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ARTICLE

Distribution of Nutrients and Antinutrients in Milled Fractions of Chickpea and Horse Gram: Seed Coat Phenolics and Their Distinct Modes of Enzyme Inhibition

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Milled fractions of chickpea (*Cicer arietinum* L.) and horse gram (*Macrotyloma uniflorum* L. Verdc.) were evaluated for their nutritional and antinutritional characteristics. Crude protein content of these fractions ranged from 22.6−23.8 g 100^{-1} g in cotyledon to 7.3−9.1 g 100^{-1} g in seed coat fractions. The fat content of chickpea fractions (1.6−7.8 g 100^{-1} g) was higher than that of horse gram fractions (0.6−2.6 g 100^{-1} g). Crude fiber content was higher in seed coat fractions of both legumes than embryonic axe and cotyledon fractions. Seed coat fractions had high dietary fiber content (28.2−36.4 g 100^{-1} g), made up of mainly insoluble dietary fiber. Most of the phytic acid and oligosaccharides were located in the cotyledon fractions, whereas phenolic compounds in higher concentrations were found in seed coats. Significantly higher concentrations of proteinaceous and phenolic inhibitors of digestive enzymes were found in cotyledon and seed coat fractions, respectively. The kinetic studies, using Michaelis−Menten and Lineweaver−Burk derivations, revealed that seed coat phenolics inhibit α-amylase activity by mixed noncompetitive (chickpea) and noncompetitive (horse gram) inhibition mechanisms. In the case of trypsin, chickpea and horse gram seed coat phenolics showed noncompetitive and uncompetitive modes of inhibition, respectively. These results suggest the wide variability in the nutrient and antinutrient composition in different milled fractions of legumes and potential utility of these fractions as ingredients in functional food product development.

KEYWORDS: Horse gram; chickpea; milled fractions; nutrients; antinutrients; mode of inhibition

INTRODUCTION

Chickpeas are one of the oldest and most widely consumed legumes in the world, particularly in tropical and subtropical areas. Horse gram is also largely cultivated in Southeast Asia and tropical Africa. These two food legumes are valuable sources of protein, minerals, and vitamins and occupy a very important place in human nutrition in many developing countries (*1*, *2*). In addition to nutritional benefits, consumption of these legumes has been associated with reduced risk of various diseases such as bronchitis, diabetes, cancer, and cardiovascular diseases (*1*, *3*). It is also well recognized that the majority of food legumes including chickpea and horse gram possess certain phytochemicals with antinutrient effects (*4*−*6*). These antinutritional factors may hinder efficient utilization, absorption, or digestion of nutrients and thus reduce their nutrient bioavailability and nutritional quality (*4*). However, in recent years, these phytochemicals with antinutrient effects have attracted more and more interest from both researchers and food manufacturers as health-promoting and disease-preventing properties were found in both in vitro and in vivo studies (*7*).

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Phenolic compounds, which are abundantly present in the seed coats of legumes, are one of the most important groups of secondary metabolites in plants having antinutrient properties. However, these compounds are believed to work synergistically to promote human health through a variety of different mechanisms, such as enhancing antioxidant activity, impacting cellular processes associated with apoptosis, platelet aggregation, blood vessel dilation, and enzyme activities associated with starch, protein, and/or lipid digestion, carcinogen activation, and detoxification (*8*−*10*). Further studies on these phenolic compounds would not only be valuable for quality control but also enhance understanding of the biological activity of legume seed coat phenolics and their benefits to human health.

Chickpea and horse gram are commonly processed by dehulling or milling to improve their cooking and nutritional properties. Dehulling of legumes in general results in variations in the content of nutrients and antinutrients in different milled fractions because the nutrients and antinutrients in legumes are unevenly distributed in the seed (*11*). The cotyledon also known as "dhal" obtained after dehulling of these legumes is consumed in a diversity of forms, depending on culture and region, and affords the advantages over un-dehulled seeds of faster cooking time, increased digestibility, and reduced antinutrient levels. However,

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dehulling of these legumes also results in the production of various types of byproducts such as seed coat, embryonic axe fraction, and powder. These byproducts have a comparatively low value, because they are used as feed material in livestock production farms (*12*).

Byproducts of the dhal milling industry have the potential to be used as ingredients in the preparation of specialty products for human consumption (*13*). However, there have been no systematic studies on the levels of various nutritional and antinutritional factors in different milled fractions of legumes. The present study was therefore carried out to elucidate the types of nutritional and antinutritional compounds and to quantify their contents in different milled fractions of chickpea and horse gram. Furthermore, the contributions of seed coat phenolics in the inhibition of digestive enzymes and the kinetics and mode of binding of these phenolics in the active sites of these enzymes were also evaluated.

MATERIALS AND METHODS

Materials. Chickpea and horse gram were purchased from a local market in Mysore, India. Care was taken to purchase all seeds from a single batch. The seeds were then taken to the laboratory in airtight polyethylene bags, cleaned, and kept in a cool and dry place prior to use. *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), porcine pancreatic α-amylase, bovine pancreatic trypsin, gallic acid, and phytic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Dehulling and Separation of Milled Fractions of Legumes. A grain-testing device (Strong-Scott Ltd., Winnipeg, MB, Canada) fitted with an abrasive wheel (36 mesh) was used for dehulling of chickpea and horse gram seeds. The samples were dehulled by maintaining abrasive wheel speed constant at 800 rpm while varying the dehulling time from 10 to 25 s (10 s for chickpea and 25 s for horse gram). The abrasive wheel speed and dehulling time were chosen after the evaluation of different wheel speeds and dehulling times on the degree of dehulling of legumes at similar moisture contents. After dehulling, seed coat and embryonic axe fractions were collected by sieving and aspiration. The aspirated fractions were sieved through a 2 mm sieve to collect embryonic axe fractions (passed through the sieve) and seed coats (hulls, remaining on the sieve). The abraded fractions were manually separated into dehulled and undehulled cotyledons. Un-dehulled cotyledons were passed through another round of dehulling to obtain dehulled cotyledons. **Figure 1** shows the process flow diagram for dehulling and separation of the milled fractions of legumes. All fractions were weighed and then expressed as a proportion of the total original sample weight. The separated dehulled cotyledons, embryonic axe, and seed coat fractions were ground into fine powder (60 mesh) using a coffee grinder. The powdered fractions were stored in airtight glass containers at room temperature for further analysis of nutrients and antinutrients.

