

The impact of four processing methods on trypsin-, chymotrypsin- and alpha-amylase inhibitors present in underutilised legumes

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Abstract The global trend in increasing plant-based protein diets due to health and ideological reasons, has created an increased demand for food legumes that exceeds current production. To meet this demand, it is timely to reduce relying solely on soybean, and explore the potential of the underutilised legumes that are cultivated regionally. Underutilised legumes are rich in protein, carbohydrates and other nutrients that are essential for consumer. However, relatively little is known about their anti-nutritional properties and processing methods. Anti-nutritional factors (ANFs) such as enzyme inhibitors are prevalent in legumes and may interfere with digestibility and nutrient absorption. Nevertheless, an optimised food processing method will overcome this challenge and warrant a safe inclusion of legume in plant-based protein diets. Hence current study aimed to optimise the food processing methods (soaking, wet heating, autoclaving and freezing) and evaluate their efficiency in eliminating the enzyme inhibitors [trypsin, chymotrypsin (CIA) and α -amylase (AIA) inhibitors] present in seven underutilised legumes. Current study showed that autoclaving at 121 °C for 15 min reduced the AIA in all underutilised legumes tested. The AIA and CIA of bambara groundnut were successfully inactivated by wet heating at 50 °C for 60 min, and by autoclaving at 121 °C

for 15 min. While the CIA of chickpea was successfully inactivated by freezing at – 80 °C for 24 h.

Keywords Food processing · Legumes · Trypsin inhibitor · Chymotrypsin inhibitor · α -Amylase inhibitor

Introduction

Globally, there is an increasing trend to move from animal-based to plant-based protein diets due to ideological and health reasons (Singh et al. 2017). This trend has created an increased demand for food legumes that exceeds current production, since only a few legumes are traded on the global market (Bhat and Karim 2009). For the past few decades we have relied solely on protein from soybean to meet the demand whilst other legumes have remained underutilised (Torres et al. 2016). Legumes are valuable source of protein, dietary fibre, carbohydrates, vitamins, minerals, phytochemicals and energy for humans and animals (Singh 2017). Unfortunately, legumes also contain anti-nutritional factors (ANFs) which are associated with defence mechanisms against predators, and help to overcome infection during seed development (Sales 2000). When consumed, ANFs such as protease and α -amylase inhibitors, may have adverse effects on digestion and trigger an allergenic reaction (Shah et al. 2016).

Protease inhibitors containing the serine residue that interacts with the protease, such as trypsin and chymotrypsin inhibitors, are found in abundance in raw legumes (Krogdahl et al. 2010). These protease inhibitors are proteins that form stoichiometric protease-inhibitor complexes with their respective enzymes and inhibit their activity in the gastrointestinal tract. They will bind to the digestive enzyme, either through competitive or allosteric

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mode of action to render the enzyme inactivate (Glencross 2015). The presence of chymotrypsin inhibitors in animal diets will cause indigestion and abdominal pain, whilst pancreatic enlargement and growth depression can be caused by the presence of trypsin inhibitors (Kumar et al. 2013).

α -Amylase inhibitors are also widely distributed in legumes and will inhibit the human and porcine pancreatic amylases by non-competitive inhibition mechanism (Santimone et al. 2004). The complex formation of α -amylase inhibitors with α -amylases reduces starch digestion by inhibiting the hydrolysis of starch α -1,4-glycosidic bonds (Singh et al. 2010). Therefore, the presence of legume α -amylase inhibitor in the human diet can cause impaired carbohydrate digestion and associated digestive issues which could ultimately result in coeliac disease, leading to weight loss (Kumar et al. 2013).

In order to promote the use of underutilised legumes for human consumption or as animal feed, it is essential that ANFs are removed. This study investigated the effectiveness of four processing methods (soaking, wet heating, autoclaving and freezing) and optimised parameters (temperatures and duration) in removing the ANFs, particularly the trypsin, chymotrypsin, and α -amylase inhibitors present in seven underutilised legumes. The outcomes of this study would help to address the world demand for alternative protein sources, and ways to process these underutilised legumes for safe consumption.

Materials and methods

Legume samples

Eight types of dried legume seeds—mung bean (*Vigna radiate* L.; MB), adzuki bean (*Phaseolus angularis*; AB), chickpea (*Cicer arietinum* L.; CP), hyacinth bean (*Lablab purpureus* L.; HB), black-eyed pea (*Vigna unguiculata* L.; BEP), pigeon pea (*Cajanus cajan*; PP), bambara groundnut (*Vigna subterranean*; BG) and soybean (*Glycine max*; SB) were used in this study. All seed samples were purchased as dried material—whole unprocessed complete with hull from a hypermarket in Semenyih, Selangor, Malaysia. Only BG was provided by the Crops For the Future Research Centre (CFFRC), Semenyih, Selangor, Malaysia.

Processing methods and parameters

Four conventional processing methods, namely soaking, wet heating, autoclaving and freezing were investigated in this study. The unprocessed dry seeds were used as a control.

Soaking

Dried seeds (10 g) were soaked in 100 mL room temperature (25 °C) distilled water for various durations (0, 6, 12, 18 and 24 h). After treatment, water was decanted, and the processed seeds were chilled and frozen at -80 °C for 24 h prior to lyophilisation.

Wet heating

Dried seeds (10 g) were immersed in 100 mL distilled water and heated immediately to 50 or 100 °C and held at this temperature for 30 or 60 min. After treatment, water was decanted and the processed seeds were chilled and frozen at -80 °C for 24 h prior to lyophilisation.

Autoclaving

Dried seeds (10 g) were immersed in 100 mL distilled water and autoclaved at 121 °C, 15 psi for 15 min. After treatment, water was decanted, and the processed seeds were chilled and frozen at -80 °C for 24 h prior to lyophilisation.

