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ORIGINAL ARTICLE

Changes in levels of enzyme inhibitors during soaking and cooking for pulses available in Canada

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Abstract The effects of processing (soaking and cooking) on enzyme inhibitors (α-amylase, trypsin and chymotrypsin inhibitors) in a range of pulses (4 peas, 9 lentils, 3 chickpeas, 2 faba beans and 4 beans) were investigated, using soybean as a control. Analysis of variance indicated that pulse type, treatment and their interaction had significant effects on levels of all enzyme inhibitors. Soybean contained the highest levels of trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity (CIA) among all seeds. α-Amylase inhibitory activity was absent from peas, lentils, chickpeas and faba beans, but was present in beans and soybean. TIA was found to be low in peas but high in beans. Beans contained relatively high CIA levels followed by chickpeas, lentils, peas and faba beans. Soaking markedly decreased the activity of enzyme inhibitors. Cooking of presoaked seeds was even more effective as greater reductions (78.7–100%) were observed for all pulses. The content of enzyme inhibitors in pulses varied widely, but levels of protease inhibitors were generally lower that those found in soybean. Processing, in particular heat treatments, drastically reduced these levels.

Keywords Pulses · α-Amylase inhibitory activity · Trypsin inhibitory activity · Chymotrypsin inhibitory activity · Soaking · Cooking

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Introduction

Pulses are important foods for human beings and domestic animals around the world, particularly in tropical and subtropical countries. As one major category of legumes, pulses, also called grain legumes, are dry edible seeds harvested from leguminous plants (Tiwari et al. 2011). Of the total world pulse production in 2014 (77.6 million tonnes), Canada contributed approximately 5.8 million tonnnes, ranking second in the world after India (~20 million tonnes) (FAOSTAT 2016).

The global significance of pulses is attributed to their nutritional quality. They contain adequate proportions of protein (21–25%) and carbohydrate (mainly starch) (35–60%), along with high levels of dietary fibre (12.7–30.5/100 g), vitamins and minerals (Tiwari and Singh 2012; Wang et al. 2008). However, utilization of pulses is often restricted due to the presence of certain heat labile and heat stable compounds, generally known as antinutritional factors (Pusztai et al. 2004). They can be classified into two groups: (1) proteins (amylase inhibitors, protease inhibitors and lectins), and (2) other substances (polyphenol compounds, non-protein amino acids and galactomannan gums) (Martín-Cabrejas et al. 2009). Undesirable flavour profiles in pulses also limit their usage, although not a focus of this study.

Proteinaceous inhibitors of α-amylase and proteases (trypsin and chymotrypsin) have been reported to interfere with starch and protein digestibility, respectively (Savelkoul et al. 1992). To improve the nutritional profile of pulses, numerous studies, using similar assays, have focused on reduction or elimination of enzyme inhibitors using different food processes, including dehulling, soaking, boiling, roasting, autoclaving, micronization, microwave cooking, extrusion cooking, fermentation and

germination (Alonso et al. 1998; Deshpande et al. 1982; Khattab and Arntfield 2009; Mubarak 2005). However, most of these studies have focused on the effect of various processing methods on selected antinutrients in only a handful of pulses. Therefore, the present research was undertaken to provide a comprehensive evaluation of enzyme inhibitors in a wide range of market classes of pulses and to evaluate the effect of limited processing (soaking and cooking) on these enzyme inhibitors using soybean for comparison.

Materials and methods

Materials

Seeds were received from AGT Foods and Ingredients (Regina, SK, Canada) and the Crop Development Centre (CDC) at University of Saskatchewan (Saskatoon, SK, Canada). Unless otherwise noted samples were from AGT. Samples included whole green pea, split green pea, whole yellow pea, split yellow pea, whole red lentil, split red lentil, football red lentil, split yellow lentil, split queen green lentil, French green lentil (CDC Marble from CDC), Spanish brown lentil (SB-2 3097-7 from CDC), large green lentil (CDC GreenStar from CDC), medium green lentil (CDC Imigreen from CDC), whole faba bean, split faba bean, whole chickpea B90, split chickpea B90, Desi chickpea (CDC Covy from CDC), pinto bean, dark red kidney bean, navy bean, black bean (CDC Expresso from CDC) and soybean (TH3303R2Y SB-Sorbia Preston from CDC). Flours for each seed were prepared at the University of Saskatchewan for analysis of raw material, while whole seeds were subjected to different physical treatments. The raw seeds and flours were stored at −40 °C in sealed plastic bags until used.

