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The trypsin inhibitors present in seed of different grain legume species and cultivar

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Abstract

Trypsin inhibitors in a selection of grain legume seeds from different species and cultivars were studied. The results showed that trypsin inhibition content ranged from negligible in *Lupinus* spp. to very high in *Glycine max*. Although there is variation among cultivars, generally the highest TIU mg^{-1} sample values occured in soybean (43–84) and common bean (21–25). Inhibitor content of different *Lathyrus* cultivars, ranged from 19–30 TIU mg^{-1} sample. This was higher than the contents in chickpea (15–19 TIU mg^{-1} sample) and pea (6–15 TIU mg^{-1} sample). Lentil and faba bean had low values in most cvs (3–8, and 5–10 TIU mg^{-1} sample, respectively). Trypsin inhibitor isoform analyses showed that the amount of TI detected, varied with legume species and variety.

Keywords: Legume seeds; Trypsin inhibitors; Zymogram gel

1. Introduction

Grain legume seeds are an important source of protein, energy, vitamins and minerals for human and animal consumption. These seeds usually contain large amounts of stored material that are used as precursors for synthetic processes during germination and seedling growth. These include starch, storage proteins and other proteinaceous compounds which are enzymatically degraded, to support early plant growth. Proteins such as amylase inhibitors, lectins and trypsin inhibitors (TIs) are likely to protect legume seed against attack by predators. However, it is not clear to what extent these proteins also have a storage function. Protease inhibitors in legume seed can have a major impact on nutritional value as they inhibit pancreatic serine proteases, thus impairing protein digestion. Legume seed protease inhibitors contain no carbohydrates and belong to two families, Kunitz and Bowman-Birk. Both types of protease inhibitors are found in soybean (*Glycine max*). In other grain legumes such as common bean (*Phaseolus vulgaris*) and lentil (*Lens culinaris*), the protease inhibitors have been characterized as members of the Bowman-Birk family (Lajolo & Genovese, 2002).

The effect of trypsin inhibitors on animal growth is not only the result of inhibition of intestinal protein digestion. When inhibitors are present in diets consisting of free amino acids, decreased growth was also observed. Kunitz and Bowman-Birk inhibitors cause enlargement of the pancreas (hypertrophy and hyperplasia) in rodents and birds, and hypersecretion of digestive enzymes. This leads to a loss of sulphur-rich endogenous proteins, trypsin and chymotrypsin, which would depress growth because legume seed proteins are generally deficient in sulphur amino acids (Lajolo & Genovese, 2002). Both trypsin inhibitors, the Kunitz and Bowman-Birk act by suppressing negative feedback regulation of pancreatic secretion through increased

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release of the hormone cholecystokinin from the intestinal mucosa (Liener, 1994).

However, the Bowman-Birk inhibitor (BBI) is effective in preventing, or suppressing, carcinogen-induced transformation in vitro and carcinogenesis in animals. It achieved investigational new drug status from the FDA in 1992 (Kennedy, 1995, 1998). Most studies on the health-promoting properties of plant protease inhibitors have used BBI from soybean. However, other grain legume seeds are the rich sources of protease inhibitors (Clemente, MacKenzie, Jonson, & Domoney, 2004).

Different research has shown that the positive or negative effect of these inhibitors depend on the level present in the different legumes and on the dose and time of consumption.

The aim of this study was to obtain more precise knowledge of trypsin inhibitors in some grain legume seeds, the variation among cultivars, quantify TI activity and to analyse qualitative variation in TI isoform patterns using the novel system of Zymogram gels.

2. Materials and methods

2.1. Plant material

The grain legume species and cultivars analysed are shown in Table 1.

Seed was ground to pass through a 1 mm screen (Tecator, Höganäs, Sweden; Cyclotec 1093). Quantitative trypsin inhibitor measurements were performed and trypsin inhibitor units (TIU) were defined using the assays described by Welham and Domoney (2000). Trypsin inhibitor was determined, using α-*N*-benzoyl-DL-arginine-*p*-nitroanilidehydrochloride (BAPNA) as the trypsin substrate. Trypsin inhibitor activity (TIA), expressed as trypsin inhibitor units/mg protein, was calculated from the absorbance read at 410 nm against a reagent blank. One unit of TIU was defined as that which gives a reduction in $A_{410\ nm}$ of 0.01, relative to trypsin control reactions, using a 10 ml assay volume (Welham, O'Neill, Johnson, Wang, & Domoney, 1998).

Protein concentration was determined by Bradford dye-binding assay Bradford (Bradford, 1976), using BSA as a standard. All assays were performed in triplicate.

