

L-Canavanine: A Natural Feed-intake Inhibitor for Pigs, (Isolation, Identification and Significance)

Dirk Enneking*, Lynne C. Giles*, Max E. Tate_* & the late Richard L. Davies**

*Waite Agricultural Research Institute, P.O. Box 1, Glen Osmond S.A. Australia, 5064. and **S.A. Dept. Agriculture, Northfield Pig Research Unit, GPO Box 1671, Adelaide, Australia, 5001.

Received 1992 Version 1.12.92

ABSTRACT

A major feed-intake inhibitor for pigs, is present in seeds of *Vicia villosa ssp. dasycarpa* Roth cv. Namoi. When the seed is fed for 4 days at only 80g kg⁻¹ of the diet, it produces a steady diminution in daily feed intake to 25% or less of their pre-treatment consumption (P<0.001), after the first feeding. The inhibitor has been isolated and identified as L-canavanine. When analytically pure L-canavanine dihydrochloride is supplied at an equivalent dietary concentration (13.0 mmol kg⁻¹) to that supplied by 80g kg⁻¹ of Namoi vetch seed, it successfully mimics the response. The response is only observed after the first meal. A review of published literature showed the diversity of animal species in the exhibiting a feed-inhibition response to canavanine-containing seeds. These reports suggest that this well documented arginine analogue will be a useful molecule for the study of post-ingestion feed-intake regulation. An ambient temperature procedure for the large scale isolation of L-canavanine dihydrochloride is described.

Key words: Canavanine dihydrochloride, deaminocanavanine, *Vicia villosa dasycarpa*, *Canavalia*, antinutritional factor, arginine analogue, feed-intake regulation, pigs, cattle, rodents, birds, monkeys, humans.

_ Corresponding author.

Citation: Enneking, D.; Giles, L. C.; Tate, M. E., and Davies, R. L. (Waite Agric. Res. Inst., P.O. Box 1, Glen Osmond, SA 5064, Aust). L Canavanine: A natural feed intake inhibitor for pigs (isolation, identification and significance). Journal of the Science of Food and Agriculture. 1993; 61(3):315-325.

INTRODUCTION

As part of a program to develop alternative grain legume crops for Australian agriculture, the aim of the present study has been to isolate and chemically identify a potent feed-intake inhibition factor for pigs, from seeds of the woolly pod vetch (*Vicia villosa* ssp. *dasycarpa* Roth cv. Namoi). This vetch is a high-yielding forage plant and is of value in dry-land agriculture. However *V.villosa* as forage can be toxic to cattle (Pancierra *et al* 1966), and its seed is toxic to poultry (Kienholz *et al* 1962) and references therein. The presence of toxic factors in the *Leguminosae* is well documented (Smolenski *et al* 1981; Rosenthal 1982). Their existence poses a significant end-use constraint for the introduction of new grain legume and forage crops with otherwise desirable agronomic features. Nevertheless it must be noted that their elimination may result in the loss of agronomically desirable attributes such as resistance to insect attack (Rosenthal 1977b, Natelson 1985 and references therein). In either case, a knowledge of the chemical structures of these biologically active factors will permit a rational decision as to whether they, or the genes that produce them should be eliminated, retained or transferred to other species.

Lepkovsky (1948) noted that "the most frequent defense used by animals against toxic compounds is to limit their food intake so that the ingestion of the compound is reduced to non-toxic levels". We have used this concept in the form of a pig feed-intake bioassay.

A three phase (pre-treatment, treatment, post-treatment) pig feed-intake bioassay was used for monitoring feed-intake inhibition during the extraction and fractionation procedures to identify and isolate the active crystalline component of *Vicia villosa* ssp. *dasycarpa* Roth cv. Namoi. With suitable controls, there was a clearcut essentially Boolean (yes/no) response in the pig feed-intake at each stage of purification.

On the basis of four such experiments, a simple cation exchange sequence was developed to isolate sufficient analytically pure crystalline canavanine dihydrochloride for a definitive experiment. This fifth experiment gave unequivocal confirmation of the pig feed-intake inhibitory activity of pure L-canavanine dihydrochloride. Providing an appropriate fractionation procedure is used, this feed intake monitoring approach should be readily adaptable for the isolation of other low MWt. (<1500) neutral or ionic feed-intake inhibitors or enhancers from grain legumes.

Because the inhibitory effect of analytically pure canavanine on feed intake has not hitherto been directly demonstrated, the relevant literature has been reviewed, to place the results in context and to delineate further areas for investigation.

MATERIALS & METHODS

Bioassay

From an operational point of view, the major limitation in using a large animal approach, is the scale of the isolation procedure necessary to obtain a clear cut bioassay response. The percentage of the dietary replacement necessary for the ($P<0.01$) level is inversely dependent upon the potency of the feed-intake inhibitory source. It is important in the treatment phase that no significant discontinuity in feed-intake response becomes apparent during the experimental period, due to an excessively large replacement by the negative control (soybean meal, in this work) in the base diet. A single four day 8% dietary replacement treatment for four pigs (each approximately 20 kg) required an extract or residue equivalent to 1.5 kg of the original Namoi meal to elicit a clearcut $P<0.01$ response. The effort necessarily involved with this bioassay is compensated to a considerable extent, because the pig is a relevant enduser as well as a useful model for human nutrition.

In expts 1-4 (briefly summarised in table 2), which relate to the fractionation and identification of the active factor, four pigs (Large White) per treatment were used. In expt 5 the number was increased to eight animals per treatment to examine the effect of analytically pure canavanine hydrochloride on feed intake. Pigs (8-9weeks old) were grouped according to weight into pens (blocks) and were individually offered two meals per 24h (1st meal 3pm, 2nd meal 9am). For each individual pig, the difference between allocated feed and the weight of the leftover food (dried if necessary) gave the absolute daily (24h) intake (kg/day), which was then (for graphical purposes) divided by that individual's mean intake during the pre-treatment phase to obtain its feed-intake ratio.

Individual daily feed allocations employed for expt 5 (table 1) as well the fractionation experiments (1-4, table 2) were calculated according to the following expression [allocation (kg)= $0.75 \times W^{0.65} \times M/D$; W= liveweight of pig (kg), M=maintenance requirement (kJ/kg), D=digestible energy of the diet (kJ/kg)]. This quantity of feed was equivalent to 75% *ad libitum* feed intake, but it has the advantage that wastage is strictly controlled. Liveweight was measured at the beginning of each phase, i.e.: pre-treatment (days 1-4),

treatment (days 5-8) and post-treatment (days 9-12). During the treatment phase, 8% of the basal pre-treatment diet was replaced (table 1) with various experimental diets. Soybean and Namoi vetch meals were used respectively as the negative and positive controls.

Details of the general feed formulation are given for experiment 5 in table 1. The corresponding formulations for experiments 1-4 differ only in the the replacement (80g kg^{-1}) of the base diet. Solid Namoi vetch residues from dialysis, extraction or chemical treatments were substituted directly for the equivalent 80g kg^{-1} content of vetch ignoring the specific activity alterations due to minor changes in weight. Likewise cation exchange fractions were adsorbed onto soybean meal (80g kg^{-1}) again ignoring the specific activity alterations due to minor changes in weight. At this stage the important question, was simply whether feed intake was inhibited or not.

