

Developments in Plant Breeding For Improved Nutritional Quality of Soya Beans
I. Protein and Amino Acid Content

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Short title: Improving protein content and quality of soya beans

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SUMMARY

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4 Soya beans, like other legumes, contain low concentrations of the nutritionally
5 essential sulphur amino acid, methionine. Cysteine, although not an essential amino
6 acid because it can be synthesized from methionine, also influences the nutritional
7 quality of soya bean products when it is only present in low levels. A low cysteine
8 content will also aggravate a methionine deficiency.

9 Soya bean lines deficient in 7S protein subunits have been identified. The 7S
10 proteins contain substantially less methionine and cysteine than the 11S proteins.
11 With the myriad of genetic null alleles for these subunits it may be possible to tailor
12 the 7S/11S storage protein ratio and their total composition in seeds to include only
13 those subunits with the richest sulphur amino acid composition.

14 Cotyledon feeding experiments, using isolated soya bean cotyledons,
15 demonstrated that addition of methionine to the culture media caused increased
16 synthesis of both proteins and free amino acids but the mechanism by which this
17 takes place is not clear.

18 Biotechnological approaches to improve nutritional value of soya beans
19 include elevated expression of genes that originate from other species which encode
20 high-sulphur proteins. High level expression of a 2S Brazil nut albumin gene in soya
21 bean resulted raised methionine concentration although the Brazil nut gene is highly
22 antigenic and therefore will not be useful in production agriculture.

23 Modification of glycinin to increase sulphur amino acid content is possible,
24 and these gene products are capable of normal assembly into trimers *in vitro*
25 although are rapidly degraded *in vivo* by the asparaginyl endopeptidase responsible
26 for post-translational modification of proglycinin.

27 Solutions to the methionine deficiency may be anticipated from a combination
28 of approaches followed in laboratories worldwide. Many of these approaches are not
29 without difficulty but, despite this, the likelihood is that soya beans with improved
30 nutritional quality (which may not be confined to sulphur-containing amino acids as
31 other nutritionally essential amino acids are also valuable) will be available in the
32 near future. It will be essential to confirm that the increased total methionine (or other
33 amino acid) is digestible to the animal to at least the same degree as conventional
34 cultivars.
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INTRODUCTION

Although soya beans are perhaps the most important legume crop worldwide, there are still problems relating to its nutritional value including deficiency of sulphur-containing and other amino acids. Enhanced nutritional quality of soya beans, achieved through increasing the concentration of nutritionally essential amino acids, improves their nutritional value and would further enhance its role as a key raw material for inclusion into diets for non-ruminants.

Although economic analyses are transient, linear programming optimization routines (University of Nottingham, unpublished data) employed in diet formulation indicate that the commercial value of a 10% improvement in concentration of lysine, methionine and threonine is associated with an increase in the value of the crop of £3.4 - £7.1, £2.0 and £4.4 / T respectively. Although value in this context is influenced by the precise circumstances under which the routines are run (for example relative price of other raw materials and nutrient content of diets), the data nevertheless indicate that it is not just an increase in methionine which would be beneficial and that a general elevation in protein content would also be useful.

There has been a considerable research effort directed towards the improvement in the quality of soya beans and this review will cover the potential for improving protein quality. Alterations of seed storage protein ratios offer the possibility of increasing amino acid content, particularly methionine, and high protein cultivars are also now being developed.

PROTEIN QUALITY

The soya bean belongs to the family *Leguminosae*, subfamily *Papilionoideae*, and the genus *Glycine* L. The cultivated form, *Glycine max* L. Merrill, grows annually. Its plant is bushy with height ranging from 0.75 to 1.25m, branching sparsely or densely, depending on cultivars and growing conditions. Most mature seeds consist of three basic parts: the seed coat, the embryo and one or more storage structures. However, the soya bean seed, as in most leguminous plants, is essentially devoid of endosperm and consists of a seed coat and a large, well-developed embryo. The embryo contains two cotyledons which function as food reserves. During seed maturation the cells of the cotyledons are filled with starch grains (about 5 μ m in

1 diameter), lipid bodies, and protein bodies. The starch grains are converted to other
2 components during the last few days of maturation, so very little starch (less than
3 10mg/g) is found in mature soya bean seeds. The proximate composition of the
4 different structural parts of soya beans varies (e.g. Wolf & Cowan 1975), for example
5 the hull contains the greatest concentration of non-starch polysaccharides (NSP but
6 have the lowest content of protein and lipid.