Freezing

Dried seeds (10 g) were frozen at -20 or -80 °C for 24 h. After treatment, the processed seeds were lyophilised to form a fine powder.

Lyophilisation

All frozen seed-samples were freeze dried. The processed seeds were lyophilised for 24 h using a freeze-dryer (Alpha 1-4 LD plus, Christ). The lyophilised seeds were ground using grinder (MX-AC210SW, Panasonic) to pass through a 1.18 μ m mesh sieve. Dried ground material was stored in air tight container at 4 °C prior to determination of enzyme inhibitor activity.

Determination of enzyme inhibitor activity

Enzyme inhibitor activity

Trypsin inhibitor activity (TIA) was determined according to the method of Marzo et al. (1998), using α -N-benzoyl-dl-arginine-p-nitroanilidehydrochloride (BAPNA) as substrate for trypsin. Chymotrypsin inhibitor activity (CIA) was determined according to the method of Marzo et al. (1998), using benzoyl-L-tyrosine ethyl ester (BTEE) as substrate. α -amylase inhibitor activity (AIA) was evaluated

according to the method of Deshpande et al. (1982), using 1% (w/w) starch as substrate.

Statistical analysis

All analyses were carried out in triplicate. Results are presented as mean $\pm$ standard error mean (SEM). IBM SPSS Statistics software (Version 22, IBM Corporation, USA) was used to perform a two-way Analysis of Variance (ANOVA) on data sets. Post-hoc analysis was carried out using Duncan's Multiple Comparison test confidence intervals of 95% with threshold for significance when $p < 0.05$.

Results and discussion

Effect of soaking on enzyme inhibitor activity

The effects of soaking duration on TIA, CIA and AIA of the seeds is presented in Table 1. Interestingly, all but two of the seeds that were soaked for 6 h showed significant increment of TIA (by 87–100%) and CIA (by 40–58%) when compared to the respective controls. The two exceptions were TIA of MB which only increased after 12 h soaking and CIA of BG which showed a reduction in CIA after 6 h with a subsequent increase after prolonged soaking. For MB, the enhancement of TIA was only spotted after soaked for 12 h. A similar increase in AIA was observed for SB and PP after soaking for 6 h (53% and 22%). In contrast, the AIA of MB, AB, CP and BEP was reduced after soaking for 6 and 12 h (by 22–36%) but increased after longer soaking for 18 and 24 h (by 18–65%).

The increase in enzyme inhibitor activity in soaked beans was in agreement with previous studies from Martín-Cabrejas et al. 2009; Shi et al. 2017 and Wang et al. 2008. These reports suggest that the increased enzyme inhibitors level in soaked seeds could be due to a low leaching-out effect during hydration, in which the loss of inhibitors to the soaking water was lower than other seed constituents such as soluble phenolic compounds (Martín-Cabrejas et al. 2009; Shi et al. 2017). Furthermore, it is possible that the structure of the intact seed could limit the removal of inhibitors in an aqueous environment, this soaking may result in retention of enzyme inhibitors in the soaked seeds (Shi et al. 2017; Wang et al. 2008).

Effect of wet heating on enzyme inhibitor activity

Table 2 shows the effects of wet heating temperatures and duration on the TIA, CIA and AIA of the seeds tested. When compared to respective controls, 30 min of heating

at 50 or 100 °C increased the TIA in all seeds. Interestingly, when the heating duration was prolonged to 60 min at 50 °C, a reduction in TIA for all seeds was recorded. Similar reduction in TIA was not found when heating at 100 °C for 60 min. Using a lower temperature over the longer heating duration may cause cleavage of the peptide bonds of protease inhibitors leading to inactivation of the TIA. The decrease in activity could be the result of the Millard reaction between the inhibitor and carbohydrate, thereby decreasing bioavailability and inactivating the nutrients.

BG showed a significant reduction of CIA (35%) after heating at 50 °C for 30 min. Prolonged heating to 60 min at 100 °C successfully reduced the CIA of BG to a non-detectable level. For SB, MB and AB, the reduction of CIA was only detected when heated at 100 °C for 30 min. While wet heating at 50 °C increased the CIA (by 2–100%) for most seeds tested, it decreased the AIA of MB, AB, CP, BEP and BG. Only SB showed more than two-fold increment of AIA after wet heating at 50 °C. When the heating temperature was increased to 100 °C, all seeds apart from BG showed an increase in AIA (by 18–65%). There are six types of α -amylase inhibitor found in higher plants, namely knottin-like inhibitor, γ -thionin-like inhibitor, CM-proteins inhibitor, kunitz-type inhibitor, thaumatin-like inhibitor and legume lectin-like inhibitor have the range of 1–8 disulphide bonds (Svensson et al. 2004). Four out of six types of α -amylase inhibitors which are CM-protein inhibitor, legume lectin-like inhibitor, thaumatin-like inhibitor and γ -thionin-like inhibitor are resistant to thermal denaturation due to the high numbers (5–8) of disulphide bonds. It is possible that the α -amylase inhibitors present in BG differ in type from the other legumes and are heat sensitive which would result in the reduction of AIA. Further studies are required to determine the type of α -amylase inhibitors present in the different legumes and the possible protein conformational changes after heat treatment.

The trypsin inhibitor activity for most of the seeds was resistant to wet heating. Wati et al. (2010) suggested that when the temperature increased from 50 to 70 °C the heat causes the trypsin inhibitor to loosen its compact structure, which is normally stabilised by numerous disulphide bonds. However, these conformational changes are reversible when the enzyme inhibitor cools down to 25 °C. Therefore, inhibitors possibly possess some degree of flexibility that allows them to regain their original conformation and inhibitor activity after being heated (Prasad et al. 2010).