TABLE 1
Diet formulations for Expt 5 (kg t⁻¹)

<i>Component -Base</i>	<i>Soy</i>	<i>Namoi</i>	<i>+38% can^{3*}</i>	<i>+100% can^{2*}</i>	<i>+lysine^{4*}</i>	<i>+lysine⁵⁺</i> <i>38% can^{3*}</i>
Wheat	790	727	727	727	727	727
Fishmeal	170	156	156	156	156	156
Soy meal	---	80	---	80	80	80
Namoi	---	---	80	---	---	---
Can HCl	---	---	---	1.23	3.25	---
Lys HCl	---	---	---	---	---	2.86
Dicalcium phosphate	5.0	4.6	4.6	4.6	4.6	4.6
Limestone	3.0	2.8	2.8	2.8	2.8	2.8
Tallow	25.0	23.0	23.0	23.0	23.0	23.0
Salt	2.5	2.3	2.3	2.3	2.3	2.3
Min/Vit/ premix ¹	2.0	1.8	1.8	1.8	1.8	1.8
Total	997.5	997.5	997.5	998.7	1000.8	1000.4

¹ Provided (per kg diet) : vitamin A, 11 000 IU; D, 2 200 IU; E, 40 IU; K, 2 mg; thiamin, 1.5 mg; riboflavin, 5 mg; pyridoxine 2 mg; calcium pantothenate, 11 mg; niacin, 20 mg; folic acid, 1 mg; biotin, 150 ug; vitamin B₁₂, 20 µg; Cu, 10 mg; Fe, 100 mg; Zn, 150 mg; Mn, 50 mg; I, 0.5 mg; Mo, 0.5 mg; Co, 0.2 mg; Se, 0.13 mg; ethoxyquin, 100 mg.

Final feed concentrations (above base diet) per kg diet: ² Canavanine 2 HCl 13.04 mmol kg⁻¹, ³ Canavanine 2.HCl 5.12 mmol kg⁻¹, ⁴ Lysine 2.HCl 13.04 mmol kg⁻¹, ⁵ Lysine 2.HCl 5.12 mmol kg⁻¹, ANALYSIS : (Base diet g kg⁻¹): Fat, 76.0; Fibre, 21.8; CP, 190.6; Arg, 10.4; His, 4.5; Ile, 7.3; Leu,

13.5; Adlys, 10.3; Met, 4.3; Cys, 3.8; Phe 8.1; Tyr, 6.3; Thr, 6.7; Try 2.2; Val 9.4; Ca 9.9; P, 6.2; Avp, 4.9. Other: DE, 15.1 MJ kg⁻¹; Adlys/DE 0.69 g MJ⁻¹.

Fractionation by bioassay

Four pig feed-intake experiments (1-4), tested various diets containing extracts of Namoi vetch, the associated residues, ion exchange fractions of the extracts as well as physical and chemical treatments of the Namoi vetch meal. The final experiment (5) was designed to establish whether crystalline L-canavanine dihydrochloride incorporated in the soybean meal at 38% and 100% of the measured canavanine content of Namoi vetch was dose dependent and at the higher level was sufficient to account for the observed feed inhibition induced by Namoi vetch. In addition measurements were made after the first feeding to more precisely determine the onset of feed-intake inhibition.

Initial extraction process

The technique (Ressler *et al.* 1961) was adapted as follows: hammer-milled vetch seed (*Vicia villosa* ssp. *dasycarpa* cv. Namoi, 10 kg) was extracted with 30% v/v ethanol/water (90 litres) in 100 litre polypropylene containers at ambient temperature (20-25°C) with intermittent stirring, settling overnight and syphoning of the supernatant extract. The container was twice more filled with 30% ethanol/water, sedimented and syphoned. The combined supernatants were filtered and the dry weight of an aliquot indicated a yield of 162 g kg⁻¹ of vetch meal.

General procedure for feed preparation

Individual ion exchange fractions or extracts equivalent to 1.5 kg of original Namoi vetch meal were concentrated to dryness at <44° and added to soybean meal as a slurry, which was made up from the minimum amount of water needed to dissolve a given fraction plus enough ethanol to wet the meal evenly. Half the soybean meal was stirred into the liquid to give a moist solid, which was then mixed with the remainder of the meal to yield a friable solid. The mixture was dried, in shallow trays with occasional stirring, in a current of warm air. The dried material (constant weight), was ground and sieved to less than 2mm prior to admixture with the remainder (92%) of the basal wheat and fishmeal diet.

The following alphabetical treatments (table 2) were tested in each experiment (a) was the (-ve) soybean control and (b) the (+ve) Namoi vetch control.

Expt 1

(c) Autoclaved Namoi vetch meal, (30 min/121° C) (d) Defatted Namoi vetch meal residue (dichloromethane, 17 h Soxhlet extraction), yield (971g kg⁻¹) (e) Concentrated solutes ex 30% Ethanol extractions (3 x 4 litre kg⁻¹), yield (162 g kg⁻¹) of defatted Namoi vetch meal residue.

Expt 2

(c) Dialyzed Namoi vetch meal retentate. Dialysis in 4 Visking tubes (1 m x 8 cm diam) containing an aqueous vetch meal slurry, (0.5 kg+1.25 litres H₂O, per tube). Tubes were knotted tightly (2x) at one end, left loose and sufficiently long at the other end and temporarily supported in a vertical position, to act as an exhaust during autoclaving (20 min/121° C). After autoclaving, the loose ends of the tubes and their sterile contents were tightly knotted (2x) prior to dialysis (4° C, 13 x 17h/17 litre dd H₂O) in a 28 litre container. A final exchange with 95% ethanol (15 litre), permitted the sterile contents to be readily filtered, washed with 50% ethanol (2.2 litre) and air dried to constant weight for bioassay. The nonsterile dialysates were discarded.

(d) Performic oxidized vetch meal.

Namoi vetch meal (2.2 kg) was suspended in water (5 litre). Performic acid was prepared by gradual addition of cold (5°) 300 g litre⁻¹ hydrogen peroxide (0.5 litre) to 98%-100% formic acid (0.5 litre) which was allowed to stand for 0.5 h. It was then gradually added with continuous stirring to the vetch seed meal suspension in an ice bath, so that the temperature did not rise above 40°C. Excess peroxide (starch/iodide detection) was destroyed by addition of sodium metabisulfite (200 g). To simplify drying, the deperoxidised slurry was filtered on a Büchner funnel and air dried. The filtrate was concentrated separately *in vacuo* before being added back to the dried filter cake. The bioassay control, for this oxidised vetch, comprised an equivalent amount of sodium sulfate added to Namoi vetch meal.

(e) Cationic fraction: identical to the total cationic treatment (c) in experiment 3 below.

Expt 3

Six cation exchange fractions (c-h) for bioassay were obtained as follows:

Namoi vetch meal (1.6 kg), was sequentially extracted with 30% ethanol (2 x 6 litre), filtered and concentrated at 40° to a dark syrup (296 g i.e. a yield of 184 g kg⁻¹). Supernatant extracts were adsorbed on Mitsubishi SK IB sulfonic acid cation exchange resin (H⁺). The column (2.5 litre) was washed with water (5x bed volume) and the neutral sugars and anionic components of this eluate were discarded. Cations were eluted with 0.5 mol litre⁻¹ NH₄OH (30 litre) and all fractions (2 litre) were monitored by paper electrophoresis (1.0 mol litre⁻¹ acetic acid/0.75 mol litre⁻¹ formic acid, pH 1.7, 37V/cm, 0.25h, ninhydrin detection). Fractions more cationic than glycine were combined as the basic amino acid fraction and contained the bulk of the canavanine, with small amounts of arginine and lysine. The neutral amino acid fractions (with electrophoretic mobilities similar in range to those of glycine to serine at pH 1.7) were bulked. These fractions contained some trigonelline (UV absorbing) and a large amount of a ninhydrin negative [guanidine positive, silver nitrate/sodium hydroxide: grey reacting] compound. Observed spectrometric properties of this compound included: fast atom bombardment mass data M-H⁺, 157, M+H⁺, 159 and ¹³C Nmr data (ppm): 179.5 (COOH), 162.7 (C guanidine), 76.5 (CH₂O), 59.6 (CHNH), 33.2 (CH₂). These data are consistent with deaminocanavanine, and the compound was found to be indistinguishable in all measured properties from an authentic sample of deaminocanavanine prepared by the alkaline degradation of canavanine (Rosenthal 1977a). Acidic amino acids were present in those fractions with electrophoretic mobilities equal to or less than glutamic acid and were partitioned according to their major electrophoretic components into three bulked fractions (5-8, 9-12, 13-15). All eluates were concentrated *in vacuo* at 37° C. Yields per kg of Namoi meal were calculated from aliquots as follows: The complete ninhydrin positive eluate (separate elution) was bulked to obtain the total cation fraction (c) (yield 33.5 g kg⁻¹). Individual bulked fractions were: (d) the acidic amino acid fractions 5-8 (yield 2.3 g kg⁻¹), (e) the acidic amino acid fractions 9-12 (yield 3.5 g kg⁻¹), (f) the acidic amino acid fractions 13-15 (yield 0.9 g kg⁻¹), (g) the neutral amino acid fraction (yield: 16.1 g kg⁻¹), (h) the basic amino acid fraction (yield 15.7 g kg⁻¹). For the bioassay the residues from each of these eluates (equivalent to 1.5 kg Namoi vetch) were dissolved in water (400 m litre), to which 95% ethanol (400 m litre) was added and the mixture gradually added to 1500 g soybean meal. For the treatment phase 80g of each adsorbate was substituted for 80g of soybean meal in the -ve control (table 1).

Expt 4

Two treatments were chosen to examine whether crude canavanine hydrochloride was active and whether there was any anion (Cl) effect.

(c) Sodium chloride $123.6 \text{ mmol kg}^{-1}$ soybean meal. (d) Crude (>95%) crystalline canavanine.2HCl 15.4 g kg^{-1} , i.e. $61.8 \text{ mmol kg}^{-1}$, soybean meal. This concentration is equivalent to 38% of the Namoi vetch seed canavanine concentration ($0.163 \text{ mol kg}^{-1}$). For isolation of the canavanine dihydrochloride, see purification method prior to recrystallisation.

Expt 5

For details of this experiment see the diet formulation in Table 1.

Analytically pure canavanine 2HCl was added to soybean meal at two concentrations equivalent to 100% and 38% of the canavanine concentration present in Namoi vetch seed ($29 \text{ g kg}^{-1} = 0.163 \text{ mol kg}^{-1}$). An additional control of an equimolar concentration of lysine 2HCl ($0.163 \text{ mol kg}^{-1}$) on soybean meal, was included to allow for either an anion (Cl) or a dibasic amino-acid effect. The sixth treatment comprised an equimolar mixture of canavanine 2HCl ($0.064 \text{ mol kg}^{-1}$) and lysine 2HCl ($0.064 \text{ mol kg}^{-1}$) on soybean meal. In this experiment the initial feed-intake at the first (3pm) feeding of the treatment diet (day 5) was measured separately in order to assess when the effect of canavanine on feed-intake became apparent.

Isolation and purification of L-Canavanine dihydrochloride

Analytically pure, crystalline canavanine 2HCl was isolated from the turbid, aqueous supernatant liquor of a sedimented aqueous extract (200 litre) of the Namoi vetch seed (20 kg) by adsorption onto a 6 litre sulfonic cation exchange (NH_4^+) column [Mitsubishi SK I B (water purification grade)].

After thorough washing with water (including backflushing to remove particulate matter), and desorption with 2M HCl (30 litre), concentration *in vacuo* at 55°C yielded a yellow solid residue. The residue was suspended in 5 mol litre^{-1} HCl (1 litre) filtered with a sintered glass (#3) funnel and washed with 5 mol litre^{-1} HCl (6 x 100 mlitre). Paper electrophoresis at pH 1.7, showed the bulk of the ferric salts and ammonium chloride were present in the mother liquor and washings. The crude crystalline canavanine hydrochloride residue was sucked dry and residual HCl was removed by storage in a vacuum dessicator over soda lime to yield crude canavanine hydrochloride (480 g, 59.1% based upon the canavanine content of Namoi). Repeated extraction (6x, $1.5 \text{ mol litre}^{-1}$) of this residue, with warm (85°) 5 mol litre^{-1} HCl, separated it into an acidic filtrate and a residue (NH_4Cl). Removal of Fe^{3+} by ether extraction of the acidic

filtrate, and charcoal decolourization, produced colourless crystals of canavanine 2HCl at 5°C. Two recrystallizations with 5 mol litre⁻¹ HCl (1.5 m litre g¹) produced 148 g (18.2%) of analytically pure canavanine 2HCl, $[\alpha]_D^{20}$ 17.4° +/- 0.3°, (H₂O, c=1, l=1, 20°C). Anal.: (C₅H₁₂N₄O₃·2HCl, requires: C, 24.1%; H,5.7%; N,22.5%; Cl,28.5%; Found: C, 24.0%; H,6.1%; N,22.3%; Cl,28.6%). HPLC (Jones and Gilligan, 1983) analysis of (0.1M HCl /35°/17h) extracts (Bell 1960) indicated a concentration of 0.163 mmol g⁻¹ (29 g kg⁻¹ D.W.) in the original Namoi vetch meal.

Statistical design and analysis

Each of the five experiments was laid out as a randomised complete block design with four blocks. Feed-intake was measured on four successive days in each of the pre-treatment, treatment and post-treatment phases. Analysis of variance was performed on the data in each of the experimental phases using the GENSTAT statistical package. In experiment five, linear contrasts were used to further investigate the relationships between the diets.

RESULTS AND DISCUSSION

Fig. 1. Daily voluntary feed-intake ratio data (experiment 3), for 20kg pigs fed wheat and fishmeal diets at approximately 75% of their *ad libitum* intake during pre- and post- treatment days (1-4 and 9-12). During the treatment days (5-8), an 8% dietary replacement with one of the four treatments, (i) -G- -ve control of soybean meal, (ii) -**H**- +ve control of Namoi ($29 \text{ g kg}^{-1} = 0.163 \text{ mol kg}^{-1}$ canavanine) (iii) -**B**- soybean + recovered total cationic amino acid fraction from Namoi vetch, (iv) -J- soybean + recovered basic amino acid fraction. SED: (day 1-4: 0.09, day 5-8 :0.10, day 9-12: 0.09)

Fig. 1 Shows the voluntary feed-intake bioassay response in pigs. These bioassay data show that after four days on an acceptable wheat and fish meal diet, replacement of 8% with soybean meal in the control diet does not induce any discontinuity. However, replacement of 8% by Namoi vetch, immediately reduced the voluntary feed-intake ratio. The ratio continued to decline to approximately one quarter of the pretreatment intake. Namoi vetch was removed on the eighth day, whereupon the pre-treatment feed-intake was resumed. Data (RL Davies,, unpublished) indicate that feed-intake depression continues as long as the vetch is incorporated into the diet.

