Antioxidant Activity and Persistence of Cottonseed Protein and Oil from Two Cultivars as Determined by Their Ability to Scavenge Peroxyl and Alkoxyl Radicals

Dipaloke Mukherjee

Zahur Z. Haque

Department of Food Science
Nutrition and Health Promotion
Mississippi State University
MAFES, MS 39762

Abstract

Short-term antioxidant activity (AA) and long-term antioxidative persistence (AP) of protein and oil fractions extracted from two cultivars of cotton, namely PIMA 3.79 056-7900 (Gossypium barbadense) and TM1 123-1452 (G. hirsutum) were investigated. This was based on their efficacy to scavenge peroxyl and alkoxyl radicals generated in-vitro by pyrolysis of 2,2'-Azobis(2-methylpropionamidine) dihydrochloride. Luminal induced chemiluminescence resulting from unquenched radicals was used to monitor the relative changes in such efficiency in real time. Results showed significantly greater (P<0.05) AA and AP of protein and oil extracts from both the cultivars compared to the control. Unlike the proteins, the oil extracts did not exhibit a significant difference among themselves regarding AA.They also showed a considerable deterioration in their AP. Such decline in the antioxidative efficacy of the hydrophobic non-protenaceous ether extractfrom cottonseeds has not been reported before.

Keywords: Reactive oxygen species, antioxidant, phytochemical.

1. Introduction

Reactive oxygen species (ROS) can be generated due to a number of extrinsic [e.g., exposure to ultra violet radiation (Pillai et al., 2005)] or intrinsic [mitochondrial respiration (Turrens, 2003)] factors. These radicals are known to cause morbidity and mortality in the humans by leading to a number of potentially lethal health consequences, including cardio-vascular diseases (Sugamura and Keaney, 2011) and cancers (Waris and Ahsan, 2006). Therefore, there is an ongoing pursuit for the identification of novel antioxidants – substances that can potentially alleviate the life threatening consequences of ROS. Cotton (*Gossypium sp.*) is a crop with significant economic importance in many counties, including the USA. Besides the use of cotton as a fiber crop, the seeds have long been used as animal feed. Nevertheless, due to their rich nutritional properties, their utilization in human diet has become a focus of interest (Gao et al., 2010;Sunilkumar et al., 2006).

The seeds contain considerable amounts of proteins (~42% crude protein in dehulled cottonseed meal) and lipid (~16%) (Nergiz, et al., 1997). Gao et al. (2010) reported the remarkable antioxidative properties of peptide fractions isolated from Neutrase hydrolysate of cottonseed protein. Lipids extracted from cottonseeds contain palmitic (20-25%), stearic (2-7%), oleic (18-30%) and linoleic (40 – 55%) acids (Aluyor and Ori-Jesu, 2008). The presence of adequate contents of saturated fatty acids makes cottonseed oil a comparatively stable one (O'Brien and Wakelyn, 2005). It possesses marked antioxidant properties due to its high content of α-tocopherols (Agarwal et al., 2003). In the current study, we have investigated the short and long term antioxidative properties (antioxidant activity, AA and persistence, AP, respectively) of crude protein and oil extracts from seeds of two cultivars of cotton based on real-time analyses of their efficacy to quench artificially generated alkoxyl and peroxyl radicals. We hypothesize that these two fractions would exhibit significantly different antioxidative properties from each other.

2. Materials and Methods

2.1 Materials

Seeds from two cotton cultivars [PIMA 3.79 056-7900 (PIMA) (*Gossypium barbadense*) and TM1 123-1452 (TM1) (*Gossypium hirsutum*)] were provided by Sukumar Saha of the Agricultural Research Service, United States Department of Agriculture, Mississippi State, MS. Diethyl ether, acetone, sodium hydroxide, sodium phosphate dibasic, citric acid, luminal and 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (ABAP) were purchased from Sigma-Aldrich Co. (Milwaukee, WI, USA). Clear bottom 96 well assay plates, polypropylene centrifuge tubes and Whatman 2 grade filter papers were from Fisher Scientific (Fair Lawn, NJ, USA). **2.2 2.2**

Extraction of Proteins

Twenty seeds from each cultivar were separately ground in a coffee grinder. One gram of the ground material was mixed with 30 mL of pre-cooled acetone (-20^{O}C). Next the mixture was stirred for 45 min using a magnetic stirrer (Thermo Fisher Scientific, Waltham, MA, USA)followed by centrifugationat 12,000 RPM for 15 min using a Sorvall^{TM} Legend^{TM} Micro 21R centrifuge (Thermo Scientific, Waltham, MA, USA). The resulting pellet was vacuum dried and re-dispersed in 10 mL of de-ionized water followed by pH adjustment to 7.5 with 1 N Na OH. Next the dispersion was subjected to continuous stirring for one hour at 4°C, followed by centrifugation at 14,000 RPM for 30 min at the same temperature to obtain a clear protein rich supernatant that was stored at -20^{O}Cforfurther analyses.

2.3 Extraction of Oil

Five grams of the ground cottonseeds from each cultivar was mixed with 15 mL of pre-cooled (2°C) diethyl ether, stirred for 30 min and filtered through What man 2 filter to obtain the oil rich organic phase. The slurry on the filter was washed twice with 5 mL of diethyl ether each time and the pooled organic phase was stored at -20°C.

