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Characterisation of trypsin and α-chymotrypsin inhibitors in Australian wattle seed (*Acacia victoriae* Bentham)

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Abstract

Crude water extracts from Australian wattle seed (*Acacia victoriae* Bentham) and their salt (ammonium sulphate)-precipitated fractions were analysed for trypsin and α-chymotrypsin (chymotrypsin) inhibitor activity, using gel electrophoresis and spectrophotometric methods. Three different bands with molecular weight 30.20, 38.03 and 39.81 kDa were active, with the 50% salt-precipitated fraction exhibiting highest activity and number of active bands. The same proteins also appeared to be responsible for both trypsin and chymotrypsin inhibitor activity. To establish conditions for the inactivation of these inhibitors, whole seed and uncoated (dehulled) cotyledon were subjected to different heat treatments. Moist heat treatment at 100 °C for 30 s was sufficient to inactivate both protease inhibitors although the trypsin inhibitor was found to be more heat-resistant than was the chymotrypsin inhibitor. Soaking overnight, before thermal treatment, improved the trypsin inhibitor activity but enhanced the efficiency of thermal inactivation in both inhibitors.
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Keywords: Wattle seed; Trypsin inhibitor; α-Chymotrypsin inhibitor

1. Introduction

Acacia victoriae Bentham (prickly wattle) is one of the most common *Acacia* species, belonging to the subgenus *Phyllodineae*, which includes approximately 950–960 species found in Australia (Hegarty & Hegarty, 2001; Maslin & McDonald, 2004). Seeds of *Acacia* have long been an important food source for the indigenous people of Australia and the prickly wattle has been recognised to have significant economic potential due to its extensive availability through cultivation and wild harvest (Ahmed & Johnson, 2000; Seigler, 2002). Commercially, acacia seeds are being used as a food flavouring agent in dairy products and as an ingredient in beverages (Maslin & McDonald, 2004; Maslin, Thomson, McDonald, & Hamilton-Brown, 1998). These products are currently limited to being processed by roasting at very high temperatures, mainly to develop the nutty flavour and aroma, before being incorporated into foods. However, since non-roasted wattle seed has been shown to contain high amounts of proteins and soluble carbohydrates (Brand, Cherikoff, & Truswell, 1985), it is conceivable that its utilisation as an ingredient or additive, for instance, as large molecular weight emulsifier/stabiliser in foods, could be even further expanded.

Many seeds in the leguminous family contain anti-nutritional factors, such as protease inhibitors, lectins, alkaloids, saponins, phenols and oxalates, that can interfere with the digestion and absorption of nutrients (Liener, 1980). Specifically, protease inhibitors inhibit the activity of the protein-digesting enzymes in the digestive tract, reducing the body's ability to utilise proteins in food. In plants they can serve as storage proteins or provide protection against insects and micro-organisms (Norton, 1991; Srinivasan, Giri, Harsulkar, Gatehouse, & Gupta, 2005). Contrary to soybean, in which different kinds of protease inhibitors have been identified and characterised (Harwood & House, 1992; Liener,

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1980; Nieblas, 1996), very little information on protease inhibitors or the processing conditions required to inactivate them is available on wattle seed. Many studies have revealed that protease inhibitors in the leguminous family exhibit considerable variations in their resistance to heat treatment. Soybean *Kunitz* trypsin inhibitor, for example, has been shown to be quite heat-stable, and a long period (30 min) of boiling is necessary to inactivate it (Concon, 1988; Markwell, Roychaudhuri, Sarath, & Zeece, 2003). Consequently, in order to establish Australian wattle seed as a viable food ingredient, utilising the significant protein and soluble carbohydrate contents, it is imperative to evaluate the presence of anti-nutritional factors such as protease inhibitors. This study thus focussed on the identification and characterisation of wattle seed protease inhibitors and the determination of minimum conditions necessary for their inactivation. This is expected to provide valuable information regarding their safety and nutritional quality when incorporating minimally processed (non-roasted) wattle seed products in food systems.

2. Materials and methods

2.1. Material

Whole wattle seeds (*Acacia victoriae* Bentham) were supplied by Outback Bushfoods, Alice Springs, Australia. All other reagents and chemicals were supplied by Sigma–Aldrich, Castle Hill, NSW, Australia, Bio-Rad Laboratories, Pty., Ltd., NSW, Australia, or by Promega Corporation, Madison, WI, USA.