TABLE 2

Expt	Treatment	Feed-intake (kg/day)	Pig weights , max-min; mean(kg) No. animals
1	a) Soybean (-ve control)	0.89	16-22.5; 18.8 20 animals
	b) Namoi vetch (+ve control)	0.40	
	c) autoclaving	0.42	
	d) defatting	0.35	
	e) aqueous ethanol extraction	0.66	
	SED	0.075	
2	a) Soybean (-ve control)	0.94	15-20; 16.7 20 animals
	b) Namoi vetch (+ve control)	0.19	
	c) dialysis retentate	0.79	
	d) peroxidation+NaHSO ₃	0.27	
	e) cationic fraction	0.60	
	SED	0.083	
3	a) Soybean (-ve control)	1.02	19-26; 22.8 32 animals
	b) Namoi vetch (+ve control)	0.30	
	c) cationic fraction	0.57	
	d) acidic amino acid fns 5-8	1.02	
	e) acidic amino acid fns 9-12	1.07	
	f) acidic amino acid fns 13-15	1.04	
	g) neutral amino acid fns 2+3 A	1.07	
	h) basic amino acid fns 2+3 B	0.67	
	SED	0.087	
4	a) Soybean (-ve control)	1.02	18-23; 20.0 16 animals
	b) Namoi vetch (+ve control)	0.40	
	c) NaCl	1.08	
	d) 0.043 M Canavanine.2HCl	0.65	
	SED	0.078	
5	a) Soybean (-ve control)	1.13	19-29; 24.6 48 animals
	b) Namoi vetch (+ve control)	0.45	
	c) 0.163 M canavanine.2HCl	0.51	
	d) 0.163 M lysine.2HCl	1.14	
	e) 0.062 M canavanine.2HCl	0.90	
	f) 0.062 M canavanine.2HCl+	0.95	
	0.062 M lysine.2HCL		
SED	0.053		

With the aid of this bioassay we have found (Experiments 1 and 2, table 2), that the active component is insoluble in dichloromethane, thermally stable (125°/30'), water soluble, resistant to performic acid oxidation, dialysable, slowly extracted by 30% ethanol, and retained with the basic amino acid fraction by cation exchange resin (NH₄⁺).

In experiment 3, elution of the total cationic and basic amino acid fractions from the resin with ammonia (Rosenthal 1977a) gave the active bioassay responses shown in Fig 1 as a function of time. In both cases the recovery of biological activity was poor by comparison with the positive control. There was no

detectable biological activity in any of the acidic or neutral amino acid fractions. By electrophoretic and ^1H NMR examination of the biologically inactive neutral amino acid fraction it was clear that it contained a considerable amount of deamino-canavanine, a well known alkaline degradation product of canavanine (Rosenthal 1977a).

By contrast, elution of the basic amino acid fraction with hydrochloric acid and fractional crystallisation under acidic conditions to separate the accompanying ammonium chloride, produced a biologically active colourless crystalline solid, whose activity was confirmed in experiment 4 (Table 2).

Electrophoretic (pH 1.7 & pH 9.2), NMR, FAB/MS and HPLC analysis of these crystals, demonstrated that they comprised more than 95% canavanine 2HCl. Recrystallization produced analytically pure L-canavanine 2HCl, the L- configuration was inferred from the similarity in sign and magnitude of the molar optical rotation of the 2HCl to the molar rotation of authentic L-canavanine sulfate. The hydrochloride salt readily crystallized from acidic aqueous solutions in marked contrast to the sulfate salt. Although the acidic elution and fractional crystallization procedure produced a lower unrecrystallized yield (59.1%) than the published low temperature ammonia elution procedure (85%, Rosenthal 1977), all operations can be carried out at room temperature on a large scale and there is no racemisation, or loss of material by deamination to form the biologically inactive deamino-canavanine. Sacrificial recrystallisation gave final isolated yield of 18.2% of analytically pure material, however recovery can be further enhanced by scavenging the mother liquors.

For verification that the biological activity of canavanine was sufficient to account for all the measured feed-intake inhibition of Namoi vetch, the concentration of pure canavanine required for incorporation into the soybean meal to simulate the Namoi vetch concentration was measured by HPLC (Jones and Gilligan 1983). Analysis of (0.1 mol litre⁻¹ HCl /35°/17h) extracts (Bell 1960) indicated a concentration of 0.163 mmol kg⁻¹ (29 g kg⁻¹DW) in the original Namoi vetch meal. This value is in agreement with the commonly reported range of 20-40 g kg⁻¹ for *Papilionoidae* seeds (Rosenthal 1982).

Detailed results and discussion of experiment five

During the pre-treatment phase, the mean intake (kg) on days 1-4 was 1.04, 1.04, 1.05 and 1.07 respectively, and differed significantly over the four days (P<0.001).

Analysis of variance of the feed-intake in the ensuing treatment period indicated that the interaction between diet and day was statistically significant ($P < 0.001$). The mean intakes for the six diets by four days are shown in table 3.

TABLE 3

Mean Daily Intake (kg) / Pig at 8% Dietary Replacement (Days5-8)

<i>Day</i>	<i>-Control</i>	<i>+Namoi*</i>	<i>+38% Namoi can_*</i>	<i>+100% Namoi can*</i>	<i>+lysine*</i>	<i>+lysine with 38% Namoi can*</i>	<i>SED Means</i>
5	1.10	0.67	0.98	0.82	1.12	1.07	0.064
6	1.14	0.46	0.87	0.48	1.15	0.96	0.064
7	1.13	0.26	0.86	0.34	1.14	0.87	0.064
8	1.14	0.41	0.88	0.39	1.14	0.88	0.064

_ canavanine abbreviated to can, *soybean+component(s)

The following linear contrasts clarify the diet x day interactions.

- (i) -Control versus modified diets containing canavanine (P<0.001)
- (ii) +Lysine versus treatments containing canavanine (P<0.001)
- (iii) +100% Namoi canavanine concentration on soybean versus +Namoi (ns)
- (iv) Diets containing 38% Namoi canavanine concentration on soybean versus 100%

Namoi canavanine concentration on soybean (P<0.001)

- (v) +38% canavanine concentration on soybean versus +lysine with 38%
Namoi canavanine concentration on soybean (ns)

These data show that Namoi vetch or canavanine at either 38% or 100% of the equivalent Namoi levels, notably affects feed-intake in the pig. Lysine 2HCl, was arbitrarily chosen as a non guanidino dibasic amino acid control additive. It was fed at the same molal concentration as the canavanine in Namoi vetch and did not emulate either the 38% or 100% canavanine inhibitory effects on feed-intake. The 100% level of canavanine added to soybean meal compared with Namoi meal resulted in similar feed-intake reductions, but the 38% canavanine-soybean diet produced a significantly smaller reduction in feed-intake compared to the treatment using 100% level of canavanine present in Namoi vetch. There is no significant difference in feed-intake depression by the 38% canavanine treatment and 38% canavanine with an equimolal addition of

lysine. Taken together, these results clearly demonstrate that the measured feed-intake inhibition produced in pigs by Namoi vetch, is a function of the L-canavanine concentration in the diet.

Analysis of the post-treatment phase in which canavanine-containing diets were no longer fed and the diet once more contained only wheat and fishmeal indicated, that the pigs' voluntary feed-intake behaviour was quickly and substantially restored as demonstrated by the nonsignificant differences ($P=0.267$) among the six post treatment diets. Both the absolute feed-intake and the dimensionless feed-intake ratio data shown in Figs. 1 and 2 are a function of the weight of the pigs.

The mean post treatment intake for each of the diets was 1.19, 1.14, 1.15, 1.10, 1.20 & 1.12 (diets in same order as in table3). The mean intakes for the final four days (9-12) of the study were 1.16, 1.15, 1.12 and 1.17. Again there was a significant day effect ($P<0.01$).