2.4 Peroxyl and Alkoxyl Radical Scavenging Assay

Radical scavenging efficacy of the protein and oil extracts were conducted by the method described by Lissi et al. (1995) as modified by Haque and Mukherjee (2015)and Haque et al.(2013a, b). Assays were run in clear bottom 96 well plates and consisted of 50 µL of each of the protein or oil fractions, 12.5 mM ABAP (as a pyrolytic agent for peroxyl and alkoxyl radical generation), 10 mM luminol (as the inducer of chemiluminescence) and oxygen saturated McIlvaine's iso-ionic buffer (pH 7.0). Chemiluminescence values for the control (only the buffer) and test samples (in relative light unit, RLU) were recorded every 1.5 min after the initiation of ABAP pyrolysis (the **1st challenge**) with a FlexStation 3 microplate reader (Molecular Devices, CA, USA). The peak values (chemiluminescence maxima) obtained from the control and individual samples were termed as L_{maxC1} and L_{maxS1}, respectively (Haque and Mukherjee, 2015). When the chemiluminescence of the control decreased indicating an apparent decline in radical proliferation, equal volume and concentration of ABAP was re-injected in the reaction mix – resulting in a second chemiluminescence curve (the **2nd challenge**). The second chemiluminescence maxima for the control and individual test samples were termed as the L_{maxC2} and L_{maxS2}, respectively (Haque and Mukherjee, 2015).

2.5 Statistical Analysis

Student's t-tests (Student, 1908) were performed to determine whether the L_{maxS1} and L_{maxS2} values for the test samples belonging to each category (protein and oil) differed significantly (α=0.05) from each other as well as from the L_{maxC1} and L_{maxC2}.

3. Results and Discussion

All samples exhibited significantly greater ($P<0.05$) AA and AP compared to the control (569 RLU). Proteins extracted from PIMA and TM1 showed 238 and 602% greater AA, respectively, (Figure 1) with respect to the control. Oil fractions extracted from the same cultivars exhibited 713 and 679% greater AA relative to the control, respectively, evident from L_{maxS1} values (Figure 1). PIMA and RM1 proteins also depicted 392 and 490% greater AP compared to the control (561 RLU), respectively, based on L_{maxS2} values (Figure 2), whereas for the oil samples this enhancement was found to be 315 and 178%, respectively (Figure 2). Proteins extracted from TM1 seeds exhibited significantly greater AA and AP compared to PIMA, elucidated by enhanced quenching of 52 and 17% radicals, following the 1st and 2nd challenges, respectively. Proteins from both the cultivars exhibited marked AP – reflecting their residual antioxidative properties.

A 32% enhancement of radical quenching persistence (compared to L_{maxS1}) was observed in case of PIMA, while in TM1, the L_{maxS2} exhibited 19% reduction relative to L_{maxS1}. Antioxidant property of protein fractions extracted from cottonseeds was reported earlier and conceivably stems from specific peptides that was reported earlier (Boboev et al., 2012). Oil fractions, on the other hand, were not significantly different from each other regarding AA. They showed declines of 90 and 176% radical quenching efficacy, respectively, during the period for estimation of persistence. The antioxidative properties of the oil extracts were plausibly due to their gossypol contents as reported earlier (Perifanova-Nemska et al., 2014). It was long been known that gossypol has distinct antioxidant property (Bickford et al.,1954). Structurally gossypol comprises of numerous aromatic rings that allow delocalization of electrons and aromaticity is expected to favor antioxidative characteristics. Unfortunately, gossypol is toxic (Sunilkumar et al., 2006; Perifanova-Nemska et al., 2014) and can not be used for human or animal consumption. Notably, its antioxidant property is not persistant and disappear easily as seen for our AP data.

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Figure Legends:

Fig 1. Determination of the point of maximal proliferation of pyrolysis induced free-radicals from the luminol emitted chemiluminescence maxima of the protein and oil fractions extracted from the seeds of two cotton cultivars [PIMA (*Gossypium barbadense*) and TM1 (*G. hirsutum*)] [calculated by averaging five data acquisitions 1.5 minutes apart (L_{maxS1}) that corresponded to five highest chemiluminescence values detected from control (the buffer by itself) (L_{maxC1}) (569 RLU) following the first pyrolysis of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP) (the 1st challenge)] . The X and Y axes indicate the cultivars under study and chemiluminescence maxima in relative light unit (RLU), respectively. Significant difference in chemiluminescence maxima between the protein and oil fractions are indicated with one and two asterisks (*), respectively.

Fig 2. Determination of the point of maximal proliferation of pyrolysis induced free-radicals from the luminol emitted chemiluminescence maxima of the protein and oil fractions extracted from the seeds of two cotton cultivars [PIMA (*Gossypium barbadense*) and TM1 (*G. hirsutum*)] [calculated by averaging five data acquisitions 1.5 minutes apart (L_{maxS2}) that corresponded to five highest chemiluminescence values detected from control (the buffer by itself) (L_{maxC2}) (561 RLU) following the second pyrolysis of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP) (the 2nd challenge)] . The X and Y axes, notations and abbreviations are as in Fig 1.