

Changes in trypsin inhibitory activity of developing seed parts and pod of red kidney bean

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Abstract

Mature red kidney bean seeds contain bovine trypsin inhibitors (TI) that could limit digestion of proteins by insects, animals or human beings. The changes in trypsin inhibitory activity of red kidney bean seed parts and pods in relation to the different stages of seed development (10 to 50 days after flower fall) were investigated using a spectrophotometric method. Specific activities of bovine trypsin inhibitor in all the different tissues increased from early on during seed development. Those in the seed pod decreased to very low levels at seed maturity suggesting that they might not be deleterious to animal nutrition as far as TI content is concerned. The high level of trypsin inhibitors found in developing seeds would be of concern from the perspective of animal or human nutrition.

Additional keywords: antinutritional proteins, azoalbumin, *Phaseolus vulgaris*, protease inhibitors

Introduction

Leguminous plants, the second most important food source for animals and human, are known to produce proteinaceous protease inhibitors that may play defence roles in these plants (Wati *et al.*, 2009; Alizadeh and Leung, 2011). These proteins might have physiological functions in the plants as well. Changes in the expression of the genes encoding them, for example, have been correlated with alterations in flower morphology, plant growth rate and seed development (Hartl *et al.*, 2010).

Protease inhibitors have been classified into 10 families on the basis of their molecular weight, amino acid sequence homology and cysteine contents (Campos *et al.*, 2004). Of all the protease inhibitors, trypsin inhibitors are the most extensively studied (Chen *et al.*, 1999) and found to be more heat-resistant than chymotrypsin inhibitors (Ee *et al.*, 2008). Most of Bowman-Birk family of protease inhibitors (belong to the MEROPS inhibitor gene family I12) have been isolated from legumes. These inhibitors have a duplicated structure that enables them to either inhibit

two distinct proteases independently or two molecules of the same protease (Campos *et al.*, 2004). The genomic DNA sequences of 23 Bowman-Birk inhibitors isolated from Italian common bean shared 92-99% homology (Piergiovanni and Galasso, 2004).

At least five different trypsin inhibitors were isolated from *Phaseolus vulgaris* L. var. Kintoki bean (Tsukamoto *et al.*, 1983). A trypsin-chymotrypsin inhibitor was also isolated from *P. vulgaris* (var. Kidney bean) with a molecular weight of 13000 Da (Jacob and Pattabiraman, 1986) but their activity during seed formation and in seed parts as well as pods has not been investigated. In *P. vulgaris* cv. Jampa the changes in trypsin inhibitory activity were assessed at different stages of seed development (including flowers, string bean and ripe bean) (Sotelo and Lucas, 1998). There are also studies on changes in some bioactive proteins during seed development of other legumes (Harsulkar *et al.*, 1997; Kokiladevi *et al.*, 2005; Kumar *et al.*, 2005). However, there is a lack of more comprehensive investigation which could include determination of trypsin inhibitory activity in different seed parts and pods of red kidney bean at different stages of seed development. The objective of this investigation was to evaluate the developmental changes in the specific inhibitor activity of bovine trypsin (as a model of serine proteinases) in different red kidney bean seed parts (seed coat, embryonic axis and cotyledons) and pods during seed formation under glasshouse conditions. Knowledge of the developmental changes and localisation of trypsin inhibitor activity is of interest to seed physiologists (to see whether trypsin

inhibitors might possess a physiological role), crop protection biotechnologists (seeking effective defence strategies against pests) and also from the perspective of gaining a better understanding of the seed and pod as food for humans and animals. In particular, the results from this work may be able to make some suggestions relevant to animal nutrition.

Materials and Methods

Seeds and chemicals

Seeds of red kidney bean (*P. vulgaris*) were purchased from a local health food store. Bovine trypsin (Cat. T-8003), azoalbumin (Cat. A-2382) and Coomassie Brilliant Blue dye (Cat. B-0770) were purchased from Sigma, Missouri, USA. Trichloroacetic acid (Cat. BSPTA321) was purchased from Biolab Australia Pty Limited, Victoria, Australia.

Preparation of bean seed extracts from developing seeds

Red kidney bean seeds were germinated in distilled water in the lab and then sown in large pots (2.5 l) of potting mix (with 8-9 months slow release fertiliser) for growing in the glasshouse at the University of Canterbury. Day and night temperatures were between 20-30°C and 15-20°C respectively. Flowers formed were tagged at anthesis. Seeds and pods (Figure 1) were harvested at 10 days from the time of flower fall and at five day intervals until seeds were fully matured (or when the seed pods were visibly desiccated) (Kumar *et al.*, 2005). After harvest, seeds and pods were stored at -80°C until they were analysed for their trypsin inhibitory activity.