Nutrient Composition. The moisture contents of chickpea and horse gram milled fractions were determined by oven-drying to a constant mass at 105 °C. The crude protein, crude lipid, crude fiber, and ash contents were determined in accordance with the standard methods of AOAC (*14*). The carbohydrate content was determined as the weight difference using moisture, crude protein, lipid, and ash content data. Soluble sugars from milled fractions were extracted into 80% (v/v) ethanol according to the procedure of McCready et al. (*15*). The reducing sugars were determined (after separation), using a modified version of the method of Nelson (*16*). The amount of nonreducing sugars was calculated as the difference between the total soluble sugars and reducing sugars. Total dietary fiber (TDF) was determined by the rapid enzymatic assay (*17*). The analytical values were evaluated from the mean of three determinations for each sample.

Antinutritional Factors. The concentration of total phenolic compounds in milled fractions of chickpea and horse gram was measured according to the method described by Yen and Hsieh (*18*). A calibration curve for gallic acid (20−100 μg) was used, and the phenolic compounds were expressed as gallic acid equivalents. Phytic acid was determined according to the method described by Haug and Lantzsch (*19*). The phytic acid content was calculated from a calibration curve using phytate phosphorus salt in the range of 10−50 μg. Oligosaccharides were first extracted from chickpea and horse gram milled fractions by treating 5 g of each sample with 25 mL of 80% ethanol at room temperature (27 ± 2 °C) by repeated shaking. The extraction was repeated three times. The extracts were pooled and concentrated using a rotary evaporator under vacuum. The residue was made up to 5 mL with deionized water, and the sugars were separated on a Kromasil NH_2 analytical HPLC column (250 × 4 mm, particle size = 5 μm; Phenomenex, Torrance, CA). The mobile phase consisted of a mixture of acetonitrile/water (70:30, v/v). The system used for the analysis was a Shimadzu HPLC system (LC-10ATVP, Shimadzu Corp., Japan), consisting of an LC-10ATVP pump. An injection volume of 20 μL of sample and a flow rate of 1 mL/min were used for the analysis. Data signals were acquired and processed on a PC running Class VP software. Oligosaccharides in the extract were detected using a refractive index detector (RID-10A) and identified by comparing their retention times with those of known standards. Standards used were raffinose, stachyose, and verbascose (Sigma Chemical Co., St. Louis, MO).

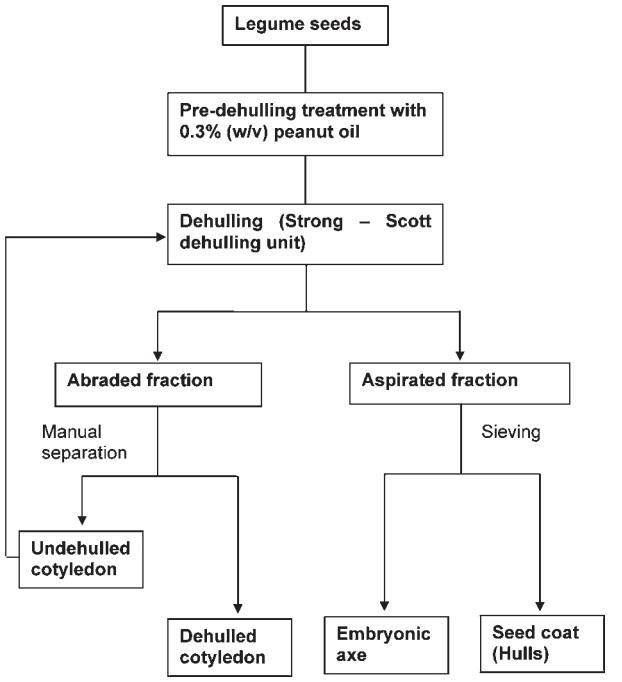

Figure 1. Flow diagram for milling and separation of milled fractions of legumes.

Inhibitory activities against α-amylase and trypsin from cotyledons, embryonic axe, and seed coat fractions of chickpea and horse gram were determined by extraction in 0.1 M Tris-HCl buffer (pH 8.2) at 4 °C according to the method of Sreerama et al. (*20*). Inhibitory activities were also extracted with methanol/water (80:20, v/v) by refluxing in a boiling water bath for 30 min. The activity of α-amylase inhibitors was determined on the basis of the amount of the reducing sugar released from soluble starch preparation under the influence of α-amylase in the presence of buffer or methanol extracts (*21*). One α-amylase activity unit is defined as the amount of enzyme that is necessary to release 1 μmol of maltose from a 1% solution of starch at pH 6.9 per minute at 37 °C. Amylase inhibitory activity (AIU) is defined in terms of amylase units inhibited per gram of sample. The amidase activity of trypsin and its inhibition was assayed using the chromogenic substrate BAPNA at pH 8.2 in 0.05 M Tris-HCl containing 0.02 M $CaCl_2$ at 37 °C according to the method of Kakade et al. (*22*). One unit of trypsin enzyme activity (TU) is defined as the increase in the absorbance of 0.01 at 410 nm under assay conditions. One trypsin inhibitory unit (TIU) is defined in terms of trypsin units inhibited per gram of sample. Appropriate controls containing buffer or methanol instead of sample were maintained.