Gender effect

Significant gender by day interactions were observed for pretreatment (Days 1-4) males: 0.99, 1.00, 1.01, 1.05 and females: 1.08, 1.08, 1.08, 1.09 ($P<0.05$), treatment (Days 5-8) males: 0.91, 0.83, 0.78, 0.77 and females: 1.01, 0.86, 0.75, 0.84 ($P<0.01$) and post treatment (Days 9-12) males: 1.14, 1.13, 1.07, 1.15 and females: 1.18, 1.18, 1.18, 1.18 ($P<0.01$) phases, with generally lower feed-intake means for boars compared with gilts, but the overall pattern of feed-intake inhibition by canavanine containing diets was unmistakable. Males were also more sporadic eaters than females in both the pre-treatment and post-treatment phases.

Analysis of variance of the pigs weights showed that the female pigs were significantly heavier than the male pigs in the pre-treatment and phases ($P<0.01$ and $P<0.05$ respectively). The gilts were, on average, 630 grams heavier in the pre-treatment phase and 730 grams heavier in the treatment phase of the experiment.

Houseman (1973) noted that boars had a lower feed intake than gilts but converted their feed more efficiently. However, Yen et al (1986) found no difference between the sexes in mean food intake except for diets with high lysine (10.4g/kg diet) and protein (160g CP/kg diet) where boars were superior to gilts and these to castrates.

Post ingestion effect (Table 4)

TABLE 4

Feed-intake (kg) for separate meals
on day five

<i>Meal</i>	<i>-Control</i>	<i>+Namoi*</i>	<i>+38% can*</i>	<i>+100% can*</i>	<i>+lysine*</i>	<i>+lysine with 38% can*</i>	<i>SED Means</i>
1st	0.52	0.48	0.47	0.49	0.54	0.56	0.043
2nd	0.58	0.19	0.51	0.33	0.58	0.51	0.053
Total	1.10	0.67	0.98	0.82	1.12	1.07	0.052

_ canavanine abbreviated to can, *soybean+component(s)

In experiment 5 only, the feed-intake was measured after the first meal in addition to the total consumption for the day at the start of the treatment period (day 5). These measurements provide the data shown in table 4. There was no significant difference among the means for the first meal ($P < 0.001$), whereas there is a clear reduction of feed-intake of the 100% canavanine containing diets during the second meal.

Taste aversion is a learned behaviour where the negative ingestional consequence of a meal is associated with a particular taste. It also requires a time period of several hours post-ingestion for such an aversion to develop (Ashe & Nachmann 1980).

From the data in table 4 we infer that for canavanine containing diets, the effect on feed-intake is mediated by a post-ingestional reaction to canavanine and this behaviour is akin to the feed aversion described for pigs by Houpt et al (1979).

Feed-intake ratios and graphical comparisons

The dimensionless feed-intake ratio is more useful than the absolute intake data for graphically comparing the time dependent patterns of different experiments as depicted in Figs 1 and 2. These feed-intake ratio patterns were an important feature of data assessment for experiments 1-4 during the isolation procedure. The data for experiment five shown in Fig 2, clearly illustrate that canavanine mimics the feed-intake inhibition of Namoi vetch.

Fig. 2. Feed-intake ratio data from experiment 5, for 20kg pigs fed wheat and fishmeal diets at approximately 75% of their *ad libitum* intake. During the treatment days (5-8), an 8% dietary replacement (c.f. Table 1) with one of the six treatments, (i) -G- (-)ve control of soybean meal, (ii) -H- (+)ve control of Namoi ($29 \text{ g kg}^{-1} = 0.163 \text{ mol kg}^{-1}$ canavanine) (iii) -J- soybean + 29 g kg^{-1} ($0.163 \text{ mol kg}^{-1}$) canavanine, (iv) -B- soybean + 11.3 g kg^{-1} ($0.064 \text{ mol kg}^{-1}$) canavanine, (v) -C- soybean + ($0.163 \text{ mol kg}^{-1}$) lysine 2HCl, (vi) -E- soybean + ($0.064 \text{ mol kg}^{-1}$) canavanine and ($0.064 \text{ mol kg}^{-1}$) lysine. SED: (day 1-4: 0.04, day 5-8 :0.06, day 9-12: 0.05)

Chemical considerations

L-Canavanine (I) is usually described as a guanidinoxy analogue of L-arginine (II). However, the analogy is flawed because the electron distribution of the guanidine portion of canavanine was unequivocally established as (I) by a crystal-structure investigation (Boyer and Marsh 1982). Replacement of the carbon-5 methylene of arginine by an electronegative oxygen alters the guanidine electron distribution as shown (I) and thereby markedly lowers the pKa of the guanidine group from 12.5 to 7.0 whilst leaving the overall dimensions of canavanine virtually unchanged (Boyer and Marsh 1982).

FIG. 3. Formulae comparing differences in guanidine electron distribution between L-canavanine and L-arginine

The resultant cationic charge diminution of canavanine compared to arginine in the physiological pH range near 7, undoubtedly has important biological implications especially when canavanine replaces arginine in proteins or peptides. Most of the well documented toxicity symptoms of canavanine can be explained by its

substitution for arginine in various arginine metabolic pathways, proteins (Rosenthal 1977b; Rosenthal 1991) and possibly bio-regulatory peptides. We can reasonably assume that its effect on pig feed-intake is also arginine related and it should provide an excellent molecule for the future study of feed-intake regulation. The indirect possibility exists, that canavanine may inhibit arginine-related nitric oxide generation (reviewed by Stuehr and Griffith 1992) which is important in blood pressure control via a mechanism analogous to the relaxation effect of nitric oxide on rat aortic rings (Schmidt et al 1988). Such an indirect mechanism could conceivably influence feed intake regulation via some as yet unidentified nitric oxide effect on peristalsis, and is worthy of further study.

Biological considerations

There are many reports of toxic effects from the feeding of seeds which contain canavanine to: rodents (Tschiersch 1962; de Muelenaere 1965; Wyss and Bickel 1988), cattle (Claughton and Claughton 1955; Shone 1961), birds (Arscott and Harper 1964, Shqueir *et al.* 1989, Leon et al 1990 and 1991) monkeys (Malinow *et al.* 1982) and humans (Roberts and Hayashi 1983). However, there appear to be only two reports confirming that acute toxicity symptoms can be induced by feeding purified canavanine salts to animals. In one case mice were fed at 2 g kg^{-1} bodyweight (Tschiersch 1962), and in the other, monkeys were fed diets containing up to 20 g kg^{-1} canavanine as the sulphate salt (Malinow *et al.* 1982). In neither case was the actual amount of ingested canavanine specified.

As far as sublethal doses of purified canavanine salts are concerned, the only report of aberrant behavioural symptoms induced by low dietary levels appears to be with mice fed $200 \text{ mg kg}^{-1} \text{ day}^{-1}$ ($1.14 \text{ mmol kg}^{-1} \text{ day}^{-1}$) bodyweight (Tschiersch 1962). Prete (1985) reported that L-canavanine fed to a "normal species" of mouse induced autoimmune phenomena and exacerbated these symptoms in a murine systemic lupus erythematosus model. However, no "substantial" decrease in protein consumption was observed. No details of feed-intake were given in any of these rodent studies. Shqueir *et al.* (1989) supplied feed which contained canavanine sulphate at 867 mg kg^{-1} ($= 3.16 \text{ mmol kg}^{-1}$) to chickens (initial av. wt. 40g) and found no statistically significant deleterious effects with a feed-intake of 18.33 g day^{-1} which equates to an initial canavanine intake per bird of $57.85 \text{ } \mu \text{mol day}^{-1}$ or $1.45 \text{ mmol kg}^{-1} \text{ day}^{-1}$ bodyweight.