Chemicals

For the enzyme assays the following enzymes were used. Type I-A α-amylase from porcine pancreas (Sigma-Aldrich A4268) was used for the α-amylase inhibitor assay. This product contains between 700 and 1400 U/mg and the lot received contained 1254 U/mg, where 1 U is defined as the amount that will liberate 1 mg of maltose from starch in 3 min at pH 6.9 at 20 °C. For the trypsin inhibitor assay, a TPCK treated trypsin from bovine pancreas (Sigma-Aldrich T1426) was used. A Type II chymotrypsin from bovine pancreas (Sigma 4129) was used for the chymotrypsin inhibitor assay. All the other chemicals used in the study were purchased from Fisher Scientific (Fair Lawn, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA).

Processing methods

Soaking

Raw seeds were soaked in distilled water at a ratio of 1:5 (seed: water, w/v) for 4 h at room temperature (~25 °C) (Khattab and Arntfield 2009). After soaking, the seeds were removed from soaking water, then rinsed with same volume of distilled water and drained.

Cooking

Presoaked seeds (~30 g) were placed in a beaker with 150 mL of distilled water. The uncovered beaker was placed in a covered 95 °C Isotemp Water Bath (Fisher Scientific, Hayward, CA, USA) and held there for 1 h. After cooking, the water was drained.

Preparation of processed seeds and raw flours

The soaked and cooked seeds were dried at 55 °C in a hot air oven (Blue M Stabil-Therm Oven, Blue Island, IL, USA) overnight (Vadivel and Pugalenthi 2008). After drying, processed seeds were ground using a Cuisinart Coffee Grinder to pass through a 500 µm sieve (USA Standard Testing Sieve #35). Raw flours were also ground to pass through the 500 µm sieve. All samples were stored at 4 °C in sealed plastic bags that were placed in a desiccator until analyzed.

Reducing fat content of soybean flour

As high fat levels interfere with some of the assays preformed, the soybean flour was treated to reduce the fat content to <3%. Basically, 10 g of full fat soybean flour were mixed with 25 mL of hexane in a 500 mL centrifuge bottle and shaken for 3 min in a Wrist Action Shaker followed by centrifugation at 600×*g* for 1 min at 25 °C. This process was repeated 2 additional times. The defatted flour was dried in a fume hood at room temperature for 1 day, and then stored at 4 °C until used. The measured oil content in the resulting flour was 2.6%.

Analytical methods

Moisture content

Moisture content in raw and processed samples was determined according to AACC International method 44-15.02 (AACC International 1999). Briefly the method involved weighing 2 g of sample into a dried pan and heating at 100 °C for 16 h. After cooling in a desiccator for

30 min, the samples were weighed and moisture content calculated as moisture loss per g of sample.

α-Amylase inhibitors

The method of Deshpande et al. (1982) was modified slightly to evaluate α-amylase inhibitory activity (AIA). One gram of ground sample was extracted with 10 mL of distilled water at 4 °C overnight (16 h) and then centrifuged at 3192×*g* for 20 min at 4 °C. If necessary, the extract was diluted so that the level of inhibition was between 40 and 60% (based on preliminary testing). An aliquot (0.25 mL) of the supernatant containing the inhibitor was incubated with 0.25 mL of α-amylase enzyme solution (diluted to 40 U/mL using 0.2 M sodium phosphate buffer pH 7.0) for 15 min at 37 °C. The α-amylase activity was then measured by adding 0.5 mL of 1% starch solution (in 0.2 M sodium phosphate buffer pH 7.0) to this mixture. After exactly 3 min the reaction was terminated by addition of 2 mL dinitrosalicylic acid DNS reagent (1 g of 3.5 dinitrosalicylic acid + 30 g sodium potassium tartrate + 20 mL 2 N NaOH and diluted to 100 mL) and heating in boiling water for 10 min. The final volume was taken to 13 mL by the addition of 10 mL of distilled water. The mixture was filtered with Whatman No. 1 filter paper before reading absorbance at 540 nm using a mixture of 0.5 mL of sodium phosphate buffer (pH 7.0), 0.5 mL of the 1% starch solution and 2 mL of the DNS reagent to zero the spectrophotometer. A blank in which the α-amylase enzyme solution was replaced by 0.2 M sodium phosphate buffer was used to account for any enzymes extracted with the α-amylase inhibitor. The blank absorbance was subtracted from the measured absorbance for the sample with the α-amylase enzyme solution prior to calculating the amount of maltose released. A standard curve of maltose (0–60 μmol/mL) was established to convert calculated absorbance into milligrams of maltose. Following the recommendation of Deshpande et al. (1982), one unit of α-amylase activity was defined as that which liberated, from soluble starch, one micromole of reducing groups (calculated as maltose) per min at 37 °C and pH 7.0 under the specified conditions. One unit of α-amylase activity inhibited was defined as one α-amylase inhibitory unit. α-Amylase inhibitory activity was reported as AIU/g on a dry basis

