Journal of Plant Biochemistry & Physiology

Aydemir and Yemenicioglu, J Plant Biochem Physiol 2013, 1:4
Doi: 10.4172/2329-9029.1000118

Research Article Open Access

Are Protein-bound Phenolic Antioxidants in Pulses Unseen Part of Iceberg?

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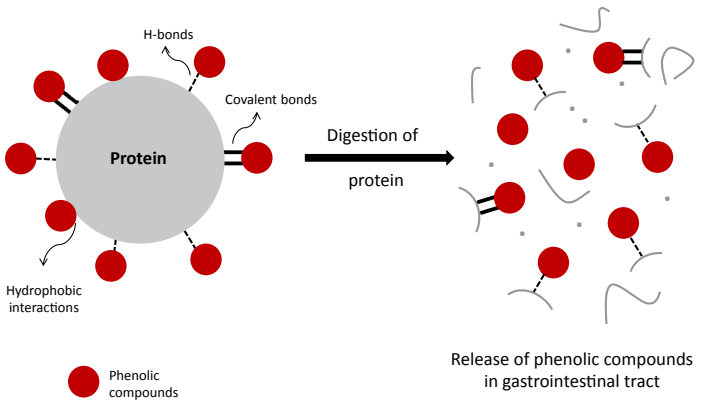
Graphical Abstract



Keywords: Legumes; Proteins; Phenolic compounds; Antioxidants; Bioactivity

Introduction

In the last ten years a particular effort has been spent to show health benefits of pulses such as lentils and chickpeas. Lentils attract significant interest since they contain higher antioxidant phenolic compounds than most other legume seeds. The studies suggest that the consumption of lentils is beneficial to prevent development of atherosclerosis, to reduce incidence of different types of cancer and type-2 diabetes, and to lower blood cholesterol levels [1-6]. Due to their lower antioxidant phenolic contents than lentils reports about bioactive properties of chickpea phenolics were limited. However, continuous reports have been published about antioxidant properties of chickpea proteins and their hydrolizates [7]. The studies on antioxidant properties of lentil proteins on the other hand are scarce.

The antioxidant activity of proteins and phenolic compounds are due basically to their free radical scavenging and iron binding capacities. The phenolic compounds owe their antioxidant activity to hydroxyl groups while proteins owe their antioxidant activity to different reactive groups at their constituent amino acids. In plants, bound phenolic compounds also made a very significant contribution to antioxidant activity of proteins [8,9]. Most of the phenolic compounds bind proteins non-covalently since phenolic hydroxyl groups are capable to form H-bonding with peptide carbonyl groups of proteins [10]. Hydrophobic interactions also cause non-covalent binding of phenolic compounds on surfaces of proteins [8], but some oxidized phenolic compounds can also bind proteins covalently [11]. In lentils, 82-85% of total antioxidant activity was formed by bound phytochemicals, while this percentage changes between 25 and 39% in many other legumes including chickpeas, yellow and green beans, and soybeans [12]. Thus, although studies related to identification of protein bound bioactive compounds in legumes scarce a high amount of bound phenolic compound and a resulting high bioactivity are expected for legume proteins. The roles of protein in bioavailability of phenolic compounds should be different than those of indigestible dietary fiber which formed by carbohydrates. The indigestible dietary fiber can bind and trap phenolic compounds and this prevents release and bioavailability of phenolic compounds during digestion process [13]. In contrast, the release of bound phenolics is possible when proteins are effectively digested in stomach and small intestines. The carrier roles of lentil protein for iron without considerable reduction in its bioavailability was shown [14], but studies are essentially needed to show bioactivity of lentil and other legume protein-bound phenolic compounds. In this study, the antioxidant activity and phenolic content of green and red lentil seeds and lentil proteins were determined. The presence of bound phenolic compounds in lentil proteins was also proved simply by showing amount of remaining phenolic compounds in protein during repeated precipitation and washing. This work aims focusing of the plant biochemists and physiologist interest in importance of protein bound antioxidant phytochemicals.

