	10.7 ± 3.8*	6.1 ± 2.8**
20% P40	+	26.7 ± 12.9**	13.1 ± 7.5	8.7 ± 3.2**	4.9 ± 2.9**
30% P40	+	22.0 ± 13.1**	12.4 ± 3.1	9.4 ± 5.7*	4.6 ± 1.3**

¥ Data were expressed as Means ± SD

€ Number of mice examined for diet groups: AIN93M/AOM+, n=5 ; AIN93M/AOM-, n=5 ; 20% O' Henry, n=6 ; 20% NC Japanese, n=6; 10% P40, n=7; 20% P40, n=7; 30% P40, n=5

*, $p<0.05$ versus AIN93M/AOM+ group; **, $p<0.01$ AIN93M/AOM+ group.

FIGURE LEGENDS

FIGURE 3.1. The effects of peonidin 3-glucose or P40 extract on cell growth in SW480 human colon cancer cell line. Cells were exposed to concentrations of major anthocyanin of purple sweetpotato, peonidin-3-glucose (■) or P40 extract with a peonidin 3-glucose equivalent doses (□) for 48h. The cell growth was assessed in adherent cells by trypan blue staining. Each value represents the mean for triplicates. [abcd] Means within a treatment without a common letter are significantly different, $p < 0.05$. Each value represents the mean for triplicates and vertical bars indicate the standard deviation.

FIGURE 3.2. The effect of peonidin 3-glucoside on cell cycle changes in SW480 human colon cancer cell line. Cells were exposed to concentrations of peonidin-3-glucoside for 48h, and analyzed by FACS analysis as described in Materials and Methods. *, $p<0.05$ versus vehicle control; **, $p<0.01$ versus vehicle control. Each value represents the mean for triplicates and vertical bars indicate the standard deviation.

FIGURE 3.3. Representative images of induced ACF in groups of animals injected Azoxymethan (AOM). (A) Normal crypt foci versus (B) aberrant crypt foci (ACF) (pointed with an arrow).

FIGURE 3.4. Protein expression of Caspase3 by immunohistochemistry staining. Representative images of caspase 3 expression; (A) AIN93M control diet or (B) 20% P40 diet group. (C) Quantification data of caspase 3 expression level in colon section was acquired by counting anti-Caspase3 stained cells. Each value represents the mean for five multiplicates and vertical bars indicate the standard deviation. *, $p<0.05$ versus vehicle control; **, $p<0.01$ versus vehicle control.

FIGURE 3.5. Protein expression of PCNA measured by immunohistochemistry staining. Representative images of PCNA expression; (A) AIN93M control diet or (B) 30% P40 diet group. (C) Quantification data of PCNA expression level in colon section was acquired by counting anti-PCNA stained cells. Each value represents the mean for five multiplicates and vertical bars indicate the standard deviation. **, $p<0.01$ versus vehicle control.