Trypsin inhibitors

Trypsin inhibitory activity (TIA) was determined colorimetrically using an UV/visible spectrophotometer in accordance with AACC International method 22-40.01 (AACC International 2000), with some modifications. Exactly 0.5 g of finely ground flour was extracted with 25 mL of 0.01 N NaOH for 3 h and the mixture was centrifuged at 14,190×*g* for 10 min. Extracts were diluted to produce 40–60% inhibition (based on preliminary testing). The supernatant (2 mL) was incubated with 2 mL of trypsin solution (20 μg/mL in 0.1 mM HCl) for 5 min at 37 °C. The substrate used was BAPA (Na-Benzoyl-D, L-arginine 4-nitroanilide hydrochloride) which was prepared by dissolving 40 mg BAPA in 1 mL of dimethyl sulfoxide and diluting to 100 mL with 0.05 M Tris Buffer at pH 8.2. Five milliliters of pre-warmed substrate solution (37 °C) was added to the extract to initiate the reaction. After exactly 10 min the reaction was stopped by the addition of 1 mL of 30% acetic acid; the mixture was then filtered using Whatman No. 2 paper. A separate blank sample was used for each extract but trypsin activity was prevented by adding the trypsin solution after acetic acid. One trypsin unit was equivalent to an increase of 0.01 absorbance unit at 410 nm per 10 mL of reaction mixture compared to the blank sample. Trypsin inhibitor activity was defined as the amount of trypsin units inhibited per mg of sample.

Chymotrypsin inhibitors

Chymotrypsin inhibitory activity (CIA) was assayed according to the method described by Makkar et al. (2007) with the following modifications. To 1 g of flour sample, 10 mL of borate buffer (0.1 M, pH 7.6) was added and then extracted for 1 h using an ATR Rotamix. The slurry was centrifuged at 3000×*g* for 10 min at 4 °C and diluted to achieve 40–60% inhibition (if necessary). The supernatant (1 mL) was incubated with 1 mL of chymotrypsin solution (40 μg/mL in 0.001 M HCl with 0.08 M $CaCl_2$) at 37 °C for 10 min and subsequently 2 mL of casein solution (1 g in 100 mL of 0.1 M borate buffer, pH 7.6), previously warmed to 37 °C, were added and mixed. At the end of 10 min, the reaction was stopped by the addition of 6 mL trichloroacetic acid (TCA) reagent (18 g TCA + 18 g anhydrous sodium acetate + 20 mL glacial acetic acid in 1 L). The suspension was left at room temperature for at least 30 min and was then filtered. The absorbance of the filtrate was recorded at 275 nm against the appropriate blank. A separate blank, in which the 6 mL of trichloroacetic acid reagent was added before the 2 mL of casein solution, was used for each sample. One chymotrypsin unit was defined as an increase of 0.01 absorbance unit at 275 nm for the reaction mixture. Chymotrypsin inhibitory activity was defined as the number of chymotrypsin units inhibited.

Statistical analysis

All treatments (soaking and cooking) were conducted in triplicate and results are expressed as mean ± SD on dry

matter basis. For the raw samples, three separate samples were taken and analyzed in triplicate. Two-way analysis of variance (ANOVA) for models with main effects and interactions were determined using the GLM procedure. Tukey's test was used to separate means and differences were that considered to be significant at $P < 0.05$. The statistical analysis was performed by SAS Program version 9.3 (SAS Institute Inc., Gary, NC, USA). As interactions were significant for all antinutritional factors, the effects of processing were evaluated for each type of pulse; comparison of the antinutritional factors in the different types of pulse was done for the raw material only.

Results and discussion

α-Amylase inhibitors

α-Amylase inhibitors in seeds can act as antinutritional factors in both human and animal nutrition (Jaffé et al. 1973; Svensson et al. 2004). Digestibility of starch is reduced due to inhibition of pancreatic and salivary α-amylase activity (Jaffé et al. 1973; Savelkoul et al. 1992; Wang et al. 2011).