For electrophoretic analysis on native gels, 1 g of flour from each sample was extracted in 10 ml of 50 mM HCl for 2 h with continuous stirring. All steps were carried out at 4 °C. Following centrifuging at 12,000*g* for 10 min, the residue was re-extracted with 2 ml of 50 mM HCl and centrifuged again. Pooled supernatants were dialysed against distilled water overnight. The supernatants were then centrifuged at 12,000*g* for 20 min and freeze-dried. Samples were prepared in Tris–Glycine native sample buffer (NOVEX) and analysed on native 4–16% Zymogram gradient gels (NOVEX Zymogram (Blue Casein) 4–16%) using the buffers supplied and a soybean TI as a standard (Type I-S, Sigma T9003). Following electrophoresis, the Zymogram gels were incubated in renaturing buffer with gentle agitation for 30 min at room temperature (Muzquiz et al., 2004).

The renaturing buffer was replaced with Zymogram developing buffer and incubated for 30 min at room temperature with gentle agitation. The developing buffer was replaced with a fresh solution of trypsin in the same buffer (20 mg trypsin per 100 ml) and incubated at 37 °C, with gentle agitation, for 90 min. The gels were treated with 5% acetic acid and placed in distilled water. Areas of the gels that remained blue indicated where trypsin had been inhibited.

2.2. Statistical analysis

A one way ANOVA analysis was applied to the obtained analytical data, as well as Duncan's multiple range test in order to stablish the statistical significance of protein and TIU variations. The computer package Statgraphics Plus 4.1 was used for this purpose.

3. Results and discussion

The results shown in Fig. 1 clearly show that trypsin inhibition among legume seeds can range from negligible,

Table 1
Legume species, cultivar, form and source of seed analysed

Legume species	Cultivar(s) and/or form	Source
Cicer arietinum	Pedrosillano, Fuentesauco, Tizón, Athenas	Servicio de Investigación Agraria, Córdoba, Spain
Glycine max	Ostrumi, BR16, Commercial raw, Defatted	Servicio de Investigación Agraria, Córdoba, Spain; EMBRAPA, Brazil; Moyresa, Spain
Lathyrus spp.	L-587124, L-591041, BG-10904, BG-27067	Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain
Lens culinaris	Ageda, Mosa, La Armuña, Magda	Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain
Lupinus albus	Multolupa	Servicio de Investigación Agraria, Badajoz Spain
Lupinus angustifolius	Saladulce	Servicio de Investigación Agraria, Badajoz Spain
Lupinus luteus	Tremosilla	Servicio de Investigación Agraria, Badajoz Spain
Lupinus mutabilis	Potosí	Servicio de Investigación Agraria, Badajoz Spain
Phaseolus vulgaris	Arthropurpurea, Cardeno, Gernikesa, Tolosana	Centro de Investigación y Mejora Agraria, Vitoria, Spain
Pisum sativum	Ucero, Esla, Luna, Coomonte	Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain
Vicia faba	BG-22387, BG-26424, Brocal, Alameda	Servicio de Investigación Agraria, Cordoba, Spain

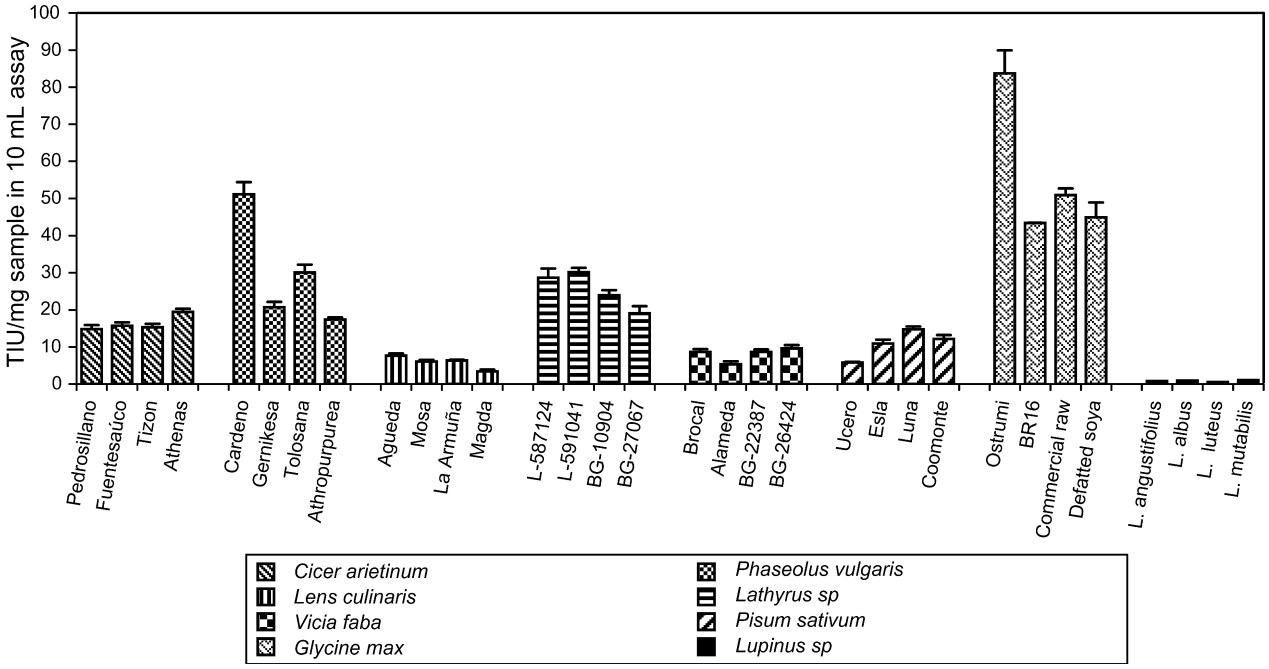

Fig. 1. Amount of TIU/mg sample in different cultivars of *Cicer arietinum*, *Phaseolus vulgaris*, *Lens culinaris*, *Lathyrus* spp., *Vicia faba*, *Pisum sativum*, *Glycine max y Lupinus* spp.