Experimental

Extraction of total water soluble antioxidants from seed

Ten grams of pulses were rehydrated in 190 ml of distilled water for 18 hrs in a refrigerator. The mixture was then first homogenized in a Waring blender for 2 minutes and then secondly in a homogenizer-disperser (IKA, Model DI 18, Basic, Brasil) at 22000 rpm for 2 minutes. The obtained homogenate was filtered through 2-layers of cheesecloth

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Received April 26, 2013; **Accepted** November 15, 2013; **Published** November 23, 2013

Citation: Aydemir LY, Yemenicioglu A (2013)Are Protein-bound Phenolic Antioxidants in Pulses Unseen Part of Iceberg? J Plant Biochem Physiol 1: 118. doi:10.4172/2329-9029.1000118

to remove insoluble residues and the filtrate was then clarified by centrifugation at 15000 g (+4°C) for 30 minutes. The extracts containing water soluble antioxidants were named as L and C when lentils and chickpeas were used in extraction, respectively.

Extraction of lentil proteins

To remove the soluble phenolic compounds, Acetone Powder (AP) obtained according to Aydemir and Yemenicioglu [15] from lentils was used in protein extraction. Briefly, 20 g of AP was suspended in 250 mL deionized water by stirring with a glass rod 100 times. The pH of the mixture was then adjusted to 9.5 with 1 N NaOH and it was extracted at room temperature for 45 min under continuous magnetic stirring. The extract was then clarified by centrifugation for 30 min at 15000×g (at 4°C), and its pH was adjusted to 4.5 with 1 N acetic acid to precipitate the lentil proteins at their isoelectric point (pI). The precipitated proteins were collected with centrifugation and resuspended in distilled water for washing soluble residues. The pH of the suspensions was once more adjusted to 4.5 and proteins were once more precipitated and collected with centrifugation for 15 min at 15000×g (at 4°C). Finally, the obtained proteins were suspended in distilled water (these processes containing 2 precipitations and one washing with water correspond to one precipitation-washing cycle specified in Figure 1) and lyophilized after adjusting the pH of the suspensions to 7.0. The lyophilized protein extracts containing almost 88% protein (w/w) (determined by the Kjeldahl method) were designated as lentil protein isolate (LP) and stored at -18°C until used in the tests.

Extract (type of pulse)[a]	TPC (mg GA/kg)	TFC (mg EC/kg)	TEAC (mmol Trolox/kg)	ORAC (mmol Trolox/kg)
C (Kabuli)	3634 ± 43db	NDc	36 ± 0.7e	ND
L (red)	5168 ± 310c	1125 ± 60 c	53 ± 0.2d	47 ± 3 d
L (green)	5655 ± 445c	1277 ± 104c	49 ± 0.9d	59 ± 9 d
LP (red)	26376 ± 607a	4121 ± 273a	133 ± 0.0a	210 ± 22a
LP (green)	19742 ± 1628b	2513 ± 132b	127 ± 0.0b	106 ± 9b

[a]C: Chickpea, L: Lentil, LP: Lentil protein; [b]Letters in columns show significant differences at P <0.05. [c]ND: Not determined.

Table 1: Antioxidant potential and phenolic contents of different pulses and pulse proteins.

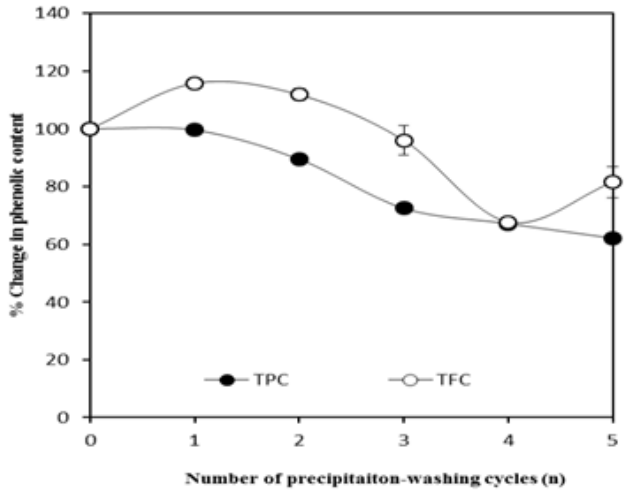

Figure 1: Remaining phenolic compounds of red lentil protein isolate during repeated precipitation-washing cycles (1 × n: precipitation at pH 4.5+ dissolving in water + precipitation at pH 4.5+ dissolving in water for determination of TPC and TFC).

