

Nutrient Interactions and Toxicity

Expression of the Insecticidal Bean α -Amylase Inhibitor Transgene Has Minimal Detrimental Effect on the Nutritional Value of Peas Fed to Rats at 30% of the Diet^{1,2}

A. Puzstai,³ G. Grant S. Bardocz, Ruben Alonso,* M. J. Chrispeels,[†] H. E. Schroeder,** L. M. Tabe** and T.J.V. Higgins**

Rowett Research Institute, Bucksburn Aberdeen AB21 9SB, Scotland, UK; *Department of Animal Physiology and Nutrition, Public University of Navarra, 31006 Pamplona, Spain; [†]Department of Biology, University of California, San Diego, La Jolla, CA 92093-0116; and **CSIRO Plant Industry, Canberra ACT 2601, Australia

ABSTRACT The effect of expression of bean α -amylase inhibitor (α -AI) transgene on the nutritional value of peas has been evaluated by pair-feeding rats diets containing transgenic or parent peas at 300 and 650 g/kg, respectively, and at 150 g protein/kg diet, supplemented with essential amino acids to target requirements. The results were also compared with the effects of diets containing lactalbumin with or without 0.9 or 2.0 mg bean α -AI, levels equivalent to those in transgenic pea diets. When 300 and 650 g peas/kg diet were fed, the daily intake of α -AI was 11.5 or 26.3 mg α -AI, respectively. At the 300 g/kg level, the nutritional value of the transgenic and parent line peas was not significantly different. The weight gain and tissue weights of rats fed either of the two pea diets were not significantly different from each other or from those of rats given the lactalbumin diet even when this was supplemented with 0.9 g α -AI/kg. The digestibilities of protein and dry matter of the pea diets were slightly but significantly lower than those of the lactalbumin diet, probably due to the presence of naturally occurring antinutrients in peas. The nutritional value of diets containing peas at the higher (650 g) inclusion level was less than that of the lactalbumin diet. However, the differences between transgenic and parent pea lines were small, possibly because neither the purified recombinant α -AI nor that in transgenic peas inhibited starch digestion in the rat small intestine in vivo to the same extent as did bean α -AI. This was the case even though both forms of α -AI equally inhibited α -amylase in vitro. Thus, this short-term study indicated that transgenic peas expressing bean α -AI gene could be used in rat diets at 300 g/kg level without major harmful effects on their growth, metabolism and health, raising the possibility that transgenic peas may also be used at this level in the diet of farm animals. *J. Nutr.* 129: 1597-1603, 1999.

KEY WORDS: • *transgenic peas* • *α -amylase inhibitor* • *nutritional value* • *growth* • *rats*

The transgenic approach to crop protection provides a novel alternative to the use of chemical pesticides and insecticides (Gatehouse and Gatehouse 1997). It offers more flexibility and promises less harm for the environment and consumers than the heavy land use of chemicals. Genes used to make pest-resistant transgenic crops are usually selected because their products in the plant will be harmful to target insects and pests by interfering with their digestion/absorption and/or reproduction, but will not harm beneficial insects or mammalian consumers. Thus, genes encoding lectins and enzyme inhibitors appear to be advantageous because their effects on the insect gut can be verified and their protective effect for the plant quantified. Indeed there has been a rapid increase in the number of major crop plants that have been made pest-

resistant by gene technology. However, there is some public concern about their release for cultivation because their effects on the environment and consumers have not been documented, particularly in the long term. Although some of the agronomic concerns have been addressed, published studies assessing the nutritional consequences of the consumption of diets based on transgenic plants are rare.

The pea is one of the world's major pulses; it is used as both food and stockfeed and is an important component in sustainable agriculture. It is well digested and has a high energy content (Savage and Deo, 1989). The lectin content of peas is low (Trowbridge, 1974); the trypsin inhibitor content is moderate (Richardson, 1991). α -Amylase inhibitor (α -AI),⁴ which occurs naturally in many food plants (Buonocore and Silano 1986), is absent in peas (Grant et al. 1995). This may explain at least in part why peas are vulnerable to insect

¹ Supported by the Scottish Office of Agriculture, Environment and Fisheries Department. M.J.C. is indebted to the U.S. Department of Agriculture for financial support.

² The development of transgenic peas was supported by the Grains Research and Development Corporation.

³ To whom correspondence should be addressed.

⁴ Abbreviations used: α -AI, α -amylase inhibitor; α -AI-LA, diets containing LA plus varying amounts of α -AI; LA, lactalbumin diet; P-PEA, pea diets containing varying amounts of parent line/kg; TR-PEA, transgenic pea diets containing varying amounts of transgenic pea/kg.