as in the *Lupinus* spp., to very intense in *Glycine max*. Although there was variation among cultivars, generally the highest values of TIU mg^{-1} sample were observed in soybean (43–84) and common bean (51–17). The TIU content of different *Lathyrus* cvs ranged from 19–30 TIU mg^{-1} sample. This was higher than in both chickpea (15–19) and pea (6–15). Most lentil and faba bean cultivars had still lower values (3–8 and 5–10 TIU mg^{-1} sample, respectively).

Trypsin inhibitor levels have been studied by Valdebouze, Bergeron, Gaborit, and Delort-Laval (1980) and compared to levels in soybean whole seed and defatted meal.

In legumes grown in Gallardo, Araya, Pak, and Tagle (1974) showed, as in the present study, a lack of trypsin inhibition by lupin seeds. However, reported differences among authors in reported values of trypsin inhibitors in legume seed are large (Elkowicz & Sosulski, 1982; Berger, Siddique, & Loss, 1999).

Griffiths (1984) also found variability in pea (0.2–4.6) and faba bean (1.4–1.6) when results were expressed in TIU mg^{-1} sample. Valdebouze et al. (1980) obtained values of 3–11 TIU mg^{-1} sample in pea and from 5.6 to 11.8 in faba bean. These values are similar to those reported here.

In soybean the commercial samples analysed gave results of the same order of magnitude as Valdebouze et al. (1980) at 57.2 TIU mg^{-1} DM in whole soybean and 64.8 TIU mg^{-1} DM in defatted soybean.

The average TIU mg^{-1}DM content of 18 *Lathyrus* seed samples (Grela, StudziOski, & Matras, 2001) was 19.64. This is slightly lower than observed in this work (25.5). The range reported by these authors was lower in *L. cicera* (12.6–20.4) than in *L. sativus* (20.1–44.1) (Hanbury, White, Mullan, & Siddique, 2000).

Trugo, Ramos, Trugo, and Souza (1990) in 10 cultivars of *Phaseolus vulgaris,* from Brazil, reported values of 71–160 TIU mg^{-1} sample which is higher that the values obtained here and Piergiovanni and Pignone (2003) found wide variation in the TI content, expressed as TIU mg^{-1} DM (14–39) in 21 local populations of common bean (*P.vulgaris*).

Singh and Jambunathan (1981) analysed samples from different chickpea cultivars (*Desi* and *Kabuli*) and found higher TIU mg^{-1} sample values in *Desi* (12.7) than in *Kabuli* (10.3). These both values are lower than reported here.

In lentil seeds, Soni, Singh, and Singh (1978) reported TIA was 25% of that in soybean. This corresponds to 5.7 TIUs. Savage (1988) reported that TIA values for lentils of between 0.2 and 5.1 units, depending on variety. Vidal-Valverde et al. (1994) reported values of 5.34 and 6.38 units for *L. culinaris* var. vulgaris and var. *variabilis*, respectively, while Frias, Diaz-Pollan, Hedley, and Vidal-Valverde (1995) obtained values of 5.0–5.1 units for *L. culinaris* var. vulgaris, cv. Magda-20, with no apparent difference between consecutive harvest years. The samples analyzed here, had values of 3 and 8 TIU mg^{-1} sample.

Part of the variation can be explained by the observed differences among cultivars. Piergiovanni and Pignone (2003) suggested that drought and thermal stress during vegetative growth may favour increased TI expression. Howard, Morton, Savage, and Russel (1996) also suggested an influence of rainfall on the expression of TIs, and an effect of drought, during seed development, on the quality and nutritive value of several legume species.

They found an increase in TI in pea cultivars that had suffered moisture stress during the growing season. Variation in the TI level, as a response to environmental stress, could be a plant strategy to increase defence mechanisms or to preserve storage products. Bacon, Lambert, Matthews, Arthur, and Duchene (1995), investigating the TI of peas, observed that some cultivars were more susceptible than others to climatic extremes.