Analysis of variance showed both pulse type and treatment (soaking or cooking) had significant effects on α-amylase inhibitory activity ($P < 0.0001$) (Table 1). The interactive effect of pulse type × soaking or cooking on α-amylase inhibitory activity was also significant (Table 1). Data on the α-amylase inhibitory activity of beans and soybean are presented in Table 2; no inhibitory activity was detected in raw peas, lentils, faba beans and chickpeas. This was similar to the results of by Jaffé et al. (1973) who stated that α-amylase inhibitor in lentils and chickpeas only showed slight activity, but *Phaseolus vulgaris* was the most active. Grant et al. (1995) and Martín-Cabrejas et al. (2009) also found inhibitory activity in beans (pinto bean, kidney bean white bean and pink-mottled cream bean), but not in lentils or peas. Amongst common beans and soybean, the α-amylase inhibitor content was in the order: dark red kidney bean (1369.75 AIU/g) > navy bean (1079.83 AIU/g) > pinto bean (1000.91 AIU/g) > soybean (938.73 AIU/g) > black bean (785.58 AIU/g). Deshpande et al. (1982) reported a range of 330–675 U/g for α-amylase inhibitor in 10 cultivars of *P. vulgaris* while only 248 U/g was detected in kidney bean by Alonso et al. (2000). α-Amylase inhibitory activity of common beans was higher than these published data and may be due to the differences in cultivars, climatic conditions, location, soil type and crop year.

There was about 4–10% reduction in α-amylase inhibitor content after soaking common beans and a 4% reduction in soybean (Table 2). Vadivel and Pugalenthi (2008) reported that the reduction in activity ranged between 25 and 28% in velvet bean seeds using a similar soaking method but at a 1:10 seed to water ratio and at 32 °C. Alonso et al. (2000) found that soaking of *Vicia faba* and *P. vulgaris* in water for 12 h at 30 °C caused 14.9 and 23.9% reduction in α-amylase inhibitory activity, respectively. Reduction in the level of α-amylase inhibitor during soaking has been attributed to the inhibitor leaching into steeping water (Vadivel and Pugalenthi 2008). The high retention of α-amylase inhibitor activity in this study may reflect the lower temperature used. Also the type of bean may be a factor as it was clear in Table 2 that the percent reduction in α-amylase inhibitor activity was not the same for all beans. The cellular structure of the intact seed could limit the removal of α-amylase inhibitors in an aqueous environment.

Cooking was more effective at decreasing α-amylase inhibitor content when compared with soaking. Approximately 80–93% reduction resulted from cooking beans; additionally, a complete inactivation of α-amylase was achieved in soybean (Table 2). Martín-Cabrejas et al. (2009) also reported that cooking of presoaked bean seeds caused a 91–95% reduction in α-amylase inhibitory activity. Jaffé and Vegna (1968) reported that amylase inhibitor activity was completely destroyed in well-cooked *P. vulgaris*. Vadivel and Pugalenthi (2008) reported reductions in α-amylase inhibitor activity of only 43–54% when soaking in water followed by cooking. Clearly the heat stability of the α-amylase inhibitors varies. The destruction or denaturation of more than 80% of the α-amylase inhibitors in this study should reduce them to a point where the antinutritional effects are minor.

Trypsin inhibitor activity

Trypsin inhibitors, which are serine protease inhibitors, are low molecular weight proteins found in a wide range of food sources including pulses (Savage 1989; Wang and

Table 1 Summary of analysis of variance (ANOVA) of effects of legume type and treatment on enzymatic inhibitory activities in pulses and soybean

Enzymatic inhibitory activity	Legume type (L)	Treatment (T)	Interaction L × T
α-Amylase inhibitory activity (AIU[1]/g)	<0.0001	<0.0001	<0.0001
Trypsin inhibitory activity (TIU[2]/mg)	<0.0001	<0.0001	<0.0001
Chymotrypsin inhibitory activity (CIU[3]/mg)	<0.0001	<0.0001	<0.0001

AIU α-amylase inhibitory unit, *TIU* trypsin inhibitory unit, *CIU* chymotrypsin inhibitory unit

Table 2 Effect of soaking and cooking on α-amylase inhibitory activity of pulses and soybean

Type	α-Amylase inhibitory activity (AIU/g dry matter)		
	Raw	Soaked	Cooked
Bean			
Dark red kidney bean	1369.75 ± 82.23^{aA}	1219.44 ± 30.18^{b} (10.97)	143.87 ± 3.46^{c} (89.50)
Pinto bean	1000.91 ± 35.99^{aC}	901.31 ± 19.23^{b} (9.95)	199.88 ± 1.21^{c} (80.03)
Navy bean	1079.83 ± 6.37^{aB}	1036.09 ± 5.77^{b} (4.05)	71.32 ± 0.42^{c} (93.40)
Black bean	785.58 ± 8.52^{aE}	731.70 ± 27.17^{b} (6.86)	71.24 ± 3.15^{c} (90.93)
Soybean	938.73 ± 19.17^{aD}	899.30 ± 4.55^{b} (4.20)	nd^{c} (100)