Another study by Morrison et al. (2007) suggested that these enzyme inhibitors may be heat stable and thus resistant to denaturation at high temperature. Different types of protease inhibitor have been isolated from legumes (soybean, tepary bean, navy bean, red kidney bean and

Table 1 Effect of soaking duration on the enzyme inhibitor activity of legumes

Legumes	Enzyme inhibitor activity at different soaking duration				
	Control	6 h	12 h	18 h	24 h
<i>Trypsin inhibitor activity (TIA unit/mg)</i>					
SB ¹	0.06 ± 0.01 ^{lmn}	0.17 ± 0.01 ^{ijklm}	0.35 ± 0.03 ^{fgh}	1.73 ± 0.14 ^a	0.99 ± 0.06 ^c
MB ²	0.17 ± 0.01 ^{ijklm}	0.15 ± 0.01 ^{ijklm}	0.64 ± 0.04 ^d	0.33 ± 0.03 ^{fghi}	0.89 ± 0.05 ^c
AB ³	0.07 ± 0.01 ^{lmn}	0.14 ± 0.01 ^{klm}	1.29 ± 0.08 ^b	0.47 ± 0.09 ^{efg}	1.04 ± 0.08 ^c
CP ⁴	0.08 ± 0.01 ^{lmn}	0.33 ± 0.02 ^{fghi}	0.36 ± 0.02 ^{fgh}	0.47 ± 0.04 ^{def}	0.29 ± 0.01 ^{ghij}
BEP ⁵	nd ⁿ	0.24 ± 0.03 ^{hijk}	0.36 ± 0.02 ^{fgh}	0.28 ± 0.03 ^{ghijk}	0.61 ± 0.01 ^d
HB ⁶	nd ⁿ	0.36 ± 0.05 ^{fgh}	0.18 ± 0.01 ^{ijkl}	0.38 ± 0.04 ^{fgh}	1.02 ± 0.04 ^c
PP ⁷	0.04 ± 0.01 ^{mn}	0.33 ± 0.03 ^{fghi}	0.37 ± 0.01 ^{fgh}	0.38 ± 0.02 ^{fgh}	0.94 ± 0.02 ^c
BG ⁸	nd ⁿ	0.33 ± 0.01 ^{fghi}	0.55 ± 0.02 ^{de}	0.36 ± 0.03 ^{fgh}	0.60 ± 0.02 ^d
<i>Chymotrypsin inhibitor activity (CIA unit/mg)</i>					
SB	1.53 ± 0.03 ^{mno}	2.53 ± 0.07 ^{fgh}	2.94 ± 0.07 ^{de}	5.32 ± 0.13 ^a	3.06 ± 0.15 ^{de}
MB	1.71 ± 0.08 ^{klmn}	3.19 ± 0.05 ^d	2.94 ± 0.07 ^{de}	4.28 ± 0.07 ^b	2.48 ± 0.07 ^{gh}
AB	1.07 ± 0.05 ^p	1.83 ± 0.07 ^{ijklm}	2.09 ± 0.07 ^{ij}	3.00 ± 0.08 ^{de}	2.21 ± 0.08 ^{hi}
CP	0.49 ± 0.04 ^r	1.61 ± 0.05 ^{lmno}	1.64 ± 0.08 ^{lmno}	2.84 ± 0.06 ^{def}	1.49 ± 0.11 ^{no}
BEP	1.15 ± 0.07 ^p	3.10 ± 0.06 ^{de}	3.06 ± 0.07 ^{de}	3.83 ± 0.10 ^c	2.76 ± 0.07 ^{efg}
HB	0.74 ± 0.05 ^q	1.75 ± 0.07 ^{klmn}	1.94 ± 0.05 ^{ijk}	1.91 ± 0.09 ^{ijkl}	1.39 ± 0.08 ^o
PP	0.78 ± 0.04 ^q	1.60 ± 0.08 ^{mno}	1.39 ± 0.08 ^o	2.03 ± 0.09 ^{ijk}	1.03 ± 0.07 ^p
BG	0.77 ± 0.04 ^q	0.58 ± 0.07 ^r	1.06 ± 0.06 ^p	0.75 ± 0.04 ^q	0.94 ± 0.07 ^{pq}
<i>α-amylase inhibitor activity (AIA unit/mg)</i>					
SB	1.99 ± 0.15 ^d	4.25 ± 0.10 ^c	4.58 ± 0.10 ^c	7.40 ± 0.27 ^b	18.06 ± 0.23 ^a
MB	0.14 ± 0.00 ^{lm}	0.09 ± 0.00 ^{qr}	0.11 ± 0.00 ^p	0.17 ± 0.01 ^{jk}	0.17 ± 0.01 ^{jk}
AB	0.12 ± 0.00 ^{no}	0.08 ± 0.00 ^{qr}	0.09 ± 0.00 ^q	0.19 ± 0.01 ^j	0.19 ± 0.01 ^j
CP	0.09 ± 0.00 ^{qr}	0.07 ± 0.00 ^t	0.09 ± 0.00 ^q	0.26 ± 0.01 ^{fg}	0.26 ± 0.01 ^{fg}
BEP	0.18 ± 0.00 ^j	0.13 ± 0.00 ^{no}	0.14 ± 0.00 ^{mn}	0.32 ± 0.01 ^e	0.32 ± 0.01 ^e
HB	0.08 ± 0.00 ^{rs}	0.08 ± 0.00 ^{qr}	0.09 ± 0.00 ^q	0.16 ± 0.01 ^{kl}	0.16 ± 0.01 ^{kl}
PP	0.07 ± 0.00 ^s	0.09 ± 0.00 ^q	0.09 ± 0.00 ^{qr}	0.12 ± 0.01 ^{op}	0.03 ± 0.00 ^u
BG	0.23 ± 0.01 ^{hi}	0.24 ± 0.00 ^{gh}	0.24 ± 0.00 ^{ghi}	0.21 ± 0.00 ⁱ	0.28 ± 0.00 ^f

