Nutritional evaluation of the trypsin (*EC* 3.4.21.4) inhibitor from cowpea (*Vigna unguiculata* Walp.)

BY A. PUSZTAI, G. GRANT, D. J. BROWN, J. C. STEWART AND S. BARDOCZ

The Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

AND S. W. B. EWEN

Department of Pathology, University of Aberdeen Medical School, Aberdeen

AND A. M. R. GATEHOUSE AND V. HILDER

Department of Biological Sciences, University of Durham, South Rd, Durham DH1 3LE

(*Received 17 June 1991 – Accepted 29 October 1991*)

The effect of feeding rats purified cowpea (*Vigna unguiculata* Walp.) trypsin (*EC* 3.4.21.4) inhibitor in a semi-synthetic high-quality diet based on lactalbumin (10 g inhibitor/kg) for 10 d was a moderate reduction in the weight gain of rats in comparison with controls, despite an identical food intake in the two groups. The reduction in the growth rate was about 20% on a live weight basis. However, the corresponding value calculated from the weight of dry carcasses was less, only about 7%, probably because the water content of the body of the two groups of rats was different. Although most of the cowpea trypsin inhibitor (CpTI) was rapidly broken down in the digestive tract, its inclusion in the diet led to a slight, though significant, increase in the nitrogen content of faeces but not of urine. Accordingly, the net protein utilization of rats fed on inhibitor-containing diets was also slightly depressed while their energy expenditure was elevated. In agreement with results obtained for the protease inhibitors of soya bean, the slight anti-nutritional effects of CpTI were probably due mainly to the stimulation of the growth and metabolism of the pancreas. Thus, the nutritional penalty for increased insect-resistance after the transfer of the cowpea trypsin inhibitor gene into food plants is slight in the short-term.

Trypsin inhibitor: Cowpea: Anti-nutritional effects: Rat

The cowpea (*Vigna unguiculata* Walp.) is a widely grown legume food crop of the tropics. Seeds of cowpea contain as much as 10 g/kg dry weight as protease inhibitors, predominantly inhibitors of trypsin (*EC* 3.4.21.4) -like enzymes (Gatehouse *et al.* 1979). These inhibitors have been characterized (Gatehouse *et al.* 1980) as members of the Bowman-Birk family of protease inhibitors (Richardson, 1981); i.e. polypeptides of approximately seventy amino acids, with a high proportion of cystine forming intramolecular disulphide bridges which are resistant to proteolysis. These polypeptides contain two separate binding sites for the inhibition of proteases which may be the same or two different enzymes. The amino acid sequence of one of the trypsin–chymotrypsin (*EC* 3.4.21.1) inhibitors of cowpea, and the isolation of cDNA clones encoding both trypsin–trypsin and trypsin–chymotrypsin inhibitors have been described (Hilder *et al.* 1989).

Extensive studies of the nutritional properties of protease inhibitors, especially of trypsin inhibitors from soya bean, have revealed that they have major anti-nutritional effects in single-stomached animals. The most important of these are a decrease in the growth rate of the animals and a damaging interference with pancreatic metabolism. However, some of the toxic effects attributed to trypsin inhibitors may have been due to contaminants

(principally lectins) in the preparations used for feeding trials. Investigation of the nutritional effects of trypsin inhibitors from sources that do not contain significant amounts of lectins, such as cowpea, is therefore a means of clarifying and extending previous results.

The use of a gene construct containing a coding sequence for cowpea trypsin inhibitor (CpTI) in the production of transgenic plants with enhanced insect resistance (Hilder *et al.* 1987) has demonstrated that protease inhibitors can have significant effects on insect metabolism, confirming earlier results of bioassays with artificial diets. It has been suggested that transgenic crops containing CpTI could provide a means of protection against insect attack that is both non-damaging to the environment and acceptable to the consumer. Cowpeas are already widely used in diets for humans and other mammals and this suggests that trypsin inhibitors in these seeds do not have major adverse nutritional effects. The present paper describes feeding trials designed to evaluate more critically the nutritional effects of this inhibitor.