α-Amylase inhibitory activity in peas, lentils, faba bean and chickpea were too low to measure in the raw material so have not been included

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test

Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test

Values in parentheses indicate % decrease over raw values

Mean ± SD of three determinations

nd not detected

Daun 2004). They are capable of binding to lysine and arginine residues in trypsin, which is a proteolytic enzyme secreted by the pancreas (Savage 1989; Mondor et al. 2009). Therefore, protein digestion is reduced and inadequate amino acids are available for good nutrition (Savage 1989). In contrast, a number of benefits have been identified for protease inhibitors such as trypsin inhibitors. In animal studies, protease inhibitors were considered as anticarcinogenic agents due to their suppressive effects on carcinogen-induced cells (Clemente and Domoney 2001; Thompson 1993).

Analysis of variance revealed that pulse type and treatment (soaking or cooking) had significant effects ($P < 0.0001$) on trypsin inhibitory activity (Table 1). The interactive effect of pulse type-by-treatment was also significant ($P < 0.0001$) (Table 3). Results of trypsin inhibitory activity of raw and treated pulses and soybean are presented in Table 3. The trypsin inhibitor content was found to be significantly higher in raw soybean (45.89 TIU/mg) than all pulses. This value was similar to that reported by Chen (2015) who detected 41.1 TIU/mg of trypsin inhibitory activity in defatted soybean flour. The data show that the content of trypsin inhibitor ranged from low, as in peas (3.16–4.92 TIU/mg), lentils (4.98–6.29 TIU/mg) and faba beans (5.96–6.10 TIU/mg), to relatively high in chickpeas (14.22–15.96 TIU/mg) and common beans (15.18–20.83 TIU/mg) (Table 3). Trypsin inhibitory activity for peas was within the range (2.80–6.32 TIU/mg) reported by Alonso et al. (1998). However, lentils in the current study had higher trypsin inhibitory activity than the 3.6 TIU/mg reported by Hernández-Infante et al. (1998) whereas the levels in faba bean and chickpea were lower than the those reported by these authors (7.2 TIU/mg in faba bean and 17.9 TIU/mg in chickpea).

When compared to whole seeds of yellow pea and chickpea B90, the split samples contained significantly higher levels of trypsin inhibitor than the corresponding whole seeds (Table 3). In this study, seed coat was removed as part of the splitting operation; therefore, the split samples were actually dehulled seeds. An increase in trypsin inhibitory activity from pulses has been reported in a number of studies after dehulling (Alonso et al. 1998, 2000; Deshpande et al. 1982; Mubarak 2005). As Deshpande et al. (1982) suggested this phenomenon might be due to the fact that trypsin inhibitors are present in the cotyledon fractions of pulses. After the seed coat is removed, the concentration of trypsin inhibitor increases on a unit weight basis.

Soaking seeds in water caused a significant reduction of trypsin inhibitor in peas (17.34–30.74%), lentils (5.57–19.35%), faba beans (12.73–22.59%), chickpeas (9.39–25.27%), common beans (4.88–9.09%) and soybean (18.58%) (Table 3). The loss of activity is more than what has been reported previously for faba bean (4.47%) and peas (1.58–12.02%) (Alonso et al. 1998, 2000). However, the loss for kidney beans (4.88%) was similar to the 5.48% loss reported by Alonso et al. (2000). This loss is believed to be associated with leaching of the trypsin inhibitors into the soak water. While the soak water was not analyzed in this study, Gatfield (1980) has identified thermostable trypsin inhibitors in commercial bean soak water. In contrast, Wang et al. (2008) reported significant increases in the levels of trypsin inhibitors due to soaking. Clearly the removal of trypsin inhibitors during soaking is limited, such that in some studies (Wang et al. 2008), loss

Table 3 Effect of soaking and cooking on trypsin inhibitory activity of pulses and soybean