TABLE 1
Composition of experimental diets¹

	Diet						
	1 Control (LA)	2 α -AI ₀₉ -LA	3 P-PEA ₃₀₀	4 TR-PEA ₃₀₀	5 α -AI ₂₀ -LA	6 P-PEA ₆₇₄	7 TR-PEA ₆₅₀
	g/kg						
Pure α -AI	0	0.9	0	0	2.0	0	0
Pea	0	0	300	0	0	674	0
Transgenic pea	0	0	0	300	0	0	650
Lactalbumin	180	179	100	98	178	0	0
Cornstarch	320	320	100	102	320	0	0
Potato starch	100	100	100	100	100	0	0
Glucose	150	150	150	150	150	76	100
Corn oil	150	150	150	150	150	150	150
Minerals ²	50	50	50	50	50	50	50
Vitamins ³	50	50	50	50	50	50	50
L-Tryptophan	0	0	0.4	0.4	0	0.8	0.8
L-Methionine	0	0	2.0	2.0	0	4.8	4.8
Silicic acid ⁴	0.4	0.4	0.4	0.4	0.4	0.4	0.4
α -AI	0	0.90	0	0.90	2.05	0	2.05

¹ All diets were isoenergetic and isoproteic. LA, lactalbumin; α -AI-LA, diets containing LA plus varying amounts of α -amylase inhibitor; P-PEA, pea diets containing various amounts of parent line/kg; TR-PEA, transgenic pea diets containing various amounts of transgenic pea/kg.

² The mineral mixture contained (per kg) 400 mg CuSO₄ · 7H₂O, 5 g FeSO₄ · 7H₂O, 4 g MnSO₄ · 4H₂O, 3.6 g ZnSO₄ · 7H₂O, 40 mg KI, 40 mg KIO₃, 120 mg NaF, 10 mg NH₄VO₃, 80 mg NiCl₂ · 6H₂O, 120 mg SnCl₄ · 5H₂O, 6 mg NaSeO₃, 0.96 mg CrK(SO₄)₂ · 12H₂O (chrome alum), 410 g CaCO₃, 314 g KH₂PO₄, 22 g KCl, 102 g MgSO₄ · 7H₂O and 142 g Na₂HPO₄.

³ The vitamin mixture contained (per kg) 200 mg thiamine, 200 mg pyridoxine, 200 mg riboflavin, 200 mg *p*-amino benzoic acid, 600 mg nicotinic acid, 400 mg calcium pantothenate, 100 mg folic acid, 100 mg biotin, 8000 mg inositol, 240 mg retinol, 50 mg cholecalciferol, 1200 mg all-*rac*- α -tocopherol, 2 mg menadione, 500 mg cyanocobalamin and 16 g choline chloride made up to 1 kg with cornstarch (Grant et al. 1993).

⁴ Silicic acid was added to the diet as a source of silicon. This is recommended if animals are kept in a clean environment (Grant et al. 1993).

damage. Kidney bean α -AI has recently been introduced into peas and azuki beans with the use of gene technology (Ishimoto et al. 1996, Schroeder et al. 1995) with the aim of improving their resistance to bruchid pests (Ishimoto and Kitamura 1989). Indeed, it was found that transgenic peas expressing α -AI were protected against various *Callosobruchus* species that attack the seed during storage (Shade et al. 1994) and against *Bruchus pisorum* that feeds on the developing pea seed (Schroeder et al. 1995). In field trials, peas expressing α -AI were protected against damage by the pea weevil (Schroeder et al., unpublished data).

α -AI occurs naturally in kidney bean seeds (Marshall and Lauda 1975, Moreno and Chrispeels, 1989). It is inactive against β -amylases and α -glucosidases; it does not bind to or inhibit higher or lower plant (bacterial or fungal) α -amylases but inhibits the corresponding enzymes in mammals, *Helix pomatia* and insects. Purified α -AI is resistant to pepsin and trypsin in vitro but not to chymotrypsin (Andriolo et al. 1984). α -AI is a member of a family of proteins that may have a role in plant defense, are encoded at a single locus and have 50–90% peptide sequence identity (Mirkov et al. 1994). The family includes phytohemagglutinin, arcelin and α -AI, each with a different mode of insecticidal action. α -AI is an anti-nutrient for humans (Bowman 1945). In clinical studies, purified α -AI inhibited intraduodenal amylase (Layer et al. 1985). Starch digestion in the rat small intestine was also inhibited, with occasional blockage of the cecum, particularly at daily α -AI intakes >20 mg, leading to losses of body nitrogen, lipids and carbohydrates and growth depression (Pusztai et al. 1995).

The main objective of this short-term nutritional study was to establish whether pest-resistant transgenic peas expressing high levels of α -AI had any adverse effects on starch and

protein digestibility and utilization, small intestinal metabolism and growth. This was accomplished by feeding rats for 10 d diets containing transgenic peas at two different levels (300 or 650 g/kg). The nutritional value of diets containing transgenic peas was compared with that of diets containing the parental pea-line at the same two dietary levels for pair-fed rats. The nutritional performance of rats fed transgenic peas was also compared with that of rats fed lactalbumin diets with or without purified bean α -AI in amounts that were equivalent to those in transgenic pea diets. Because purified bean α -AI included in the lactalbumin diet depressed rat growth, particularly at the higher dietary level (Pusztai et al. 1995), this demonstration that the short-term nutritional effects of transgenic and parent peas at moderate levels (300 g/kg) were indistinguishable gives rise to cautious optimism that it should be possible to use transgenic peas containing α -AI in animal feeding, particularly at the low dietary levels normally recommended for peas.