Figure 1: Seed development in red kidney bean. From left to right; seeds and pods at 10, 15, 20, 25, 30, 35, 45 days after flower fall and at the mature dry seed stage.

Water contents

Water contents of whole seeds, seed and seedling parts, expressed as percentages, were calculated from the difference between their fresh weights and their dry weights after heating at 70°C for five days (Ramos *et al.*, 1998).

Protein extraction

Seeds (35 g) were soaked in distilled water (100 ml) in a beaker for 15 hours at room temperature and seed parts (cotyledons, embryonic axes and seed coat)

were separated. Ten and 15-day old seeds were too small to be separated into seed parts. Moreover, the seed parts including the seed coat, embryonic axis and cotyledons were found to adhere together tightly at these ages that made it difficult to separate them. Therefore, whole red kidney bean seeds harvested at these two time points were used to prepare extracts for evaluation of their bioactive protein contents, while those harvested at subsequent times were separated into the different seed parts and extracted.

The method of Le Berre-Anton *et al.* (1997) with some modifications was followed to extract heat stable and low-pH soluble proteins from the seed parts. Briefly, each type of seed part was homogenised separately with distilled water in a mortar and the homogenates were centrifuged at 6000 g for 10 minutes. The volume of supernatant was measured and then 0.11 ml of succinate buffer (pH 3.8) containing 100 mM CaCl₂.2H₂O per ml of the supernatant was added. The mixture was heated at 70°C for 10 minutes and centrifuged at 10,000 g for 10 minutes. After this, the supernatant was recovered and its pH was adjusted to 5.6 by adding 0.1 M NaOH. Samples were freeze dried, dissolved and dialysed in 200 mM Tris-HCl buffer at pH 8 containing 0.2 M CaCl₂.H₂O.

Protein contents

The amounts of soluble proteins in the different extracts were quantified using the principle of quantitative binding of proteins (Bradford, 1976) with the Coomassie Brilliant Blue dye. Bovine serum albumin was used as a standard.

Assay of trypsin inhibitory activity

Extracts of seeds or seed parts were tested for trypsin (EC 3.4.21.4) inhibitory (TI) activity against bovine trypsin. This assay was carried out with crude seed extracts (containing 10 µg soluble proteins) and using 1% (w/v) azoalbumin dissolved in distilled water as substrate following a previously described method with some modifications (Sarath *et al.*, 1989). Briefly, trypsin (25 µg in 100 µl 200 mM Tris-HCl buffer at pH 8 containing 0.2 M CaCl₂.H₂O) and seed or seedling extract (10 µg protein in 100 µl of the same buffer) were incubated for eight min at 37°C. Then 300

µl of substrate was added and reaction was allowed to proceed for 25 minutes at the same temperature before it was terminated with addition of one ml of trichloroacetic acid (5%, w/v). The difference in absorbance due to breakdown products at 450 nm between trypsin reaction with the substrate in the presence or absence of a seed extract was determined after centrifugation of the reaction mixtures at 10,000 g for 10 min. One trypsin inhibitor unit (TIU) was defined as the amount of inhibitor that reduced absorbance at 450 nm by 0.1 unit relative to trypsin control reactions for 25 minutes.

Data analysis

All experiments were repeated at least two times and all treatments were carried out with at least in three replicates. Data were subjected to one-way analysis of variance (ANOVA, $P \leq 0.05$) followed by comparison of mean values of the treatments using Duncan's Multiple Range test at 5% level of significance (Clewer and Scarisbrick, 2001).

Results

Visible changes in seeds and pods during seed development

The size of red kidney bean seed increased more than four fold from 10 days after flower fall to maturation (data not shown). The colour of immature seeds was green up to 20 days after flower fall and then started changing to reddish (Figure 1). No visible fibres were found on pods up to 15 days after flower fall but they appeared at day 20. Overall, the water contents of developing seeds increased up to 15 days after flower fall and then started decreasing significantly ($P < 0.05$, Table 1). Water contents in seed, seed parts and pods

dropped to very low levels at day 50 (the seed maturation stage).