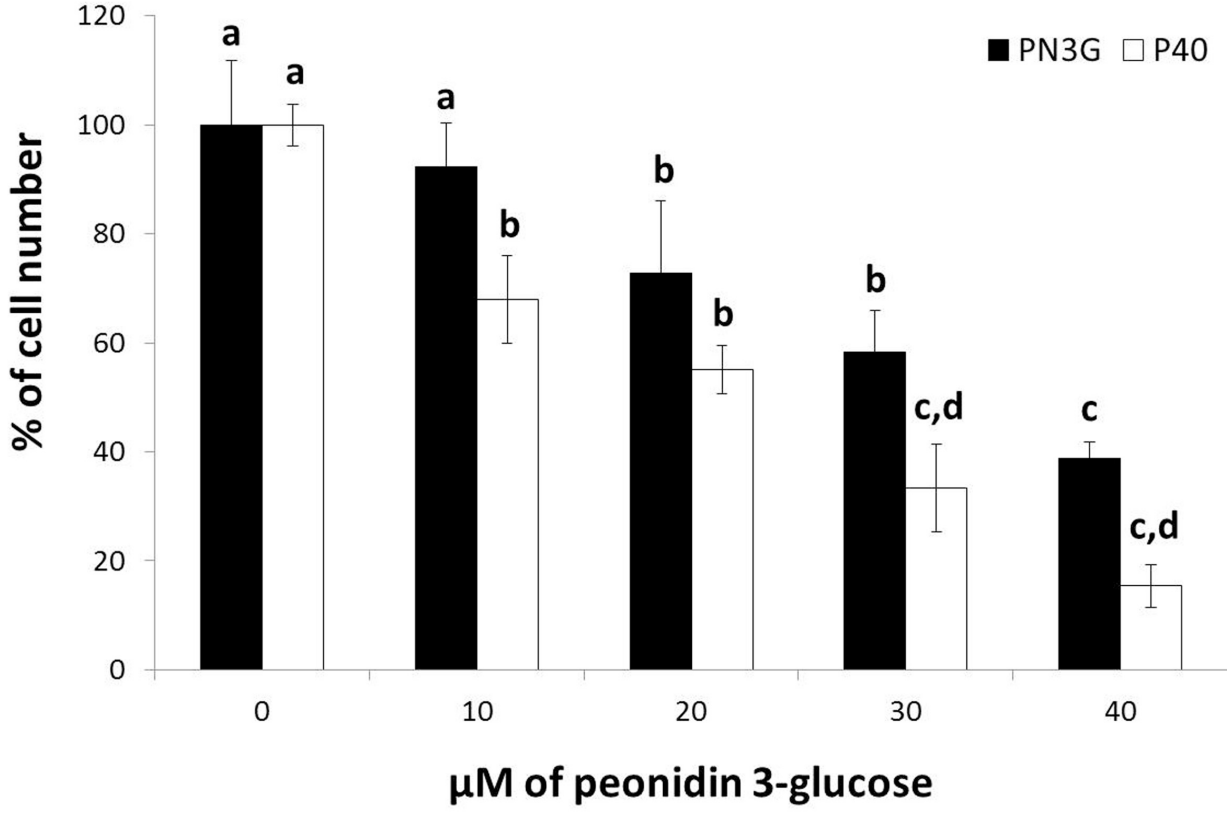
120
100
80
60
40
20
0
% of cell number
PN3G
P40
a
a
a
b
b
b
b
c,d
c
c,d
0
10
20
30
40
µM of peonidin 3-glucose