2.1.1. Extraction of wattle seed samples

Whole wattle seeds or cotyledons (dehulled with a coffee grinder) were ground separately using a ZM 100 ultra centrifugal mill (Retsch GmbH, Germany) to pass through a 0.11 mm mesh and extracted with distilled water or 0.1 M NaOH in a 1:10 (flour to solvent) ratio, by stirring for one hour at room temperature. To examine the influence of soaking and heating on protease activity in extracts, whole wattle seeds were soaked in distilled water overnight, after which fresh distilled water in a ratio of 1:10 (original seed weight to distilled water) was added. The seeds were blended for 2 min into a smooth slurry and stirred for one hour at room temperature (25 °C). In a further treatment, wattle seeds that were soaked overnight were heated at 100 °C in a water bath (Julabo Labortechnik GmbH, Germany) for varying periods of time. The treated seeds were blended with fresh distilled water in a 1:10 ratio, as above, for 2 min and stirred for one hour at room temperature. Each treatment was followed by centrifugation (Phoenix Equipment, Inc, New York) at 3000*g* for 10 min. Supernatant for each treatment, that included extracts from unsoaked whole seed, unsoaked cotyledon, soaked seed and soaked-heated seed, was collected and freeze-dried on a Christ-Alpha 1–4 freeze dryer (Biotech International, Germany).

2.1.2. Ammonium sulphate fractionation

The supernatant obtained from whole seed water extraction, as described above, was fractionated by using increasing concentrations of ammonium sulphate [$(NH_4)_2SO_4$] according to the method described by Bollag, Rozycki, and Edelstein (1996). The precipitates obtained at 25%, 50% and 75% (w/v) $(NH_4)_2SO_4$ were labelled AS-1, AS-2 and AS-3, respectively, while the supernatant obtained from the final precipitation (75%) was collected as the 4th fraction (AS-4). All fractions were dialysed (molecular weight cut-off 10,000 Da) against Tris-$CaCl_2$ buffer solution (0.023 M $CaCl_2$ and 0.092 M Tris-HCl, pH 8.1) for 24 h with three changes of buffer at 4°C and freeze-dried using a Christ Alpha 1–4 Freeze Dryer (Biotech International, Germany).

2.1.3. Extraction of protease inhibitors

Extraction buffer solution, containing 0.023 M $CaCl_2$ and 0.092 M Tris-HCl, pH 8.1, was added to all extracts and salt-precipitated fractions to a final concentration of 20 mg/ml. All of the samples were kept overnight at 4 °C before they were clarified by centrifugation at 10,000*g* for 2 min. The supernatant of each sample was transferred to fresh micro-centrifuge tubes and assayed immediately for protease inhibitor activity or stored at −20 °C.

2.1.4. Protease inhibitor activity assays

Protease inhibitor activity assays were carried out according to the methods described by Kollipara and Hymowitz (1992) and Beynon and Bond (2001), with slight modifications. Substrates used were *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) for trypsin and chymotrypsin inhibitor activity assays, respectively.

2.1.5. Trypsin inhibitor assay

An inhibitor assay buffer was prepared which contained 10.3 mM $CaCl_2$, 41.4 mM Tris-HCl, at pH 8.1. TAME substrate (10 mM) was prepared fresh on the same day by dissolving 37.9 mg TAME in 10 ml of assay buffer. An aliquot (2.6 ml) of assay buffer and 0.3 ml of TAME substrate was measured into a quartz cuvette (10 mm path length, 3.5 ml) after which 0.1 ml of bovine trypsin (20 μg/ml in 1 mM HCl) was added and mixed. The absorbance at 247 nm (A_{247}) was monitored immediately and continuously on a spectrophotometer (Shimadzu Scientific Instruments, Australia). The slope of enzyme activity was recorded as change in absorbance for 6 min and the linear progression of the reaction curve was confirmed. A mixture of the assay buffer (2.6 ml), substrate (0.3 ml), and 0.1 ml 1 mM HCl without enzyme was used as a reference blank. For inhibitor activity assay, 2–6 μl of each wattle seed extract prepared above was mixed with 2.6 ml of assay buffer and 0.1 ml of bovine trypsin in a quartz cuvette, and incubated at room temperature (25°C) for 6 min. An aliquot (0.3 ml) of TAME substrate was added after the incubation period and the absorbance reading was recorded at