IC_{50} Values of Enzyme Inhibitors. Inhibition of the activities of α-amylase and trypsin by methanolic extracts of chickpea and horse gram seed coats was determined in the presence of various concentrations of seed coat methanolic extracts (4−20 μg). IC_{50} was the concentration of

Table 1. Distribution of Major Nutrients in Different Milled Fractions of Chickpea and Horse Gram (Percent of Dry Matter)

	chickpea			horse gram		
constituent	cotyledon	embryonic axe	seed coat	cotyledon	embryonic axe	seed coat
moisture	6.5 ± 0.26b	9.4 ± 0.18a	4.6 ± 0.08c	5.8 ± 0.31b	8.4 ± 0.21a	3.9 ± 0.05c
protein	23.8 ± 1.05a	14.4 ± 0.72b	7.3 ± 0.69c	22.6 ± 1.23a	18.6 ± 0.90b	9.1 ± 0.35c
fat	4.2 ± 0.09b	7.8 ± 0.08a	1.6 ± 0.05c	1.8 ± 0.06b	2.6 ± 0.04a	0.6 ± 0.02c
ash	3.9 ± 0.05a	2.9 ± 0.03b	2.6 ± 0.04b	2.9 ± 0.02b	2.2 ± 0.04b	3.8 ± 0.05a
crude fiber	1.8 ± 0.01c	9.4 ± 0.09b	17.6 ± 1.1a	1.6 ± 0.02c	11.2 ± 0.26b	21.8 ± 1.6a
total carbohydrate[a]	61.6 ± 1.5b	65.5 ± 1.09b	83.9 ± 1.2 a	66.9 ± 2.6b	68.2 ± 1.9b	82.6 ± 1.1a
soluble sugars	5.8 ± 0.38a	5.2 ± 0.43 a	1.02 ± 0.08b	6.4 ± 0.15a	4.8 ± 0.19b	0.96 ± 0.06c
reducing sugar (mg/100 g)	464.5 ± 10.6a	261.9 ± 9.1b	129.4 ± 3.7c	538.3 ± 16.2a	211.7 ± 6.3b	108.6 ± 5.1c
nonreducing sugar	5.4 ± 0.13a	4.94 ± 0.06a	0.89 ± 0.04b	5.86 ± 0.15a	4.6 ± 0.08b	0.85 ± 0.03c
dietary fiber	14.2 ± 0.35c	18.1 ± 0.46b	28.2 ± 0.62a	16.7 ± 0.27c	22.6 ± 0.18b	36.4 ± 0.90a
soluble	1.06 ± 0.03b	2.4 ± 0.03a	2.8 ± 0.04a	1.32 ± 0.04b	3.1 ± 0.04a	3.9 ± 0.05a
insoluble	13.14 ± 0.41b	15.7 ± 0.52b	25.4 ± 0.91a	15.38 ± 0.16c	19.5 ± 0.28b	32.5 ± 1.1a

[a] By difference as 100 − (moisture + protein + ash + fat). Values are mean ± standard deviation of three independent determinations. Means with the same letter (a, b, c) within the same row for each legume do not differ ($P > 0.05$).

phenolics required for 50% inhibition of the enzyme activity under the assay conditions and obtained graphically by an inhibition curve.

Kinetics of Enzyme Inhibition. The mode of inhibition of α-amylase and trypsin by methanolic extracts of chickpea and horse gram seed coat phenolics was determined by analyzing Michaelis−Menton and Lineweaver−Burk equations. Soluble starch in the concentration range of 0.25−3.0% and BAPNA in the range of 0.025−0.3 mM were used as substrates for α-amylase and trypsin, respectively. Enzyme activities were determined in the absence or presence of different concentrations of phenolic compounds. Controls containing methanol instead of sample were maintained. The concentrations of chickpea and horse gram phenolic compounds used for the inhibitory kinetics of α-amylase were 8, 16, and 24 μg/mL and 10, 15, and 20 μg/mL, respectively. However, the same concentrations of 5, 10, and 15 μg/mL of both chickpea and horse gram seed coat phenolics were used for trypsin inhibitory kinetics. The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) in the Michaelis−Menten equation were determined by ploting $1/V$ versus $1/[S]$, where [S] is the substrate concentration and V is the initial velocity of the substrate cleavage. The inhibition constant (K_i) of phenolic compounds in α-amylase and trypsin inhibition was obtained by Dixon plots (*23*). The results shown are the average of three experiments.

Statistical Analysis. All results in this study are reported as means of three replicate analyses. One-way analysis of variance (ANOVA) was carried out to compare the mean values of nutrients and antinutritional factors in different milled fractions of legumes followed by Duncan's multiple-range test.

RESULTS AND DISCUSSION

Dehulling and Separation of Milled Fractions. Dehulling of chickpea and horse gram seeds with a Strong-Scott grain-testing device resulted in three fractions (**Figure 1**). These are the seed coat, the cotyledon, and the embryonic axe. Chickpea provided 13.8, 1.6, and 84.6% and horse gram provided 11.2, 2.7, and 86.1% of seed coat, embryonic axe, and cotyledon fractions, respectively, of the seed content (relative amount of each fraction). The cotyledon fraction, which is the main reserve for proteins and carbohydrates, was the major fraction in both legumes. Higher yield of brown-colored thick seed coat was recovered in chickpea, whereas dehulling of horse gram produced comparatively thin and lower yield of brown-colored seed coat. The small seed of horse gram possesses a higher proportion of embryonic axe than chickpea.

Nutrient Distribution in Milled Fractions. The nutritional compositions of mechanically separated cotyledons, embryonic axe, and seed coat fractions of chickpea and horse gram are presented in **Table 1**. The moisture contents of seed coat fractions were lower than cotyledon and embryonic axe fractions in both legumes. However, higher moisture values were recorded in the embryonic axe fractions (9.4 and 8.4% for chickpea and horse gram, respectively). Wide variations were observed in the protein content of different milled fractions. Protein content of chickpea fractions ranged from 23.8% in cotyledon to 7.3% in seed coat fraction. Similarly, protein content of horse gram fractions also ranged from 22.6% in cotyledon to 9.1% in seed coat fraction. The protein content of embryonic axe fractions in both legumes is higher than seed coat fractions and lower than cotyledons. Seed coat fractions had the lowest recorded protein content of 7.3% in chickpea and 9.1% in horse gram. Fat content of chickpea fractions ranged from 1.6% in the seed coat to 7.8% in the embryonic axe fraction (**Table 1**). All three chickpea fractions showed higher fat content than horse gram fractions. Embryonic axe fractions of both legumes showed higher fat content compared to their respective seed coat and cotyledon fractions. This might be disadvantageous in terms of the shelf life and keeping qualities of these fractions. However, utilization of this fraction in food products will enhance the ability of flour to absorb and retain oil, improve binding of the structure, enhance flavor retention, improve mouthfeel, and reduce moisture and fat losses of food products (*20*). The flour from embryonic axe fractions may be useful in ground meat formulations, meat replacers and extenders, pancakes, baked goods, and soups, in which oil-holding capacity is of prime importance. In view of their low lipid content in horse gram fractions, they may be suitable as ingredients of weight restriction diets.