MATERIALS AND METHODS

Plant and other materials. Seeds of the green pea (*Pisum sativum* L.), cultivar Greenfeast and the transgenic line F-10 that was derived from Greenfeast (Schroeder et al. 1995) were obtained from plants grown in the greenhouse. Seeds were allowed to mature and dry on the plants before harvesting and then ground to a fine powder for inclusion in the diet at two levels (Table 1). Nitrogen contents were 35.6 and 37.0 g N/kg seed meal, equivalent to 223 and 230 g protein/kg (N × 6.25) for Greenfeast and transgenic F-10 line, respectively.

The functional α -AI content of the transgenic line F-10 was equivalent to 3.0 g bean inhibitor/kg seed meal as estimated from the inhibition of α -amylase (Pusztai et al. 1995) by aqueous extracts of the F-10 line. Samples of α -AI were purified from both kidney bean

TABLE 2

Body weight, composition, nutritional performance and relative weights of selected organs of rats fed for 10 d diets containing 300 g/kg parent or transgenic peas or lactalbumin diet alone or with pure kidney bean α -amylase inhibitor (α -AI) to match its level in the transgenic pea diet^{1,2}

	Diet				Pooled SD
	1 Control (LA)	2 α -AI ₀₉ -LA	3 P-PEA ₃₀₀	4 TR-PEA ₃₀₀	
Food intake, g/10 d	128	128	128	128	
N intake, mg/10 d	2950	3004	2998	3075	
α -AI, mg/10 d	0	115	0	115	
Initial weight, g	83.5	84.3	83.5	83.0	0.6
Final weight, g	153.1	156.5	160.0	155.4	4.8
Weight gain, g/10 d	69.6	72.2	76.5	72.4	4.2
Body water, g	99.5 ^a	104.0 ^b	105.8 ^b	101.8 ^a	1.4
Dry body weight, g	48.0	46.8	49.0	47.5	1.2
Body N, g	4.4	4.6	4.6	4.5	0.1
Body lipid, g	15.2 ^b	11.3 ^a	12.8 ^{ab}	13.4 ^{ab}	1.2
Feces, g/10 d	7.9 ^a	9.5 ^a	15.0 ^b	24.1 ^c	1.9
Fecal N, mg/10 d	358 ^a	378 ^a	586 ^b	678 ^b	61
Urine N, mg/10 d	322	344	311	369	55
N balance, mg/10 d	2270 ^b	2282 ^b	2101 ^{ab}	2028 ^a	80
DM ³ digestibility, %	95.0 ^c	91.3 ^{bc}	87.9 ^b	81.7 ^a	2.5
N digestibility, %	91.1 ^b	88.3 ^b	81.6 ^a	79.8 ^a	2.6
Small intestine, mg/100 g DBW	2527 ^a	2891 ^b	2357 ^a	2578 ^a	136
Cecum, mg/100 g DBW	320 ^a	519 ^b	367 ^a	585 ^b	60

1 Results are given as means of 4 rats with pooled SD. Values in a row with no common superscripts differ significantly ($P \leq 0.05$).

2 Diet abbreviations as in Table 1.

3 DBW, dry body weight; DM, dry matter.

and transgenic peas and were shown to have comparable inhibitory activity against crystalline α -amylase. Moreover, the in vitro α -amylase inhibitory activity of equivalent amounts of purified bean α -AI (Glycoprotein I; Pusztai, 1966) was not affected by its incorporation into either lactalbumin or parent pea line control diets.

Enzyme assays and chemical analysis. Trypsin, chymotrypsin and α -amylase enzyme assays in luminal washings of small intestinal contents and freeze-dried pancreas samples after zymogen activation were performed as described previously (Pusztai et al. 1995). The results of these assays correlated well with the in vivo nutritional performance of the rats (Pusztai et al. 1995 and 1997).

Dried tissues and carcass samples were combined and ground in a mincer. Lipid was extracted from the ground material (1 g:100 mL solvent) with chloroform/methanol (2:1, v/v), the solvent removed by filtration and the residue dried under reduced pressure. Lipid content was calculated from the weight difference as before (Grant et al. 1986). Nitrogen estimations were done on the defatted carcass material, diets, feces and urine samples by using a Foss Heraeus Macro N automated system (Foss Electric, Bishopthorpe, UK). Starch was measured with an iodine reagent (Piergiorganni 1992).

Samples of pancreas, small intestine, cecum and colon were extracted with perchloric acid (100 g/L; 15 g tissue/L) for 30 min at 0°C and centrifuged (10,000 \times g for 10 min). Protein in the residue that was insoluble in perchloric acid was determined by a modified Lowry method (Schachterle and Pollack 1973) after solubilization with 0.3 mol/L NaOH. RNA and DNA were estimated as before (Pusztai et al. 1992).

Animal experiments. All management and experimental procedures in this study were conducted in strict accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986.

Male Hooded Lister (Rowett) rats weaned at 19 d of age were individually housed in metabolism cages and adapted to experimental conditions by prefeeding them a fully balanced semisynthetic lactalbumin diet (LA; 150 g protein/kg diet; Table 1) for 10 d. In the experiments, groups of rats, (4 rats randomly selected for each diet), average weight of 83.3 (SD 1) g, were pair-fed for 10 d (12.8 g/d; two meals daily, 4 g in the morning and 8.8 g in the evening) control and experimental diets, respectively.