FIGURE 3.1

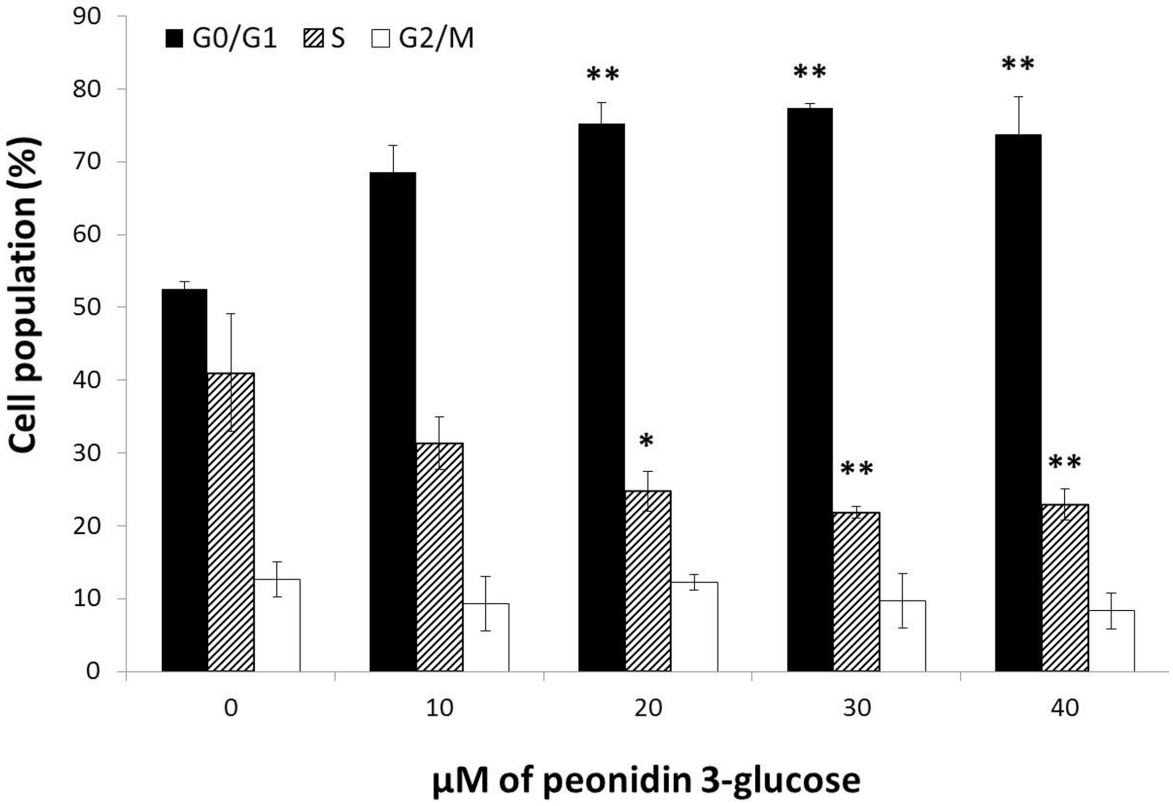
90
80
70
60
50
40
30
20
10
0
Cell population (%)
G0/G1
S
G2/M
**
**
**
*
**
**
0
10
20
30
40
µM of peonidin 3-glucose