247 nm (A_{247}) immediately and continuously for at least 3 min. The slope of inhibitor activity was recorded as change in absorbance for 3 min. The trypsin inhibitor activity was calculated in trypsin inhibitor units per gramme of extract (TIU/g extract) as follows:

$$\text{TIU/g extract} = \frac{(\text{T}\Delta A_{247}/\text{min} - \text{I}\Delta A_{247}/\text{min}) \times 3 \times 1000}{540 \times \text{gramme seed extract}}$$

where TΔ A_{247}/min is the change in A_{247}/min in the absence of inhibitor (substrate and trypsin only), IΔ A_{247}/min is the change in A_{247}/min in the presence of inhibitor. The factor 540 is the molar extinction coefficient at A_{247}, empirically estimated, given the assay buffer composition and light path length of 10 mm in the cuvette. A trypsin unit (TU) is defined as the amount of trypsin that catalyses the hydrolysis of 1 μmol of substrate per min and a TIU is the reduction in activity of trypsin by 1 TU.

2.1.6. Chymotrypsin inhibitor assay

In this assay, 1 mM substrate solution was prepared fresh by dissolving 15.7 mg BTEE in 50 ml of 50% (w/w) aqueous, spectral grade methanol. An aliquot (0.1 ml) of bovine chymotrypsin (20 μg/ml in 1 mM HCl) was mixed with 1.4 ml assay buffer (0.1 M $CaCl_2$, 0.1 M Tris-HCl, pH 7.8) and 1.5 ml of substrate solution immediately before the initiation of absorbance reading at 256 nm wavelength (A_{256}). The progress of the reaction was monitored continuously, as described above, for 6 min and the linearity of the reaction curve was confirmed. The reference blank contained the same solutions as above except that 0.1 ml 1 mM of HCl was used in place of the enzyme solution. For inhibitor activity assay, 10–15 μl of each of the extract solutions was mixed with 1.4 ml assay buffer and 0.1 ml of bovine chymotrypsin in a quartz cuvette, and incubated at room temperature (25 °C) for 6 min. BTEE substrate (1.5 ml aliquot) was added after the incubation period and the absorbance reading was recorded at 256 nm (A_{256}) immediately and continuously for at least 3 min. The slope of inhibitor activity was recorded as change in absorbance for 3 min. The chymotrypsin inhibitor activity was calculated in chymotrypsin inhibitor units per gramme of seed extract (CIU/g extract) as follows:

$$\text{CIU/g extract} = \frac{(\text{C}\Delta A_{256}/\text{min} - \text{I}\Delta A_{256}/\text{min}) \times 3 \times 1000}{964 \times \text{grammes of seed extract}}$$

where, CΔ A_{256}/min is the change in A_{256}/min in the absence of inhibitor (substrate and enzyme only), IΔ A_{256}/min is the change in A_{256}/min in the presence of inhibitor. The factor 964 is the molar extinction coefficient at A_{256}, empirically estimated, given the assay buffer composition and light path length of 10 mm in the cuvette. A chymotrypsin unit (CU) is defined as the amount of trypsin that catalyses the hydrolysis of 1 μmol of substrate per min and a CIU is the reduction in activity of chymotrypsin by 1 TU.

2.1.7. Polyacrylamide gel electrophoresis (PAGE)

Samples for native (non-denaturing conditions) PAGE were prepared by mixing freeze-dried wattle seed extract and sample buffer {80% (v/v) glycerol, 0.4% (v/v) bromophenol blue, Tris-$CaCl_2$ extraction buffer, pH 8.1}, in a 7:3 ratio. However, samples of dialysed and freeze-dried ammonium sulphate fractions, AS-1, AS-2, AS-3, and AS-4 were mixed with sample buffer in a 2:1 ratio. They were incubated at room temperature for 20 min before they were loaded into the sample wells of the gel. The polyacrylamide gel system was comprised of 15% resolving and 4% stacking gel on a Bio-Rad PROTEAN II xi Cell slab gel electrophoresis unit (Bio-Rad Laboratories, Pty., Ltd., NSW, Australia), using the Laemmli discontinuous buffer system (Laemmli, 1970). Gels were normally run at a constant current of 16 mA/gel for 15–16 h at 10 °C.