7 Based on biological function in plants, seed proteins are of two types:
8 metabolic and storage. Metabolic proteins include enzymatic and structural proteins
9 and are involved in normal cellular activities, including synthesis of storage proteins
10 which, together with oil reserves, occurs during seed development. Following seed
11 germination, they provide a source of nitrogen and carbon skeletons for the
12 developing seedling. The majority of soya bean protein is storage protein.

13 Legume seed proteins are divided into albumins and globulins, a distinction
14 which is based on solubility patterns. Albumins are soluble in water, whereas an
15 early detailed study of soya bean globulins carried out by Osborne & Campbell (1898)
16 demonstrated that a substantial proportion of the seed protein of legumes could be
17 extracted by dilute saline solutions which were referred to as globulins in accordance
18 with the empirical nomenclature devised by Osborne (1924). Under this classification,
19 most protein in soya bean protein is globulin. Two types of globulin were found in
20 soya bean and these were named glycinin and conglycinin.

21 22 Soya bean globulins

23
24 The invention of the ultracentrifuge and subsequent studies by Danielsson (1949) led
25 to the demonstration that glycinin and conglycinin had sedimentation coefficients of
26 about 11S and 7S, respectively. Ensuing reports, from researchers in both Japan
27 and the United States, established that glycinin and conglycinin were multi-subunit
28 proteins and account for approximately 0.7 of total protein in a typical soya bean
29 seed (Hill & Breidenbach 1974; Meinke *et al.* 1981).

30 31 *∃-Conglycinin (7S fraction)*

32
33 In the literature, reference is made to ∇-, ∃- and ∑-conglycinin. These terms were

1 used originally by Catsimpoalas & Ekenstam (1969) to refer to three immunologically
2 distinct fractions separated from a crude conglycinin preparation. However, a
3 subsequent report by Catsimpoalas (1969) showed that ∇ -conglycinin contains
4 enzymatic activities characteristic of 2S fractions. Ξ - and Θ -conglycinins contain no
5 enzymatic activities and are differentiated from one another on the basis of their
6 capacity to undergo reversible polymerization at neutral pH in response to lowering
7 ionic strength from 0.5 to 0.1 (Catsimpoalas 1969); Ξ -conglycinin exhibits a
8 sedimentation coefficient of about 7S at high ionic strengths and one of 9-10S at the
9 lower ion concentration, whereas Θ -conglycinin fails to undergo this change. In
10 addition, based on the method of fractional isoelectric precipitation, Thanh &
11 Shibasaki (1976a) reported that the former was precipitated at pH 4.8 whereas the
12 latter remained soluble. Because of its highest proportion in the 7S fraction,
13 Ξ -conglycinin has been studied extensively (See Table1).

14
15 (Table 1 about here)

16
17 It is now generally agreed that Ξ -conglycinin is a trimer with a molecular
18 weight of about 180 kDa. It has three prevalent types of subunits, designated as ∇' ,
19 ∇ and Ξ . The individual subunits can be resolved from one another by ion exchange
20 chromatography after denaturation of the native complex with urea (Thanh &
21 Shibasaki 1976a) or by sodium dodecyl sulphate (SDS) polyacrylamide gel
22 electrophoresis (PAGE). Based on the electrophoretic mobility of the second method,
23 the molecular weight of the ∇' , ∇ and Ξ subunits were estimated at 57, 57, and 42
24 kDa, respectively (Thanh & Shibasaki 1976b).

25 Another subunit, named Ξ' , is present only in some soya bean varieties
26 (Coates *et al.* 1985; Morita *et al.* 1996). The primary structure of the Ξ' subunit is still
27 unknown, but the data suggested that this subunit is rich in sulphur-containing amino
28 acids (Coates *et al.* 1985). All three subunits are rich in aspartate, glutamate, leucine
29 and arginine. The two subunits ∇' , ∇ are very similar in amino acid composition. Both
30 are devoid of cysteine and have low levels of methionine. In contrast, the Ξ subunit
31 contains no methionine (Nielson 1985). All of the Ξ -conglycinin subunits are
32 glycoproteins and contain 40-50g carbohydrate per kg. Thus the 7S globulin is
33 considered to be glycosylated (Pernollet and Mosse 1983).