Analysis of the protein content (mg g^{-1} flour) showed significant differences among the cvs of the different legumes (Table 2). Average values were: 146.56 mg g^{-1} in *Cicer*, 79.32 mg g^{-1} in *Phaseolus*, 161.49 mg g^{-1} in *Lens*, 175.13 mg g^{-1} in *Lathyrus,* 162.65 mg g^{-1} in *Vicia*, 162.65 mg g^{-1} in *Pisum*, 135.22 mg g^{-1} in *Glycine* and the highest protein content was found in *Lupinus*, 257.87 mg g^{-1}. There was no change in the proportion of TIA (TIU mg^{-1}protein) in the different legume species in relation to the TIU mg^{-1} sample (Table 2 and Fig. 1). The statistical analysis showed significant differences in TIA among the species studied. Statistically, 3 groups were identified according to the TIA values (Fig. 2). The first one corresponded to the highest content (*G. max*); the second one corresponded to *P. vulgaris* with a medium content and the other seeds formed the last group with a low TIA content.

Table 2
Protein content (mg g^{-1} flour) and TIA (TIU mg^{-1} protein) in different cultivars of *Cicer arietinum*, *Phaseolus vulgaris*, *Lens culinaris*, *Lathyrus* spp., *Vicia faba*, *Pisum sativum*, *Glycine max y Lupinus* spp.

Seed/cultivar	Protein	TIA
Cicer arietinum		
Predosillano	146^{efgh}	101^{F*}
Fuentesaúco	134^{cdef}	118^{G}
Tizón	150^{ghi}	102^{F}
Athenas	157^{hij}	124^{G}
Phaseolus vulgaris		
Cardeno	88.73^{b}	576
Gernikesa	73.5^{ab}	282
Tolosana	66.5^{a}	458
Athropurpurea	88.5^{b}	197
Lens culinaris		
Ageda	140^{def}	54.8
Mosa	156^{ghij}	38.5^{B}
La Armuña	185^{l}	34.7^{B}
Magda	165^{ijk}	20.6
Lathyrus spp.		
L-587124	180^{kl}	160^{H}
L-591041	184^{l}	164^{H}
BG-10904	170^{jkl}	141
BG-27067	168^{jk}	114^{G}
Vicia faba		
Brocal	133^{cdef}	64.6^{DE}
Alameda	129^{cd}	41.2^{B}
BG-22387	156^{ghij}	55.7^{CD}
BG-26424	149^{fghi}	64.4^{DE}
Pisum sativum		
Ucero	148^{efgh}	39.1^{B}
Esla	149^{fgh}	73.4^{E}
Luna	1753^{kl}	83.8
Coomonte	178^{kl}	67.9^{E}
Glycine max		
Ostrumi	155^{ghij}	540
BR16	132^{cde}	328
Commercial raw	118^{c}	430
Defatted soya	29.7	1512
Lupinus spp.		
L. angustifolius	241	3.32^{A}
L. albus	212	4.25^{A}
L. luteus	280	1.78^{A}
L. mutabilis	298	3.69^{A}

* Means followed by the same superscript are not significant ($p > 0.05$) by Duncan multiple range test.

Extracts of different cultivars of *P. vulgaris* seed on Zymogram gel, showed three isoforms, corresponding to major, equivalent and minor electronegativity than the soybean inhibitor used as a standard (Fig. 3A). The same three bands were also observed in the soybean cvs but in the soybean there were others bands in zones of higher electronegativity (Fig. 3A). The different *P. vulgaris* cultivars had the same isoforms. In defatted soybean there were a lower number of isoforms than in the other soybean samples. Defatted soybean is used in animal feed compounding. Trypsin inhibitor activity and the number of isoforms varies with the source of the beans and their processing conditions as TIs are heat labile (Clarke & Wiseman, 2005).

A large number of isoforms of the Bowman-Birk inhibitor have been reported in soybean cultivars. Generally, the properties of the inhibitor are attributable to different isoforms. Nulls for both Bowman-Birk and Kunitz trypsin inhibitors have been identified allowing new low trypsin inhibitor cultivars to be produced. However, research into the breeding of low trypsin inhibitor cultivars, currently has limited application as trypsin inhibitors contribute a major proportion of soybean seed methionine (Clarke & Wiseman, 2000). Isoforms were classified by Tan-Wilson

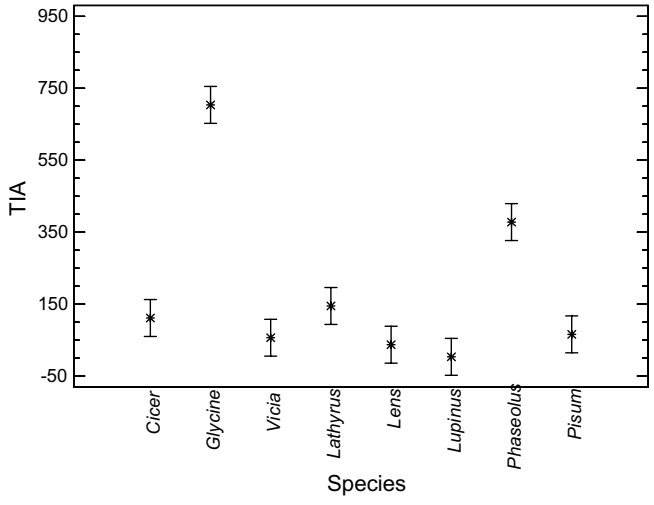

Fig. 2. Distribution of mean TIA values for species and 95% intervals based on Fisher's least significant difference procedure.