Molecular weight analysis was carried out, under denaturing conditions, using sodium dodecyl sulphate (SDS) PAGE. Sample buffer, containing 2% (w/v) SDS, 50 mM DTT, 10% (v/v) glycerol, 0.4% (v/v), bromophenol blue in Tris-$CaCl_2$ extraction buffer (pH 8.1), was used. The stacking and resolving gels contained 10% (w/v) SDS while the reservoir buffer contained 0.1% (w/v) SDS. Wattle seed extract samples, as well as molecular weight standards ranging between 6.5 and 205 kDa (SigmaMarker^{TM}), were resolved on the same gel.

After the run, the stacking gel was removed and discarded and the resolving gel was subjected either to Coomassie Brilliant Blue R 250 (CBBR) (Ahmed, 2005) or acid silver nitrate staining methods (Promega Corporation, Madison, WI, USA). Molecular weight of proteins separated by SDS-PAGE was determined against the molecular weight standards.

2.1.8. Trypsin and chymotrypsin inhibitor activity gels

Native PAGE gels were stained for trypsin and chymotrypsin inhibitor activities as described by Kollipara and Hymowitz (1992). The principle behind this method relied on the separation of the seed extract into protein bands using native PAGE. The inhibitor bands, on exposure to trypsin or chymotrypsin, reacted with the respective enzyme and were depleted, showing up as clear bands on the gel after staining and comparison with gels unexposed to the enzymes. Immediately after separation, the gels were washed in 0.1 M sodium phosphate buffer (pH 7.5) in a glass tray placed on an orbital shaker, for three 5 min washes to remove excess chemicals and to equilibrate with phosphate buffer. This was followed by incubation in 0.1 M phosphate buffer containing 15 mg/ml of bovine trypsin or chymotrypsin for 15–20 min on the shaker at room temperature. The gels were rinsed three times, for 2 min each, in distilled water before incubating in staining solution for 4 h without shaking. For each gel, the staining solution was prepared fresh by dissolving 25 mg of *N*-acetyl-DL-phenylalanine β-naphthyl ester in 10 ml of *N*,*N*-dimethylformamide, and 50 mg tetrazoitized (zinc chloride complex) *o*-dianisidine (Fast blue *B* salt) in

100 ml of 50 mM Tris-HCl (pH 8.0), separately. These solutions were mixed immediately before they were poured onto the gel. The stained gels were rinsed in distilled water and stored in 7.5% (w/v) acetic acid. The presence of trypsin or chymotrypsin inhibitors was visualised as clear bands in a dark violet or pink background.

2.1.9. Statistical analysis

All extractions and analyses were carried out at least in triplicates and the means (with standard deviations) reported. Data collected were subjected to analysis of variance, and means of treatments were subjected to Fisher's least significant difference test. Significant difference was reported at $p < 0.05$.

3. Results and discussion

3.1. Protease inhibitor activity in wattle seed flour and extracts

Protease inhibitor activity assays of whole wattle seed flour, de-hulled cotyledon flour, freeze-dried water extract and freeze dried alkali extract revealed varying trypsin and chymotrypsin inhibitor activity levels (Fig. 1A and B). The protein content of the extracts and fractions (data not shown) did not appear to correlate with protease inhibitor activity. This is in spite of the fact that the inhibitors are also proteins. Apparently, the extraction of inhibitors is not proportional to the overall amount of protein that is present in each sample. In general, all treatments exhibited higher TIU than CIU per gramme of extract. Extracts exhibiting trypsin inhibitor activity also exhibited chymotrypsin inhibitor activity, with the exception of the freeze-dried alkali extract that only showed chymotrypsin inhibitor activity, probably indicating destruction of trypsin inhibitor when wattle seed was extracted under alkaline conditions. The ammonium sulphate fraction, precipitated at fifty percent saturation (AS-2), exhibited the highest trypsin inhibitor activity (347 TIU/g) among all the samples. However, no chymotrypsin inhibitor activity was obtained in this or in any of the three other fractions.

Protease inhibitory activities in wattle seed flour samples and their extracts appeared to be much lower (highest values were 235 TIU/g for wattle cotyledon flour and 347 TIU/g for AS2) than those reported for the commonly consumed legume seeds, such as soy bean, suggesting that the inhibitors would be amenable to easy removal using milder treatments (Hamerstrand & Black, 1981). Friedman, Brandon, Bates, and Hymowitz (1991) reported up to 7136 TIU/g of soy flour from a standard cultivar which decreased to 1030 TIU/g after dry heating in an autoclave for 30 min. Seigler (2002) also hypothesised that wattle seed anti-nutritional factors must be mild, owing to long term successful utilisation as food by the Australian Aboriginal communities. Our results suggest, therefore, that the functional properties of the untreated wattle seed extracts would not be significantly different from those obtained after the expected mild heat treatment requirement. Moderate heating prior to application may actually improve functionality of proteins, such as increased molecular flexibility and surface hydrophobicity, accompanied by greater participation of proteins in emulsion formation and the stability of newly formed emulsion droplets, and nutritional improvement of the food products (Halling, 1981). Conversely, overheating may cause protein aggregation and reduction of functionalities (Damodaran, 1996; Sikorski, 1997).