Canavanine and feed-intake

This report represents the first example of voluntary feed-intake inhibition in a monogastric animal by an analytically pure naturally occurring amino-acid analogue. On a porcine bodyweight basis, an initial canavanine intake of less than $43 \text{ mg kg}^{-1} \text{ day}^{-1}$ ($= 0.24 \text{ mmol kg}^{-1} \text{ day}^{-1}$) was sufficient to induce an easily measurable feed-intake depression of 20%. Such control of *ad libitum* feed-intake in pigs has been seen as commercially desirable by Fowler (1985).

By contrast, Shqueir *et al* (1989) found no statistically measurable effects on chickens fed canavanine sulfate at $1.45 \text{ mmol kg}^{-1} \text{ day}^{-1}$ bodyweight. Hence on a bodyweight basis, some chickens can apparently tolerate much higher levels of purified canavanine than pigs. Nevertheless, the acute toxicity to poultry of diets which comprised mainly *Vicia villosa* seeds, is well documented (Arscott and Harper 1964; Kienholz *et al* 1962). These seeds can reasonably be expected to contain canavanine approaching the level ($163 \text{ mmols kg}^{-1}$) found in *V.villosa* ssp. *dasycarpa* in the present study. Hence, the canavanine dosage ($\text{mmol kg}^{-1} \text{ day}^{-1}$ bodyweight) for acute toxicity symptoms in poultry has not yet been established. Leon *et al* (1991) have established that although a short term feed-intake inhibition in chickens fed jack bean *Canavalia ensiformis* (L) DC meal, could be explained in terms of the presence of an uncertain concentration of the thermolabile Concanavaline A. Nevertheless, a thermostable residual feed intake depressant activity for poults and chicks still remained in jack bean meal which had been extruded (135°C) (Leon *et al* 1990 and 1991). The thermostable residual feed intake depressant activity was tentatively ascribed to canavanine, but the concentration of canavanine in the extruded material was not reported.

The literature records similar feed-intake inhibitory behaviour by diets incorporating seeds of *C. ensiformis* fed to rats (de Muelenaere 1965) and cattle (Addison, 1958), and of *Vicia villosa* fed to cattle (Pancierra *et al.* 1966). These seeds are now well known to contain canavanine; for its qualitative distribution see Bell (1960) and for its quantitation see Turner and Harbourne (1967); Rosenthal (1977) and Natelson (1985).

From these diverse reports, virtually all of which directly or indirectly associate this arginine analogue with feed-intake inhibition, it is clear that the effect is not necessarily limited to monogastric animals. Future comparative studies of tolerance and adaption to canavanine containing diets in different species are clearly warranted. In the mean time, considerable care should be exercised in formulating feedstuffs incorporating seeds with a high canavanine content.

In cattle (Addison, 1958), the unpalatability of jack bean meal (Canavanine content 26.5 g kg^{-1} , Natelson, 1985) in the diet, was effectively masked by the addition of molasses ($\sim 18 \text{ litre t}^{-1}$). In unpublished work by the late R L Davies of these laboratories, it was observed that a neutralized hydrochloric acid hydrolysate of a turbid aqueous extract of Namoi vetch seed was less inhibitory to pigs than an equivalent amount of unhydrolyzed extract. Examination of the hydrolysate showed the presence of much glucose from starch present in the original turbid extract. From these two reports, and a personal communication from E A Bell, which indicated that rats could be induced to eat canavanine-containing feed when mixed with chocolate spread, it seems that a canavanine induced feed-intake inhibition may be at least partly reversed by including sweet tasting additives in the diet.

Human dietary implications

In view of the reproducible and readily observable feed-intake inhibition induced in both pigs (this work), and rats (de Muelenaere 1965), by diets incorporating *Vicia villosa* (canavanine, 29 g kg^{-1}) and *Canavalia* (canavanine, 26.5 g kg^{-1} , Natelson 1985), the possible deleterious effects to human dietary sources of canavanine needs to be considered. Natelson (1985) has reported the canavanine content of various alfalfa seeds to be in the range 8.33 g kg^{-1} to 13.6 g kg^{-1} , and adverse human reactions to alfalfa tablets are documented (Roberts and Hayashi 1983). Although canavanine is rapidly lost during germination (Bell 1960) the possibility exists, that hyper-sensitivity in some human individuals to low residual levels of naturally occurring canavanine in alfalfa sprouts could have physiological and dietary implications. Such effects may well be exacerbated in individuals suffering from malnutrition or hepatic disease.

Nevertheless, for the vast majority of the human population, we support the view expressed by Rosenthal (1978), that although "some canavanine seeds do comprise a dietary nitrogen source in certain human cultures (Shone 1961; Nout and Rombouts 1990; Obizoba and Obiano 1988)" ... " these legumes are [soaked (Obizoba and Obiano 1988); drained (Nout and Rombouts 1990) and] cooked extensively "and in general" none of the commonly consumed table legumes, nor legumes such as soya bean, which are employed as protein supplements contain significant canavanine." The water solubility of canavanine as well as the usage of alkaline conditions for cooking *Canavalia* (Obizoba and Obiano 1988, D'Mello and Walker 1991), would certainly enhance the destruction of any residual non-extracted canavanine by

conversion to deaminocanavanine. In experiment 3, alkaline degradation of canavanine (recovery = 15.7g kg⁻¹, 54%) yielded deaminocanavanine as a major component in the neutral amino acid fraction which eluted with ammonia. Assuming that the unrecovered canavanine was substantially converted to the observed deaminocanavanine we can estimate that this fraction (recovery = 16.1g kg⁻¹) could account for up to nearly half the initial canavanine concentration (= 29g kg⁻¹). However the experiment 3 pig bioassay (Fig1 and table 2) demonstrated that in contrast to a similar level of recovered canavanine, this neutral amino acid fraction containing deaminocanavanine, showed no significant feed-intake inhibition. It is evident, that with an understanding of the chemistry of anti-nutritional factors, detoxification strategies can be explained; or newly devised in order to render many potentially toxic leguminous foods safe to eat, which could be a matter of considerable importance in famine situations and for future food resources.

CONCLUSION

The factor present in *V.villosa* ssp. *dasycarpa* which inhibits feed-intake in pigs at a voluntarily ingested dose of < 43 mg kg⁻¹ day⁻¹ has been identified with the help of a pig feed-intake bioassay as L-canavanine and may be useful in the study of feed intake regulation.

The close structural resemblance of canavanine to arginine suggests that a re-examination of the role of arginine in feed intake regulation could be rewarding. This is especially so, if recently discovered properties such as its role in blood pressure control via the generation of the nitric acid radical (reviewed by Stuehr and Griffith 1992) were to be replicated in other phenomena related to feeding such as peristalsis.

In principle, in a protein deficient world, more leguminous high protein food could be made available for animal and human consumption with a knowledge of the content of undesirable biologically active components such as canavanine. In particular, their stability to heat, hydrolysis, pH, endogenous enzymes and ease of extraction can logically explain or suggest effective post harvest processing methods such as the simple extraction and alkaline heat treatment for jack bean (Obizoba and Obiano 1988, D'Mello and Walker 1991). This post harvest processing can now be seen to be thermally destroying the Concanavaline A content which is already known to affect feed intake in poultry (Leon et al 1990, and 1991) in addition to destroying any residual unextracted canavanine by conversion to the cyclic deamino-canavanine (Rosenthal et al 1977a).