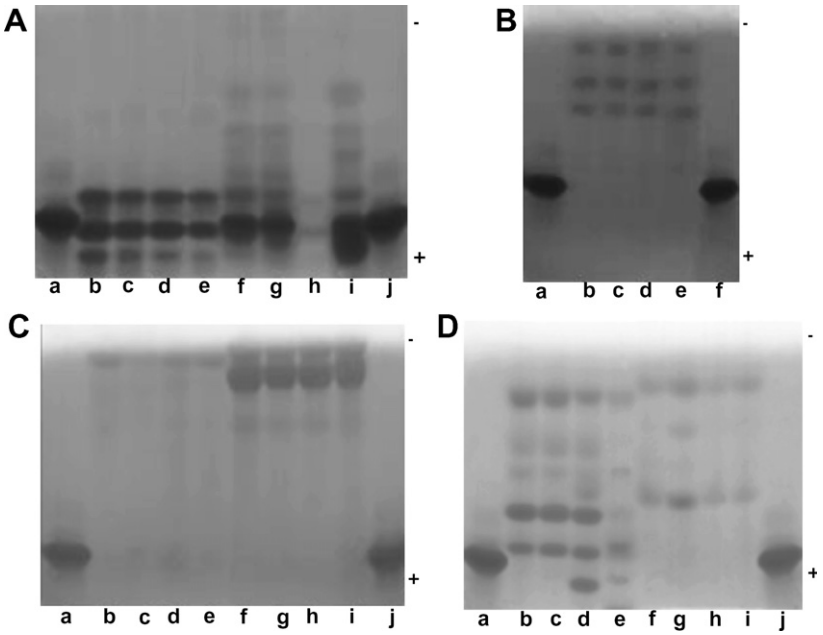

Fig. 3. Trypsin inhibitor isoform patterns of *Phaseolus vulgaris* (A): Cardeno, Gernikesa, Tolosana and Arthropurpurea (lanes b-e) (15 μg protein per well); *Glycine max* (A): Commercial raw, BR-16, commercial defatted and Ostrumi (lanes f-i) (60 μg protein per well); *Lathyrus* spp.(B): L-587124, L-591041, BG-10904 and BG-27067 (lanes b-e) (10 μg protein per well); *Vicia faba* (C): Alameda, Brocal, BG-22387 and BG-26424 (lanes b-e) (120 μg protein per well); *Cicer arietinum* (C):Athenas, Fuentesauco, Pedrosillano and Tizón (lanes f-i) (60 μg protein per well); *Pisum sativum* (D): Coomonte, Esla, Luna and Ucero (lanes b-e) (60 μg protein per well) and *Lens culinaris* (D): Ageda, La Armuña, Mosa and Magda (lanes f-i) (80 μg protein per well) analysed by non-denaturing Zymogram gel, using a soybean tripsin inhibitor as standard (A, C, D lanes a and j; B lanes a and f).

et al. (1987) into four sub-groups based on their distinctive amino acid compositions, molecular weights, spectrum of enzyme inhibitor activity and immunochemical cross-reactivity.

Genovese and Lajolo (1998) using gel electrophoresis on *P. vulgaris* extracts stained for trypsin inhibitory activity, showed that the 46 cultivars they analysed could be placed into three groups: two groups with a three-band profile and one group with a four band profile. Whitaker and Sgarbieri (1981) and Wu and Whitaker (1990) also observed four isoinhibitors in different varieties of *P. vulgaris.*

When extracts of the *Lathyrus* cvs were analysed on Zymogram gels, three isoforms were also observed (Fig. 3B), that were in zones of higher electronegativity than the soybean inhibitor used as a standard and in *P. vulgaris.* There was no difference among cultivars.

In *Vicia faba* cvs there was only one band of high electronegativity and no variation among cultivars. This band also appeard in the different *C. arietinum* cultivars although in these cultivars another isoform of high electronegativity was observed (Fig. 3C).

Genotypic variation in chickpea protease inhibitor levels was reported by Singh and Jambunathan (1981) and Saini (1989). Others studies on trypsin inhibitors in chickpea showed the existence of six to eight isoinhibitors (Belew, Porath, & Sundberg (1975); Chavan & Hejgaard (1981); Hamza, Saeta, & Stegemann (1986).

Saini, Weder, & Knights (1992) with a range of *Desi* and *Kabuli* chickpea genotypes, developed and grown in Australia examined their ability to inhibit bovine, porcine and human trypsin/chymotrypsin, and compared their isoinhibitor patterns. After staining for trypsin inhibitors, seven isoinhibitors were detected in all of the samples. The isoelectric points (pI) of chickpea inhibitors CPI-1 to CPI-7 (denoted according to increasing pI) were 4.9, 6.8, 7.1, 7.5, 8.4, 8.6 and 9.3, respectively. Inhibitors CPI-2, CPI-5 and CPI-6 were most pronounced and CPI-4 was least pronounced. Differences in intensity among samples were mainly due to differing inhibitor activities of individual samples.