Type	Trypsin inhibitory activity (TIU/mg dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	3.16 ± 0.04^{aO}	2.62 ± 0.11^{b} (17.34)	0.59 ± 0.02^{c} (81.25)
Split yellow pea	4.18 ± 0.13^{aN}	2.90 ± 0.08^{b} (30.74)	nd^{c} (100)
Whole green pea	4.65 ± 0.09^{aMN}	3.78 ± 0.07^{b} (18.56)	0.99 ± 0.00^{c} (78.74)
Split green pea	4.92 ± 0.12^{aLMN}	3.86 ± 0.09^{b} (21.61)	nd^{c} (100)
Lentil			
Whole red lentil	5.99 ± 0.19^{aHIJ}	5.42 ± 0.23^{b} (9.59)	nd^{c} (100)
Split red lentil	6.29 ± 0.35^{aH}	5.07 ± 0.36^{b} (19.35)	nd^{c} (100)
Football red lentil	5.88 ± 0.26^{aHIJK}	4.94 ± 0.58^{b} (15.89)	nd^{c} (100)
Spanish brown lentil	4.98 ± 0.08^{aLM}	4.39 ± 0.22^{b} (11.90)	nd^{c} (100)
Split yellow lentil	5.17 ± 0.21^{aKLM}	4.32 ± 0.03^{b} (16.51)	nd^{c} (100)
French green lentil	5.14 ± 0.07^{aKLM}	4.24 ± 0.14^{b} (17.64)	nd^{c} (100)
Large green lentil	6.21 ± 0.21^{aHI}	5.67 ± 0.10^{b} (8.71)	nd^{c} (100)
Medium green lentil	5.40 ± 0.09^{aJKLM}	5.10 ± 0.14^{b} (5.57)	nd^{c} (100)
Split queen green lentil	5.46 ± 0.10^{aIJKL}	4.21 ± 0.10^{b} (22.93)	nd^{c} (100)
Faba bean			
Whole faba bean	5.96 ± 0.27^{aHIJ}	5.20 ± 0.10^{b} (12.73)	nd^{c} (100)
Split faba bean	6.10 ± 0.34^{aHIJ}	4.72 ± 0.31^{b} (22.59)	nd^{c} (100)
Chickpea			
Whole chickpea B90	14.22 ± 0.13^{aG}	12.89 ± 0.26^{b} (9.39)	2.29 ± 0.07^{c} (83.92)
Split chickpea B90	16.24 ± 0.24^{aDE}	12.14 ± 0.39^{b} (25.27)	1.89 ± 0.03^{c} (88.37)
Desi chickpea	15.96 ± 0.22^{aEF}	13.85 ± 0.21^{b} (11.19)	1.88 ± 0.21^{c} (87.95)
Bean			
Dark red kidney bean	17.77 ± 0.27^{aC}	16.90 ± 0.18^{b} (4.88)	1.32 ± 0.06^{c} (92.58)
Pinto bean	15.18 ± 0.10^{aF}	13.80 ± 0.54^{b} (9.09)	0.99 ± 0.07^{c} (93.46)
Navy bean	16.44 ± 0.45^{aD}	15.02 ± 0.73^{b} (8.63)	1.36 ± 0.03^{c} (91.72)
Black bean	20.83 ± 0.33^{aB}	19.55 ± 0.26^{b} (6.18)	1.31 ± 0.08^{c} (93.70)
Soybean	45.89 ± 0.51^{aA}	37.37 ± 0.57^{b} (18.58)	3.29 ± 0.23^{c} (92.83)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test

Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test

Values in parentheses indicate % decrease over raw values

Mean ± SD of three determinations

nd not detected

of trypsin inhibitors to the soaking water was lower than other seed constituents, resulting in increased trypsin inhibitors in the soaked product. As there are several types of trypsin inhibitor, this may contribute to the noted variation. Further investigation into the characteristics of pulse trypsin inhibitors may be warranted. Also porosity of the seed coat may have limited trypsin inhibitor extraction. The percent reduction in trypsin was about 10% higher for split (dehulled) material (Table 3), although the levels of trypsin inhibitors in the soaked split and whole products were similar.

Cooking brought a total removal of trypsin inhibitory activity in split yellow and green peas, lentils and faba beans. A relatively high reduction was also observed in common beans (92.58–93.70%) and soybean (92.83%), with lower reductions for chickpeas (83.92–88.37%) and whole yellow and green peas (78.74–81.25%) (Table 3). Complete removal of trypsin inhibitor activity due to boiling of whole seeds has been reported previously for peas, while reductions of 83.3–100% and 79.6–93.5% have been reported for beans and chickpeas, respectively (Khattab and Arntfield 2009; Martín-Cabrejas et al. 2009;

Table 4 Effect of soaking and cooking on chymotrypsin inhibitory activity of pulses and soybean