ACKNOWLEDGMENTS

We thank the Australian Grain Legumes Council for financial support, Northfield Pig Research Unit staff for animal care and Mr. H. Gockowiak of the Australian Wine Research Institute for the HPLC analysis.

REFERENCES

- Addison K B 1958 The Jack Bean. *Rhodesian Farmer* **28** 17-18.
- Arcscott G H, Harper J A 1964 Evidence for a difference in toxicity between common and hairy vetch seed for chicks. *Poult Sci* **43** 271-273.
- Ashe J H, Nachmann M 1980 Neural mechanisms in taste aversion learning. *Prog Psychobiol Physiol Psychol* **9** 233-262.
- Bell E A 1960 Canavanine in the *Leguminosae*. *Biochem J* **75** 618-620.
- Bell E A, Lackey J A, Polhill R M 1978 Systemic significance of canavanine in the *Papilioideae* (*Faboideae*). *Biochem Syst Ecol* **6** 201-212.
- Boyer A, Marsh R E 1982 L-Canavanine, a paradigm for the structures of substituted guanidines. *J Am Chem Soc* **104** 1995-1998.
- Claughton W P, Claughton, H.P 1955 Vetch seed poisoning. *Auburn Veterinarian* **10** 125-126.
- D'Mello J P F, Walker A G 1991 Detoxification of jack beans (*Canavalia ensiformis*): studies with young chicks. *Anim Feed Sci Technol* **33** 117- 127.
- Fowler V R 1985 The importance of voluntary feed intake in pigs. *Proc Nutr Soc* **44** 347-353.
- Haupt K A, Zahorik D M, Anika S M, Haupt T R 1979 Taste aversion learning in suckling and weanling pigs. *Vet Sci Commun* **3** 165-169.
- Houseman R A 1973 A comparison of the voluntary intakes of boars, gilts and castrates (Abstr). *Proc Brit Soc Anim Prod* **2** 90-91.
- Jones B N, Gilligan J P 1983 o-Phthaldialdehyde precolumn derivatization and reversed-phase high performance liquid chromatography of polypeptide hydrolysates and physiological fluids. *J Chromatogr* **266** 471-482.
- Kienholz E W, Jensen L S, McGinnis J 1962 Evidence for chick growth inhibitors in several legume seeds. *Poult Sci* **41** 367-371.
- Leon A, Carre B, Larbier M, Lim F, Ladjali T, and Picard M, 1990 Amino acids and starch digestibility and true metabolizable energy content of raw and extruded jackbeans (*Canavalia ensiformis*) in adult cockerels. *Ann Zootech* **39** 53-61.

- Leon A M, Caffin J-P, Plassart M, Picard M L 1991 Effect of concanavalin A from jackbean seeds on short-term food intake regulation in chicks and laying hens. *Animal Feed Science and Technology* **32** 297-311.
- Lepkovsky S 1948 Physiological basis of voluntary food intake (appetite?). *Adv Food Res I* **1** 105-148.
- Muelenaere de H J H 1965 Toxicity and haemagglutinating activity of legumes. *Nature* **206** 827-828.
- Malinow M R, Bardana Jr E J, Pirofsky B, Craig S, McLaughlin P 1982. Systemic Lupus Erythematosus-like syndrome in monkeys fed alfalfa sprouts: role of a nonprotein amino acid. *Science* **216** 415-417.
- Natelson S 1985 Canavanine to arginine ratio in alfalfa (*Medicago sativa*), clover (*Trifolium*), and the jack bean (*Canavalia ensiformis*). *J Agric Food Chem* **33** 413-419.
- Nout M J R, Rombouts F M 1990 Recent developments in Tempe research. *J Appl Bact* **69** 609-633.
- Obizoba I C, Obiano N 1988 The nutritive value of jackbean (*Canavalia ensiformis*). *Ecol Food Nutr* **21** 265-270.
- Pancierra R J, Johnson L, Osbourne B I 1966 A disease of cattle grazing hairy vetch pasture. *J Am Vet Med Ass* **148** 804-808.
- Prete P E 1985 Effects of L-canavanine on immune function in normal and autoimmune mice: disordered B-cell function by a dietary amino acid in the immunoregulation of autoimmune disease. *Can J Physiol Pharmacol* **63** 843-854.
- Ressler C, Redstone P A, Erenberg R H 1961 Isolation and identification of a neuroactive factor from *Lathyrus latifolius*. *Science* **134** 188-189.
- Roberts J L, Hayashi J A 1983 Exacerbation of SLE associated with alfalfa ingestion. *New England Journal of Medicine* **308** 1361.
- Rosenthal GA 1977a Preparation and colorimetric analysis of L-canavanine. *Anal Biochem* **77** 147-151.
- Rosenthal G A 1977b The biological effects and mode of action of L- canavanine, a structural analogue of L-arginine. *Q Rev Biol* **52** 155-178.

- Rosenthal G A 1978 Is canavanine a natural component of mammalian metabolism?. *J Theor Biol* **71** 265-273.
- Rosenthal G A 1982 *Plant Nonprotein Amino and Imino acids. Biological, Biochemical and Toxicological Properties*. Academic Press, New York, pp57-157.
- Rosenthal G A 1991 The biochemical basis for the deleterious effects of L- canavanine. *Phytochemistry (OXF)* **30** 1055-1058.
- Schmidt H H H W, Klein M M, Niroomond F, Bohme E 1988 "Is arginine a physiological precursor of endothelium-derived nitric oxide?". *Europ J Pharmac* **148** 293-295.
- Shone D K 1961 Toxicity of the Jack Bean. *Rhod Agric J* **58** 18-20.
- Shqueir A A, Brown D L, Klasing K C 1989 Canavanine content and toxicity of *Sesbania* leaf meal for growing chickens. *Anim Feed Sci Technol* **25** 137-147.
- Smolenski S J, Kinghorn A D, Balandrin M F 1981 Toxic constituents of legume forage plants. *Econ Bot* **35** 321-355.
- Stuehr D J, Griffith O W 1992 Mammalian nitric oxide synthases. in *Advances in Enzymology*. John Wiley & Sons, New York, pp287-346.
- Tschiersch B 1962 Zur toxischen Wirkung der Jackbohne. *Pharmazie* **17** 621-623.
- Turner B L, Harborne J B 1967 Distribution of canavanine in the plant kingdom *Phytochemistry (OXF)* **6** 863-866.
- Wyss U, Bickel H 1988 Ripe beans of *Canavalia ensiformis* (jackbean) as feed ingredient for monogastric animals. *Anim Feed Sci Technol* **20** 325-326.
- Yen H T, Cole D J A, Lewis D 1986 Amino acid requirements of growing pigs. 7. The response of pigs from 25 to 55 kg live weight to dietary ideal protein. *Anim Prod* **43** 141-154

FIG.1

ENNEKING *et al.* : "L-Canavanine : A Natural Feed-Intake Inhibitor for Pigs"

FIG. 2

ENNEKING *et al.* : "L-Canavanine : A Natural Feed-Intake Inhibitor for Pigs"

FIG. 3
ENNEKING *et al.* : "L-Canavanine : A Natural Feed-Intake Inhibitor for Pigs"