4. Effect of soaking and heat treatment on protease inhibitor activity

Soaking whole wattle seed overnight resulted in a significant increase in trypsin inhibitor activity but led to a

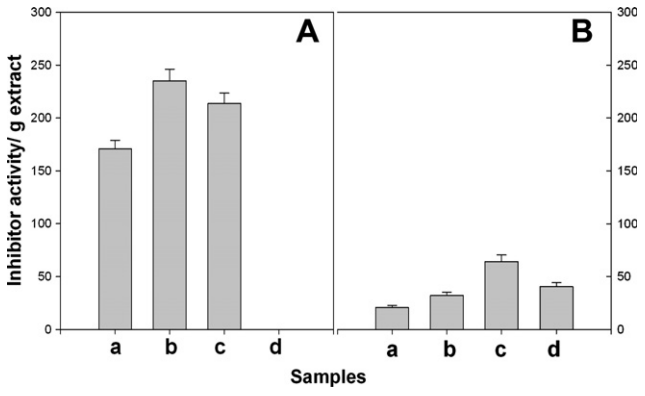

Fig. 1. (A) Trypsin and (B) chymotrypsin inhibitor activities of wattle seed and extracts. Samples are (a) whole seed flour or control, (b) uncoated cotyledon flour, (c) water extract from whole seed and (d) alkali (0.1 M NaOH) extract from whole seed. Data are means of three replicates.

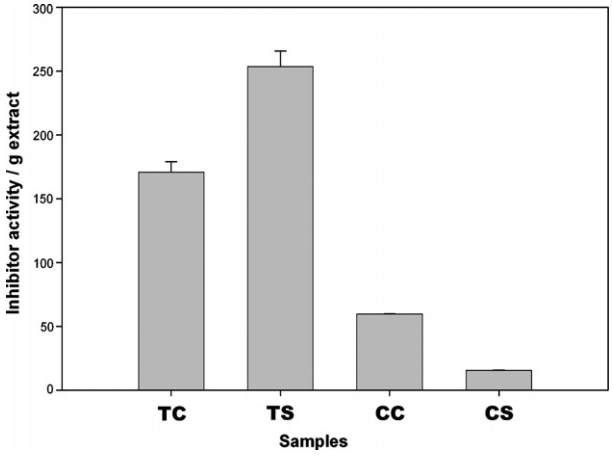

Fig. 2. Effect of soaking (overnight) on the trypsin and chymotrypsin inhibitor activities (units) in whole wattle seed. TC: trypsin activity of unsoaked sample; TS: trypsin activity of sample soaked overnight at room temperature; CC: chymotrypsin activity of unsoaked sample; CS: chymotrypsin activity of sample soaked overnight at room temperature. Data are means of three replicates.

decrease in chymotrypsin inhibitor activity (Fig. 2). Comparatively, heating at 100 °C (boiling) was very effective (Fig. 3) in reducing both trypsin and chymotrypsin inhibitor activity, especially after soaking overnight. Fig. 3 shows CIU and TIU curves of soaked seeds that were boiled for varying time periods, up to two min. Both trypsin and chymotrypsin inhibitor activity were completely destroyed after boiling for 30 s and 15 s, respectively. Interestingly, contrary to TIU, CIU increased sharply at 10 s before the inhibitor was completely inactivated shortly thereafter, at 15 s.

Results are in agreement with Liu (1997) who recognised moist heat treatment as being necessary for complete inactivation of lipoxygenase, amylase and inhibitors of protease and other enzymes during commercial protein preparations. This study concentrated mainly on the inactivation of trypsin and chymotrypsin inhibitors because other enzymes, such as lipoxygenase and amylase, are usually less stable to heat treatment (Liener, 1980; Whitaker, 1996).