Changes in bovine trypsin inhibitory activity during seed formation

Specific activity of bovine trypsin inhibitor was detectable in extracts of immature red kidney bean seeds at day 10 after flower fall and it increased significantly by day 15 (Figure 2A). Specific activity of bovine trypsin inhibitory was also found in extracts of red kidney bean cotyledons at day 20 after flower fall. It then increased with a transient peak at day 30 followed by a lower but constant level over the next 15 days. There was a further and significant drop in specific trypsin inhibitory activity by day 50

(Figure 2B). In the embryonic axis specific trypsin inhibitory activity increased from day 20 and peaked at day 35 and 40. Then it continued to decrease (Figure 3C). The level of specific trypsin inhibitory activity in the seed coat at day 20 was relatively high compared with that in the cotyledons and embryonic axis at the same time (compare Figures 2B, C and D). Then specific trypsin inhibitory activity increased to peak levels at day 25 and 30 before it decreased (Figure 2D). In pods, specific trypsin inhibitory activity increased from day 10 after flower fall and reached peak levels from day 20 to day 30. Then it started to decline until it was not detectable in pods at the seed maturation stage (Figure 2E).

Table 1: The percentage of water in developing seeds, seed parts and pods of red kidney bean (10-50 days after flower fall). Means in each row labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P < 0.05$).

Seed parts and pod	Percentage of water in developing seeds, seed parts and pods (%)								
	10d	15d	20d	25d	30d	35d	40d	45d	50d
Whole seed	81.57±0.3b	85.73±0.35a	80.59±0.26c	73.68±0.46d	63.92±0.40e	59.73±0.65f	48.18±0.34g	35.21±0.45h	11.08±0.39i
Cotyledon	n/a	n/a	81.14±0.60a	71.19±0.60b	60.01±0.18c	55.07±0.66d	44.65±0.60e	31.49±0.66f	11.38±0.46g
Embryonic axis	n/a	n/a	78.1±0.42a	71.15±0.79b	65.56±0.59c	63.9±0.47d	53.85±0.64e	41.32±0.77f	9.46±0.28g
Seed Coat	n/a	n/a	79.61±0.56a	79.7±0.55a	77.47±0.55b	76.23±0.75c	64.42±0.71d	52.85±0.87e	7.52±0.44f
Pod	90.19±0.442b	92.52±0.58a	90.54±0.68b	88.49±0.60c	86.96±0.66d	85.28±0.58e	74.13±0.74f	66.92±0.46g	8.44±0.49h