Type	Chymotrypsin inhibitory activity (CIU/mg dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	2.84 ± 0.09^{aL}	2.56 ± 0.21^{b} (9.83)	nd^{c} (100)
Split yellow pea	3.23 ± 0.20^{aJKL}	2.89 ± 0.09^{b} (10.28)	nd^{c} (100)
Whole green pea	3.13 ± 0.03^{aKL}	2.87 ± 0.07^{b} (8.35)	nd^{c} (100)
Split green pea	3.34 ± 0.17^{aIJKL}	2.88 ± 0.08^{b} (13.80)	nd^{c} (100)
Lentil			
Whole red lentil	4.14 ± 0.33^{aGHIJ}	3.87 ± 0.34^{b} (6.62)	nd^{c} (100)
Split red lentil	4.89 ± 0.17^{aG}	4.58 ± 0.24^{b} (6.40)	nd^{c} (100)
Football red lentil	4.66 ± 0.13^{aGH}	4.19 ± 0.45^{b} (10.21)	nd^{c} (100)
Spanish brown lentil	3.51 ± 0.10^{aIJKL}	3.15 ± 0.30^{b} (10.23)	nd^{c} (100)
Split yellow lentil	4.58 ± 0.26^{aGHI}	4.00 ± 0.24^{b} (12.68)	nd^{c} (100)
French green lentil	3.71 ± 0.12^{aHIJKL}	3.42 ± 0.18^{b} (7.82)	nd^{c} (100)
Large green lentil	4.55 ± 0.11^{aGH}	4.10 ± 0.11^{b} (9.78)	nd^{c} (100)
Medium green lentil	3.89 ± 0.09^{aHIJK}	3.42 ± 0.19^{b} (12.03)	nd^{c} (100)
Split queen green lentil	4.02 ± 0.03^{aGHIJK}	3.79 ± 0.25^{b} (5.64)	nd^{c} (100)
Faba bean			
Whole faba bean	1.12 ± 0.09^{aM}	0.99 ± 0.09^{b} (11.43)	nd^{c} (100)
Split faba bean	1.67 ± 0.11^{aM}	1.37 ± 0.10^{b} (17.51)	nd^{c} (100)
Chickpea			
Whole chickpea B90	12.29 ± 0.34^{aF}	10.83 ± 0.14^{b} (11.85)	nd^{c} (100)
Split chickpea B90	13.59 ± 0.26^{aE}	11.06 ± 0.26^{b} (18.58)	nd^{c} (100)
Desi chickpea	11.78 ± 0.22^{aF}	10.47 ± 0.32^{b} (11.14)	nd^{c} (100)
Bean			
Dark red kidney bean	21.00 ± 0.88^{aC}	18.81 ± 0.19^{b} (10.45)	nd^{c} (100)
Pinto bean	17.77 ± 0.07^{aD}	16.50 ± 0.18^{b} (9.68)	nd^{c} (100)
Navy bean	18.67 ± 0.12^{aD}	17.25 ± 0.59^{b} (7.60)	nd^{c} (100)
Black bean	24.48 ± 0.97^{aB}	21.42 ± 0.61^{b} (12.40)	nd^{c} (100)
Soybean	30.16 ± 0.17^{aA}	28.14 ± 0.45^{b} (6.71)	nd^{c} (100)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test

Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test

Values in parentheses indicate % decrease over raw values

Mean ± SD of three determinations

nd not detected

Wang et al. 2010). While Trypsin inhibitors tend to be heat sensitive and therefore inactivated by cooking due to denaturation (Vidal-Valverde et al. 1994), there have been reports of heat stable trypsin inhibitors. Two types of soybean trypsin inhibitors, Kunitz (KTI) and Bowman Birk (BBI) have been extensively studied and both show some heat stability as a result of the disulfide bonds (2 in KTI and 7 in BBI) present (van der Ven et al. 2005). Some heat stable trypsin inhibitors have also been identified in pulses. Rayas-Duarte et al. (1992) examined the heat-stable trypsin inhibitors in beans and found that stability increased from 2.5 to 5% trypsin inhibitor retention in whole seed to 28–58% in ground bean flours. This supports the observation in the current study that some of the trypsin inhibitors are heat stable. However, for those samples that were treated as both whole seed and dehulled split seed (peas and chickpeas), there was no advantage to having a dehulled seed (Table 3).

Chymotrypsin inhibitor activity

The role of chymotrypsin inhibitors is similar to trypsin inhibitors in that they limit protein digestibility. However, the site of activity is different in that chymotrypsin targets

hydrophobic residues such as tyrosine, tryptophan and phenylalanine rather than lysine and arginine.