When extracts of different cultivars of *Pisum sativum* were analysed on Zymogram gels, five isoforms were observed in the cultivars Coomonte and Esla, corresponding to major isoforms (Fig. 3D) of the soybean inhibitor used as a standard. In cultivar Luna there were six major isoforms and one minor to the standard and in cultivar Ucero there were four major and two minor to the soybean inhibitor standard.

Welham et al. (1998) analyzed mature seed cotyledonary extracts of *P. sativum* cv. Birte on non-denaturing gels and found two predominant TI isoforms and two additional minor forms. Of these four isoforms, only the two predominant ones were present in extracts of immature axes and cotyledons at mid to late developmental stages. However, extracts of axes from mature dry seed showed, in addition to the predominant cotyledonary forms, a much higher proportion of the isoforms that were relatively minor in mature pea cotyledons.

In different lentil cultivars, there were two isoforms appeared except cv. La Armuña where another isoform

of higher electronegativity than the soybean inhibitor was detected (Fig. 3D).

Inhibition of trypsin by lentil seed extracts was first reported by Jaffé (1950) and that of chymotrypsin by Mansfeld, Ziegelhoffer, Horakova, & Hladovec (1959). In a comparative study of the inhibitory activity of the seeds of 18 legumes, lentils were found to inhibit human chymotrypsin much more than bovine chymotrypsin, human trypsin and bovine trypsin (Belitz, Lynen, & Weder, 1982). Further, there were four isoinhibitors in 38 lentil samples tested by Weder, Mueller, Mato, & Hegarty (1985), Weder, Hegarty, Holzner, & Kern-Dirndorfer (1983) who isolated and characterized the principal inhibitor from a local market lentil sample. Two trypsin-chymotrypsin inhibitors from Italian red lentils have been reported by Mueller & Weder (1989).

Weder & Kahley (1998) reported that the four lentil inhibitors were similar in amino acid composition, because of their high cystine content, (five to seven disulphide bonds), their molecular weight (M_r) values and their ability to inhibit trypsin and chymotrypsin. The four lentil inhibitors belonged to the Bowman-Birk inhibitor family.

Usually gel intensity of inhibitors can be correlated with total TIU. However, band intensity in our gels could not be correlated with total TIU due to the different charge applied to the gel for the legumes (Fig. 3A–D).

4. Conclusion

These results show that the quantity of TI in legume seed varies with species and variety. It is therefore necessary, to analyze initial material which may be used in animal feeding. It is important to know the seed TI content, as active inhibitors can pass through the stomach unaltered as they are stable against pepsin and low pH (Weder & Kahley, 2003). As discussed above Kunitz and Bowman-Birk inhibitors cause enlargement of the pancreas and hypersecretion of digestive enzymes (sulphur-rich proteins), causing a loss of sulphur-rich endogenous proteins. This would depress growth as legume seed proteins are deficient in sulphur amino acids (Lajolo & Genovese, 2002).

On the other hand, protease inhibitors have been linked, over the last two decades, to health-promoting properties (Champ, 2002). They are considered natural bioactive substances (Hill, 2004). Protease inhibitors can act as anticarcinogenic agents (Clemente et al., 2004). Bowman-Birk inhibitors (BBI) are effective in preventing or suppressing carcinogen-induced transformation in vitro and carcinogenesis in animal assays.

It is possible that the beneficial or injurious effect of TIs would depend on the dose of the inhibitor ingested. Clemente et al. (2004) found that the TI of peas was significantly more effective at inhibiting cell proliferation (human colon adenocarcinoma cells), compared with BBI from soybean, with an IC50 value of less than 50 μM in the case of pea TI.

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References

Bacon, J. R., Lambert, N., Matthews, P., Arthur, A. E., & Duchene, C. (1995). Genotype, environment and genotype × environment effects on trypsin inhibitor in peas. *Journal of the Science of Food and Agriculture, 67*, 101–108.

Belew, M., Porath, J., & Sundberg, L. (1975). The trypsin and chymotrypsin inhibitors in chick pea (*Cicer arietinum* L.). Purification and properties of the inhibitors. *European Journal of Biochemistry, 60*, 247–258.

Belitz, H. D., Lynen, F., & Weder, J. K. P. (1982). Comparative studies on the inhibitory action of some legume seeds, potato tubers, and bran against human and bovine proteinases. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung, 174*, 442–446.

Berger, J. D., Siddique, K. H. M., & Loss, S. P. (1999). Cool season grain legumes for Mediterranean environments: species × environment interaction in seed quality traits and anti-nutritional factors in the genus *Vicia*. *Australian Journal of Agricultural Research, 50*, 389–401.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Champ, M. M. (2002). Non-nutrient bioactive substances of pulses. *British Journal of Nutrition, 88*, 307–319.