4.1. Polyacrylamide gel electrophoresis (PAGE) and native PAGE

The trypsin and chymotrypsin inhibitor activity gels revealed the presence of several inhibitor bands with varying intensities (clear bands against dark background) in all of the samples examined. Fig. 5 shows the trypsin (a) and chymotrypsin (b) inhibitor activity as analysed by native PAGE. Lanes 2 (soaked seeds extract), 3 (soaked-heated seeds extract), 5 (AS-2) and 6 (AS-3) showed high intensity of activity and up to five bands, although not very well resolved, appeared in each lane. It also shows that most trypsin inhibitors were also found to be chymotrypsin inhibitors, in agreement with Kollipara and Hymowitz (1992), who studied the trypsin and chymotrypsin inhibitors in wild perennial *Glycine* species. However, the chymotrypsin inhibitor bands were generally weaker than the trypsin inhibitor bands. Results for trypsin inhibition activity were compatible with the spectrophotometric analysis of protease inhibitors where the samples showed higher intensity (clearer bands) in soaked seeds. However, while chymotrypsin inhibitor activity did not appear in spectrophotometric analysis of ammonium sulphate fractions, some inhibitory activity appeared in the equivalent native gel (Fig. 5c). Similarly, the soaked-heated (at 100 °C, 30 s) sample (Fig. 5, lane 3) also exhibited considerable protease inhibitor activity in the native PAGE gel. To further analyse the heat resistance of these inhibitors, the samples were heated for a longer time of two min at 100 °C. The current during the run was also reduced from 16 mA (Fig. 5) to 10 mA in order to slow down the process and thereby improve the resolution of the inhibitor bands.

Results show that five clear bands from AS-2 (Fig. 6b, lane 3) would now be seen, which corresponded to the AS-2 native PAGE (untreated with substrate) in Fig. 6a. Results of activity gel (Fig. 6b and 6c) also show that, although the chymotrypsin inhibitors had been largely destroyed by the longer duration of boiling, considerable amounts of trypsin inhibitor activity still remained. It would appear that protease inhibitors were renatured in the native gel because the inhibitory activities were fully deactivated after heating (100 °C, 30 s), as shown in Fig. 3. Although it has been reported that soybean *Kunitz* trypsin inhibitor renatured after heating to 90 °C and subsequent cooling (Markwell et al., 2003), the renaturation of protease inhibitor activity in wattle seed extracts might have occurred during sample handling and preparation for gel electrophoresis since this was not observed during the spectrophotometric measurement of inhibitor activity (Fig. 3). It is also possible that the activity staining method is sensitive to the presence of the denatured proteases. According to Norton (1991), the method can pick up inhibitors at levels that are as low as 1 ng. Alternative methods

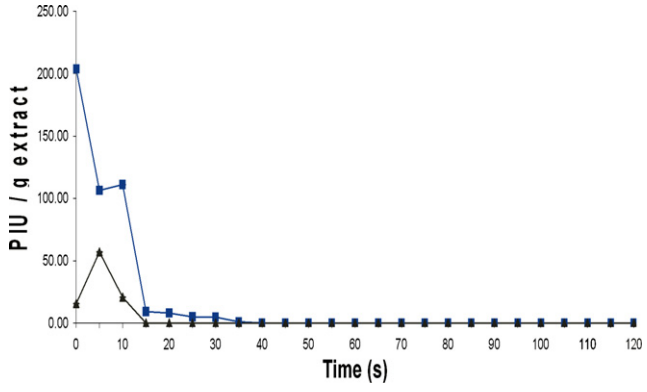

Fig. 3. Effect of moist heat treatment (100 °C) for various periods on the trypsin (■) and chymotrypsin (▲) inhibitor activities (units) in whole wattle seed flour. PIU is protease inhibitor units defined as either trypsin or chymotrypsin inhibitor units (see Methods section). Data are means of three replicates.