In Trial 1, at the lower pea inclusion level, groups of rats ($n = 4$ /group) were fed for 10 d one of the following diets: lactalbumin control (LA, diet 1); LA + 0.9 mg α -AI/g diet (α -AI₀₉-LA, diet 2); a low pea diet containing 300 g parent line/kg diet (P-PEA₃₀₀, diet 3); and a low transgenic pea diet containing 300 g transgenic pea/kg diet (TR-PEA₃₀₀, diet 4). In Trial 2, at the higher α -AI level, the following diets were used: lactalbumin control (LA, diet 1); LA + 2.0 mg α -AI/g diet (α -AI₂₀-LA, diet 5); high pea diet containing 674 g parent line/kg diet (P-PEA₆₇₄, diet 6); and high transgenic pea diet containing 650 g transgenic pea/kg diet (TR-PEA₆₅₀, diet 7). All diets contained 150 g total protein/kg diet; the formulation of the pea diets was such that at the higher pea inclusion level, all protein in the diet was derived from pea proteins but at the lower level, appropriate amounts of lactalbumin were used to reach a total of 150 g protein/kg diet (Table 1). With the daily intake of 12.8 g diet, the test rats were fed 11.5 mg α -AI/d when fed the lower level diet and 26.3 mg with the higher level of transgenic pea diet; these were matched for α -AI in the α -AI₀₉-LA and α -AI₂₀-LA diets. Water was freely available. The rats were weighed, and urine and feces samples were collected daily and stored at -20°C until required. Fecal samples were then freeze-dried and ground in a mortar for analysis. On the morning of d 10, rats were given 2 g of their respective diets and killed by halothane overdose 2 h later. The abdomen was cut open, the gut removed and the rest of the body dissected. The small intestine was rinsed with saline and the washings were kept frozen for enzyme activity measurements. Stomach and small intestinal washings were used for estimations of starch content. Pellets obtained after centrifugation were resuspended in 0.1 mol/L Tris-HCl, pH 6.9, heated at 100°C for 60 min and centrifuged (4500 g for 15 min at 4°C); the supernatant was reacted with an iodine reagent and the color developed was read at 565 nm (Piergiorganni 1992). Soluble potato starch was used as a standard. The tissues, including the pancreas, spleen, small intestine, cecum, colon, liver, kidneys, thymus, heart, testes, prostate plus vesicular and coagulating glands, brain, lungs and hind-leg muscles of soleus, plantaris and gastrocnemius were rinsed with water, blotted dry and weighed. All tissues and the carcass were freeze-dried to constant weight.

Possible differences in the effects of α -AI purified from beans or

TABLE 3

Body weight, composition, nutritional performance and relative weights of selected organs of rats fed for 10 d diets containing 675 g/kg parent or 650 g/kg transgenic peas or lactalbumin diet alone or with pure kidney bean α -amylase inhibitor (α -AI) to match that in the transgenic pea diet^{1,2}

	Diet				Pooled SD
	1 Control (LA)	5 α -AI ₂₀ -LA	6 P-PEA ₆₇₅	7 TR-PEA ₆₅₀	
Food intake, g/10 d	128	128	128	128	
N intake, mg/10 d	2950	2945	3124	3214	
α -AI, mg/10 d	0	263	0	263	
Initial weight, g	83.5	83.2	83.7	82.2	1.1
Final weight, g	150.1	150.5	157.0	151.0	3.5
Weight gain, g/10 d	67.6	67.3	73.3	68.8	4.5
Body water, g	97.6a	100.5b	105.3c	101.0b	1.4
Dry body weight, g	48.5b	43.1a	46.4ab	45.0ab	1.8
Body N, g	4.6	4.4	4.7	4.5	0.3
Body lipid, g	14.3b	9.0a	11.4ab	10.7ab	1.7
Feces, g/10 d	7.5a	25.5c	18.0b	21.4b	2.0
Fecal N, mg/10 d	295a	381a	659b	754c	45
Urine N, mg/10 d	365	351	315	360	47
N balance, mg/10 d	2290b	2218ab	2150a	2100a	61
DM digestibility, %	93.9c	80.0a	85.9b	83.3ab	1.7
N digestibility, %	90.0b	86.9b	78.9a	76.5a	2.8
Small intestine, mg/100 g DBW	2500a	3273b	2553a	2602a	144
Cecum, mg/100 g DBW	325a	568c	470b	485b	36
Testes, mg/100 g DBW	472a	565b	516a	496a	30

¹ Results are given as means of 4 rats with pooled SD. Values in a row with no common superscripts differ significantly ($P \leq 0.05$).

² Diet abbreviations as in Table 1.

