Studies on the biological responses of rats to seed trypsin inhibitors using near-isogenic lines of *Pisum sativum* L (pea)

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Abstract: In order to determine the true antinutritional status of pea seed trypsin inhibitor (TI) proteins, pea lines are being produced that are near-isogenic except for the genetic locus, *Tri*, containing the TI structural genes. These lines are based on selection from the progeny of a cross between lines showing quantitative variation in TI content, as well as TI isoform and gene polymorphisms that serve as markers. Chemical analyses revealed that the composition of seeds from lines of each near-isogenic pair was extremely similar, except for a more than five-fold difference in TI content. Such lines provide material that is superior to the diverse lines previously used for nutritional assessment of pea TI. The specific biological effects of pea TI were studied by including the near-isogenic lines in standardised rat diets. The results indicated that TI content was correlated with a significant negative effect on protein digestibility and biological value. The difference in TI content of the pea seeds was reflected in the relative activity of pancreatic chymotrypsin whereas the activities of trypsin, lipase and amylase were less clearly affected.

Keywords: near-isogenic; pancreatic enzymes; *Pisum sativum* (pea); protein quality; rats; seeds; trypsin inhibitor

INTRODUCTION

In common with other legumes, the nutritional quality of pea seed protein is perceived as being influenced not only by the amino acid composition of the protein itself but also by the amounts of the antinutritional compounds that potentially can interfere with protein digestion in animals. In pea seeds the principal antinutritional compounds are the enzyme inhibitors, commonly referred to as trypsin inhibitors (TI). The effects of such proteins have been well-documented for soya bean seed TI in a number of animal species but these have been extrapolated to inhibitors from other legumes and to other animals, with a resulting limitation to the uses of protein from such legumes. Since inhibitor genes and proteins have become a focus of attention for plant protection strategies,[1] the real antinutritional effects of such proteins need to be determined to allay concerns over their introduction into crop plants.[2]

It is clear from analyses of the major pea inhibitors that they are homologous to the Bowman–Birk family of inhibitors.[3,4] Many of this family of inhibitors are simultaneous inhibitors of chymotrypsin and trypsin and all have a high content of disulphide bridges originating from cysteine. On the other hand, cysteine in these inhibitors can make an appreciable contribution to the total content of sulfur-containing amino acids, which are generally very low in legume seeds. However, the pea inhibitors have distinct characteristics, both in terms of their sequences and their behaviour in an in-vitro digestion system, when compared with the soya bean Bowman–Birk inhibitor.[4,5] Conflicting information on the antinutritional effects of pea TI has resulted from the comparison of genotypes of pea that differ not only in their TI content, but also in many other seed components, including storage proteins. Observed effects cannot be reliably attributed, therefore, to differences in TI content. The results of studies where purified TI proteins have been added to diets are useful[6] but can be criticised, as the TI are added in a different form to that in seed meal.

Genetic studies of the inheritance of qualitative and quantitative TI variants in pea[7] suggested that the production of near-isogenic lines differing only in quantitative expression of genes at the *Tri* locus,

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containing the seed-expressed TI genes, was possible. The use of such lines in feeding trials will permit more meaningful assessments of the effects of pea TI proteins in animals to be carried out.

This paper describes the production and analysis of initial selections of near-isogenic lines having high or low amounts of seed TI, and the biological responses in animals that can be specifically related to the difference in dietary pea TI from these near-isogenic lines.

MATERIALS AND METHODS

Plant material

The pairs of near-isogenic pea (*Pisum sativum* L) lines, HA5/LA5 and HB5/LB5, were derived through heterozygote selection from the progeny of a cross between JI 516 (round seed having high TI activity; TIA) and JI 868 (wrinkled seed having low TIA), respectively.[7] The selection of true heterozygotes among seeds with medium TIA was through genomic DNA analyses of leaf DNA, when the seeds had germinated, by hybridisation to a probe corresponding to seed TI;[7] heterozygosity was verified by analyses of segregating TI variants in the subsequent generation. Seeds having high or low TI were selected from the progeny of a single round seed that was heterozygous at the *Tri* locus at the F_5 generation; the selected seeds were verified as being homozygotes for seed-expressed TI genes (JI 516- and JI 868-like, respectively) and were bulked up to give rise to the near-isogenic lines, HA5 and LA5. Similarly, the selection of progeny from a single wrinkled seed, heterozygous at the *Tri* locus at the F_5 generation, gave rise to the near-isogenic lines, HB5 and LB5.