1 Ξ -conglycinin is heterogeneous with regard to subunit composition. Among
2 the ten theoretically possible multiple forms, six (B_1 to B_6) have been demonstrated
3 to occur (Thanh & Shibasaki 1976b). The subunit compositions of the six
4 components are as follows: B_1 is composed of 1 ∇' and 2 Ξ ; B_2 is composed of 1 ∇
5 and 2 Ξ ; B_3 is composed of 1 ∇ , 1 ∇' and 1 Ξ ; B_4 is composed of 2 ∇ and 1 Ξ ; B_5 is
6 composed of 2 ∇ and 1 ∇' and B_6 is composed of 3 ∇ . Furthermore, Ξ -conglycinin
7 undergoes a complicated association-dissociation phenomenon in response to
8 changes in ionic strength and pH. The phenomenon was initially reported by
9 Naismith (1955) and then confirmed by many other workers. According to Thanh &
10 Shibasaki (1979), the six forms of trimers are able to dimerise reversibly at low ionic
11 strength or in the pH region of 4.8-11.0. The resulting 9S form is a super dimer of
12 two trimers facing each other and becomes a hexamer. At extreme pH, 2.0 or 12.0,
13 dissociation into subunits (∇' , ∇ and Ξ) are also reversible so that six molecular
14 species B_1 to B_6 can be reconstituted by mixing the three subunits in urea solution
15 and subsequently dialyzing against a phosphate buffer. Subsequent studies by
16 other workers led to the identification of the B_0 form of Ξ -conglycinin. It contains three
17 subunits (Sykes & Gayler 1981, Yamauchi *et al.* 1981).

18 Most recently, Morita *et al.* (1996) succeeded in isolation and purification of
19 four major molecular components of Ξ -conglycinin, 3∇ , $2\nabla 1\Xi$, $1\nabla 2\Xi$ and 3Ξ , from
20 seeds of an ∇' subunit-deficient strain. By using such cultivars with a simple subunit
21 composition and chromatography at intermediate ionic strengths over a period
22 sufficient to reach an effective dissociation equilibrium between the monomer and
23 dimer, they also succeeded in crystallization of single Ξ -conglycinin components.
24 Consequently they were able to compare 3∇ and 3Ξ components regarding their
25 secondary structure and found that the 3∇ component showed a higher ∇ -helix
26 content than 3Ξ . Moreover, they also found two novel components from the same
27 seeds: $2\Xi 1\Xi'$ and $2\Xi 1 des (V1R126)\nabla$. The former contained a subunit Ξ' , pointed out
28 by Coates *et al.* (1985), and the latter contained a sub-component which could be
29 derived artificially from limited hydrolysis of the ∇ -subunit by a novel serine protease
30 found previously in some soya bean strains (Morita *et al.* 1994). The proteolysis may
31 occur in the course of extraction and purification.

32
33 *Glycinin (11S fraction).*

1
2 Glycinin is the purified form of the 11S globulin. It is the largest single fraction of the
3 total seed protein (250-350g/kg protein) and accounts for over 0.4 of the total seed
4 globulin (Murphy & Resurreccion 1984). In contrast to \exists -conglycinin, only a small
5 portion of glycinin is glycosylated (Lei & Reeck 1987).

6 The currently accepted model of glycinin is a hexamer with molecular weight
7 of around 360kDa. Its monomeric subunits have a generalized structure A-S S-B,
8 where A represents an acidic polypeptide of 34-44 kDa; B is a basic polypeptide of
9 around 20kDa; and S-S is a single disulphide bond that links the two polypeptides.
10 The subunits and polypeptides can be disassembled by use of urea plus a disulphide
11 reductant such as \exists -mercaptoethanol. The reduced and denatured acidic
12 polypeptide components can be resolved by chromatography with DEAE-sephadex,
13 whereas the basic components can be separated by chromatography with
14 CM-Sephadex (Moreira *et al.* 1979). Some of the peptide units of \exists -conglycinin,
15 together with other seed proteins or subunits can also be separated by SDS-PAGE.

16 Five major subunits have been characterized, namely $A_{1a}B_2$, $A_{1b}B_{1b}$, A_2B_{1a} , A_3B_4
17 and $A_4A_5B_3$ (Staswick *et al.* 1981). Based on physical properties, these subunits can
18 be separated into two distinct groups. Group I includes the first three subunits whilst
19 group II includes the two remaining subunits. The group I subunits have more
20 uniform apparent MW and contain more methionine than members of group II (see
21 Table 2). The last feature is important for breeders to increase the methionine
22 content in seeds.

23
24 (Table 2 about here)

25
26 Among the five major subunits, the G5 subunit ($A_4A_5B_3$) is an exception from
27 the general structure. Its acidic component consists of two polypeptides, A_5A_6 , and
28 contains a proteolytic cleavage site approximately 100 amino acids from the amino
29 terminal of the precursor polypeptide. Consequently the A_4 -component separates
30 from the disulphide linked A_5A_6 component upon denaturation.