Chavan, J. K., & Hejgaard, J. (1981). Detection and partial characterisation of subtilisin inhibitors in legume seeds by isoelectric focusing. *Journal of the Science of Food and Agriculture, 32*, 857–862.

Clarke, E. J., & Wiseman, J. (2000). Developments in plant breeding for improved nutritional quality of soya beans II. Anti-nutritional factors. *Journal of Agricultural Science, Cambridge, 134*, 125–136.

Clarke, E. J., & Wiseman, J. (2005). Effects of variability in trypsin inhibitor content of soya bean meals on true and apparent ileal digestibility of amino acids and pancreas size in broiler chicks. *Animal Feed Science and Technology, 121*, 125–138.

Clemente, A., MacKenzie, D. A., Jonson, I. T., & Domoney, C. (2004). Investigation of legume seed protease inhibitors as potential anticarcinogenic proteins. In M. Muzquiz, G. D. Hill, C. Cuadrado, M. M. Pedrosa, & C. Burbano (Eds.), *Proceedings of the fourth international workshop on antinutritional factors in legume seeds and oilseeds* (pp. 137–141). Wageningen: EAA Publications.

Elkowicz, K., & Sosulski, F. W. (1982). Antinutritive factors in eleven legumes and their air-classified protein and starch fractions. *Journal of Food Science, 47*, 1301–1304.

Frias, J., Diaz-Pollan, C., Hedley, C. L., & Vidal-Valverde, C. (1995). Evolution of trypsin inhibitor activity during germination of lentils. *Journal of Agricultural and Food Chemistry, 43*, 2231–2234.

Gallardo, F., Araya, H., Pak, N., & Tagle, M. A. (1974). Factores toxicos de leguminosas cultivadas en Chile. II. Inhibitor de tripsina. *Archivos Latinoamericanos de Nutrición., 24*(2), 183–189.

Genovese, M. I., & Lajolo, F. M. (1998). Trypsin inhibitors from beans (*Phaseolus vulgaris* L.): isoforms and thermal property studies by differential scanning calorimetry. In: Third European conference on grain legumes. Proceedings of the 3rd European conference on grain legumes. AEP (eds), pp. 174–176.

Grela, E. R., StudziOski, T., & Matras, J. (2001). Antinutritional factors in seeds of *Lathyrus sativus* cultivated in Poland. *Lathyrus Lathyrism Newsletter, 2*, 101–104.

Griffiths, D. W. (1984). The trypsin and chymotrypsin inhibitor activities of various pea (*Pisum* spp.) and field bean (*Vicia faba*) cultivars. *Journal of the Science of Food and Agriculture, 35*, 481–486.

Hamza, M. A., Saeta, A. M. E. T., & Stegemann, H. (1986). Studies on α-amylase and trypsin inhibitors in legume seeds using agar diffusion and isoelectric focusing techniques.*Qualitas plantarum*. *Plant Foods for Human Nutrition, 36*, 139–146.

Hanbury, C. D., White, C. L., Mullan, B. P., & Siddique, K. H. M. (2000). A review of the potential of *Lathyrus sativus* L. and *L. cicera* L. grain for use as animal feed. *Animal Feed Science and Technology, 87*, 1–27.

Hill, G. D. (2004). Grain legumes and oilseeds-the way ahead. In: Muzquiz, M., Hill, G. D., Cuadrado, C., Pedrosa, M. M., Burbano, C. (Eds.), In: Proceedings of the fourth international workshop on antinutritional factors in legume seeds and oilseeds, EAAP publication No. 110 (pp. 353–364). The Netherlands: Wageningen.

Howard, T. M., Morton, J. D., Savage, G. P., & Russel, A. C. (1996). Trypsin inhibitors in New Zealand pea cultivars: quantification and isoforms. *Proceedings Nutrition-Society of New Zealand, 21*, 120–124.

Jaffé, W. G. (1950). Protein digestibility and trypsin inhibitor activity of legume seeds. *Proceedings of the Society for Esperimental Biology and Medicine, 75*, 219–220.

Kennedy, A. R. (1995). The evidence for soybean products as cancer preventive agents. *Journal of Nutrition, 125*, 733S–743S.

Kennedy, A. R. (1998). The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *American Journal of Clinical Nutrition, 68*, 1406S–1412S.

Lajolo, F. M., & Genovese, M. I. (2002). Nutritional significance of lectins and enzyme inhibitors from legumes. *Journal of Agricultural and Food Chemistry, 50*, 6592–6598.

Liener, I. E. (1994). Implications of antinutritional components in soybean foods. *Critical Reviews of Food Science & Nutrition, 34*, 31–67.

Mansfeld, V., Ziegelhoffer, A., Horakova, Z., & Hladovec, J. (1959). Isolierung der trypsin-inhibitoren aus einigen hulsenfruchten. *Naturwiss, 46*, 172.

Mueller, R., & Weder, J. K. P. (1989). Isolation and characterization of two trypsin-chymotrypsin inhibitors from lentil seeds (*Lens culinaris* Medik). *Journal of Food Biochemistry, 13*, 39–63.