Soya bean seeds were obtained commercially from Central Soya Aarhus A/S, Denmark.

Feeding trials

Rat feeding trials were performed under essentially the same conditions as described by Eggum,[8] in an experiment designed to study seed protein quality of the pea lines. Four experimental diets, each containing unprocessed seed meal from one of the four pea lines as the only nitrogen (N) source, and three control diets containing unprocessed soya bean seeds, either alone or supplemented with methionine, or casein as the only source of N, were formulated to contain 93 g protein kg^{-1} diet. The N content of the diets was kept constant by adjusting with a N-free mixture consisting of 807 g autoclaved maize starch, 89 g sucrose, 52 g cellulose powder and 52 g rapeseed oil kg^{-1}. A group of five rats, each weighing approximately 68 g, was allocated to each of the seven diets. Every animal received 10 g dry matter (DM) and 150 mg N per day with free access to water supply. A preliminary four-day adaptation period to the experimental diets was followed by a four-day balance period, during which separate collection of urine and faeces was carried out. The balance period was followed by another three-day period with the same diets. Body weight and feed intake were monitored during the trial and balance period, respectively. At the end of the experiment, the animals were fasted for 12 h and then killed by asphyxiation. The pancreas was removed, dissected free of fat and weighed. The pancreas was stored at −20 °C for subsequent analyses.

True protein digestibility (TD) and biological value (BV) were determined for the different diets, and net protein utilisation (NPU) calculated as the product of TD and BV. Dry matter digestibility (DMD) in the diets was also determined.

Analytical methods

Bulked seeds from the near-isogenic pairs of lines were analysed for total protein profile by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and for TI isoform pattern on non-denaturing gels (PAGE) as described by Domoney *et al.*[3] Quantitative measurement of legumin and vicilin amounts was based on rocket immunoelectrophoresis of seed extracts. Meal was extracted in 0.05 M Tris-HCl, pH 8.0 containing 0.5 M NaCl and extracts analysed by electrophoresis in agarose gels containing antibodies to legumin or vicilin and by reference to purified protein standards analysed in the same gels.[9] Three extracts were prepared for every sample and analyses of total protein (Bio Rad assay kit), legumin and vicilin were performed in duplicate for every extract. For chemical analyses, DM content of seeds was determined by drying at 105 °C for 20 h and ash determined by the method of the Association of Official Analytical Chemists.[10] Nitrogen was analysed by the Kjeldahl method using a Kjell-Foss 16200 autoanalyser. Amounts of crude fat,[11] starch and low-molecular-weight sugars,[12] amino acids[13,14] and fatty acids[15] were determined for every sample. Gross energy was determined using a LECO AC 300 automated calorimeter system 789-500 (LECO, St Joseph, Michigan, USA). TI activities were determined by two methods;[16,17] a modified version of the quantitative assay described by the latter authors was employed, where seeds were extracted in 0.05 M HCl (1 ml 25 mg^{-1} meal). In-vitro enzyme digestibility of organic matter was determined as described by Boisen and Fernández.[18]

Pancreatic tissue was homogenised in 10 ml of buffer (0.1 M Tris-HCl, pH 7.9, 0.02 M $CaCl_2$) using a glass homogeniser and a Teflon pestle. In order to activate the inactive zymogens in the homogenate, enterokinase (500 µl; 1 mg ml^{-1} buffer) was added to each sample and the samples were incubated at 4 °C for 24 h. After activation, the samples were centrifuged (14000 *g* for 15 min at 4 °C) and the supernatant was used for determination of protein content and enzyme activities. Soluble protein and trypsin, chymotrypsin, amylase and lipase activities were determined as previously described.[19] All chemical analyses were performed in duplicate.