31 There is a genetic polymorphism of the 11S globulin in soya beans. The 11S
32 globulin of standard varieties can be classified into two types, A_5 - and A_4 - types
33 (Kitamura *et al.* 1980), depending on the presence or absence of the A_4A_5 acidic

1 polypeptide component and its paired basic polypeptide. The absence of the subunit
2 is controlled by a single recessive allele (Harada *et al.* 1983). Almost all the USA
3 varieties examined to date contain all five 11S subunits whilst around 0.2 of
4 Japanese varieties lacked the A₄A₅B₃ subunit (Kitamura 1995).

5 6 *Differences between glycinin and \exists -conglycinin globulins.*

7
8 Due to differences in composition and structure, glycinin and \exists -conglycinin exhibit
9 differences in both nutritional quality and functional properties. In general, the 11S
10 globulin (glycinin) contains 3-4 time more methionine per unit protein than 7S
11 globulin (\exists -conglycinin) (Kitamura 1995). As soya bean protein is deficient in sulphur
12 amino acids, glycinin protein is more valuable from a nutritional perspective.

13 14 *Variation in total seed protein composition.*

15
16 A typical soya bean contains approximately 120g non-structural carbohydrate/kg,
17 200g oil/kg and 400g protein/kg but both genetic and environmental factors strongly
18 influence seed composition. Soya bean varieties grown commercially in the United
19 States typically contain 360-390g protein/kg, although there are accessions in the
20 US soya bean collection that approach 500g protein/kg (Juvik *et al.* 1989a; b). The
21 latter generally have unacceptably low yields and are not suited for production
22 agriculture. Because seeds with high protein content are commercially important,
23 attempts have been made to develop high protein soya bean lines. Provar (Probst *et*
24 *al.* 1971) and Protana, (Weber & Fehr 1970) are examples of two early varieties
25 selected for high protein content. The varieties were not readily accepted by farmers
26 because of the decreased yield compared to other readily available varieties. In
27 general, protein content is inversely related to yield (Brim & Burton 1979). Negative
28 correlations are also often observed between soya bean oil or carbohydrate and
29 protein content (Serretti *et al.* 1994)

30 Backcrossing, a plant breeding approach used to introgress simply inherited
31 traits into a designated parent, has been used with limited success to improve the
32 protein concentration of seeds (Hartwig & Hinson 1972; Wehrman *et al.* 1987). A
33 recent example of this approach is reported by Wilcox and Cavins (1995). The high

1 protein trait originated from Pando (498g protein/kg), the unadapted donor parent.
2 Cutler 71, the adapted parent with 408g protein/kg, was the other parent. After
3 several backcross generations, multiple lines were identified that contained greater
4 than 470g protein/kg and had yields similar to Cutler 71. One line from a backcross-3
5 population was identified that contained 472g protein/kg and produced a greater
6 yield than the adapted parent. Thus, while often not of value, it is possible to transfer
7 a high protein content trait even in the face of a strong negative correlation between
8 protein content and yield. A better understanding of the physiological and molecular
9 events responsible for the inter-relationships between oil, protein and yield should
10 enhance the ability of plant breeders to develop high protein soya bean varieties.

11 A recent breakthrough has demonstrated that the apparent negative
12 correlation between protein, oil and yield can be overcome. Development of the
13 cultivar *Prolina* has provided a more promising avenue towards improving soyabean
14 protein quality. Achieving higher protein concentration in this variety also affected
15 subtle changes in 11S and 7S composition that resulted in enhanced functional
16 properties. Given that 11S proteins are enriched in sulphur-containing amino acids
17 compared to 7S proteins, the association of a higher 11S/7S ratio with expression of
18 the high-protein trait in *Prolina* provided opportunity to test the hypothesis that
19 genetic manipulation of storage protein composition may affect nutritional quality.
20 Work by Kwanyuen *et al.* (1998) showed that when expressed as a function of total
21 mass per tonne meal, pure 11S protein from *Prolina* contained approximately 9.0kg
22 more methionine, cysteine and lysine than pure 11S protein from cultivar *Dare*, a
23 1.6-fold increase. Thus, improved nutritional quality may be achieved in soyabean
24 meal by a combination of elevated 11S/7S ratio plus increased total protein content.
25 In *Prolina*, the overall gain in essential amino acid content may be enough to obviate
26 need for nutritional supplements in feedstocks.