Values are presented in mean ± SEM (n = 3)

Control represents unprocessed dry bean

nd represent not detectable

^{a–n} are significantly different at $p < 0.001$

¹SB—Soybean, ²MB—Mung bean, ³AB—Adzuki bean, ⁴CP—Chickpea, ⁵BEP—Black-eyed pea, ⁶HB—Hyacinth bean, ⁷PP—Pigeon pea, ⁸BG—Bambara groundnut

P value for Legume: $p < 0.001$

P value for Soaking duration: $p < 0.001$

P value for Legume × Soaking duration: $p < 0.001$

bambara groundnut) and characterised from legumes according to their primary structure (Benjakul et al. 2000; Campos et al. 2004; Shi et al. 2017). It is evident that although the protease inhibitors from different sources have relatively diverse structures, molecular weights and chemical compositions, they all have a similar active site (Vidal-Valverde et al. 1997).

Effect of autoclaving on enzyme inhibitor activity

Table 3 shows the effects of autoclaving at 121 °C, 15 psi for 15 min on the TIA, CIA and AIA of the eight seeds. When compared to the respective control, the autoclaved seeds showed an increase in TIA level. The only exception was MB, which showed a 35% reduction. No TIA was detected in the respective control and autoclaved BG. Previous studies reported that the autoclaving of kidney bean, peas, lentils and tepary bean resulted in a reduction or complete inactivation of the trypsin inhibitors (Habiba

Table 2 Effect of wet heating on the enzyme inhibitor activity of legumes

Legume	Enzyme inhibitor activity at different wet heating temperatures and duration				
	Control	50 °C, 30 min	50 °C, 60 min	100 °C, 30 min	100 °C, 60 min
<i>Trypsin inhibitor activity (TIA unit/mg)</i>					
SB ¹	0.06 ± 0.01 ^{klm}	0.20 ± 0.02 ^{gh}	0.05 ± 0.01 ^{klm}	0.22 ± 0.01 ^{fgh}	0.34 ± 0.01 ^{cd}
MB ²	0.17 ± 0.01 ^{hi}	1.39 ± 0.04 ^a	0.12 ± 0.01 ^{ij}	0.24 ± 0.01 ^{fgh}	0.20 ± 0.01 ^{gh}
AB ³	0.07 ± 0.01 ^{jkl}	0.34 ± 0.03 ^{cd}	0.17 ± 0.01 ^{hi}	0.19 ± 0.01 ^h	0.46 ± 0.04 ^b
CP ⁴	0.08 ± 0.01 ^{jkl}	0.28 ± 0.02 ^{def}	0.11 ± 0.01 ^{jk}	0.24 ± 0.01 ^{fgh}	0.27 ± 0.02 ^{efg}
BEP ⁵	nd ^m	0.49 ± 0.02 ^b	0.12 ± 0.01 ^{ij}	0.21 ± 0.01 ^{fgh}	0.36 ± 0.01 ^c
HB ⁶	nd ^m	0.23 ± 0.01 ^{fgh}	0.10 ± 0.01 ^{jk}	0.20 ± 0.01 ^{gh}	0.17 ± 0.02 ^{hi}
PP ⁷	0.04 ± 0.01 ^{lm}	0.26 ± 0.01 ^{efg}	0.11 ± 0.01 ^{jk}	0.20 ± 0.01 ^{gh}	0.20 ± 0.01 ^{gh}
BG ⁸	nd ^m	0.31 ± 0.02 ^{cde}	0.23 ± 0.02 ^{fgh}	0.09 ± 0.01 ^{ijkl}	0.04 ± 0.01 ^{lm}
<i>Chymotrypsin inhibitor activity (CIA unit/mg)</i>					
SB	1.53 ± 0.03 ^{gh}	1.56 ± 0.04 ^{fgh}	1.84 ± 0.09 ^{def}	0.92 ± 0.11 ^{mno}	0.79 ± 0.05 ^{nop}
MB	1.71 ± 0.08 ^{fg}	3.45 ± 0.06 ^a	2.98 ± 0.05 ^b	1.35 ± 0.06 ^{hij}	2.02 ± 0.08 ^d
AB	1.07 ± 0.05 ^{klm}	1.16 ± 0.08 ^{ijklm}	1.72 ± 0.05 ^{efg}	0.32 ± 0.03 ^r	0.96 ± 0.12 ^{lmno}
CP	0.49 ± 0.04 ^q	0.77 ± 0.09 ^{nop}	0.78 ± 0.09 ^{nop}	0.67 ± 0.07 ^{pq}	0.64 ± 0.07 ^{pq}
BEP	1.15 ± 0.07 ^{ijklm}	2.41 ± 0.06 ^c	2.00 ± 0.05 ^{de}	1.16 ± 0.05 ^{ikl}	1.08 ± 0.05 ^{klm}
HB	0.74 ± 0.05 ^{nop}	1.47 ± 0.05 ^{ghi}	1.11 ± 0.06 ^{ijklm}	0.97 ± 0.04 ^{lmn}	0.84 ± 0.06 ^{nop}
PP	0.78 ± 0.04 ^{nop}	0.83 ± 0.09 ^{nop}	1.26 ± 0.05 ^{ijk}	0.69 ± 0.04 ^{opq}	0.64 ± 0.05 ^{pq}
BG	0.77 ± 0.04 ^{nop}	0.50 ± 0.06 ^q	nd ^s	nd ^s	nd ^s
<i>α-amylase inhibitor activity (AIA unit/mg)</i>					
SB	1.99 ± 0.15 ^d	4.55 ± 0.11 ^c	5.49 ± 0.10 ^b	8.45 ± 0.19 ^a	7.99 ± 0.06 ^a
MB	0.14 ± 0.00 ^{ij}	0.08 ± 0.00 ^{qrs}	0.10 ± 0.00 ^{no}	0.17 ± 0.01 ^h	0.17 ± 0.01 ^h
AB	0.12 ± 0.00 ^{kl}	0.09 ± 0.00 ^{pqr}	0.10 ± 0.00 ^{nop}	0.19 ± 0.01 ^h	0.19 ± 0.01 ^h
CP	0.09 ± 0.00 ^{pqr}	0.08 ± 0.00 ^{stu}	0.07 ± 0.00 ^{tu}	0.26 ± 0.01 ^f	0.26 ± 0.01 ^f
BEP	0.18 ± 0.00 ^h	0.13 ± 0.00 ^{kl}	0.14 ± 0.00 ^{jk}	0.32 ± 0.01 ^e	0.32 ± 0.01 ^e
HB	0.08 ± 0.00 ^{rst}	0.09 ± 0.00 ^{opqr}	0.09 ± 0.00 ^{opq}	0.16 ± 0.01 ^{hi}	0.16 ± 0.01 ^{hi}
PP	0.07 ± 0.00 ^{stu}	0.08 ± 0.00 ^{qrs}	0.07 ± 0.00 ^u	0.12 ± 0.01 ^{lm}	0.12 ± 0.01 ^{lm}
BG	0.23 ± 0.01 ^g	0.17 ± 0.00 ^h	0.21 ± 0.00 ^g	0.11 ± 0.00 ^{mn}	0.12 ± 0.00 ^{lm}