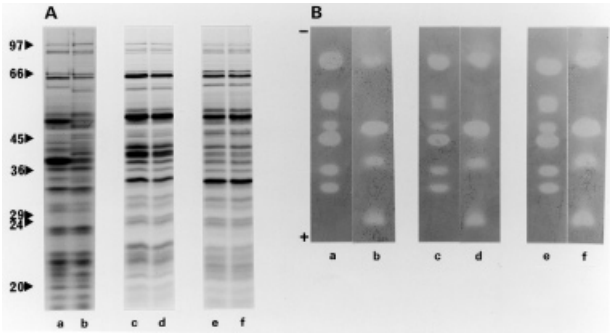

Figure 1. A: An analysis by SDS-PAGE of total protein from seeds of the lines (a) JI 516. (b) JI 868, (c) HA5, (d) LA5, (e) HB5 and (f) LB5, extracted in 0.05 M Tris-HCl, pH 8.0, containing 0.5 M NaCl; protein extracted from 0.3 mg meal is shown. The positions of molecular weight markers ($\times 10^{-3}$) are indicated with arrows. B: An analysis by PAGE of the TI isoform pattern of protein from seeds of the lines (a) JI 516, (b) JI 868, (c) HA5, (d) LA5, (e) HB5 and (f) LB5, extracted using 0.05 M HCl. Amounts loaded for L lines were eight times greater than for H lines. Gels were incubated in trypsin after electrophoresis and zones of inhibition detected as previously described.[3]

Statistical analysis

Results were subjected to a one-way analysis of variance using the SAS statistical program.[20] In cases where the overall effect was significant ($P < 0.05$), differences between the means were compared by the Fisher's least significant difference procedure.[21]

RESULTS

The development of the near-isogenic pairs of lines HA5/LA5 and HB5/LB5 resulted in lines of each pair having identical seed protein profiles on SDS-PAGE (Fig 1A), but with TIA (Table 1A) and TI isoform patterns characteristic of the high (H) or low (L) TI parent line, JI 516 and JI 868, respectively (Fig 1B). During development and selection of the near-isogenic lines, provided plants were not stressed, quantitative variation in TI activity co-segregated with qualitative variation in TI isoforms and TI gene polymorphisms, in agreement with previous observations.[7]

Chemical and biochemical analyses verified that seeds from the lines of each pair were otherwise very similar to each other in composition (Table 1A, B). Quantitative immunochemical analysis indicated that the lines contained very similar amounts of the storage proteins, legumin and vicilin (Table 1B). The differences between A and B lines that are evident in Table 1A reflect their round-and wrinkled-seeded status, with the latter having lower amounts of starch but higher amounts of low-molecular-weight sugars and marginally higher protein. An approximately five-fold difference in TI activity was apparent between the high and low TI lines of each pair (Table 1A), whether