FIGURE 3.2

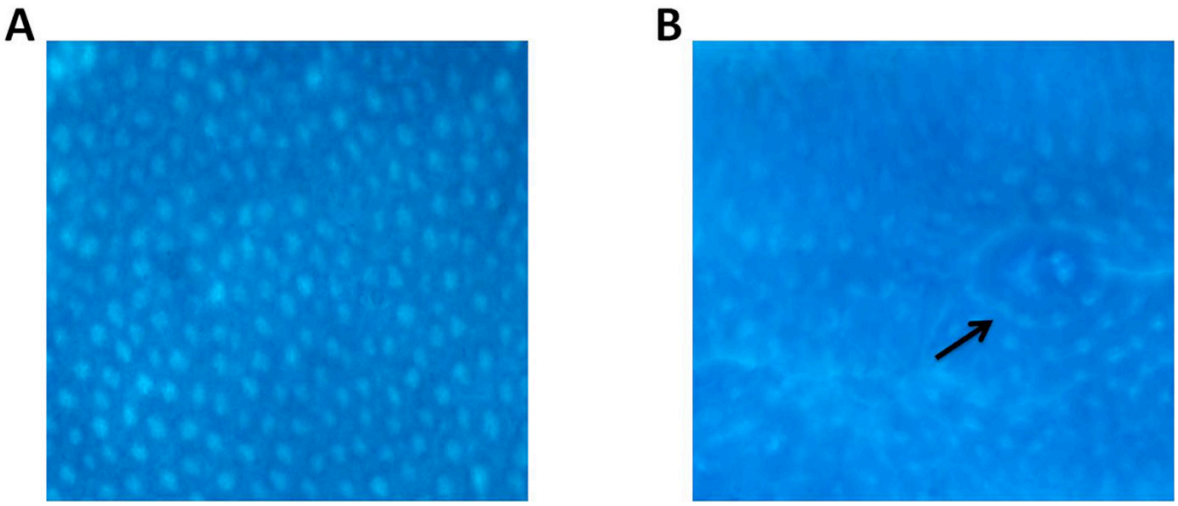

FIGURE 3.3

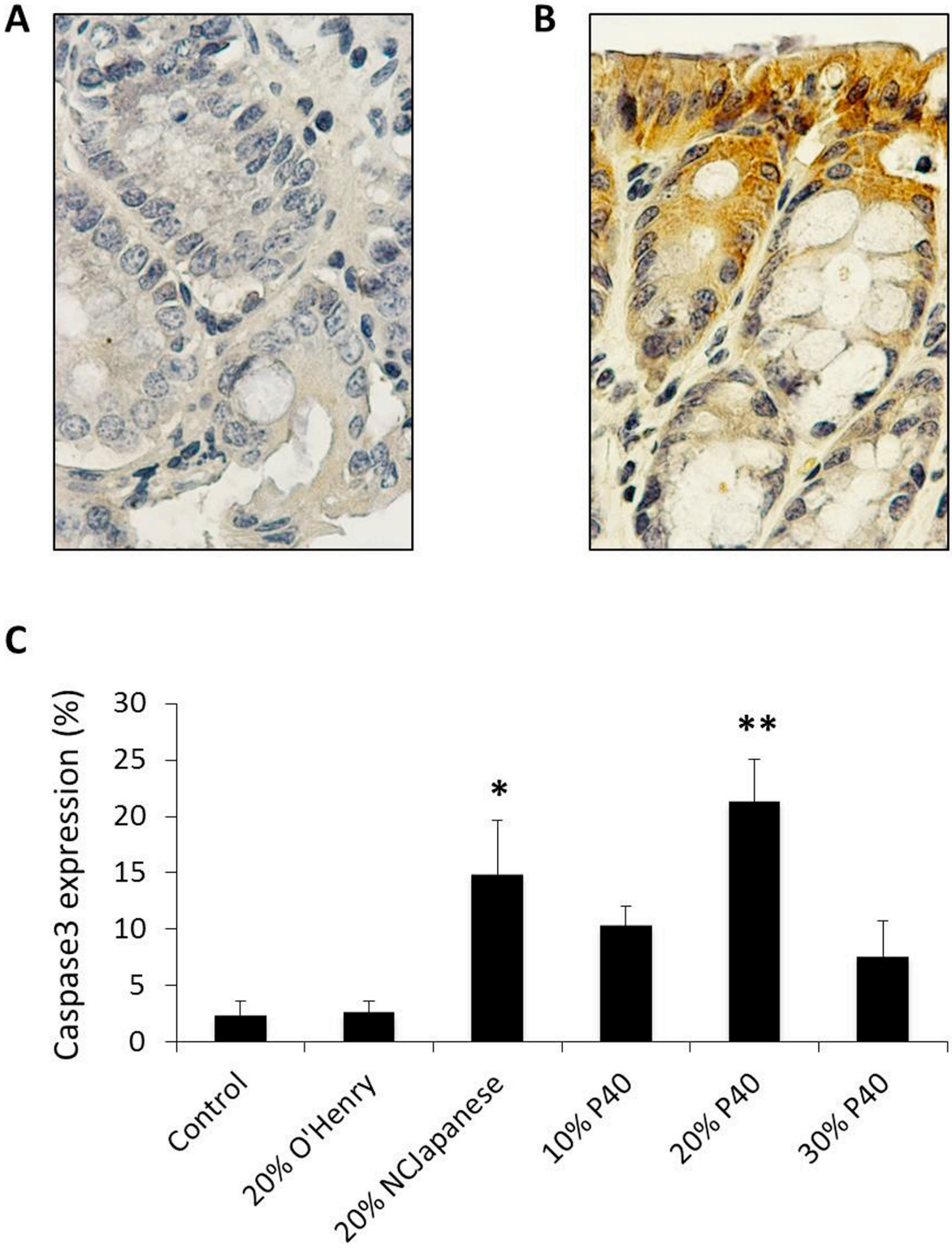
A
B
C
Caspase3 expression (%)
30
25
20
15
10
5
0
*
**
Control
20% O'Henry
20% NCJapanese
10% P40
20% P40
30% P40