MATERIALS AND METHODS

Purification of CpTI

A CpTI preparation which contained a mixture of isoinhibitors of various specificities but no other protein components was prepared by affinity chromatography on a trypsin–Sepharose column, by a scaled up version of the method described previously (Gatehouse *et al.* 1980). Cowpeas (200 g) were ground to a fine powder in a rotary mill and extracted in 500 ml buffer 1 (0·1 M-sodium acetate, pH 4·0, containing 0·3 M-sodium chloride, 0·01 M-calcium chloride) overnight with stirring at 15°. After centrifugation (12000 ***g***, 20 min) at 15° the clear supernatant fraction was loaded onto a column of trypsin–Sepharose (500 ml volume, 32 mm diameter) containing approximately 1·2 g trypsin coupled to Sepharose-4B by activation with cyanogen bromide. The column was washed with 640 ml buffer 1, followed by 600 ml buffer 2 (0·3 M-NaCl, 0·01 M-$CaCl_2$) before elution with 800 ml buffer 3 (0·01 M-hydrochloric acid, containing 0·3 M-NaCl, 0·01 M-$CaCl_2$). The eluted peak of trypsin inhibitors was detected by u.v. absorbance, pooled and precipitated by addition of solid ammonium sulphate to 95% saturation (65 g/l). The precipitate was collected by centrifugation (12000 ***g***, 20 min, 4°), redissolved in approximately 10 ml 0·1 M-ammonium bicarbonate and desalted by passage through a column of Sephadex G-25. The pooled and lyophilized protein peak yielded 150–200 mg trypsin inhibitor. Analysis by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis confirmed its purity (results not shown). Batches of inhibitor, produced by repeating the procedure described previously, were combined and used for rat diets.

Animals and diets

Male rats of the Hooded Lister (Rowett) strain were weaned at 19 d of age and fed on stock diet (Biosure; Special Diets Services, Manea, Cambridgeshire) for 10 d. Groups of four rats per treatment, matched by weight (86·3 (SD 1·0) g), were housed singly in metabolism cages with plastic separators for urine and faeces collection, as described previously (Rubio *et al.* 1991). The amount of diets fed to the rats was restricted to a total food consumption of 80·0 g per animal for the 10 d experimental period. Distilled water was available *ad lib.* The animals were weighed and urine and faeces were collected daily. Faeces samples were freeze-dried and ground for analyses.

The semi-synthetic control diet used in the trial was based on lactalbumin (LA; 100 g protein/kg). The test diet contained 10 g CpTI/kg and 90 g lactalbumin protein/kg. The composition of the diets is given in Table 1. For calculation of approximate net protein

utilization (NPU) values a group of four rats was also fed on a protein-free diet (NPC). However, the voluntary feed intake of these rats was only 55·4 g over 10 d and, therefore, they could not be paired with the test or the control (LA) animals.

Chemical and other analyses

Protein. Diets, faeces, urines and carcasses were analysed for total nitrogen (Davidson *et al.* 1970). Urine samples were analysed for urea and creatinine by standard Technicon methods using a modified carbamido-diacetyl reaction and the Jaffe reaction respectively (Technicon Instruments Corporation, 1963*a*, *b*). Ammonia was determined by a standard Unicam automated method (Unicam Instruments Ltd 1960). Free α-amino-N was determined by reaction with 2,4,6-trinitrobenzene sulphonate (Palmer & Peters, 1969). All the assays were carried out by the Analytical Services Department of The Rowett Research Institute using Technicon AutoAnalyzers. SDS–polyacrylamide gel electrophoresis was performed by a method similar to that described by Laemmli (1970).

Pancreas samples were extracted with perchloric acid (100 g/l; 15 mg tissue/ml) for 30 min at 0° and the supernatant fraction was used for polyamine determinations (Seiler & Knödgen, 1980). The residue solubilized in 0·3 M-sodium hydroxide was used for protein estimation by a modified Lowry method (Schachterle & Pollack, 1973). The remainder of the alkaline solution was incubated at 37° for 90 min, protein and DNA were precipitated by perchloric acid (Johnson & Chandler, 1973) and the ribose content of the supernatant fraction was determined by the orcinol reaction (Sneider, 1957). The protein and DNA precipitate was resuspended in perchloric acid (50 g/l) and heated at 80° for 45 min. The protein precipitate was removed by centrifugation and the deoxyribose content of the supernatant fraction was measured by the diphenylamine reagent (Lovtrup, 1962). Bovine serum albumin, calf liver RNA and calf thymus DNA were used as protein, RNA and DNA standards respectively.

The concentration of 3-hydroxybutyrate in serum was measured by Technicon AutoAnalyzer (Zivin & Snarr, 1973).