Values are presented in mean ± SEM (n = 3)

Control represents unprocessed dry bean

nd represent not detectable

^{a–s} are significantly different at $p < 0.001$

¹SB—Soybean, ²MB—Mung bean, ³AB—Adzuki bean, ⁴CP—Chickpea, ⁵BEP—Black-eyed pea, ⁶HB—Hyacinth bean, ⁷PP—Pigeon pea, ⁸BG—Bambara groundnut

P value for Legume: $p < 0.001$

P value for Wet heating: $p < 0.001$

P value for Legume × Wet heating: $p < 0.001$

2002; Hefnawy 2011; Osman et al. 2002; Shimelis and Rakshit 2007). This study only MB demonstrated similar reduction. All remaining legumes exhibited an increment of TIA after autoclaving.

Several studies report that the stability of proteinase inhibitors could be due to several factor. One likely explanation is the presence of crude fibre, phytate and tannins in the legumes that could provide some protection to the inhibitors against heat treatment (Benjakul et al. 2000). In addition, the nature of the protein conformation and bonding involved, such as the number of disulphide

bridges, could contribute to the stability of TIA which allows it to be resistant to being denatured at high temperature (Cheftel et al. 1985; Dokka et al. 2015).

Interestingly, all autoclaved seeds except for BEP and PP exhibited a reduction of CIA (by 25–100%) and AIA (by 29–99%). Autoclaving successfully reduced the CIA of BG to a non-detectable level, and the AIA of SB was reduced by 99%. These results suggested that the CIA and AIA inhibitors are thermosensitive, the autoclaving treatment promotes the breakage of intermolecular bonds responsible of holding the structure of the inhibitors that

Table 3 Effect of autoclaving on the enzyme inhibitor activity of legumes

Legumes	Enzyme inhibitor activity after autoclaving at 121 °C, 15 psi for 15 min	
	Control	Autoclaved
<i>Trypsin inhibitor activity (TIA unit/mg)</i>		
SB ¹	0.06 ± 0.01 ^{gh}	0.09 ± 0.01 ^{def}
MB ²	0.17 ± 0.01 ^c	0.11 ± 0.01 ^d
AB ³	0.07 ± 0.01 ^{fgh}	0.10 ± 0.01 ^{de}
CP ⁴	0.08 ± 0.01 ^{efg}	0.10 ± 0.01 ^{def}
BEP ⁵	nd ⁱ	0.22 ± 0.01 ^b
HB ⁶	nd ⁱ	0.23 ± 0.01 ^b
PP ⁷	0.04 ± 0.01 ^h	0.41 ± 0.01 ^a
BG ⁸	nd ⁱ	nd ⁱ
<i>Chymotrypsin inhibitor activity (CIA unit/mg)</i>		
SB	1.53 ± 0.03 ^a	0.98 ± 0.05 ^{cd}
MB	1.71 ± 0.08 ^a	1.18 ± 0.07 ^b
AB	1.07 ± 0.05 ^{bc}	0.60 ± 0.05 ^{gf}
CP	0.49 ± 0.04 ^g	0.98 ± 0.05 ^{cd}
BEP	1.15 ± 0.07 ^{bc}	0.49 ± 0.04 ^g
HB	0.74 ± 0.05 ^{ef}	0.56 ± 0.05 ^g
PP	0.78 ± 0.04 ^e	0.81 ± 0.05 ^{de}
BG	0.77 ± 0.04 ^e	nd ^h
<i>α-amylase inhibitor activity (AIA unit/mg)</i>		
SB	1.99 ± 0.15 ^a	0.02 ± 0.00 ^g
MB	0.14 ± 0.00 ^{cd}	0.04 ± 0.00 ^f
AB	0.12 ± 0.00 ^d	0.04 ± 0.01 ^f
CP	0.09 ± 0.00 ^e	0.02 ± 0.00 ^f
BEP	0.18 ± 0.00 ^{bc}	0.04 ± 0.00 ^g
HB	0.08 ± 0.00 ^e	0.03 ± 0.00 ^f
PP	0.07 ± 0.00 ^e	0.04 ± 0.01 ^f
BG	0.23 ± 0.01 ^b	0.16 ± 0.00 ^{cd}