Table 1A. Chemical analysis of seeds from two pairs of near-isogenic peas

	LA5	*HA5*	*LB5*	*HB5*
Dry matter ($g\,kg^{-1}$ seed meal)	918.9	921.9	923.6	927.6
$g\,kg^{-1}$ DM				
Ash	39.1	42.5	40.0	49.7
Crude protein (N × 6.25)	288.1	299.4	310.0	339.4
Crude fat	26.2	25.4	34.6	36.0
Starch + sugars	424.9	392.0	354.3	309.5
Low-molecular-weight sugars:				
Glucose	0.3	0.4	0.5	0.6
Fructose	0.3	0.4	0.5	0.6
Sucrose	36.3	46.9	58.4	78.1
Raffinose	1.8	2.2	4.3	3.3
Stachyose	15.9	19.9	23.0	30.4
Verbascose	29.5	23.0	27.8	38.1
Amino acids:				
Lysine	19.8	20.0	22.3	24.5
Methionine	2.7	2.5	3.0	3.2
Cystine	3.7	3.6	4.0	4.4
Threonine	10.4	10.2	12.1	12.9
Tryptophan	2.4	2.3	2.7	2.7
Isoleucine	12.0	11.8	13.1	14.3
Leucine	19.6	19.3	20.8	23.1
Histidine	7.3	7.2	7.8	8.2
Phenylalanine	12.8	12.3	13.5	15.1
Valine	13.6	13.5	15.0	16.5
Arginine	28.3	31.7	27.4	32.1
Fatty acids:				
C16:0	2.9	4.1	4.3	4.9
C18:0	0.8	0.9	1.1	1.1
C18:1	6.6	8.4	9.7	8.1
C18:2	6.8	6.2	9.8	10.4
C18:3	0.9	0.9	1.6	1.6
Other analyses				
Gross energy ($MJ\,kg^{-1}$ DM)	18.5	18.4	18.8	18.8
Trypsin inhibitor units ($U\,g^{-1}$ DM)[a]	0.5	2.7	0.6	3.9
Trypsin inhibitor units ($U\,mg^{-1}$ DM)[b]	1.5	8.7	1.8	7.4
EDOM (%)[c]	90.3	90.0	91.7	90.9

[a] One trypsin inhibitor unit is that which inhibits 1 mg trypsin[16].
[b] One trypsin inhibitor unit is that which gives a decrease in absorbance of 0.01 under standard assay conditions,[17] approximately equivalent to the inhibition of 2 μg trypsin.
[c] EDOM = enzyme digestibility of organic matter determined from an in-vitro digestibility assay.

Table 1B. Amounts of the major storage proteins, legumin and vicilin, in seeds from two pairs of near-isogenic peas, determined by immunoelectrophoresis

	LA5	*HA5*	*LB5*	*HB5*
Legumin ($g\,kg^{-1}$ DM)	47.3 (±4.5)	55.8 (±10.6)	53.6 (±10.4)	53.6 (±8.7)
Vicilin ($g\,kg^{-1}$ DM)	56.3 (±6.4)	57.0 (±9.8)	55.7 (±5.6)	64.7 (±3.8)
Legumin ($g\,kg^{-1}$ protein)	240.6 (±31.0)	289.8 (±59.2)	260.5 (±53.0)	262.4 (±42.9)
Vicilin ($g\,kg^{-1}$ protein)	289.4 (±33.9)	326.5 (±78.9)	270.2 (±23.9)	315.5 (±17.6)

Table 2. Body weight (g) at various stages of the trial and feed intake (g DM day^{-1}) during the balance period for the seven diets

	LA5	*HA5*	*LB5*	*HB5*	*SOYA*	*SOYA+MET*	*CASEIN*	*SEM*[1]
Initial	67.6	67.7	67.8	68.3	68.4	68.4	67.4	1.30
Beginning of balance	70.4^{b}	69.8^{bc}	70.9^{b}	69.7^{bc}	66.6^{c}	69.7^{bc}	80.2^{a}	1.25
End of balance	75.2^{cd}	70.6^{be}	78.3^{d}	74.2^{bc}	66.9^{e}	78.5^{fd}	93.0^{a}	1.30
At slaughter	71.9^{cd}	65.7^{de}	74.4^{bc}	67.4^{cde}	63.0^{e}	82.2^{b}	92.2^{a}	2.80
Feed intake[2]	7.5^{bc}	6.7^{bc}	9.7^{a}	7.5^{bd}	5.4^{c}	8.6^{ade}	9.8^{a}	0.46

[1] Standard error of the mean ($n=5$).
[2] Feed intake during the balance period.
Values in the same row with different superscripts are significantly different ($P<0.05$).