Organ and tissue weight. At the end of the 10 d experimental period the animals were fasted overnight, refed with 1·5 g diet, killed 2 h later and dissected. The stomach, intestines, pancreas, kidneys, liver, adrenals, spleen, thymus and the hind-leg muscles, soleus, plantaris and gastrocnemius were excised, blotted dry and weighed. For the determination of dry weights the rat carcasses and tissues were freeze-dried and dried to constant weight.

The degree of survival of immunoreactive CpTI in the small intestine was estimated in both acute and chronic trials. In the acute test, 8·8 mg CpTI dissolved in 1 ml saline (9 g NaCl/l) was administered by intragastric intubation to a single rat. After 1 h the rat was killed, its stomach and small intestine were removed and washed. To control further proteolytic breakdown the stomach contents were washed out with about 10 ml 0·05 M-phosphate buffer in saline, pH 7·6. The buffer used for washing the small intestine also contained 0·1 mg aprotinin/ml (Sigma). In the chronic test, four rats fed for 10 d on the test CpTI diet were given 1·5 g of the same diet containing 15 mg trypsin inhibitor 2 h before being killed. The procedures used for rinsing were the same as in the acute test. The freeze-dried contents of the washing solutions were reconstituted with water to about 40 μg protein/ml (Bradford, 1976) and the CpTI content of the samples were determined by dot immunobinding assay (Jahn *et al.* 1984) at duplicate loadings of 0·4 and 4 μg protein in each sample using rabbit anti-CpTI primary antiserum and donkey ^{125}I-labelled anti-rabbit IgG (Amersham International plc, Amersham, Bucks.) secondary antibody.

Table 1. *Composition of diets (g/kg)*

Diet...	Lactalbumin	Cowpea (*Vigna unguiculata*) trypsin inhibitor*	Protein-free
Ingredients			
Maize starch	382	383	500
Potato starch	100	100	100
Glucose	150	150	150
Maize oil	150	150	150
Vitamins + minerals	100	100	100
Silicic acid	0·4	0·4	0·4
Lactalbumin	118	107	—
Cowpea trypsin inhibitor	—	10	—

* Supplemented with amino acids (g/kg diet): tyrosine 0·2, phenylalanine 0·2, methionine 0·3, leucine 0·4.

Table 2. *Nutritional performance of rats fed on diets containing cowpea* (Vigna unguiculata) *trypsin inhibitor and control diets*

(Mean values and standard deviations for four rats/treatment)

Diet*...	Lactalbumin		Cowpea trypsin inhibitor		Protein-free	
	Mean	SD	Mean	SD	Mean	SD
Wt change (g/rat per 10 d)	$23·9^{a}$	1·8	$18·6^{b}$	2·0	$-13·6^{c}$	0·3
Food intake (g/10 d)	$80·0^{a}$	0·0	$80·0^{a}$	0·0	$55·4^{b}$	3·0
Nitrogen intake (g/10 d)	1·29	—	1·27	—	0·03	
Faeces (g/10 d)	$5·03^{a}$	0·22	$5·35^{a}$	0·89	$3·35^{b}$	0·43
Faecal N (g/10 d)	$0·18^{a}$	0·01	$0·27^{b}$	0·02	$0·12^{c}$	0·01
Urine (ml/10 d)	$19·0^{a}$	2·5	$17·6^{a}$	3·1	$9·1^{b}$	2·0
Urinary N (mg/10 d)	111^{a}	18	134^{a}	19	$81^{a,b}$	13
Urea-N (mg/10 d)	12^{a}	2	24^{a}	10	$28^{a,b}$	5
Ammonia-N (mg/10 d)	13^{a}	3	14^{a}	2	$7^{a,b}$	2
Creatinine-N (mg/10 d)	4^{a}	1	5^{a}	1	3^{a}	1
α-Amino-N	3^{a}	1	3^{a}	1	2^{a}	1
Urinary 3-hydroxybutyrate (μmol/10 d)	$63·2^{a}$	9·0	$177·4^{b}$	27·0	$13·9^{c}$	2·4
Urinary glucose (mg/10 d)	$10·2^{a}$	2·6	$9·2^{a}$	1·9	$7·4^{a}$	1·6
Dry body-wt (g)	$32·35^{a}$	0·26	$29·91^{b}$	0·40	$21·81^{c}$	0·26
Carcass N (g)	$3·30^{a}$	0·11	$3·07^{a}$	0·06	$2·15^{b}$	0·08
N balance (g/10 d)	$1·00^{a}$	0·01	$0·87^{b}$	0·02	$-0·17^{c}$	0·01
Net protein utilization	$0·93^{a}$	0·06	$0·75^{b}$	0·04	—	—
N digestibility	$1·00^{a}$	0·00	$0·93^{b}$	0·02	—	—
Biological value	$0·93^{a}$	0·06	$0·80^{b}$	0·05	—	—

^{a,b,c} Values in the same row with different superscript letters were significantly different ($P < 0·01$).