Values are presented in mean ± SEM (n = 3)

Control represents unprocessed dry bean

nd represent not detectable

^{a–i} are significantly different at $p < 0.001$

¹SB—Soybean, ²MB—Mung bean, ³AB—Adzuki bean, ⁴CP—Chickpea, ⁵BEP—Black-eyed pea, ⁶HB—Hyacinth bean, ⁷PP—Pigeon pea, ⁸BG—Bambara groundnut

P value for Legume: $p < 0.001$

P value for Autoclaving: $p < 0.001$

P value for Legume × Autoclaving: $p < 0.001$

consequently causes changes over the active site conformation that lead to the inactivation of the enzyme inhibitors. Sathya and Siddhuraju (2015) and Tan et al. (1984) reported that autoclaving *Parkia* seeds and *Psophocarpus tetragonolobus* either reduced or completely inactivated the CIA. Similar reduction or complete inactivation of AIA was also reported for autoclaved peas, mung bean and mucuna seeds (Habiba 2002; Kavitha et al. 2015; Siddhuraju et al. 1996).

Effect of freezing on enzyme inhibitor activity

Table 4 shows the effect of freezing on TIA, CIA and AIA of the tested seeds. The TIA, CIA and AIA of all tested seeds were increased after being stored at -20 °C, except for the CIA and AIA of SB, which were reduced by 27% and 88%, respectively. Reducing the temperature to -80 °C, an increase resulted in the enzyme inhibitor activity in the majority of legumes, except for CIA. Five (SB, AB, CP, PP and BG) out of the eight seeds showed a reduction in CIA by 20–100% when frozen at -80 °C.

Table 4 Effect of freezing on the enzyme inhibitor activity of legumes

Legumes	Enzyme inhibitor activity at different freezing temperatures		
	Control	– 20 °C	– 80 °C
<i>Trypsin inhibitor activity (TIA unit/mg)</i>			
SB ¹	0.06 ± 0.01 ^{hi}	0.18 ± 0.02 ^{efg}	0.17 ± 0.01 ^{fg}
MB ²	0.17 ± 0.01 ^{fg}	0.55 ± 0.04 ^a	0.23 ± 0.01 ^{cd}
AB ³	0.07 ± 0.01 ^{hi}	0.19 ± 0.01 ^{defg}	0.32 ± 0.01 ^b
CP ⁴	0.08 ± 0.01 ^{hi}	0.09 ± 0.01 ^h	0.20 ± 0.01 ^{cdef}
BEP ⁵	nd ^j	0.18 ± 0.01 ^{efg}	0.16 ± 0.01 ^g
HB ⁶	nd ^j	0.24 ± 0.01 ^c	0.08 ± 0.01 ^h
PP ⁷	0.04 ± 0.01 ⁱ	0.22 ± 0.01 ^{cde}	0.28 ± 0.01 ^b
BG ⁸	nd ^j	0.16 ± 0.01 ^{fg}	0.16 ± 0.01 ^{fg}
<i>Chymotrypsin inhibitor activity (CIA unit/mg)</i>			
SB	1.53 ± 0.03 ^{bc}	1.12 ± 0.10 ^{de}	1.23 ± 0.09 ^d
MB	1.71 ± 0.08 ^b	3.30 ± 0.09 ^a	2.99 ± 0.10 ^a
AB	1.07 ± 0.05 ^{de}	1.37 ± 0.11 ^{cd}	0.91 ± 0.08 ^{ef}
CP	0.49 ± 0.04 ^{hij}	0.52 ± 0.22 ^{ij}	nd ^k
BEP	1.15 ± 0.07 ^{de}	1.57 ± 0.07 ^{bc}	1.23 ± 0.07 ^d
HB	0.74 ± 0.05 ^{fgh}	0.63 ± 0.07 ^{ghi}	0.62 ± 0.08 ^{ghi}
PP	0.78 ± 0.04 ^{fg}	0.68 ± 0.07 ^{fghi}	0.33 ± 0.04 ^j
BG	0.77 ± 0.04 ^{fg}	1.64 ± 0.10 ^{bc}	0.59 ± 0.07 ^{shi}
<i>α-amylase inhibitor activity (AIA unit/mg)</i>			
SB	1.99 ± 0.15 ^a	0.23 ± 0.00 ^{fg}	0.23 ± 0.00 ^{ef}
MB	0.14 ± 0.00 ^j	0.27 ± 0.00 ^d	0.27 ± 0.00 ^d
AB	0.12 ± 0.00 ^k	0.25 ± 0.00 ^{de}	0.26 ± 0.00 ^d
CP	0.09 ± 0.00 ^l	0.21 ± 0.00 ^{gh}	0.21 ± 0.00 ^h
BEP	0.18 ± 0.00 ⁱ	0.30 ± 0.00 ^b	0.30 ± 0.00 ^b
HB	0.08 ± 0.00 ^m	0.29 ± 0.01 ^{bc}	0.27 ± 0.00 ^{cd}
PP	0.07 ± 0.00 ^m	0.31 ± 0.00 ^b	0.26 ± 0.00 ^d
BG	0.23 ± 0.01 ^{fg}	0.23 ± 0.00 ^{fg}	0.21 ± 0.00 ^h

Values are presented in mean ± SEM (n = 3)

Control represents unprocessed dry bean

nd represent not detectable

^{a–m} are significantly different at $p < 0.001$

¹SB—Soybean, ²MB—Mung bean, ³AB—Adzuki bean, ⁴CP—Chickpea, ⁵BEP—Black-eyed pea, ⁶HB—Hyacinth bean, ⁷PP—Pigeon pea, ⁸BG—Bambara groundnut

P value for Legume: $p < 0.001$

P value for Freezing: $p < 0.001$

P value for Legume × Freezing: $p < 0.001$

Under the same conditions, the CIA of CP was reduced to a non-detectable level. A reduction of 9% was also observed for the AIA of BG.