Table 3. Protein quality and dry matter digestibility (% of intake) of the experimental diets, as determined by a rat feeding trial

	*LA5**	*HA5**	*LB5*	*HB5**	*SOYA*	*SOYA+MET*	*CASEIN*	*SEM*[1]
TD	96.0^{b}	93.6^{c}	95.7^{b}	90.0^{d}	85.2^{e}	86.4^{e}	101.4^{a}	0.69
BV	68.3^{c}	55.0^{d}	68.8^{c}	55.6^{d}	60.5^{d}	82.5^{b}	95.6^{a}	2.47
NPU	65.5^{b}	51.4^{c}	65.9^{b}	50.1^{c}	51.6^{c}	71.3^{b}	96.9^{a}	2.35
DMD	90.7^{b}	89.8^{b}	89.5^{bc}	88.1^{c}	89.5^{bc}	90.4^{b}	92.9^{a}	0.50

TD: True protein digestibility. BV: Biological value. NPU: Net protein utilisation. DMD: Dry matter digestibility.
[1] Standard error of the mean ($n=5$) * ($n=4$). TD, SE=0.77; BV, SE=2.76; NPU, SE=2.63; DMD, SE=0.56. Values in the same row with different superscripts are significantly different ($P<0.05$).

determined as in Boisen and Djurtoft[16] or by the quantitative assay described by Domoney and Welham.[17] The absolute values determined by the two methods differ but both are given in Table 1A to avoid the difficulties that arise in comparing the results of different authors. When the appropriate conversions are performed (see Footnote to Table 1A), the two sets of determinations for pea TI are comparable. The soya bean seeds used in some experimental diets in the present work were determined to have 22.6 TI units[16] g^{-1} DM.

The initial body weight of the rats was similar among groups (Table 2), but differences were observed after only four days on the diets. By the end of the balance period, the rats fed the casein diet showed the highest body weight, followed by those fed the supplemented soya bean, the LB5 and LA5 diets, whereas feeding unsupplemented soya or peas with high levels of protease inhibitors (HA5 or HB5) resulted in lower body weights. The type of diet fed also affected feed intake during the balance period. The highest feed intake was observed when the rats were fed the casein diet, the supplemented soya bean diet, and the diet containing LB5, whereas the rats fed unsupplemented soya or HA5 showed a lower feed intake.

The results determined for protein quality and DM digestibility of the experimental diets are presented in Table 3. The TD of the diets containing LA5 and LB5 were similar, and decreased values were observed when feeding peas with high TIA. Moreover, the diet containing HB5 showed a lower TD than that containing HA5. Feeding unsupplemented soya beans resulted in a significantly lower TD compared to all the pea diets. The BV of LA5 and LB5 diets were similar (68.3–68.8%) and significantly lower values were measured when feeding HA5 and HB5 (55.0–55.6%). Supplementation of soya beans with methionine resulted in a considerable increase of the BV with respect to the unsupplemented diet.

There was no effect of TIA on DMD within pea lines. However, HB5 showed a lower digestibility than HA5. The diet containing casein showed the highest values for all the parameters measured. Supplementation of the soya bean diet with methionine did not increase the DM digestibility compared to the unsupplemented soya bean diet.

The effects of feeding diets with different protein source and TI content on pancreatic weight relative to body weight and the activities of digestive enzymes in the pancreatic tissue are shown in Table 4. The weight of the pancreas relative to the body weight was significantly higher in rats fed soya supplemented with methionine than in rats fed casein or peas, whereas no significant difference was observed between the rats fed unsupplemented soya beans and the other groups. Trypsin activities were highest when HA5 or HB5 were fed although the differences were not significant. Chymotrypsin activity tended to be higher in animals fed HA5 when compared to LA5 ($P=0.06$) and in those fed HB5 compared to LB5 ($P=0.05$). The activity of amylase was generally higher in the groups fed peas when compared to the other groups. The level of TI in the diets had no significant effect on the activity of lipase.