³ DBW, dry body weight; DM, dry matter.

transgenic peas on starch digestibility in the rat small intestine were tested in an acute in vivo trial. By measuring the amount of starch remaining undigested in the small intestinal lumen, this experiment was designed to establish whether the α -AI purified from transgenic peas was as effective or less effective in reducing starch digestibility in rats gavaged with starchy diets as the LA control diets to which α -AI purified from beans was added. Three groups of 4 rats were deprived of food overnight; in the morning, they were given 1.5 g different pea diets, killed 2 h later, dissected and their stomach and small intestine were removed and washed with saline to recover their luminal contents for starch determination. All three diets contained 1 g parent pea meal, 0.27 g cornstarch and 0.23 g corn oil with a total starch content of 950 mg. The first diet contained no α -AI, whereas the second and third contained 2 mg α -AI from transgenic pea and kidney bean, respectively.

Statistics. One-way ANOVA was performed on nutritional performance, organ weight, organ composition data and enzyme level measurements using the Minitab statistical software package (Minitab, New York, NY); multiple comparisons were done by the Tukey test using the InStat statistical package (Graphpad Software, San Diego, California). In the tables, the results are given as means with pooled SD.

RESULTS

Rat trials. The nutritional performance variables of the rats given transgenic or parent line peas were remarkably similar. Thus, with the exception of a lower body water content and dry matter digestibility (hence correspondingly higher fecal output of rats fed transgenic pea diets) the major performance indicators such as the weight gain, final dry body weight and body N values of rats given parent line or transgenic peas in their diet at the level of 300 g/kg (diets 3 and 4) were not significantly different. Neither were these values different from those of rats fed the LA diet (diet 1), even

though the output of feces and fecal N was higher and digestibilities of N and dry matter and overall N balance were slightly but significantly lower in pea-fed rats than those given LA diet (diet 1) or LA diet with α -AI (diet 2). The growth and other nutritional performance variables did not differ in rats fed the LA diet (diet 1) or LA diet containing 0.9 g purified α -AI/kg (diet 2), with the exception of body water and lipid contents (Table 2). The presence of α -AI, whether in purified form (diet 2) or expressed in the pea (diet 4), had no significant effects on organ weights except for higher relative cecal weights (Table 2). The relative weight of the small intestine was also slightly greater in rats fed purified α -AI.

When peas supplied all of the dietary protein, there were no significant differences between the two pea groups, except for a slightly but significantly higher fecal N excretion and lower body water in rats given transgenic peas. However, output of both feces and fecal N of pea-fed rats was significantly higher than that of LA control rats, resulting in significantly poorer N digestibility values for the pea diets than those for the LA control diet regardless of the absence or presence of α -AI (Table 3). The relative weight of the small intestine in pea-fed rats was not different from that of LA controls but it was significantly less than that of the control rats fed diet to which α -AI was added. The cecum of the pea-fed rats was significantly heavier than that of the LA controls. However, in the presence of α -AI in the LA diet, the relative weights of both the cecum and testes were significantly higher than those of all other groups.

The dry body and lipid weights and dry matter digestibility values were lower but the water content of the body was higher in rats fed LA diet containing 2 g purified α -AI/kg (diet 5) than in rats given LA diet without α -AI (diet 1; Table 3). The

TABLE 4

Chemical composition of selected organs and enzyme levels in the small intestinal lumen and pancreas of rats fed for 10 d diets containing 300 g/kg parent line peas or transgenic peas or lactalbumin diet either alone or supplemented with pure kidney bean α -amylase inhibitor to a level equivalent to that in the transgenic pea diet^{1,2}

	Diet				Pooled SD
	1	2	3	4	
	Control (LA)	α -AI ₀₉ -LA	P-PEA ₃₀₀	TR-PEA ₃₀₀	
<i>mg α-AI/10 d</i>					
	0	115	0	115	
	<i>mg</i>				
Cecum					
DNA	1.8 ^a	3.8 ^c	2.8 ^b	3.6 ^c	0.3
RNA	3.2 ^a	5.9 ^b	4.9 ^b	6.1 ^b	0.8
Protein	70 ^a	124 ^c	90 ^b	126 ^c	12
Pancreas					
DNA	5.4	5.1	5.2	4.9	0.9
RNA	39	33	38	36	7
Protein	178	160	198	176	34
Trypsinogen	6.7 ^a	5.6 ^a	8.4 ^{ab}	10.0 ^b	1.3
Chymotrypsinogen	13.0	12.9	12.2	11.2	2.2
α -Amylase	4.3	4.6	5.5	5.8	1.4
Small intestinal lumen					
Trypsin	0.85 ^b	0.72 ^b	0.21 ^a	0.30 ^a	0.19
Chymotrypsin	0.21 ^b	0.25 ^b	0.09 ^a	0.09 ^a	0.03
α -Amylase	0.20 ^b	0.01 ^a	0.18 ^b	0.21 ^b	0.03

¹ Values are given for tissues that had significant differences; the chemical composition of small intestine, colon and liver of rats of the 4 groups was not significantly different. The results are given as means of 4 rats with pooled SD. Values in a row with no common superscripts differ significantly ($P \leq 0.05$).