Slow freezing of fresh wet foods encourages the formation of non-uniform ice crystals and produces larger ice crystals through the condensation of solutes. This causes cell membranes to break and results in irreversible cell wall collapse and tissue breakage (Mok et al. 2015; Xue et al. 2017). Conversely, dried seeds have a relatively low water content, this freezing causes minimal structural damage (Li and Sun 2002). Contradictory findings were observed in

this study, in which freezing resulted in an increase in TIA and AIA, and reduction of CIA when frozen at – 80 °C.

Conclusion

Enzyme inhibitors are complex and prevalent in legumes. Different enzyme inhibitors have been identified with ability to inhibit many diverse enzymes, including animal digestive proteases and amylases. However, their degree of inactivation by food processing methods are varied and

highly dependent on the legume source. No single processing method is capable to inactivate all three enzyme inhibitors present in the seven underutilised legumes tested. The four processing methods showed different degree of inactivation in enzyme inhibitor activity. The soaking, wet heating and autoclaving methods were effective in reducing AIA in legumes, whereas autoclaving and freezing methods were able to reduce CIA in legumes. Only autoclaving method able to reduce the TIA in MB.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

References

- Benjakul S, Visessanguan W, Thummaratwasik P (2000) Isolation and characterization of trypsin inhibitors from some Thai legume seeds. *J Food Biochem* 24:107–127. <https://doi.org/10.1111/j.1745-4514.2000.tb00689.x>
- Bhat R, Karim AA (2009) Exploring the nutritional potential of wild and underutilized legumes. *Compr Rev Food Sci Food Saf* 8:305–331. <https://doi.org/10.1111/j.1541-4337.2009.00084.x>
- Campos JE, Whitaker JR, Yip TT et al (2004) Unusual structural characteristics and complete amino acid sequence of a protease inhibitor from *Phaseolus acutifolius* seeds. *Plant Physiol Biochem* 42:209–214. <https://doi.org/10.1016/j.plaphy.2003.12.002>
- Cheftel J, Cuq J, Lorient D (1985) Amino acids, peptides and proteins. In: Fennema O (ed) *Food chemistry*, 2nd edn. Marcel Dekker Inc., New York, pp 245–369
- Deshpande SS, Sathe SK, Salunkhe DK, Cornforth DP (1982) Effects of dehulling on phytic acid, polyphenols, and enzyme inhibitors of dry beans (*Phaseolus vulgaris* L.). *J Food Sci* 47:1846–1850. <https://doi.org/10.1111/j.1365-2621.1982.tb12896.x>
- Dokka MK, Seva L, Davuluri SP (2015) Isolation and purification of trypsin inhibitors from the seeds of *Abelmoschus moschatus* L. *Appl Biochem Biotechnol* 175:3750–3762. <https://doi.org/10.1007/s12010-015-1542-1>
- Glencross B (2015) Understanding the nutritional and biological constraints of ingredients to optimize their application in aquaculture feeds. In: Nates SF (ed) *Aquafeed formulation*. Nikky Levy, Oxford, pp 39–42
- Habiba RA (2002) Changes in anti-nutrients, protein solubility, digestibility, and HCl-extractability of ash and phosphorus in vegetable peas as affected by cooking methods. *Food Chem* 77:187–192. [https://doi.org/10.1016/S0308-8146\(01\)00335-1](https://doi.org/10.1016/S0308-8146(01)00335-1)
- Hefnawy TH (2011) Effect of processing methods on nutritional composition and anti-nutritional factors in lentils (*Lens culinaris*). *Ann Agric Sci* 56:57–61. <https://doi.org/10.1016/j.aoas.2011.07.001>
- Kavitha S, Myrene D, Yogitha R (2015) Nutrient content and in vivo reduction of anti-nutrients of Mung Bean (*Vigna radiate* L.) under various processing methods. *J Chem Biol Phys Sci* 5:1627–1638
- Krogdahl Å, Penn M, Thorsen J et al (2010) Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquac Res* 41:333–344. <https://doi.org/10.1111/j.1365-2109.2009.02426.x>
- Kumar S, Verma A, Das M et al (2013) Clinical complications of kidney bean (*Phaseolus vulgaris* L.) consumption. *Nutrition* 29:821–827. <https://doi.org/10.1016/j.nut.2012.11.010>
- Li B, Sun D-W (2002) Novel methods for rapid freezing and thawing of foods—a review. *J Food Eng* 54:175–182. [https://doi.org/10.1016/S0260-8774\(01\)00209-6](https://doi.org/10.1016/S0260-8774(01)00209-6)
- Martín-Cabrejas MA, Aguilera Y, Pedrosa MM et al (2009) The impact of dehydration process on antinutrients and protein digestibility of some legume flours. *Food Chem* 114:1063–1068. <https://doi.org/10.1016/j.foodchem.2008.10.070>
- Marzo F, Alonso R, Oru E (1998) Effects of extrusion and conventional processing methods on protein and antinutritional factor contents in pea seeds. *Food Chem* 63:502–512
- Mok JH, Choi W, Park SH et al (2015) Emerging pulsed electric field (PEF) and static magnetic field (SMF) combination technology for food freezing. *Int J Refrig* 50:137–145. <https://doi.org/10.1016/j.jrefrig.2014.10.025>
- Morrison SC, Savage GP, Morton JD, Russell AC (2007) Identification and stability of trypsin inhibitor isoforms in pea (*Pisum sativum* L.) cultivars grown in New Zealand. *Food Chem* 100:1–7. <https://doi.org/10.1016/j.foodchem.2005.07.062>
- Osman MA, Reid PM, Weber CW (2002) Thermal inactivation of tepary bean (*Phaseolus acutifolius*), soybean and lima bean protease inhibitors: effect of acidic and basic pH. *Food Chem* 78:419–423. [https://doi.org/10.1016/S0308-8146\(02\)00144-9](https://doi.org/10.1016/S0308-8146(02)00144-9)
- Prasad ER, Dutta-Gupta A, Padmasree K (2010) Purification and characterization of a Bowman–Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*). *Phytochemistry* 71:363–372. <https://doi.org/10.1016/j.phytochem.2009.11.006>
- Sales MP (2000) Do legume storage proteins play a role in defending seeds against Bruchids? *Plant Physiol* 124:515–522. <https://doi.org/10.1104/pp.124.2.515>
- Santimone M, Koukiekolo R, Moreau Y et al (2004) Porcine pancreatic α -amylase inhibition by the kidney bean (*Phaseolus vulgaris*) inhibitor (α -AI1) and structural changes in the α -amylase inhibitor complex. *Biochim Biophys Acta - Proteins Proteomics* 1696:181–190. <https://doi.org/10.1016/j.bbapap.2003.11.001>
- Sathya A, Siddhuraju P (2015) Effect of processing methods on compositional evaluation of underutilized legume, *Parkia roxburghii* G. Don (yongchak) seeds. *J Food Sci Technol*. <https://doi.org/10.1007/s13197-015-1732-4>
- Shah KR, Patel DK, Pappachan A et al (2016) Characterization of a Kunitz-type serine protease inhibitor from *Solanum tuberosum* having lectin activity. *Int J Biol Macromol* 83:259–269. <https://doi.org/10.1016/j.ijbiomac.2015.11.068>
- Shi L, Mu K, Arntfield SD, Nickerson MT (2017) Changes in levels of enzyme inhibitors during soaking and cooking for pulses available in Canada. *J Food Sci Technol* 54:1014–1022. <https://doi.org/10.1007/s13197-017-2519-6>
- Shimelis EA, Rakshit SK (2007) Effect of processing on antinutrients and in vitro protein digestibility of kidney bean (*Phaseolus vulgaris* L.) varieties grown in East Africa. *Food Chem* 103:161–172. <https://doi.org/10.1016/j.foodchem.2006.08.005>
- Siddhuraju P, Vijayakumari K, Janardhanan K (1996) Chemical composition and protein quality of the little-known legume, Velvet bean (*Mucuna pruriens* (L.) DC.). *J Agric Food Chem* 44:2636–2641. <https://doi.org/10.1021/JF950776X>
- Singh N (2017) Pulses: an overview. *J Food Sci Technol* 54:853–857. <https://doi.org/10.1007/s13197-017-2537-4>