DISCUSSION

The production of near-isogenic lines, differing only at, or close to, the *Tri* locus that controls quantitative and qualitative variation in expression of TI genes, permits the effects of variation in TI content to be

Table 4. Effect of feeding diets with different protein source and TI content on weight of the pancreas relative to the body weight and the content of soluble protein and digestive enzymes in the pancreas

		LA5	*HA5*	*LB5*	*HB5*	*SOYA*	*SOYA+MET*	*CASEIN*	*SEM*[1]
Pancreas	($mg\,g^{-1}$ BW)	7.9^{bc}	8.8^{bc}	8.4^{bc}	8.1^{bc}	10.2^{ac}	11.7^{a}	9.1^{bc}	0.8
Soluble protein	($\mu g\,mg^{-1}$ pancreatic tissue)	59.1	60.4	56.0	62.4	61.9	66.9	56.9	4.4
Trypsin	($U \times 10^{-3} mg^{-1}$ pancreatic tissue)	2.2	3.6	2.1[2]	2.7	2.0[2]	1.2	1.5[3]	0.6
Chymotrypsin	($U \times 10^{-3} mg^{-1}$ pancreatic tissue)	57^{bc}	92^{ab}	59^{abc}	96^{a}	82^{ab}	64^{abc}	30^{c}	13
Amylase	($U\,mg^{-1}$ pancreatic tissue)	4.2^{ab}	2.9^{abc}	2.6^{abc}	5.5^{a}	0.6^{c}	0.9^{c}	1.9^{bc}	1.0
Lipase	($U \times 10^{-3} mg^{-1}$ pancreatic tissue)	198	186	300	202	152	366	330	64

[1] Standard error of the mean ($n=5$).

[2] $n=4$, SE = 0.7.

[3] $n=3$, SE = 0.8.

Values in the same row with different superscripts are significantly different ($P<0.05$).

assessed in animal diets, in isolation from effects caused by variation in other seed components. The near-identity of the lines within each pair was evident on total protein analyses (Fig 1A) and through chemical composition and biochemical analyses (Table 1). It is likely, therefore, that the differences in biological value observed in the results from feeding trials are attributable to the TI contents of the respective pea lines.

The protein quality of the diets containing peas with low levels of TI determined in the present study is in the range of those reported in the literature.[22,23] In similar studies, Christiansen and Larsen[22] observed an average TD of 91.7%, a BV of 66.7% and a NPU of 64.1% when investigating eight varieties of peas. Canibe *et al*[23] measured a BV of 67.0% when feeding rats with peas, cultivar Solara, with a TIA of 0.33 units[16] g^{-1} DM.

The digestibility of pea protein appears to be significantly reduced by the presence of pea TI, probably caused by inhibition of the pancreatic protease activity in the intestinal lumen with a consequent increased amount of pea protein escaping digestion. In agreement with this, the higher level of TI in HB5 compared to HA5, as measured by the method of Boisen and Djurtoft,[16] could account for the lower digestibility observed in diets containing the first. On the other hand, TI preparations have been shown to retard growth of rats when incorporated into a diet containing pre-digested proteins or free amino acids,[24] indicating that impaired intestinal proteolytic digestion of dietary protein is not the only factor to explain the TI effect. A high amount of endogenous N in the faeces due to an increased secretion of enzymes or a decreased proteolysis and absorption of endogenous protein in the small intestine could partially explain the results presented here.

The reduction of BV by higher TI levels in the pea diets could be due to a deficiency of the sulfur-containing amino acids in these diets. Pancreatic enzymes are rich in sulfur-containing amino acids.[25] Enhanced pancreatic secretions caused by TI divert methionine and cysteine to additional production of pancreatic enzymes. This diversion would further aggravate the sulfur amino acid deficiency of peas, with a consequent decrease of BV. The dramatic increase of BV of the soya diet after supplementation with methionine can be explained by this mechanism, although an improved amino acid balance itself due to methionine supplementation can result in a higher BV of soya. It has previously been observed in fasted rats that the activities of the proteases, trypsin and chymotrypsin, in pancreatic tissue increase upon feeding diets containing protease inhibitors.[26] By lowering the duodenal concentration of trypsin and chymotrypsin, the protease inhibitor triggers the cholecystokinin-mediated negative-feedback mechanism and increased amounts of the proteases are synthesised and secreted,[27] with consequent pancreatic hypertrophy in some cases.[28,29]