Total phenolic and flavonoid content

The Total Phenolic Content (TPC) of samples was determined spectrophotometrically at 765 nm using the Folin-Ciocalteu method of Singleton and Rossi [16]. The Total Flavonoid Content (TFC) of samples was determined spectrophotometrically at 510 nm by the aluminum chloride colorimetric method given by Zhishen et al. [17]. The TPC and TFC were expressed as milligrams of Gallic Acid (GA) and Epicatechin (EC) equivalents per kg of dry pulse (for L or C) or per kg of Lyophilized Protein (for LP), respectively. All measurements were conducted three times.

Determination of antioxidant capacity

Antioxidant potentials of samples were determined by the Oxygen Radical Absorbance Capacity (ORAC) and Trolox Equivalent Antioxidant Capacity (TEAC) methods given by Xu and Chang [4], Re et al. [18]. The results were expressed as mmol Trolox/kg pulse (for L or C) or mmol Trolox/kg Lyophilized Protein (LPs). All measurements were conducted for three times.

Results and Discussion

Antioxidant capacities and phenolic contents of lentil seeds

The antioxidant capacities of green and red lentils seeds were given in Table 1. The TEAC and TPC values of different pulses clearly showed 1.4-1.5 fold higher antioxidant capacity and phenolic contents of lentils than chickpea. However, no significant differences exist in ORAC, TEAC, TPC and TFC of aqueous extracts of green and red lentils.

Antioxidant capacities and phenolic contents of lentil proteins

The ORAC, TEAC, TPC and TFC values clearly showed the significantly higher phenolic content and antioxidant capacity per kg of lentil proteins than per kg of lentil seeds. In fact, it is worth to note that the proteins of green and red lentils showed 2 to 3.5 higher phenolic content, and 1.8 to 4.5 fold higher antioxidant capacities than lentil seeds. On the other hand, the results also showed the significantly higher phenolic content and antioxidant capacity of red lentil proteins than the green lentil proteins. These results suggest the presence of significant amount of phenolic compounds in lentil protein isolates. However, it does not give any information whether the measured phenolic compounds are free or protein-bound.

Identification of bound phenolics in lentil proteins

To understand whether phenolic compounds in protein extract are free or protein-bound a simple experiment was conducted by exploiting precipitation of proteins at their pI. The isoelectric precipitation already employed during extraction and purification of proteins used in this work (see methods) was repeatedly applied for many times. The TPC and TFC were determined after each precipitation-washing cycle which refers to precipitation of protein, redissolve it in water to remove soluble phenolic residues, precipitation for the second time and redissolve in water for analysis of phenolic content. The results given in Figure 1 clearly show the presence of phenolic compound in bound form. The precipitated proteins maintained almost 100% of their TFC and almost 75% of their TPC after three repeated precipitation-washing cycles. This finding shows the binding of phenolic compounds by lentil protein. Slight to moderate reductions and increases occurred in TPC and TFC of protein in following precipitation and washing, but 60% of bound TPC and 80% of bound TFC were maintained at the end of five precipitation-washing cycles. It appears that the reduced phenolic content during repeated cycles was due to solubilization and removal of

bound phenolics or precipitated protein during washing step, but the overall results showed the presence of considerable amounts of protein bound phenolics in lentils.

In conclusion, this work clearly showed the presence of significant amount of antioxidant phenolic compounds bound onto surfaces of lentil proteins. The protein may act as carrier for the phenolic compounds along the digestive system. The binding of phenolic compounds by protein and then release of bind phenolic compounds following protein digestion could be the major factor responsible for the health benefits of phenolic rich pulses and other legume seeds including soy beans. Further studies on roles of protein in bioavailability of phenolic compounds can help understanding the unseen part of the iceberg.

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