² Diet abbreviations as in Table 1.

fecal output but not fecal N was also significantly higher in the presence of α -AI in the LA diet, as were some organ relative weights such as the small intestine, cecum and testes. There were some relatively slight differences in the performance of rats fed the transgenic pea diet (diet 7) and the LA diet containing an equivalent amount of α -AI (diet 5). Thus, fecal N content was significantly lower and, correspondingly, N digestibility higher in rats given purified α -AI (diet 5). These rats also had greater small intestinal, cecal and testes relative weights (Table 3).

Tissue composition and effects on pancreatic enzyme levels. DNA, RNA and protein contents of the cecum of rats given different diets reflected the differences in the size of this tissue. Thus, all of these values were moderately higher in rats given the parent pea line at the 300 g/kg level (diet 3) than in those fed the LA-diet. Moreover, the cecum of rats fed α -AI-supplemented LA diet (diet 2) or transgenic pea diet (Table 4) contained more DNA and protein but not RNA than that of the other two groups. However, the composition of tissues such as the small intestine, colon and liver of these groups of rats (Trial 1) was not different. In general, the tissue composition of rats fed the LA diet containing 2 g purified α -AI/kg (diet 5) or pea diets (diets 6 and 7) did not differ (Table 5). However, the small intestine of rats given the LA diet supplemented with α -AI had significantly higher protein; the cecum of these rats also had higher protein, RNA and DNA contents than that of rats in any other group.

The dietary treatments had no effect on the concentrations of digestive enzymes in the pancreas but they did affect these enzymes in the small intestinal lumen. Thus, feeding pea diets at both levels of inclusion and with both pea lines drastically

reduced the luminal concentration of trypsin and chymotrypsin but not that of α -amylase. In contrast, feeding both levels of purified α -AI had no effect on trypsin and chymotrypsin levels but almost completely eliminated α -amylase activity in the small intestine. Consequently, the high small intestinal starch digestibility value of $96.9 \pm 0.4\%$ in rats fed the LA diet (diet 1) was reduced to $50.7 \pm 27.3\%$ in the presence of purified α -AI at the higher level (diet 5). It was also reduced at the lower level (diet 2) to $90.4 \pm 0.3\%$, but the reduction was significantly smaller. In contrast, in rats given pea diets whether from parent or transgenic peas, starch digestibility values remained high, 95.8 ± 2.0 and $93.0 \pm 1.0\%$, respectively, indicating that α -AI in the transgenic pea diets did not or only slightly inhibited starch digestion. This was confirmed in the acute experiment in which the small intestinal starch digestibility value of $94.5 \pm 1.3\%$ of parent pea-fed rats was similarly high, $95.3 \pm 1.8\%$, when the diet contained 2 mg of purified pea α -AI, whereas in the presence of 2 mg of bean α -AI, starch digestibility was reduced to $86.4 \pm 0.3\%$.

DISCUSSION

The nutritional performance of rats fed diets containing transgenic peas at the 300 g/kg level of inclusion, which is equivalent to the maximal inclusion rate in most commercial pig and poultry diets, was not significantly different from that of rats given the parent pea or LA diet, even when the LA diet contained 0.9 g purified bean α -AI/kg (Table 2). This confirmed previous findings that a daily dose of 11.5 mg α -AI is well tolerated by the rats (Pusztai et al. 1995). Although digestibilities of proteins and dry matter, and therefore N

TABLE 5

Composition of organs and enzyme levels in the small bowel lumen and pancreas of rats given 674 g/kg parent line or 650 g/kg transgenic pea diets or lactalbumin diet either alone or with pure α -amylase inhibitor to match that in the transgenic pea diet^{1,2}

	Diet				Pooled SD
	1	5	6	7	
	Control (LA)	α -AI ₂₀ -LA	P-PEA ₆₇₄	TR-PEA ₆₅₀	
	<i>mg α-AI intake/10 d</i>				
	0	263	0	263	
	<i>mg</i>				
Small intestinal tissue					
DNA	17	17	15	14	2
RNA	40	46	37	36	4
Protein	635 ^a	759 ^b	639 ^a	599 ^a	42
Small intestinal lumen					
Trypsin	0.83 ^b	0.63 ^b	0.21 ^a	0.23 ^a	0.16
Chymotrypsin	0.19 ^b	0.23 ^b	0.07 ^a	0.10 ^a	0.05
α -Amylase	0.20 ^b	0.01 ^a	0.22 ^b	0.16 ^b	0.03
Protein	60 ^a	148 ^b	120 ^b	155 ^b	17
Starch	18 ^a	315 ^b	15 ^a	25 ^a	50
Cecum					
DNA	2.8 ^a	3.4 ^b	2.7 ^a	2.8 ^a	0.2
RNA	5.4 ^a	7.8 ^b	4.9 ^a	5.1 ^a	0.6
Protein	90 ^a	116 ^b	96 ^a	92 ^a	13
Pancreas					
DNA	4.2	3.2	3.7	4.2	0.7
RNA	29	23	25	30	6
Protein	138	130	130	176	26
Trypsinogen	5.6	9.0	7.6	8.0	2.0
Chymotrypsinogen	13.1 ^{ab}	16.6 ^b	10.1 ^a	9.4 ^a	2.3
α -Amylase	4.0	5.1	5.4	5.4	1.4

¹ Values are given for tissues that had differences. The results are given as means of 4 rats with pooled SD. Values in a row with no common superscripts differ significantly ($P \leq 0.05$).