Crude fiber content was higher in seed coat fractions than in embryonic axe and cotyledon fractions. These results are similar to the values reported for beach pea (*11*). The presence of high crude fiber in food material is reported to decrease dry matter digestibility in animals (*24*). Furthermore, seed coat fractions of legumes with high fiber and low protein may be useful in food product formulations to improve gastrointestinal health and satiety changes. Chickpea cotyledon showed higher ash content (3.9%) than embryonic axe (2.9%) and seed coat (2.6%) fractions. Similar results for ash content in cotyledons and seed coats were reported for cowpea, green pea, pigeon pea, and beach pea (*11*). However, the horse gram seed coat fraction had the highest ash content followed by cotyledon and embryonic axe fractions. Ash content is an index of the quality of feeding materials used for poultry and cattle feeding (*25*). Because the ash content of embryonic axe and seed coat fractions of chickpea and horse gram is lower, these fractions are suitable for feed materials in livestock production farms.

Total carbohydrate, soluble sugars, reducing sugars, and nonreducing sugars were present in higher amounts in cotyledon

Table 2. Concentration of Antinutritional Factors in Different Milled Fractions of Chickpea and Horse Gram (Percent of Dry Matter)[a]

		chickpea			horse gram		
	antinutritional factor	cotyledon	embryonic axe	seed coat	cotyledon	embryonic axe	seed coat
a	phytic acid (mg/g)	9.82 ± 0.46a	3.43 ± 0.20b	0.79 ± 0.06c	8.42 ± 0.41a	3.81 ± 0.11b	1.02 ± 0.09c
b	phenolic compounds (mg of GA/g)	15.24 ± 1.05c	46.18 ± 3.6b	75.94 ± 5.8a	13.81 ± 0.81c	159.43 ± 9.4b	484.60 ± 13.9a
c	flatulence factors (mg/g)						
	raffinose	7.42 ± 0.42a	3.44 ± 0.21b	0.75 ± 0.06c	6.35 ± 0.26a	2.86 ± 0.09b	0.96 ± 0.03c
	stachyose	17.51 ± 0.75a	9.30 ± 0.54b	0.92 ± 0.05c	14.84 ± 0.91a	6.38 ± 0.38b	0.70 ± 0.05c
	verbascose	9.49 ± 1.3a	2.61 ± 0.10c	1.93 ± 0.26c	3.75 ± 0.26a	1.38 ± 0.04b	1.05 ± 0.04c
d	enzyme inhibitors (units/g)						
	buffer extract						
	trypsin inhibitor activity	6090 ± 12.4a	938 ± 9.6b	861 ± 11.4c	9856 ± 16.1a	2018 ± 12.9b	1134 ± 8.2c
	α-amylase inhibitor activity	461.5 ± 1.6a	75.2 ± 0.7b	26.0 ± 0.6c	56.9 ± 1.1a	12.3 ± 0.4b	4.1 ± 0.2c
	methanol extract						
	trypsin inhibitor activity	1542 ± 6.5c	1964 ± 9.4b	2170 ± 15.2a	2474 ± 11.8c	3663 ± 13.1b	10434 ± 21.4a
	α-amylase inhibitor activity	209.6 ± 1.0c	223.3 ± 0.5b	263.4 ± 1.2a	632.2 ± 2.4b	639.5 ± 2.0b	937.3 ± 2.7a

[a] Results are mean ± standard deviation of triplicate determinations. Mean values bearing different letters (a, b, c) in the same row for each legume are significantly different ($P < 0.05$) on application of Duncan's multiple-range test.

fractions. Seed coat fractions contain highest amount of TDF in the range from 28.2% (chickpea) to 36.4% (horse gram) (**Table 1**). Similarly, the insoluble dietary fiber (IDF), which comprises lignin, cellulose, and some hemicelluloses and soluble dietary fiber (SDF), was also higher in seed coat fractions (**Table 1**). In both legumes, lowest values of TDF, SDF, and IDF were observed for cotyledon fractions. These values are similar to the values reported for chickpea (*26*) and horse gram (*20*). The dietary fibers present various physiological effects in the gastrointestinal tract of humans. These include alteration of the gastrointestinal transit time, satiety changes, influence on the levels of body cholesterol, after-meal serum glucose and insulin levels, flatulence, and alteration in nutrient bioavailability. These physiological effects are due to the physicochemical properties and chemical components of which they are composed (*27*). The legume fractions with variations in the contents of TDF, SDF, and IDF might find applications in different specialty food products for target populations.

Antinutritional Factors. The concentrations of the antinutritional factors in different milled fractions of chickpea and horse gram are shown in **Table 2**. Although a significant quantity of phytic acid was found in embryonic axe fractions of chickpea and horse gram, most of it was located in the cotyledon fractions (**Table 2**). Seed coat fractions showed lowest phytic acid content (0.79 mg/g in chickpea and 1.02 mg/g in horse gram). By comparison to the phytic acid contents of other common food legumes, cotyledon fractions showed similar values reported for chickpea (*28*) and horse gram (*20*). However, the phytate content in cotyledon fractions of both legumes is lower than the values reported for black gram (11 mg/g), lentil (12.5 mg/g), red kidney bean (14.4 mg/g), and white kidney bean (12.3 mg/g) (*28*) and higher than the levels reported for pigeon pea (2.2 mg/g) and bambara groundnut (2.9 mg/100 g) (*29*). Besides lowering the bioavailability of minerals and inhibiting the digestibility of proteins, phytic acid is also implicated in the "hard-to-cook" phenomenon of legumes (*30*). However, its presence is also beneficiary because it may have a positive nutritional role as an antioxidant and anticancer agent (*31*).