- Singh J, Dartois A, Kaur L (2010) Starch digestibility in food matrix: a review. *Trends Food Sci Technol* 21:168–180. <https://doi.org/10.1016/j.tifs.2009.12.001>
- Singh B, Singh JP, Shevkani K et al (2017) Bioactive constituents in pulses and their health benefits. *J Food Sci Technol* 54:858–870. <https://doi.org/10.1007/s13197-016-2391-9>
- Svensson B, Fukuda K, Nielsen PK, Bønsager BC (2004) Proteinaceous α -amylase inhibitors. *Biochim Biophys Acta - Proteins Proteomics* 1696:145–156. <https://doi.org/10.1016/j.bbapap.2003.07.004>
- Tan NH, Rahim ZHA, Khor HT, Wong KC (1984) Chymotrypsin inhibitor activity in winged beans (*Psophocarpus tetragonolobus*). *J Agric Food Chem* 32:163–166. <https://doi.org/10.1021/jf00121a038>
- Torres J, Rutherford SM, Muñoz LS et al (2016) The impact of heating and soaking on the in vitro enzymatic hydrolysis of protein varies in different species of tropical legumes. *Food Chem* 194:377–382. <https://doi.org/10.1016/j.foodchem.2015.08.022>
- Vidal-Valverde C, Frias J, Diaz-Pollan C et al (1997) Influence of processing on trypsin inhibitor activity of Faba beans. *Agric Food Chem* 45:3559–3564
- Wang N, Hatcher DW, Gawalko EJ (2008) Effect of variety and processing on nutrients and certain anti-nutrients in field peas (*Pisum sativum*). *Food Chem* 111:132–138. <https://doi.org/10.1016/j.foodchem.2008.03.047>
- Wati RK, Theppakorn T, Benjakul S, Rawdkuen S (2010) Trypsin inhibitor from 3 legume seeds: fractionation and proteolytic inhibition study. *J Food Sci* 75:C223–C228. <https://doi.org/10.1111/j.1750-3841.2010.01515.x>
- Xue Z, Hao J, Yu W, Kou X (2017) Effects of processing and storage preservation technologies on nutritional quality and biological activities of edible fungi: a review. *J Food Process Eng* 40:e12437. <https://doi.org/10.1111/jfpe.12437>