* For details, see Table 1.

RESULTS

Nutritional data

The presence of CpTI in the diet depressed the live-weight gain of the rats by about 20% (Table 2). Similarly, the NPU value of this diet was about 20% less than that of the lactalbumin control diet. This reduction in growth occurred despite the identical food intakes in the two groups of rats. Although the weight of faeces in the two groups was

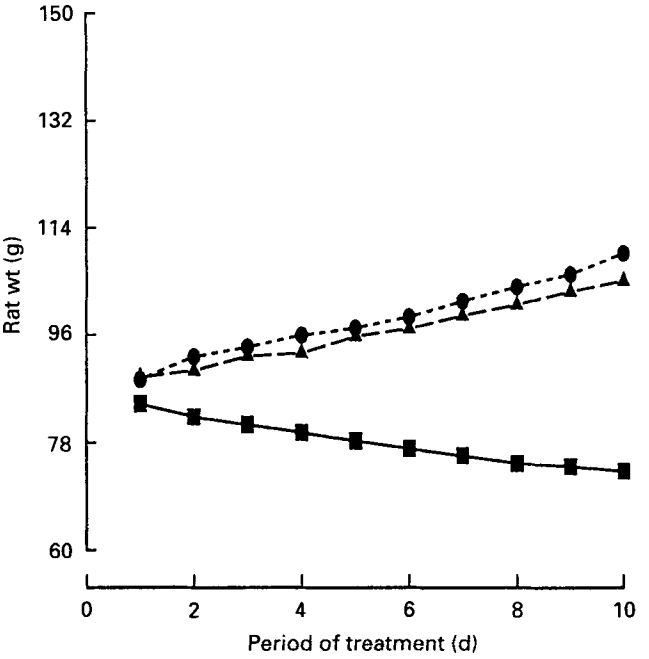

Fig. 1. Growth curve of rats fed on diets containing cowpea (*Vigna unguiculata*) trypsin inhibitor (10 g/kg) + 90 g lactalbumin/kg (▲) and control diets: protein-free (■) and 100 g lactalbumin protein/kg (●). For details of diets, see Table 1.

similar, the faecal N loss of the CpTI group was significantly higher. However, after correction for N loss of rats fed on protein-free diet the true N digestibility of the CpTI group was only about 7% less than that of the controls (Table 2).

Rats in the CpTI group lost slightly, though not significantly, more N through urine than the control (LA) group. Urinary urea was also slightly higher in the CpTI group, but other N-containing components were similar in both groups (Table 2). The overall net N gain of the CpTI group was about 13% less than that of the control (LA) group. The urine of rats in the CpTI group contained nearly three times as much 3-hydroxybutyrate as that of the LA controls, while the urinary glucose contents in the two groups were similar.

Body and tissue weights

The dry body-weight of rats given CpTI was 7% less than that of the controls, with virtually identical body N concentration in both groups (Table 2).

Both the small intestine and the pancreas of the CpTI group were about 30% heavier on a wet tissue weight (g/100 g live weight) basis than those of the LA controls (results not given). No other tissues were significantly affected by the presence of CpTI in the diet. However, after drying the tissues and the body, the difference between the two groups in the weight of the small intestine (g/100 g body-weight) was reduced to only 10%. In contrast, the dry weight of pancreas (g/100 g dry body-weight) of the CpTI group was still about 30% above that of the control (LA) group, with correspondingly higher contents of protein, RNA, DNA and polyamines in the CpTI group (Table 3).