Muzquiz, M., Welham, T., Altares, P., Goyoaga, C., Cuadrado, C., Romero, C., et al. (2004). The effect of germination on seed trypsin inhibitors in *Vicia faba* and *Cicer arietinum*. *Journal of the Science of Food and Agriculture, 84*, 556–560.

Piergiovanni, A. R., & Pignone, D. (2003). Effect of year-to-year variation and genotype on trypsin inhibitor level in common bean (*Phaseolus vulgaris* L) seeds. *Journal of the Science of Food and Agriculture, 83*, 473–476.

Saini, H. S. (1989). Activity and thermal inactivation of protease inhibitors in grain legumes. In: Huisman, J., van der Poel, T. F. B., Liener, I. E. (Eds.), Recent advances of research in Antinutritional factors in legume seeds (pp. 249–253). The Netherlands: Wageningen.

Saini, H. S., Weder, J. K. P., & Knights, E. J. (1992). Inhibitor activities of chickpeas (*Cicer arietinum* L.) against bovine, porcine and human trypsin and chymotrypsin. *Journal of the Science of Food and Agriculture, 60*, 287–295.

Savage, G. P. (1988). The composition and nutritional value of lentils (*Lens culinaris*). *Nutr. Abstr. Rev. (Ser.A), 58*, 319–343.

Singh, U., & Jambunathan, R. (1981). Studies on desi and kabuli chickpea (*Cicer arietinum* L.) cultivars: levels of protease inhibitors, levels of polyphenolic compounds and in vitro protein digestibility. *Journal of Food Science, 46*, 1364–1367.

Soni, S. G., Singh, T. P., & Singh, R. (1978). Comparative studies on the effect of certain treatments on antitrypsin activity of the common Indian pulses. *Indian Journal of Nutrition and Dietetics, 15*, 341–347.

Tan-Wilson, A. L., Chen, J. C., Duggan, M. C., Chapman, C., Obach, R. S., & Wilson, K. A. (1987). Soybean Bowman-Birk trypsin isoinhibitors: classification and report of a glycine-rich trypsin inhibitor class. *Journal of Agriculture and Food Chemistry, 35*, 974–981.

Trugo, L. C., Ramos, L. A., Trugo, N. M. F., & Souza, M. C. P. (1990). Oligosaccharide composition and trypsin inhibitor activity of *P. vulgaris* and the effect of germination on the α-galactoside composition and fermentation in the human colon. *Food Chemistry, 36*, 53–61.

Valdebouze, P., Bergeron, E., Gaborit, T., & Delort-Laval, J. (1980). Content and distribution of trypsin inhibitors and haemaglutinins in some legume seeds. *Canadian Journal of Plant Science, 60*, 695–701.

Vidal-Valverde, C., Frias, J., Estrella, I., Gorospe, M. J., Ruiz, R., & Bacon, J. (1994). Effect of processing on some antinutritional factors of lentils. *Journal of Agriculture and Food Chemistry, 42*, 2291–2295.

Weder, J. K. P., Hegarty, M. P., Holzner, M., & Kern-Dirndorfer, M. L. (1983). Trypsin and chymotrypsin inhibitors in *Leguminosae* VII. Isolation and some properties of the principal inhibitor from lentils (*Lens culinaris* Medik). *Zeitschrift Fur Lebensmittel-Untersuchung Nud-Forschung, 177*, 109–113.

Weder, J. K. P., & Kahley, R. (1998). Isolation and characterisation of four trypsin-chymotrypsin inhibitors from lentil seeds. *Journal of Science Food Agriculture, 78*, 429–434.

Weder, J. K. P., & Kahley, R. (2003). Reaction of lentil trypsin-chymotrypsin inhibitors with human and bovine proteinases. *Journal of Agriculture and Food Chemistry, 51*, 8045–8050.

Weder, J. K. P., Mueller, R., Mato, I. C., & Hegarty, M. P. (1985). Proteinase inhibitor activities and electrophoretic inhibitor patterns of lentils. *Lens Newsletter, 12*(1), 29–32.

Welham, T., & Domoney, C. (2000). Temporal and spatial activity of a promoter from a pea enzyme inhibitor gene and its exploitation for seed quality improvement. *Plant Science, 159*, 289–299.

Welham, T., O'Neill, M., Johnson, S., Wang, T. L., & Domoney, C. (1998). Expression patterns of genes encoding seed trypsin inhibitors in *Pisum sativum*. *Plant Science, 131*, 13–24.

Whitaker, J. R., & Sgarbieri, V. C. (1981). Purification and composition of trypsin/chymotripsin inhibitors of *Phaseolus vulgaris* var. Rosinha G2. *Journal of Food Biochemistry, 5*, 197–213.

Wu, C., & Whitaker, J. R. (1990). Purification and partial characterization of four trypsin/chymotrypsin inhibitors from red Kidney Beans (*Phaseolus vulgaris*, var. Linden). *Journal of Agriculture and Food Chemistry, 38*, 1523–1529.