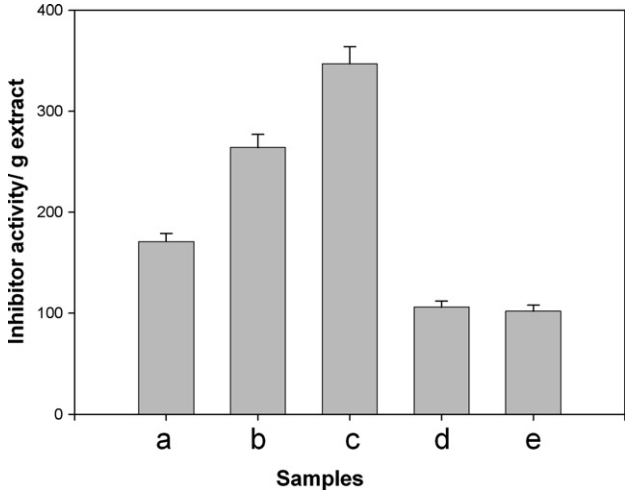

Fig. 4. Trypsin inhibitor activities (units) of ammonium sulphate fractions of whole seed flour (water) extract. Samples are (a) whole seed flour or control, (b) 25% precipitate (AS-1), (c) 50% precipitate (AS-2), (d) 75% precipitate (AS-3) and (e) supernatant after 75% precipitate (AS-4). Data are means of three replicates.

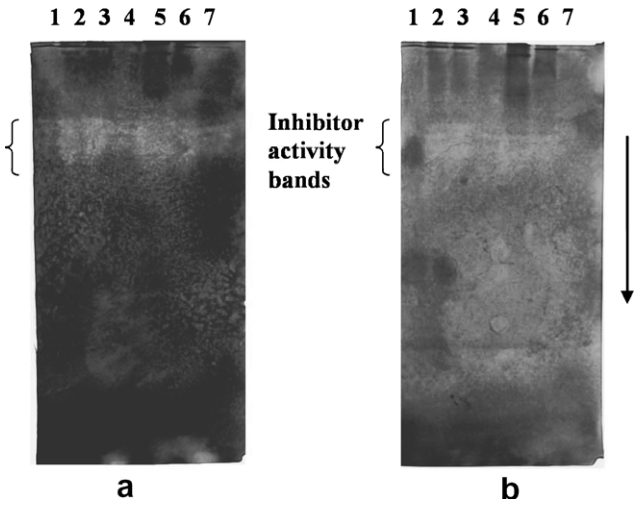

Fig. 5. Electrophoretograms of wattle seed extracts showing trypsin (a) and chymotrypsin (b) inhibitor activities. Lane 1: whole seed extract without treatment; lane 2: soaked seed extract; lane 3: soaked-heated (100 °C, 30 s) seed extract; lane 4 to lane 7: ammonium sulphate precipitation fractions 1 to 4 [25% (AS-1), 50% (AS-2), 75% (AS-3), and supernatant of 75% (AS-4), respectively]. Both gels were run at a constant current 16 mA at constant temperature (10 °C) for 15–16 h. The sequence of samples is similar in both gels. Arrow shows direction of protein migration.

should be considered in order to confirm the results that were obtained by gel staining.

Results also revealed that, among the ammonium sulphate precipitated fractions, the one obtained at 50% precipitation (AS-2) had the highest inhibitor activity (Fig. 4), and its activity gels showed the highest number of bands and highest band intensities (Figs. 5 and 6). Consequently, it was chosen as the sample for molecular characterisation of protease inhibitors in further studies. Results suggested that most of the slower migrating inhibitors in all samples were specific to both trypsin and chymotrypsin. The bands that exhibited protease inhibitor activity (Fig. 6a) were cut individually from the normal stained native gel, treated with denaturing sample buffer, and analysed by SDS-PAGE to ascertain the molecular weight of the protease inhibitors.

Fig. 7 shows the molecular weight of each band that was cut from the native gel and loaded onto the SDS-PAGE gel and stained using an acid silver staining method. From observation of the SDS-PAGE gel, the first four protease inhibitor bands showed molecular weights of either 30,199.52 Da or 39,810.72 Da, or both, the final native gel band (lane 6) being the only one with a different molecular weight of 38,018.04 Da. Although there were different bands in the native gel, they appeared, in general, to have similar molecular weights and inhibition activities. Therefore, the protease inhibitors most likely represent a heterogeneous group, consisting of a number of iso-inhibitors with a range of molecular weights between 30,199.52 and 39,810.72 Da.