Recovery of CpTI from the small intestine

The total CpTI content of the stomach in the acute experiment was less than 0·5 μg and was just detectable. From the small intestine 88·4 μg immunoreactive CpTI was recovered,

Table 3. *The weight and composition of the pancreas of rats fed on diets containing cowpea* (Vigna unguiculata) *trypsin inhibitor and control diets*

(Mean values and standard deviations for four rats/treatment, calculated for total tissue)

Diet*...	Lactalbumin		Cowpea trypsin inhibitor		Protein-free	
	Mean	SD	Mean	SD	Mean	SD
Dry wt of pancreas (g/100 g dry body-wt)	0·44[a]	0·02	0·58[b]	0·04	0·35[c]	0·03
Protein (mg)	79[a]	3	89[a]	5	39[b]	4
RNA (mg)	10·2[a]	0·7	17·2[b]	1·8	4·1[c]	0·7
DNA (mg)	2·0[a]	0·1	2·4[a]	0·3	1·7[a,b]	0·2
Polyamines						
Putrescine (nmol)	31[a]	5	33[a]	7	28[a]	9
Spermidine (nmol)	2303[a]	198	2691[a]	257	1449[b]	280
Spermine (nmol)	479[a]	36	675[b]	64	148[c]	18
Dry wt of small intestine (g/100 g dry body-wt)†	2·40[a]	0·10	2·63[b]	0·05	2·24[a]	0·12

[a,b,c] Values with different superscript letters in the same row were significantly different ($P < 0·01$).

* For details, see Table 1.

† Values for the small intestine are given for comparison.

Table 4. *Recovery of immunoreactive cowpea* (Vigna unguiculata) *trypsin inhibitor (CpTI) from the stomach and the small intestine of rats on the last day of a 10 d feeding experiment with CpTI (10 g/kg diet), 2 h after dosing rats with 15 mg inhibitor*

(Mean values and standard deviations)

	Stomach		Small intestine	
	Mean	SD	Mean	SD
Protein* (mg)	1·25	0·45	8·67	1·06
Trypsin inhibitor* (μg)	380	204	276	44
Recovery	0·025		0·018	

* Total content.

giving a survival rate of 1·0% overall. The recoveries of CpTI from the stomach and the small intestine in the corresponding tests carried out after feeding rats for 10 d (Table 4) were somewhat higher, giving an average survival rate of 4·3 (SD 1·3)%.

DISCUSSION

Protease inhibitors have long been known to interfere with the proper nutritional utilization of proteins (Pusztai, 1967; Liener & Kakade, 1980; Gallaher & Schneeman, 1984). However, most of these studies have been carried out with the Kunitz or Bowman-Birk inhibitors obtained from soya bean.

Cowpeas and other members of the *Vigna* family contain high and variable levels of trypsin inhibitors (Gatehouse *et al.* 1979; Kochar *et al.* 1988). Their biological importance is underlined by the observation that seeds containing high concentrations of protease

inhibitors have increased resistance to insect predation (Gatehouse *et al.* 1979; Gatehouse & Boulter, 1983).

The nutritional effects of cowpea seeds on rats have been evaluated previously. The results indicated that, although the lectin content was low, the presence of other antinutritional factors and the deficiency of methionine in the protein components of the seed depressed its nutritional value. Typically, proteins from different cowpea cultivars gave NPU values of 0·37 in rat assays (Grant *et al.* 1983). These results have recently been confirmed and it has also been shown that amino acid supplementation to target levels (Coates *et al.* 1969) increased NPU and digestibility values of the seed proteins to only about 0·67 (A. Pusztai, G. Grant and A. M. R. Gatehouse, unpublished results).

It is difficult to evaluate the specific influence of trypsin inhibitors on the nutritional value of diets from experiments with seed meals or partially purified fractions containing protease inhibitors. Therefore, in the present study the effect on rats of inclusion of purified CpTI on the nutritional value of a lactalbumin-based semi-synthetic diet was tested at a concentration (10 g/kg diet) which is similar to that encountered in cowpea-based diets. The main finding was that CpTI caused only slight depression of weight gain by the rats (Table 2). Although the reduction in live-weight gain was about 20%, the corresponding reduction in carcass dry weight was only about 7% and, therefore, similar to the reduction of 7% in protein digestibility caused by CpTI. Although the difference in carcass N between the two groups was about the same as that in dry body-weight, it did not reach significance at the level of $P < 0{\cdot}01$. The urine N loss with CpTI in the diet was also fractionally, but not significantly, higher. However, the difference of about 13% in N balance between the CpTI and control rats over the 10 d period was significant and reflected the differences in the N content of the body, faeces and urine. The 3-hydroxybutyrate content of urine of the CpTI group was also almost three times higher than that of the LA control suggesting a higher energy metabolism in these rats.