TABLE 1

Diet formulations kg t⁻¹

<i>Component -Base</i>	<i>Soy</i>	<i>Namoi</i>	<i>+38% Namoi can³*</i>	<i>+100% Namoi can²*</i>	<i>+lysine⁴*</i>	<i>+lysine⁵+ 38% can³*</i>
Wheat	790	727	727	727	727	727
Fishmeal	170	156	156	156	156	156
Soy meal	---	80	---	80	80	80
Namoi	---	---	80	---	---	---
Can HCl	---	---	---	1.23	3.25	---
Lys HCl	---	---	---	---	---	2.86
Dicalcium phosphate	5.0	4.6	4.6	4.6	4.6	4.6
Limestone	3.0	2.8	2.8	2.8	2.8	2.8
Tallow	25.0	23.0	23.0	23.0	23.0	23.0
Salt	2.5	2.3	2.3	2.3	2.3	2.3
Min/Vit/ premix ¹	2.0	1.8	1.8	1.8	1.8	1.8
Total	997.5	997.5	997.5	998.7	1000.8	1000.4

¹ Provided (per kg diet) : vitamin A, 11 000 IU; D, 2 200 IU; E, 40 IU; K, 2 mg; thiamin, 1.5 mg; riboflavin, 5 mg; pyridoxine 2 mg; calcium pantothenate, 11 mg; niacin, 20 mg; folic acid, 1 mg; biotin, 150 ug; vitamin B₁₂, 20 ug; Cu, 10 mg; Fe, 100 mg; Zn, 150 mg; Mn, 50 mg; I, 0.5 mg; Mo, 0.5 mg; Co, 0.2 mg; Se, 0.13 mg; ethoxyquin, 100 mg.

Final feed concentrations per kg diet: ² Canavanine 2 HCl 13.04 mmol kg⁻¹, ³ Canavanine 2.HCl 5.12 mmol kg⁻¹, ⁴ Lysine 2.HCl 13.04 mmol kg⁻¹, ⁵ Lysine 2.HCl 5.12 mmol kg⁻¹, ANALYSIS : (Base diet g kg⁻¹): Fat, 76.0; Fibre, 21.8; CP, 190.6; Arg, 10.4; His, 4.5; Ile, 7.3; Leu, 13.5; Adlys, 10.3; Met, 4.3;

Cys, 3.8; Phe 8.1; Tyr, 6.3; Thr, 6.7; Try 2.2; Val 9.4; Ca 9.9; P, 6.2; Avp, 4.9. Other: DE, 15.1 MJ kg⁻¹; Adlys/DE 0.69 g MJ⁻¹.

TABLE 2

Expt	Treatment	Feed-intake (kg/day)	Pig weights , max-min; mean(kg) No. animals
1	a) Soybean (-ve control)	0.89	16-22.5; 18.8 20 animals
	b) Namoi vetch (+ve control)	0.40	
	c) autoclaving	0.42	
	d) defatting	0.35	
	e) aqueous ethanol extraction	0.66	
	SED	0.075	
2	a) Soybean (-ve control)	0.94	15-20; 16.7 20 animals
	b) Namoi vetch (+ve control)	0.19	
	c) dialysis retentate	0.79	
	d) peroxidation+NaHSO ₃	0.27	
	e) cationic fraction	0.60	
	SED	0.083	
3	a) Soybean (-ve control)	1.02	19-26; 22.8 32 animals
	b) Namoi vetch (+ve control)	0.30	
	c) cationic fraction	0.57	
	d) acidic amino acid fns 5-8	1.02	
	e) acidic amino acid fns 9-12	1.07	
	f) acidic amino acid fns 13-15	1.04	
	g) neutral amino acid fns 2+3 A	1.07	
	h) basic amino acid fns 2+3 B	0.67	
	SED	0.087	
4	a) Soybean (-ve control)	1.02	18-23; 20.0 16 animals
	b) Namoi vetch (+ve control)	0.40	
	c) NaCl	1.08	
	d) 0.043 M Canavanine.2HCl	0.65	
	SED	0.078	
5	a) Soybean (-ve control)	1.13	19-29; 24.6 48 animals
	b) Namoi vetch (+ve control)	0.45	
	c) 0.163 M canavanine.2HCl	0.51	
	d) 0.163 M lysine.2HCl	1.14	
	e) 0.062 M canavanine.2HCl	0.90	
	f) 0.062 M canavanine.2HCl+ 0.062 M lysine.2HCL	0.95	
	SED	0.053	

ENNEKING *et al.* : "L-Canavanine : A Natural Feed-Intake Inhibitor for Pigs"

TABLE 3

Mean Daily Intake (kg) / Pig at 8% Dietary Replacement (Days5-8)

<i>Day</i>	<i>-Control</i>	<i>+Namoi*</i>	<i>+38% Namoi can_*</i>	<i>+100% Namoi can*</i>	<i>+lysine*</i>	<i>+lysine with 38% Namoi can*</i>	<i>SED Means</i>
5	1.10	0.67	0.98	0.82	1.12	1.07	0.064
6	1.14	0.46	0.87	0.48	1.15	0.96	0.064
7	1.13	0.26	0.86	0.34	1.14	0.87	0.064
8	1.14	0.41	0.88	0.39	1.14	0.88	0.064

_ canavanine abbreviated to can, *soybean+component(s)

TABLE 4

Feed-intake (kg) for separate meals
on day five

<i>Meal</i>	<i>-Control</i>	<i>+Namoi*</i>	<i>+38% Namoi can_*</i>	<i>+100% Namoi can*</i>	<i>+lysine*</i>	<i>+lysine with 38% Namoi can*</i>	<i>SED Means</i>
1st	0.52	0.48	0.47	0.49	0.54	0.56	0.043
2nd	0.58	0.19	0.51	0.33	0.58	0.51	0.053
Total	1.10	0.67	0.98	0.82	1.12	1.07	0.052

_ canavanine abbreviated to can, *soybean+component(s)

LEGENDS:

ENNEKING *et al.* : "L-Canavanine : A Natural Feed-Intake Inhibitor for Pigs"

Fig. 1. Daily voluntary feed-intake ratio data (experiment 3), for 20kg pigs fed wheat and fishmeal diets at approximately 75% of their *ad libitum* intake during pre- and post- treatment days (1-4 and 9-12). During the treatment days (5-8), an 8% dietary replacement with one of the four treatments, (i) -G- -ve control of soybean meal, (ii) -H- +ve control of Namoi ($29 \text{ g kg}^{-1} = 0.163 \text{ mol kg}^{-1}$ canavanine) (iii) -B- soybean + recovered total cationic amino acid fraction from Namoi vetch, (iv) -J- soybean + recovered basic amino acid fraction. SED: (day 1-4: 0.09, day 5-8 :0.10, day 9-12: 0.09)

Fig. 2. Feed-intake ratio data from experiment 5, for 20kg pigs fed wheat and fishmeal diets at approximately 75% of their *ad libitum* intake. During the treatment days (5-8), an 8% dietary replacement (c.f. Table 1) with one of the six treatments, (i) -G- (-)ve control of soybean meal, (ii) -H- (+)ve control of Namoi ($29 \text{ g kg}^{-1} = 0.163 \text{ mol kg}^{-1}$ canavanine) (iii) -J- soybean + 29 g kg^{-1} ($0.163 \text{ mol kg}^{-1}$) canavanine, (iv) -B- soybean + 11.3 g kg^{-1} ($0.064 \text{ mol kg}^{-1}$) canavanine, (v) -C- soybean + ($0.163 \text{ mol kg}^{-1}$) lysine 2HCl, (vi) -E- soybean + ($0.064 \text{ mol kg}^{-1}$) canavanine and ($0.064 \text{ mol kg}^{-1}$) lysine. SED: (day 1-4: 0.04, day 5-8 :0.06, day 9-12: 0.05)

FIG. 3. Formulae comparing differences in guanidine electron distribution between L-canavanine and L-arginine (Boyar and Marsh 1982)