² Diet abbreviations as in Table 1.

balance, were significantly lower with both the transgenic and parent pea diets than those with the LA diet (Tables 2 and 3), this was probably the result of the presence of protease inhibitors in pea seeds (Richardson 1991). However, these differences were not large; the parent line was ~10% and the transgenic pea ~15% less digestible than the LA diet, probably accounting for the similar weight gain values of all four groups in Trial 1. Because fecal N loss was higher with pea than with the LA diet, particularly at the higher inclusion level (Table 3), the lower N balance with pea diets was possibly due at least in part to naturally occurring trypsin inhibitors present in both transgenic and parent peas.

Despite a significantly higher fecal N loss with transgenic pea diets at the higher inclusion level in which peas supplied all of the dietary protein, the nutritional performance of rats fed transgenic or parent pea diets was similar. Although the growth and N accretion of rats fed pea diets (Table 3) was significantly less than that of LA-fed rats, it was similar for both pea lines and was therefore due to the nutritional characteristics of peas rather than to the presence of the α -AI gene in the transgenic pea line.

One of the most important findings of this work was that although the transgenic peas contained 0.3 g α -AI/kg seed meal, which was functionally active in vitro and inhibited the amylolytic activity of crystalline bovine α -amylase, it had only marginal inhibitory effects on rat amylase in vivo. Indeed, starch digestion proceeded largely unimpeded in the small intestinal lumen of rats fed transgenic pea diets. This was the

more remarkable because the same amount of purified bean α -AI included in the LA diet did inhibit rat amylase in the small intestinal lumen in vivo, resulting in the passage of large amounts of dietary undigested starch into the cecum. The effect of the inclusion of purified bean α -AI in the diet was tested only with LA but not the parent pea line because its in vitro activity was the same whether it was tested in the presence of LA or parent pea extracts. However, because starch digestion in the small intestinal lumen was significantly more extensive when the rats were gavaged with parent peas supplemented with recombinant pea α -AI than with equivalent amounts of bean α -AI, it is unlikely that the lack of the in vivo inhibition of α -amylase by the transgenic pea was the result of the neutralization of its α -AI activity by some unidentified pea component. It is more likely that the α -AI gene product in peas is structurally and/or conformationally different from the bean inhibitor protein and therefore less resistant to degradation by serine proteases in the small intestinal lumen but not by cysteine proteases in the brush border digestive system (Gatehouse et al. 1985). Indeed, the polypeptide subunit patterns of α -AI in beans and peas are different, which could reflect differences in post-translational processing and/or glycosylation (Schroeder et al. 1995). The precise reason(s) for the relative inactivity of α -AI in transgenic peas can be established only by isolating the inhibitor from transgenic peas in sufficient amounts for full nutritional testing. However, this does not alter the fact that the nutritional value of diets

containing transgenic or parent line peas was similar at the moderate levels of inclusion used in practice.

In conclusion, this work has shown that the nutritional value of diets containing transgenic or parent peas was remarkably similar. Moreover, after supplementation with essential amino acids, irrespective of whether parent or transgenic lines were used, pea diets were only slightly, though significantly, inferior to semisynthetic lactalbumin-based diets. Although the nutritional value of the diet decreased with increasing pea inclusion in comparison with that of the lactalbumin control diet, the differences between transgenic and parent pea lines remained relatively small even at the highest inclusion level. This was probably due to the lack of inhibition of starch digestion in the small intestine *in vivo* by the α -AI expressed in the transgenic peas. From this short-term study, we conclude that transgenic peas may be used in the diet of mammals, including farm animals, particularly at the moderate levels of dietary inclusion recommended in commercial practice. However, this nutritional study with transgenic peas expressing α -AI cannot at this stage be taken as proof that transgenic peas are fit for human consumption. This may be established only with the use of further and more specific risk assessment testing procedures, which must be designed and developed with human consumers in mind.

ACKNOWLEDGMENTS

We wish to thank Stephanie Gollasch and Andy Moore for their help with this work.