Analysis of variance indicated that pulse type, treatment (soaking or cooking) and their interaction exerted significant effects ($P < 0.0001$) on chymotrypsin inhibitory activity (Table 1). The results of chymotrypsin inhibitory activity in raw and processed pulses and soybean are summarized in Table 4. Chymotrypsin inhibitor was widely distributed in the pulses and soybean; soybean had the highest content (30.16 CIU/mg) among the investigated seeds. Common beans (17.77–24.48 CIU/mg) and chickpeas (11.78–13.59 CIU/mg) contained relatively high inhibitory activity, followed by lentils (3.51–4.89 CIU/mg) and peas (2.84–3.34 CIU/mg). Faba beans had the lowest chymotrypsin inhibitor content (1.12–1.67 CIU/mg). Deshpande et al. (1982) reported a range of 217–345 U/mg for chymotrypsin inhibitor content in 10 cultivars of *P. vulgaris*. The range for inhibitory activity in peas was 2.73–4.85 U/mg (Alonso et al. 1998). Alonso et al. (2000) also reported levels of 3.56 and 3.97 CIU/mg of chymotrypsin inhibitor in *V. faba* and *P. vulgaris*, respectively. Singh and Jambunathan (1981) found that chymotrypsin inhibitory activity was 7.6–8.8 CIU/mg for desi chickpeas and 6.1–8.0 CIU/mg for kabuli. The considerable variations noted could mainly be attributed to the different chymotrypsin inhibitor assays used. From Tables 3 and 4, it can be seen that the activity of trypsin inhibitor is generally higher than that of chymotrypsin inhibitor in all seeds, except for common beans. These results are in agreement with those reported by Alonso et al. (1998, 2000), Deshpande et al. (1982) and Singh and Jambunathan (1981) in peas, faba beans, common beans and chickpeas. Similar to trypsin inhibitor, chymotrypsin inhibitor content was significantly higher in split chickpea B90 when compared to the whole seed sample (Table 4). No significant differences were found between split and whole seeds of green and yellow peas, and faba bean. Alonso et al. (1998, 2000) and Deshpande et al. (1982) reported that for *P. vulgaris*, *Pisum sativum* and *V. faba*, the dehulling process increased chymotrypsin inhibitory activity, by concentrating the inhibitors associated with the cotyledon. It appears that for samples other than chickpea (*Cicer arietinum*) the concentration effect was not great enough to create a significant difference.

Soaking resulted in significant losses of chymotrypsin inhibitor content in peas (8.35–13.80%), lentils (5.64–12.68%), faba beans (11.43–17.51%), chickpeas (11.14–18.58%), beans (7.60–12.40%) and soybean (6.71%) (Table 4). Alonso et al. (1998) and Alonso et al. (2000) reported that 13.4–17.6, 8.43 and 15.1% reductions in chymotrypsin inhibitory activity in pea, faba bean and kidney bean, respectively. The observed decreases in chymotrypsin inhibitory activity during soaking are likely due to leaching into the soak water, as was seen for trypsin inhibitors; evaluation of the soak water would be needed to confirm this. As was the case with trypsin inhibitors, losses were generally greater for the split seeds, although the difference was much less than that seen for trypsin inhibitor. The seed structure was able to inhibit the removal of chymotrypsin inhibitors. Complete removal of chymotrypsin inhibitory activity was found for all studied seeds after cooking (Table 4) indicating chymotrypsin inhibitors are more sensitive to heat than trypsin inhibitors. These results agree with those reported by Martín-Cabrejas et al. (2009) who found that cooked seeds of chickpea, white bean and pink-mottled cream bean exhibited 100% reduction in chymotrypsin inhibitory activity. Other studies have also shown that cooking is an effective processing method to inactivate protease inhibitors in pulses (Gatta et al. 1989; Wang et al. 1997). As stated by Wang et al. (2010), pulses can be softened by sufficient cooking and the levels of protease inhibitors may also be inactivated or reduced simultaneously. Therefore, cooking enables improvement of the nutritional value of pulses. However, over-cooking may decrease nutritional quality due to loss of essential amino acids in pulses (Wang et al. 2008, 2010; Youssef et al. 1986).

Conclusion

As shown in this study, pulse type, treatment (soaking or cooking) and their interaction had significant effects on the levels of all enzyme inhibitors (α-amylase, trypsin and chymotrypsin inhibitors) in pulses and soybean. Considerable differences in these enzyme inhibitors were detected among all investigated seeds. Soybean contained relatively high contents of protease inhibitors when compared with the pulses. For those using pulse flours for animal feed, where no heat treatments are applied, this is particularly a concern with beans which had the highest level of enzyme inhibitors for all the pulses, although levels of protease inhibitors were not as high as soybean. For human consumption, the cooking step is often included prior to consuming pulses. In this work the combination of soaking and cooking was more effective than soaking alone in reducing enzyme inhibitors in these materials. Chymotrypsin inhibitors were denatured or degraded as were the trypsin inhibitors in lentils, faba beans and some peas. The levels of trypsin inhibitors in cooked chickpeas and beans and amylase inhibitors in cooked beans were considerably lower than in the raw product. Information obtained from the present study should help the pulse industry and regulatory bodies in identifying the appropriate pulse and treatment for effective further utilization of these materials.

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