The levels of phenolic compounds were higher in seed coat fractions than cotyledon and embryonic axe fractions of both legumes. Highest concentrations of phenolic compounds were found in horse gram seed coat fraction (484.6 mg of gallic acid equiv/g). Cotyledon fractions contain very low concentrations of phenolics (**Table 2**). Similar results were observed for chickpea fractions, although the levels of phenolic compounds were much lower. These results indicate that the phenolic compounds are mostly concentrated in the seed coat fractions and might be easily removed by dehulling. The content of phenolic compounds in cotyledon fractions of these legumes is lower than those reported for beach pea (*11*), cowpea, pea, pigeon pea, and chickpea (*32*). Phenolic compounds usually form insoluble complexes with protein, thereby interfering with their bioavailability (*33*). However, these phenolic compounds have been reported to act as antioxidants by preventing oxidative stress that causes diseases such as coronary heart disease, some types of cancer, and inflammation (*9*). Because the content of phenolic compounds is higher in seed coat and embryonic axe fractions of chickpea and horse gram, they are likely to have antioxidant activity.

The contents of oligosaccharides in the three milled fractions of chickpea and horse gram are shown in **Table 2**. The oligosaccharides, such as raffinose, stachyose, and verbascose, were detected in all of the samples and represent a large portion of the total soluble sugars present in the milled fractions. All three chickpea fractions contain higher amounts of oligosaccharides than horse gram fractions. Cotyledon fractions contain higher concentrations of these oligosaccharides, accounting for about 60 and 39% of the total soluble sugars in chickpea and horse gram, respectively. The lowest amount of α-galactosides was detected in seed coat fractions. Within the oligosaccharides, stachyose was present in higher amounts in both cotyledon and embryonic axe fractions, whereas verbascose was the major oligosacharide in seed coat fractions of both legumes (1.93 mg/g in chickpea and 1.05 mg/g in horse gram). However, substantial amounts of raffinose and verbascose were also found in cotyledon and embryonic axe fractions. The total amount of stachyose in all of the milled fractions of chickpea (2.8%) was within the range of 1.60−3.10% reported previously for whole chickpea (*34*). However, the total contents of raffnose (1.16%) and verbascose (1.40%) in chickpea fractions seem to be higher when compared to 0.46−0.92 and 0.27−0.70% reported previously for whole chickpea (*34*). Similarly, the total contents of raffinose (0.99%) and stachyose (2.17%) in horse gram fractions were also higher than the content of these oligosaccharides reported for whole horse gram (*35*). Raffinose, stachyose, and verbascose have a tendency to induce flatulence. However, it is well-known that balance of intestinal bacterial flora is important for human health, especially bifidobacterium, which could dominate pathogenic organisms and thus invigorate human health. It has been reported that the growth of intestinal bifidobacteria is facilitated by galacto-oligosaccharides (*36*, *37*). In fact, many prebiotic oligosaccharides are used as ingredients in various products, such as soft drinks, cookies, cereals, candies, and infant foods (*38*, *39*). The cotyledon and

embryonic axe fractions of chickpea and horse gram, which contain the highest amounts of oligosaccharides, may be used as ingredients in functional foods.

Typical inhibitors of digestive enzymes, which are present in legume seeds, are protein substances. However, it is known that the ability to inhibit the activity of proteases and α-amylases is also attributed to phenolic compounds (*10*, *40*). This study evaluated the ability of proteinaceous inhibitors and phenolic compounds present in different milled fractions of legumes to inhibit the digestive enzymes such as trypsin and α-amylase. The mean values for the trypsin and amylase inhibitory activities of milled fractions of chickpea and horse gram are presented in **Table 2**. Trypsin and α-amylase inhibitory activities in the buffer extracts of cotyledon fractions (consisting of mainly proteinaceous inhibitors) were significantly higher than those in the embryonic axe and seed coat fractions. However, methanol extracts of seed coat fractions (consisting of mainly phenolic compounds) have higher inhibitory activities compared to cotyledon and embryonic axe fractions, thus showing a direct relationship between the inhibitory activity and the concentration of phenolic compounds. Horse gram fractions contain higher levels of proteinaceous inhibitors inhibiting trypsin, where as chickpea fractions showed higher levels of proteinaceous inhibitors inhibiting α-amylase. Phenolic compounds of horse gram methanol extracts had higher trypsin and α-amylase inhibitory activities than chickpea methanol extracts. Although the trypsin inhibitor activity has been studied in a number of legumes, the results obtained in the present investigation cannot be compared because the expressions of trypsin inhibitor activity, nature, and concentration of the substrates, etc., are different. However, on the basis of the investigations carried out by Chau et al. (*41*) under similar experimental conditions for trypsin inhibitor activity in whole legumes such as cowpea (2240 TIA/g), field bean (3400 TIA/g), and *Phaseolus calcaratus* (2280 TIA/g), it may be inferred that the TIA observed in cotyledon fractions in the present study (6090 TIA/g in chickpea and 9856 TIA/g in horse gram) appears to be very high. Amylase inhibitory activity (AIU) in the buffer extract of chickpea cotyledon fraction (461.5 AIU/g) and methanol extracts of all chickpea (209.6−263.4 AIU/g) and horse gram (632.2−937.3 AIU/g) fractions seems to be higher than that in the broad bean varieties (115−147.8 AIU/g) (*42*). However, when compared to different varieties of chickpea (*43*) and *Mucuna pruriens* (*44*), the presently investigated chickpea and horse gram milled fractions show comparable levels of AIU activity.