FIGURE 3.4

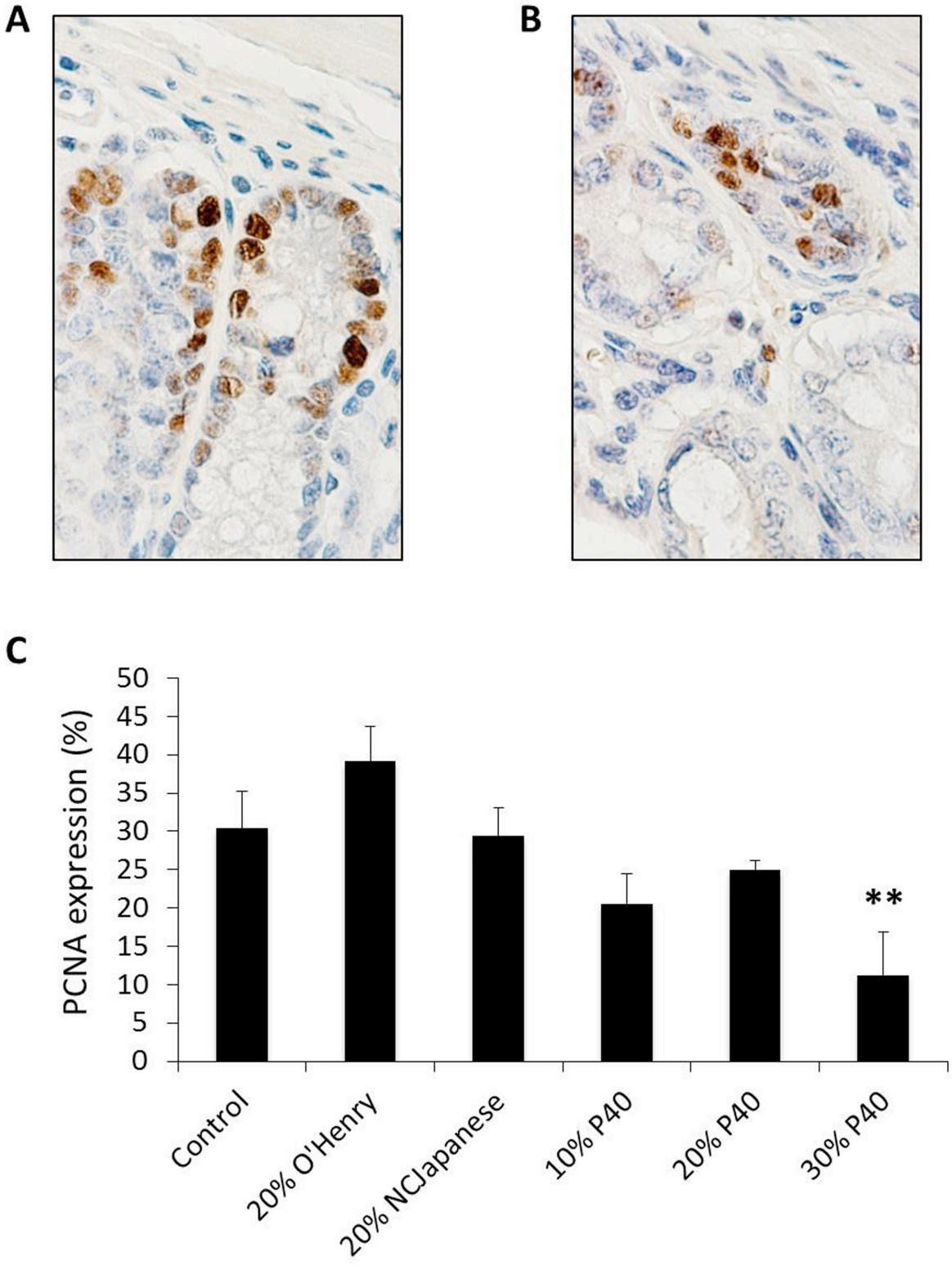
A
B
C
PCNA expression (%)
50
45
40
35
30
25
20
15
10
5
0
**
Control
20% O'Henry
20% NCJapanese
10% P40
20% P40
30% P40

FIGURE 3.5