It is tempting to suggest that the reduction by about 10% in the nutritional value of a lactalbumin-based diet by the inhibitor was the result of the substitution of one-tenth of the lactalbumin with the corresponding amount of an indigestible ballast such as CpTI. Indeed, reduction of the protein concentration in the diet from 100 g/kg to 90 g/kg depressed the growth of rats by about 7% (A. Pusztai, unpublished results). However, the CpTI clearly did not behave as a ballast as it was extensively hydrolysed during its passage in the digestive tract (Table 4). Therefore, as the CpTI diet of the present study also contained 100 g well-digestible protein/kg the reduction in weight gain in the presence of the inhibitor could not be explained by differences of the protein intake of the two groups. It is more likely that, as with other Bowman-Birk inhibitors, (Abbey *et al.* 1979*a*, *b*; Madar *et al.* 1979; Friedman, 1986), the effects of CpTI are transitory and not due directly to its interference with the digestion of dietary proteins.

The most striking consequence of the inclusion of CpTI in the diet of rats for 10 d was the enlargement of the pancreas by about 30%, with corresponding increases in its protein, RNA, DNA and polyamine contents (Table 3). Accordingly the N loss was probably the result of increased metabolism of this organ leading to hypersecretion of pancreatic enzymes rich in sulphur-containing amino acids. The increased polyamine content of the pancreas, particularly the elevated spermidine and spermine levels, was in full accord with such an increased metabolic activity and the hypertrophic growth of the tissue (Bardocz *et al.* 1989). Although the secreted pancreatic enzymes are in part recycled and re-absorbed from the small intestine, this process is unlikely to be 100% efficient and increased loss of endogenous N will occur.

The CpTI gene has recently been successfully transferred into plants, which then became more resistant to insect attack (Hilder *et al.* 1987). The success of this gene transfer and the

expectation that transgenic crop plants expressing appreciable levels of CpTI will increasingly find their way into food and feedstuffs underline the importance of the present study. It is clear that although there is a nutritional penalty for the gains in insect resistance obtained with the transfer of the CpTI gene into food crop plants, this is likely to be modest, at least in the short-term. The negative effects of the presence of CpTI on the nutritional value of food or feeds may be further reduced by heat processing. However, long-term effects on nutrition and health of continuous dietary exposure to CpTI are still rather unpredictable at present.

REFERENCES

Abbey, B. W., Neale, R. J. & Norton, G. (1979*a*). Nutritional effects of field bean (*Vicia faba* L.) proteinase inhibitors fed to rats. *British Journal of Nutrition* **41**, 31–38.

Abbey, B. W., Norton, G. & Neale, R. J. (1979*b*). Effects of dietary trypsin inhibitors from field bean (*Vicia faba* L.) and field bean meal on pancreatic function in the rat. *British Journal of Nutrition* **41**, 39–45.

Bardocz, S., Grant, G., Brown, D. S., Ewen, S. W. B. & Pusztai, A. (1989). Involvement of polyamines in *Phaseolus vulgaris* lectin-induced growth of rat pancreas *in vivo*. *Medical Science Research* **17**, 309–311.

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.

Coates, M. E., O'Donoghue, P. N., Payne, P. R. & Ward, R. J. (1969). *Laboratory Animal Handbooks. 2. Laboratory Standards for Laboratory Rats and Mice*, p. 15. London: Laboratory Animals Ltd.

Davidson, J., Mathieson, J. & Boyne, A. W. (1970). The use of automation in determining nitrogen by the Kjeldahl method, with final calculations by computer. *Analyst* **95**, 181–193.

Friedman, M. (1986). *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods*, [M. Friedman, editor]. New York and London: Plenum Press.

Gallaher, D. & Schneeman, B. O. (1984). Nutritional and metabolic response to plant inhibitors of digestive enzymes. In *Nutritional and Metabolic Aspects of Food Safety. Advances in Experimental Medicine and Biology*, pp. 299–320 [M. Friedman, editor]. New York and London: Plenum Press.

Gatehouse, A. M. R. & Boulter, D. (1983). Assessment of the antimetabolic effects of trypsin inhibitors of cowpea (*Vigna unguiculata*) and other legumes on the development of the bruchid beetle *Callosobruchus maculatus*. *Journal of Science of Food and Agriculture* **34**, 345–350.