Water-soluble extracts from wattle seed contain both trypsin and chymotrypsin inhibitors. They have been characterised and studied under various extraction and processing conditions. However, it is not clear why ammonium sulphate fractions which showed significant trypsin inhibitor activity did not have any chymotrypsin activity, given that the molecular characterisation confirmed that the same molecules were most likely responsible for both. Similarly, we do not know why some processing methods (e.g. soaking) increased trypsin inhibitor activity but not chymotrypsin inhibitor activity. It is possible that the wattle seed protease inhibitors are similar to the soybean Bowman-Birk inhibitor, which is capable of binding both trypsin and chymotrypsin at two independent sites (Norton, 1991). It is also conceivable that different factors control the activation of these sites on the inhibitor protein molecules. Investigations continue on how to elucidate the differences in the binding ability of the inhibitors to either trypsin or chymotrypsin, as

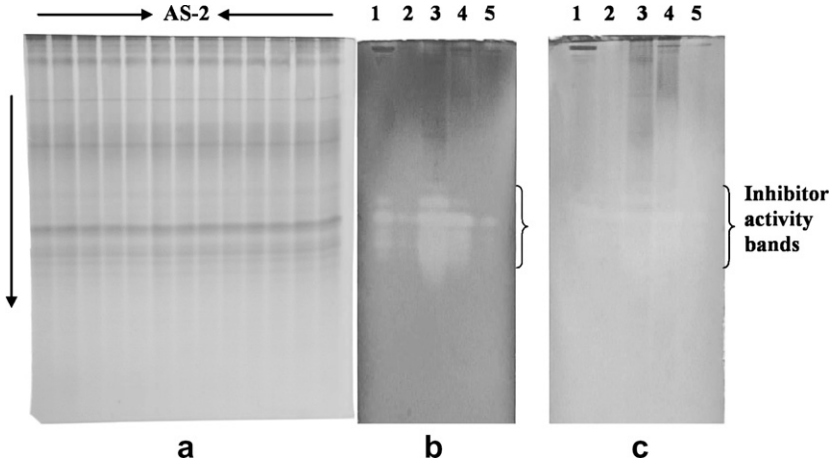

Fig. 6. (a) Native PAGE gel showing trypsin and chymotrypsin activities of AS-2 fraction run in all lanes; (b) trypsin inhibitor and (c) chymotrypsin inhibitor activity gels of ammonium sulphate-precipitated fractions of whole wattle seed extract. All gels were run at a constant current 10 mA at constant temperature (10 °C) for 15–16 h. Lane 1: Soaked-heated (100 °C, 2 min) whole seed extract; lanes 2 to lane 5: AS-1 to AS-4, respectively. Arrow shows direction of protein migration.

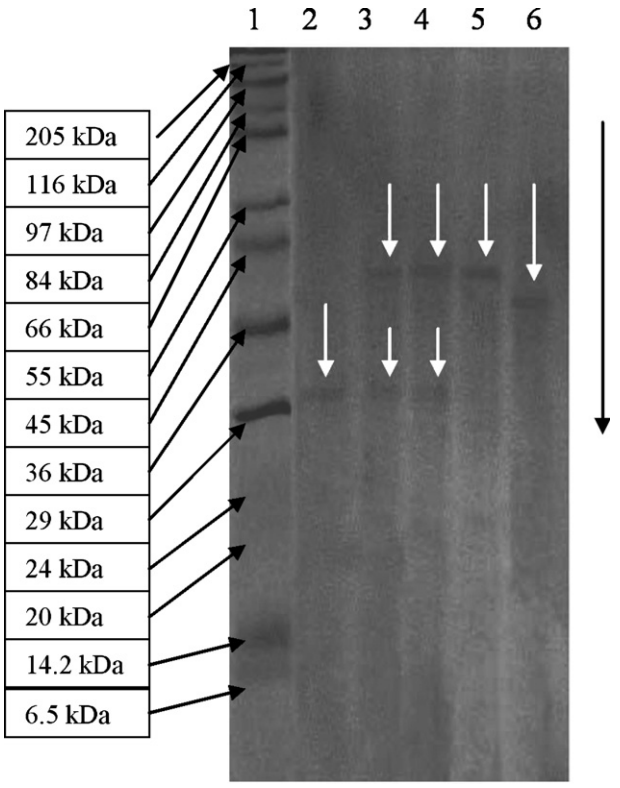

Fig. 7. Molecular weight analysis of five inhibitor-active bands cut from AS-2 native gel (Fig. 6a) by SDS-PAGE (run at constant current (10 mA) and temperature (10 °C) overnight. Lane 1: Sigma (molecular weight) markers from 6.5 kDa to 205 kDa as labelled; lane 2: first band; lane 3: second band; lane 4: third band; lane 5: fourth band; lane 6: fifth band. Major arrow shows direction of protein migration while minor arrows indicate position of inhibitor protein bands in each lane.

well as on the effect of heat processing on the functional properties of wattle seed extracts.

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