The property of trypsin inhibitors to form bonds with proteolytic enzymes actually decreases the digestibility of protein but, on the other hand, the results of the research done in recent years indicate that such forms of protein present in food can suppress carcinogenesis in both in vivo and in vitro model systems (*45*). The anticarcinogenic property of protease inhibitors is attributed to Bowman−Birk type of protease inhibitors (BBI). Previously, we have purified and characterized four double-headed trypsin/chymotrypsin isoinhibitors (Bowman−Birk type) from horse gram. The reactive sites and antigenic determinants in the major isoinhibitor were also identified, providing a new strategy in the design of ideal, smaller protease inhibitors as cancer preventive agents (*46*). Therefore, horse gram fractions having higher TIA could be used as functional food ingredients similar to soybean BBI concentrate (*47*). A favorable influence on health was also shown in the case of α-amylase inhibitors; that is, they contribute to the limitation of a postprandial increase in the concentration of blood glucose, which alleviates the course of insulin-dependent diabetes (*48*). These protease and α-amylase inhibitors could be inactivated when subjected to hydrothermal processing techniques (*49*). Such processed milled fractions of legumes could be a valuable addition to monogastric diets when supplemented with cereal protein, specifically wheat flour.

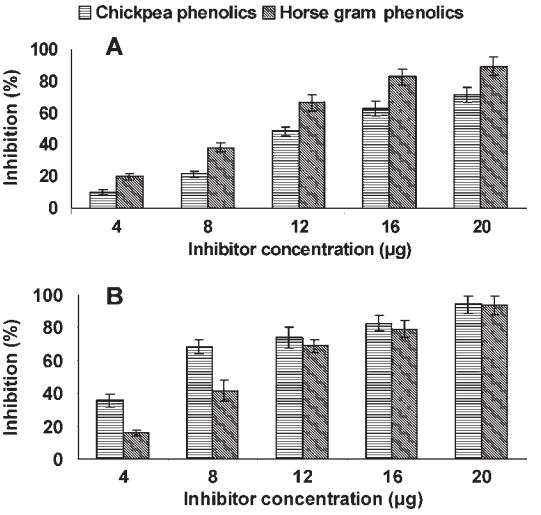

Figure 2. Inhibitory effect of chickpea and horse gram seed coat phenolics on the activities of α-amylase (**A**) and trypsin (**B**). Both α-amylase and trypsin were pre-incubated with different concentrations of phenolic compounds at 37 °C for 10 min and assayed for remaining activity. The data points are the means ± SD from three experiments.

Mode of Enzyme Inhibition by Seed Coat Phenolics. Seed coat fractions of chickpea and horse gram contained the highest amounts of phenolics and possessed strong inhibitory activities against α-amylase and trypsin (**Table 2**). Their inhibitory effects were further evaluated on different doses, and the IC_{50}, the concentration that provides 50% inhibition, was obtained for each seed coat extract. These seed coat phenolics inhibited α-amylase and trypsin in a dose-dependent manner (**Figure 2**). Both chickpea and horse gram seed coat phenolics had low IC_{50} values for trypsin (5.9 and 8.6 μg, respectively), whereas for α-amylase IC_{50} values were found to be 12.6 and 9.8 μg, respectively (**Table 3**). These results suggested that chickpea seed coat phenolics had the strongest trypsin inhibitory activity, whereas the horse gram seed coat phenolics were the most effective inhibitors of trypsin and also α-amylase.

Kinetic studies were performed using Michaelis−Menten and Lineweaver−Burk derivations to ascertain the mode of inhibition of chickpea and horse gram seed coat phenolics. The rate of α-amylase activity was determined at various substrate concentrations (0.25−3.0% starch) in the presence and absence of fixed seed coat phenolics as indicated in **Figure 3**. In the presence of chickpea seed coat phenolics, the slope of the straight lines in the double-reciprocal plot increased with increasing concentrations of phenolics. The straight lines were intercepted at a single point in the second quadrant (**Figure 3A**). The fact that intercepts occur in the second quadrant in the Lineweaver−Burk plots is better explained by the mixed noncompetitive inhibition mechanism proposed earlier for the porcine pancreatic α-amylase−acarbose complex (*50*). This type of inhibition indicates that the phenolics can bind to E or the ES complex rather than a catalytic site. Binding of inhibitor changes the affinity for the substrate, resulting in increasing K_m and decreasing V_{max} proportionate to the concentration of phenolics. However, in the case of horse gram, the binding of the seed coat phenolics also affected the velocity of the reaction catalyzed by α-amylase proportionately to the concentration of the phenolic compound in the reaction mixture but without affecting the K_m (**Figure 3B**). Noncompetitive inhibition can account for these results. Noncompetitive inhibitors form strong noncovalent bonds with enzymes, and this

Table 3. Kinetic Properties for the Inhibition of α-Amylase and Trypsin by Chickpea and Horse Gram Seed Coat Phenolics

	chickpea phenolics		horse gram phenolics	
kinetic property	α-amylase	trypsin	α-amylase	trypsin
K_m [a]	1.15%	1.32×10^{-4} M	1.15%	1.32×10^{-4} M
V_{max} [b]	90×10^{-2}	10.0×10^{3}	90×10^{-2}	10.0×10^{3}
V_{max}' [c]	83×10^{-2} (8.0 μg)	9.09×10^{3} (5.0 μg)	56×10^{-2} (10.0 μg)	8.3×10^{3} (5.0 μg)
V_{max}' [c]	57×10^{-2} (16.0 μg)	7.10×10^{3} (10.0 μg)	37×10^{-2} (15.0 μg)	6.71×10^{3} (10.0 μg)
V_{max}' [c]	47×10^{-2} (24.0 μg)	5.78×10^{3} (15.0 μg)	29×10^{-2} (20.0 μg)	5.40×10^{3} (15.0 μg)
K_i (μg)	1.37	2.25	0.75	3.96
IC_{50} (μg)	12.60	5.90	9.80	8.60
mode of inhibition	mixed noncompetitive	noncompetitive	noncompetitive	uncompetitive

[a] Starch was used as a substrate for α-amylase, whereas the substrate for trypsin was BAPNA. [b] Amylase activity was expressed as micromoles of maltose equivalents released per minute. Trypsin activity was expressed as the increase in the absorbance of 0.01 at 410 nm under assay conditions. [c] Concentration of seed coat phenolic compounds used for the kinetics of inhibition is given in parentheses.