LITERATURE CITED

- Andriolo, S., Rouanet, J. M., Lafont, J. & Besancon, P. (1984) Inactivation of phaseolamine, an alpha-amylase inhibitor from *Phaseolus vulgaris* by gastric acid and digestive proteases. *Nutr. Rep. Int.* 29: 149–156.
- Bowman, D. E. (1945) Amylase inhibitor of navy bean. *Science* (Washington, DC) 102: 358–359.
- Buonocore, V. & Silano, V. (1986) Biochemical, nutritional and toxicological aspects of alpha-amylase inhibitors from plant foods. *Adv. Exp. Med. Biol.* 199: 483–507.
- Gatehouse, A.M.R., Butler, K. J., Fenton, A. A. & Gatehouse, J. A. (1985) Presence and partial characterization of a major proteolytic enzyme in the larval gut of *Callosobruchus maculatus*. *Entomol. Exp. Appl.* 39: 129–286.
- Gatehouse, A.M.R. & Gatehouse, J. A. (1997) Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. *Pestic. Sci.* 52: 165–175.
- Grant, G., Dorward, P. M. & Pusztai, A. (1993) Pancreatic enlargement is evident in rats fed diets containing raw soyabean (*Glycine max*) or cowpea (*Vigna unguiculata*) for 800 days but not in those given diets based on kidney bean (*Phaseolus vulgaris*) or lupinseed (*Lupinus angustifolius*). *J. Nutr.* 123: 2207–2215.
- Grant, V., Edwards, J. E. & Pusztai, A. (1995) α -Amylase inhibitor levels in seeds generally available in Europe. *J. Sci. Food Agric.* 67: 235–238.
- Grant, G., McKenzie, N. H., Watt, W. B., Stewart, J. C., Dorward, P. M. & Pusztai, A. (1986) Nutritional evaluation of soya beans (*Glycine max*): nitrogen balance and fractionation studies. *J. Sci. Food Agric.* 37: 1001–1010.
- Ishimoto, M. & Kitamura, K. (1989) Growth inhibitory effects of an α -amylase inhibitor from kidney bean, *Phaseolus vulgaris* (L.) on three species of bruchids (Coleoptera: Bruchidae). *Appl. Entomol. Zool.* 24: 281–286.
- Ishimoto, M., Sato, T., Chrispeels, M. J. & Kitamura, K. (1996) Bruchid resistance of transgenic azuki bean expressing seed α -amylase inhibitor of common bean. *Entomol. Exp. Appl.* 79: 309–315.
- Layer, P., Carlson, G. L. & DiMagno, E. P. (1985) Partially purified white bean amylase inhibitor reduces starch digestion *in vitro* and inactivates intraduodenal amylase in humans. *Gastroenterology* 88: 1985–1992.
- Marshall, J. J. & Lauda, C. M. (1975) Purification and properties of phaseolamine, an inhibitor of α -amylase, from the kidney bean, *Phaseolus vulgaris*. *J. Biol. Chem.* 250: 8030–8037.
- Mirkov, T. E., Wahlstrom, J. M., Hagiwara, K., Finardi-Filho, F., Kjemtrup, S. & Chrispeels, M. J. (1994) Evolutionary relationships among proteins in the phytohemagglutinin-arcelin- α -amylase inhibitor family of the common bean and its relatives. *Plant Mol. Biol.* 26: 1103–1113.
- Moreno, J. & Chrispeels, M. J. (1989) A lectin gene encodes the α -amylase inhibitor of the common bean. *Proc. Natl. Acad. Sci. U.S.A.* 86: 7885–7889.
- Piergiorganni, A. R. (1992) Effects of some experimental parameters on the activity of cowpea α -amylase inhibitors. *Lebensm.-Wiss. Technol.* 25: 321–324.
- Pusztai, A. (1966) The isolation of two proteins, glycoprotein I and a trypsin inhibitor, from the seeds of kidney bean (*Phaseolus vulgaris*). *Biochem. J.* 101: 379–384.
- Pusztai A., Grant G., Bardocz S., Baintner K., Gelencser E. & Ewen, S.W.B. (1997) Both free and complexed trypsin inhibitors stimulate pancreatic secretion and change duodenal enzyme levels. *Am. J. Physiol.* 35: G340–G350.
- Pusztai, A., Grant, G., Duguid, T., Brown, D. S., Peumans, W. J., Van Damme, E.J.M. & Bardocz, S. (1995) Inhibition of starch digestion by α -amylase inhibitor reduces the efficiency of utilization of dietary proteins and lipids and retards the growth of rats. *J. Nutr.* 125: 1554–1562.
- Pusztai, A., Grant, G., Stewart, J. C., Bardocz, S., Ewen, S.W.B., Gatehouse, A.M.R. & Hilder, V. (1992) Nutritional evaluation of the trypsin (EC 3.4.21.4) inhibitor from cowpea (*Vigna unguiculata* Walp.). *Br. J. Nutr.* 68: 783–791.
- Richardson, M. (1991) Seed storage proteins: the enzyme inhibitors. *Methods Plant Biochem.* 5: 259–305.
- Savage, G. P. & Deo, S. (1989) The nutritional value of peas (*Pisum sativum*). A literature review. *Nutr. Abst. Rev. (Series A)* 59: 66–86.
- Schachterle, G. R. & Pollack, R. L. (1973) A simplified method for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem.* 51: 654–655.
- Schroeder, H. E., Gollasch, S., Moore, A., Tabe, L. M., Craig, S., Hardie, D., Chrispeels, M. J., Spencer, D. & Higgins, T.J.V. (1995) Bean α -amylase inhibitor confers resistance to the pea weevil, *Bruchus pisorum*, in genetically engineered peas (*Pisum sativum* L.). *Plant Physiol.* 107: 1233–1239.
- Shade, R. E., Schroeder, H. E., Pueyo, J. J., Tabe, L. M., Murdock, L. L., Higgins, T.J.V. & Chrispeels, M. J. (1994) Transgenic pea seeds expressing the α -amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio/Technology* 12: 793–796.
- Trowbridge, I. S. (1974) Isolation and chemical characterisation of a mitogenic lectin from *Pisum sativum*. *J. Biol. Chem.* 249: 6004–6012.