Gatehouse, A. M. R., Gatehouse, J. A. & Boulter, D. (1980). Isolation and characterisation of trypsin inhibitors from cowpea (*Vigna unguiculata*). *Phytochemistry* **19**, 751–756.

Gatehouse, A. M. R., Gatehouse, J. A., Dobie, P., Kilminster, A. & Boulter, D. (1979). Biochemical basis of insect resistance in *Vigna unguiculata*. *Journal of Science of Food and Agriculture* **30**, 948–958.

Grant, G., More, L. J., McKenzie, N. H., Stewart, J. C. & Pusztai, A. (1983). A survey of the nutritional and haemagglutination properties of legume seeds generally available in the UK. *British Journal of Nutrition* **50**, 207–214.

Hilder, V. A., Barker, R. F., Samour, R. A., Gatehouse, A. M. R., Gatehouse, J. A. & Boulter, D. (1989). Protein and cDNA sequences of Bowman-Birk protease inhibitors from cowpea (*Vigna unguiculata* Walp.). *Plant Molecular Biology* **13**, 701–710.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. & Boulter, D. (1987). A novel mechanism of insect resistance engineered into tobacco. *Nature* **330**, 156–159.

Jahn, R., Schliebler, W. & Greengard, P. A. (1984). A quantitative dot immunobinding assay for proteins using nitrocellulose membrane filters. *Proceedings of the National Academy of Sciences U.S.A.* **81**, 1684–1687.

Johnson, L. R. & Chandler, A. M. (1973). RNA and DNA of gastric and duodenal mucosa in antrectomized and gastrin-treated rats. *American Journal of Physiology* **224**, 937–940.

Kochar, N., Walker, A. F. & Pike, D. J. (1988). Effect of variety on protein content, amino acid composition and trypsin inhibitor activity of cowpeas. *Food Chemistry* **29**, 65–78.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227**, 680–685.

Liener, I. E. & Kakade, M. L. (1980). Protease inhibitors. In *Toxic Constituents of Plant Foodstuffs*, 2nd ed., pp. 7–71 [I. E. Liener, editor]. New York: Academic Press.

Lovtrup, S. (1962). Chemical determination of DNA in animal tissues. *Acta Biochimica Polonica* **9**, 411–424.

Madar, Z., Gertler, A. & Birk, Y. (1979). The fate of the Bowman-Birk trypsin inhibitor from soybeans in the digestive track of chicks. *Comparative Biochemistry and Physiology* **62**A, 1057–1061.

Palmer, D. W. & Peters, T. Jr. (1969). Automated determination of free amino groups in serum and plasma using 2,4,6-trinitrobenzene sulfonate. *Clinical Chemistry* **15**, 891–901.

Pusztai, A. (1967). Trypsin inhibitors of plant origin, their chemistry and potential role in animal nutrition. *Nutrition Abstracts and Reviews* **37**, 1–9.

Richardson, M. (1981). Plant protein inhibitors of enzymes; their role in animal nutrition and plant defence. *Journal of Biological Education* **15**, 178–182.

Rubio, L. A., Grant, G. Bardocz, S., Dewey, P. & Pusztai, A. (1991). Nutritional response of growing rats to faba beans (*Vicia faba* L., minor) and faba bean fractions. *British Journal of Nutrition* **66**, 533–542.
Schachterle, G. R. & Pollack, R. L. (1973). A simplified method for the quantitative assay of small amounts of protein in biological material. *Analytical Biochemistry* **51**, 654–655.
Seiler, N. & Knödgen, B. (1980). HPLC procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. *Journal of Chromatography* **221**, 227–235.
Sneider, W. C. (1957). Determination of nucleic acids in tissue by pentose analysis. *Methods in Enzymology* **3**, 680–684.
Technicon Instruments Corporation (1963*a*). *Creatinine*; Technicon AutoAnalyzer Method File, N-11a.
Technicon Instruments Corporation (1963*b*). *Urea nitrogen*; Technicon AutoAnalyzer Method File, N-11c.
Unicam Instruments Ltd. (1960). *Ammonia*; Methodology Sheet, No. 51.
Zivin, J. A. & Snarr, J. F. (1973). An automated colorimetric method for the measurement of 3-hydroxybutyrate. *Analytical Biochemistry* **52**, 456–461.

Printed in Great Britain