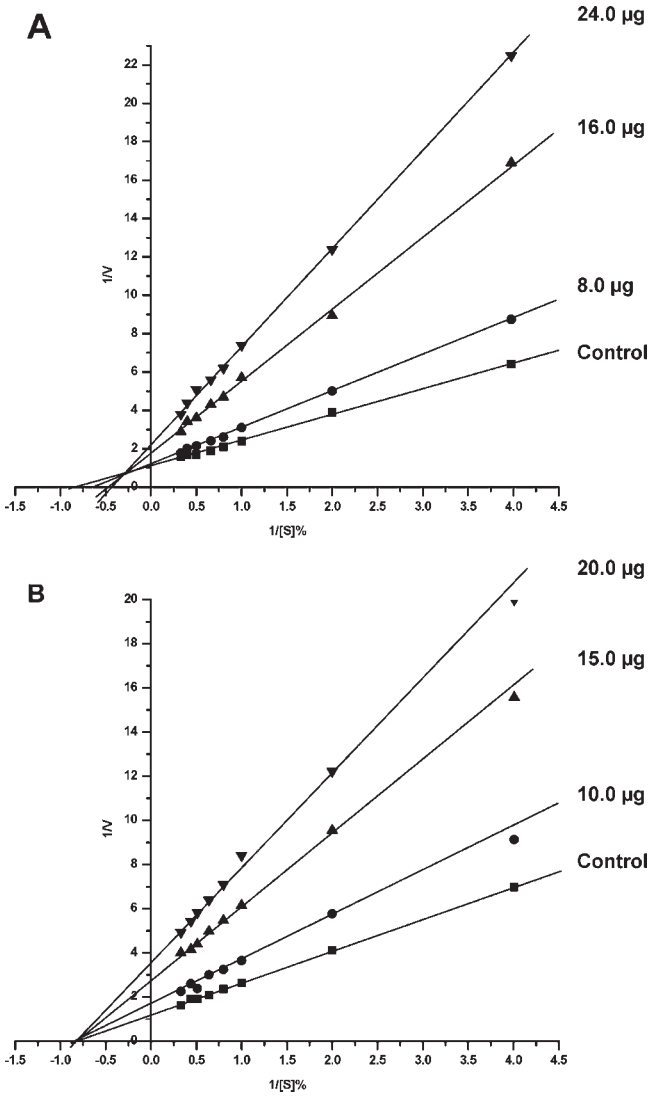

Figure 3. Lineweaver−Burk double-reciprocal plots for the inhibition of α-amylase. Amylase hydrolysis reactions were performed with variable starch concentrations (0.25−3.0%) and at different fixed concentrations of chickpea (**A**) and horse gram (**B**) phenolic compounds as indicated on the right side of the graph. The inhibitor was pre-incubated at 37 °C for 10 min prior to the substrate addition. The data points represent the mean of three independent determinations.

effect can only be partially overcome by high concentrations of the substrate. Inhibition of pancreatic α-amylase by different classes of phenolic compounds is described in the literature. Pancreatic amylase was effectively inhibited by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/(−)-epicatechin, diadzein, and epigallocatechin gallate (*51*). These flavonoids exhibited a mixed and close to noncompetitive type of inhibition on α-amylase. Mixed noncompetitive inhibition of finger millet malt amylase (*52*) and noncompetitive inhibition of pancreatic α-amylase by finger millet seed coat phenolics (*53*) were recently reported. The seed coat extracts of chickpea and horse gram exhibited an inhibitory activity against α-amylase similar to that of acarbose, a synthetic amylase inhibitor currently being used therapeutically to control non-insulin-dependent diabetes mellitus (*54*).

To study the mechanism of inhibition of seed coat phenolics on trypsin activity, the inhibitory effect was investigated as a function of substrate concentration (0.025−0.3 mM BAPNA) and the results were plotted as $1/V$ versus 1/[S]. In the presence of chickpea seed coat phenolics, double-reciprocal plots for the uninhibited and partly inhibited enzyme exhibited different slopes and intercepted at different locations in the vertical axis without affecting the K_m, indicating noncompetitive inhibition (**Figure 4A**). In the presence of horse gram seed coat phenolics, the best-fit lines of the double-reciprocal plots for the substrate cleavage by trypsin were parallel to the best-fit line of the plots in the absence of the inhibitor (**Figure 4B**), indicating the uncompetitive mechanism of inhibition. In this case, the inhibitor binds only to the enzyme−substrate complex, resulting in a change in K_m and V_{max}. Only a few works have been devoted to the inhibition of proteases involved in digestive processes by polyphenols. Extracts of cocoa, pears, and lentils have been found to inhibit trypsin; however, the composition of these extracts and the structure of the phenolic inhibitors involved were not described (*55*). He et al. (*40*) reported inhibitory effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin, and lipase. Oleuropein, a major phenolic compound present in olive oil, was found to be a competitive inhibitor for trypsin with an inhibitory constant of 0.85 mM (*56*). Moreover, according to our knowledge, the results presented here are the first showing noncompetitive (chickpea) and uncompetitive (horse gram) patterns of inhibition of trypsin by legume seed coat phenolics.