A significant enlargement of the pancreas weight relative to body weight was not detected in the present study when feeding peas or unsupplemented soya beans compared to casein. When soya beans were supplemented with methionine, hypertrophy was observed. The hypertrophy with respect to casein could be due to the presence of TI in soya whereas with respect to unsupplemented soya it could be explained by the more adequate supply of amino acids to meet the requirement of the pancreas to secrete more proteases and to maintain an adequate synthesis in the tissue. Gumbmann and Friedman[28] suggested that in animals fed raw soya bean diets, where the lack of sulfur amino acids is limiting growth, dietary supplements of L-cysteine and its derivatives may be utilised preferentially by the pancreas for the synthesis of new protein. The present results would partially support this suggestion since addition of methionine to the soya bean diet resulted in a higher pancreatic, relative to body, weight. On the other hand, the remarkable increase in BV observed after addition of methionine indicates that the more balanced amino acid profile was additionally used for body protein synthesis.

Pea protein is also characterised by low levels of sulfur-containing amino acids.[23] The lack of hypertrophic effect of the pea diets on the pancreas could be due to shortage of sulfur-containing amino acids, to

the lower level of protease inhibitors in the pea-containing diets compared to those containing soya beans and/or to the short period of time the animals were fed these diets. Gumbmann *et al*[30] observed increased pancreatic weights in rats fed a diet with high TIA for four weeks. In the present experiment the animals were fed the experimental diets for only 11 days and the TIA of the peas was low compared to that of raw soya beans, although sufficient to result in weight differences in the animals. The activity of chymotrypsin tended to change in accordance with the content of TI in the pea lines. Chymotrypsin has been shown to be more sensitive than trypsin to changes in protein source[31] and the results of the present study suggest a similar difference when introducing TI to the diet.

The higher amylase activities in the pancreatic tissue of rats fed the pea diets compared to those receiving soya bean or casein are probably due to the pea starch being more slowly digested and at a lower level than maize starch.[32]

The results reported here using the A5 and B5 lines should be reproduced ideally using lines derived from heterozygotes selected at a later generation when near-isogenicity will be even greater than in the lines utilised here. As a result of the close linkage [3–6 map units[7]] on linkage group V between *Tri* and *Vc*-2 (a genetic locus encoding a sub-set of vicilin polypeptides), heterozygosity at the *Vc*-2 locus has been maintained in the selection process to date (data not shown). It is possible, therefore, that the observed differences in biological value between lines having high and low TI contents (Table 3) can be attributed to residual variation at the *Vc*-2 or another closely linked locus. However, this seems unlikely since neither quantitative nor qualitative variation in the small vicilin polypeptides (M_r 12 000–30 000) that derive from the *Vc*-2 locus is apparent on SDS-PAGE (Fig 1A); quantitative immunochemical analyses have indicated that the near-isolines contain very similar amounts of vicilin. The production of near-isogenic lines based on the selection of seeds heterozygous for *Tri*, but homozygous for *Vc*-2, that result from a recombination event between *Tri* and *Vc*-2 offers superior material for nutritional assessment; the development of such lines is underway.

It can be concluded from these studies utilising near-isogenic lines that native pea TI affects various biological parameters when fed to rats. Ultimately, however, to be meaningful, the presence or absence of antinutritional effects should be demonstrated in appropriate target animal species as it is evident that the biological response of different animal species to antinutritional compounds may vary considerably due to their having distinct digestive enzymes[33] and extrapolation of results could be invalid. Relevant nutritional data will permit assessment of the consequences of high-level expression of particular inhibitor proteins in crops, whether for crop protection[34] or for their possible beneficial (anti-tumorigenic) properties.[35]

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