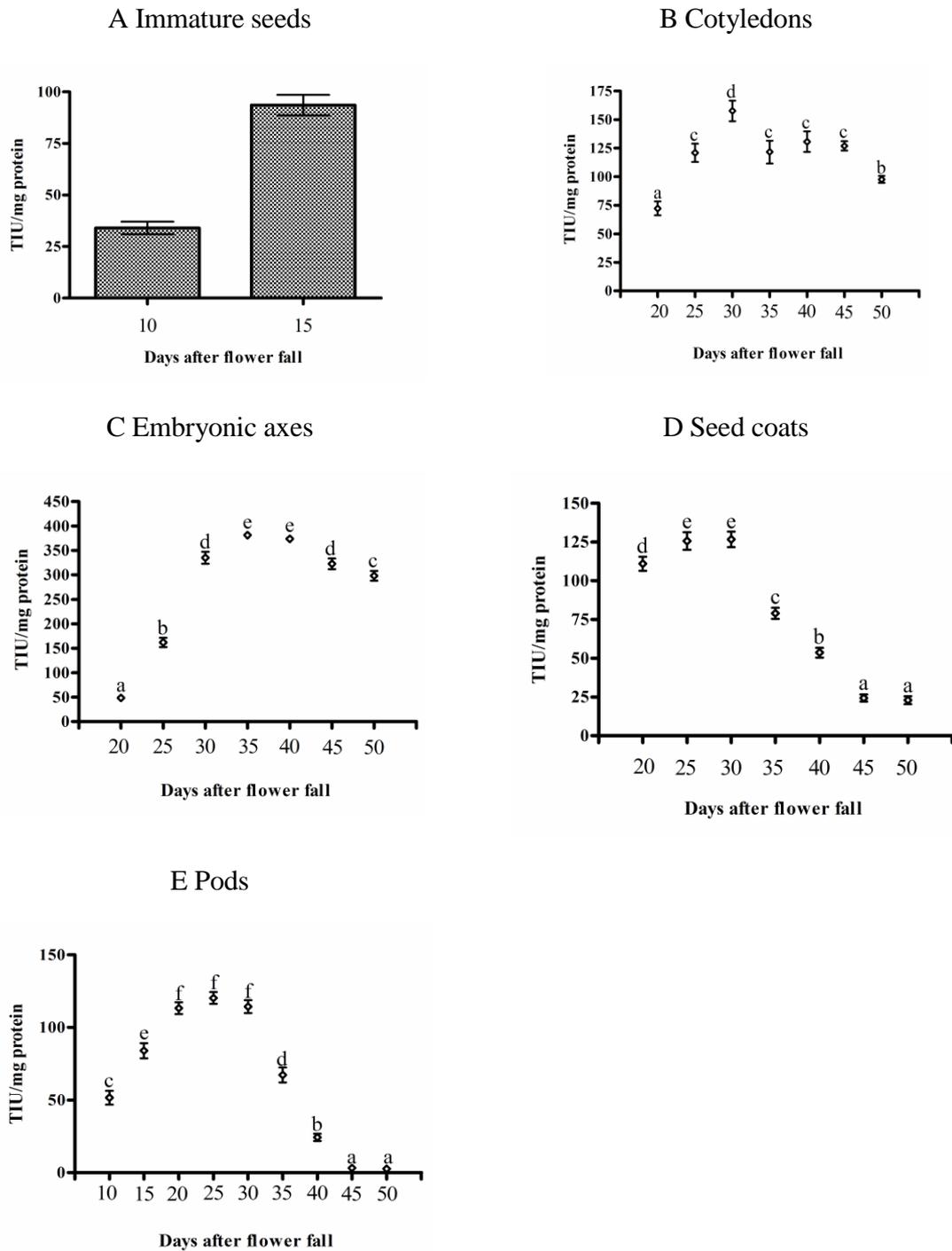


Figure 2: Changes in specific activity of bovine trypsin inhibitor in red kidney bean pods and seed parts during seed formation. Means labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P < 0.05$).

Discussion

The highest water content in seed parts was observed at an early stage of seed formation (day 15). In contrast trypsin inhibitory activity was generally at its highest level by day 30 or 35. Therefore, changes in TI activity during seed formation are discussed in terms of specific activity rather than fresh weight. Bovine trypsin inhibitory activity was observed in all seed parts as well as the pod of red kidney bean. Presence of trypsin inhibitors can block trypsin activity in either seed or pod eaters which is consistent with their potential defence role against predators. These results confirm and extend the findings of previous studies (Fernandez *et al.*, 1982; Texier *et al.*, 1989 and Domash *et al.*, 2006). The presence of trypsin inhibitors has been detected in the soybean (Santos *et al.*, 2008) but not the cowpea seed coat (Carasco and Xavier-Filho, 1981), pods of common bean and winged bean (Tadera *et al.*, 1984; Texier *et al.*, 1989).

Among the different seed parts and pod of red kidney bean, the embryonic axis had the highest level of TI activity, suggesting that TI activity is particularly important during development of the embryonic axis. It might play in a role in regulation of endogenous proteolytic activity involved in protein turnover therein. The highest specific TI activity in the cotyledons and embryonic axis of red kidney bean was detected at day 30 and day 35 after flower fall, respectively. The physiological significance, if any, of this difference is not readily apparent. TI activity in the seed coat at seed maturation was the lowest in comparison with that at the other seed developmental stages. Also, the seed coat was darker, probably due to increased tannin accumulation therein. This might help protect the mature seeds against

predators and pathogens along with the low level of TI activity therein. The lower activity in mature seeds can also be attributed to preparation of seed for dormancy while the activity can increase later during germination (Pusztai, 1972). Future work will focus on isolation and purification of bovine trypsin inhibitors from pods and seed parts to determine whether they are the same isoinhibitors or not.

At day 20 after flowering no trypsin inhibitory activity was detectable in two varieties of chickpeas while one of the varieties (Annigeri) showed the highest activity at 40 days after flowering (Harsulkar *et al.*, 1997). This is contrary to the result obtained in this study which showed that trypsin inhibitor activity was detectable at day 20 after flower fall. Different levels of trypsin inhibitor content in four different genotypes of Indian soybean were also observed at a very early stage of seed development (Kumar *et al.*, 2005). Similarly, research on the inhibitory activity of papain (another type of protease inhibitor) in white bean seeds was detectable at early stages during seed development (Santino *et al.*, 1998).

TI activity was found in all seed parts and appeared at an early stage of seed formation, suggesting there was no tissue specific distribution of trypsin inhibitors and the inhibitors might have some biological significance in the different parts particularly at early stages of seed formation. The general pattern of changes in TI activity observed is consistent with the overall metabolic changes during seed development in favour of net accumulation of seed reserves including storage proteins from early stages till seed maturation. The low levels of TI in mature seeds and pods

correlated with loss of water and the characteristic decline in metabolic activity therein. As levels of TI were relatively high in immature red kidney bean pods, from a nutritional point of view it is suggested that they should be inactivated after thorough cooking before consumption. By contrast, dry mature pods could be fed to animals without prior cooking as no or very low TI activity was observed in mature pods.

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