It is clear from the above results that, chickpea and horse gram seed coat phenolics inhibited α-amylase and trypsin via distinct mechanisms. Compositional analysis of seed coat phenolics by reversed-phase high-performance liquid chromatography revealed that distribution of flavonols, isoflavones, phenolic acids, and anthocyanins in these legume seed coat fractions had obvious differences (unpublished results). Horse gram seed coat fraction contains higher amounts of flavonols such as quercetin, kaempferol, and myricetin than chickpea seed coat fraction. The isoflavone genistin was detected only in the chickpea seed coat fraction, but a small quantity of diadzein was detected in the horse

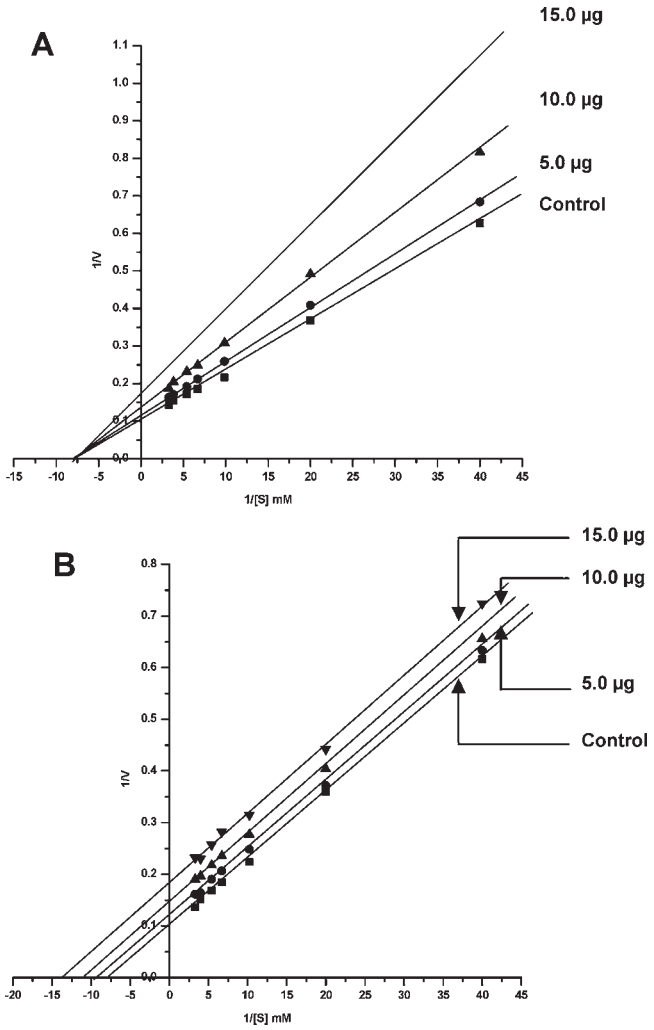

Figure 4. Lineweaver−Burk double-reciprocal plots for the inhibition of trypsin. Trypsin hydrolysis reactions were performed with variable BAPNA concentrations (0.025−0.3 mM) and at different fixed concentrations of chickpea (**A**) and horse gram (**B**) phenolic compounds as indicated on the right side of the graph. The inhibitor was pre-incubated at 37 °C for 10 min prior to the substrate addition. The data points represent the mean of three independent determinations.

gram seed coat fraction. Vanillic, *p*-hydroxybenzoic, and ferulic acids were the principal phenolic acids in the seed coat fraction of horse gram, whereas in chickpea seed coat fraction *p*-hydroxybenzoic, ferulic, and *p*-coumaric acids were the predominant phenolic acids. Furthermore, cyanidin, petunidin, and delphinidin were detected in seed coat fractions of both legumes. In addition to these three anthocyanins, malvidin was exclusively found in the horse gram seed coat fraction. This heterogeneity of phenolics having different structural features in seed coat phenolic extracts may be the reason for the observed modes of inhibition. Kinetic constants derived from Lineweaver−Burk and Dixon plots for the inhibition of α-amylase and trypsin are listed in **Table 3**. The α-amylase has a Michaelis−Menton constant (K_m) of 1.15% for starch and a V_{max} value of 90×10^{-2} µmol of maltose. The K_m for trypsin under the reaction conditions was found to be 1.32×10^{-4} M BAPNA with a V_{max} value of 10.0×10^3 µmol of *p*-nitrophenol (**Table 3**). The apparent V_{max} values for both enzymes decreased with increasing concentrations of seed coat phenolics. The inhibitory constants (K_i) determined from Dixon plots and presented in **Table 3** indicate that chickpea and horse gram phenolics have higher affinity for α-amylase (K_i values of 1.37 and 0.75 µg, respectively) than trypsin (K_i values of 2.25 and 3.96 µg, respectively).

The kinetics and patterns of α-amylase and trypsin inhibition described above suggest that phenolic compounds present in seed coat fractions of legumes may play a key role in the inhibition of starch- and protein-digesting enzymes. The inhibition of starch-digesting enzymes reduces starch digestion and absorption, consequently lowering the postprandial hyperglycemic response. This hypoglycemic effect is an established and effective target for type 2 diabetes prevention and treatment (*54*, *57*). Proteases also present an attractive target for pharmaceutical research (*58*, *59*). Proteolytic processes are necessary for normal physiological functions in the body, including digestion, normal blood vessel maintenance, new vessel formation (angiogenesis), clot formation and dissolution, bone remodeling, and ovulation (*60*). Their breakdown can lead to many pathological processes, such as pancreatitis, coagulation diseases, and cancer. One of the most interesting strategies seems to be the development of selective low molecular weight inhibitors from natural sources. Phenolic compounds, which inhibit the activities of α-amylases and proteases, provide an attractive target for the development of potential therapeutic agents to treat diabetes, pancreatitis, coagulation, and neoplastic diseases.

In conclusion, the physical separation of different morphological parts in legumes has the greatest impact on the nutrient and antinutrient contents of the grain legumes. Cotyledon fractions are the main reserve for proteins and carbohydrates. However, substantial quantities of various antinutritional factors, such as proteinaceous enzyme inhibitors, flatulence-producing factors, and phytic acid, are also present in higher quantities in cotyledon fractions. The embryonic axe fraction, having a higher fat content, may help in fat/water phase stability in many baked food products. Phenolic compounds are mostly concentrated in the seed coat fractions and might be easily removed by dehulling. However, these seed coat phenolics are potent inhibitors of digestive enzymes and showed distinct inhibitory mechanisms against α-amylase and trypsin. The inhibition of these digestive enzymes by dietary phenolics may represent a biochemical rationale or mechanism for delivering some of the health benefits attributed to a diet rich in phenolics. Further elucidation of the full potential of the health-promoting capabilities of legume seed coat phenolics continues to enhance and advance the discipline of functional foods and nutraceutical research.

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