27 Although seed composition is controlled genetically, environmental factors
28 have a strong modulating effect on seed content. For example, the number of pods
29 per plant can be increased 30-40% by increasing light intensity by 60% during late
30 flowering and early pod development. (Schou *et al.* 1978) (see Table 3). Although
31 the total seed weight per plant is increased, the increased number of pods per node
32 is accompanied by a decrease in seed size and a lower rate of seed fill. The
33 increased seed weight is not the result of a light-induced increase in the production
34 of photosynthate in leaves (Streeter & Jeffer 1979). Instead, the substrates for

1 synthesis of storage compounds are withdrawn from the nearest source (i.e. pods,
2 stems and petioles). Compositional changes also occur when environmental
3 conditions result in either an extension or premature termination of seed fill
4 compared to the interval that is normal for a given soya bean genotype. The result of
5 the extended seed development is a larger seed with increased protein and
6 decreased oil and carbohydrate content. The opposite trends occur when
7 environmental conditions, such as drought, hasten seed maturation (Sato & Ikeda
8 1979). The physiological and biochemical events responsible for these changes are
9 not known, although the availability of genetic probes for specific seed proteins
10 makes exploration of these phenomena increasingly possible.

11
12 (Table 3 about here)

13
14 Whereas the preponderance of seed proteins in soya bean is either glycinin or
15 β -conglycinin, genetic background controls the contribution of these two proteins to
16 total protein. In addition to glycinin and β -conglycinin, certain other proteins such as
17 the Bowman-Birk and Kunitz trypsin inhibitors, lectin, lipoxygenase and urease can
18 each account for 20-50g/kg of the total seed in some soya bean cultivars. Medeiros
19 (1982) used an immunological approach to quantify the proportion of total seed
20 protein contributed by glycinin and β -conglycinin in 32 lines derived from a high
21 protein breeding population. The glycinin plus β -conglycinin contents among these
22 cultivars ranged from 377g/kg and 882g/kg of the total seed protein content with a
23 mean of 630g/kg. The mean total protein content of the seeds in the test was
24 453g/kg, but no statistically significant relationship between total protein and the
25 relative amount of glycinin or β -conglycinin was reported. The ratio of
26 glycinin/ β -conglycinin ranged from 1.04 to 2.91, with a mean of 1.63. Even more
27 extreme ratios of glycinin/ β -conglycinin were described by Ogawa *et al.* (1989), who
28 also used an immunological response to quantify the amounts of seed storage
29 proteins. Soya beans were described which contained abnormally low amounts of
30 β -conglycinin, and whose seed protein had ratios of glycinin to β -conglycinin as high
31 as 6.03 (Kitamura 1993). Because a strong negative statistical correlation existed
32 between glycinin and β -conglycinin content in the lines, Ogawa *et al.* (1989)
33 suggested that the 11S globulins might be overproduced to compensate for the

1 reduction in amount of the 7S globulins. Thus, reports in the literature indicate that
2 there is considerable genetic variation in the amounts of glycinin and β -conglycinin
3 present in soya beans. Such variation may be useful in these traits can be controlled
4 in a predictable manner.

5
6 *High protein soya beans.*

7
8 Currently there is interest in increasing the protein concentrations of soya bean
9 seeds in order to improve nutritional value. Potentially this could change the amino
10 acid composition of the protein, perhaps even negating the improved nutritional
11 value associated with the increased protein concentration. AC Proteus is a new high
12 protein cultivar recently developed and registered as germplasm line OT89-16. It is
13 conventional in animal nutrition to view protein more in terms of its amino acid
14 balance as well as total amounts. Fig 1 shows that the protein quality of Maple Arrow
15 is superior to AC Proteus (expression of the content of an amino acid relative to
16 lysine is a conventional means of expressing protein quality for non-ruminant
17 livestock) although the total lysine content was much lower. AC Proteus has been
18 bred for increased lysine content but this appears to have been achieved at the
19 expense of other amino acids. An additional consideration to cultivation of high
20 protein soya bean lines content is that selection for increased protein usually results
21 in decreased yield (Helms & Orf 1998). In barley, high N nutrition resulted in a
22 greater protein concentration in the seed and a differential accumulation of seed
23 storage protein types (Kirkman *et al.* 1982), although the net effect was a reduction
24 in protein quality (Guise & Hopp 1984).

25
26 (Figure 1 about here)

27
28 In soya bean lines from a single cross, ranging in protein concentration from
29 370-500g/kg, methionine and protein concentration showed a minimal negative
30 correlation (Burton *et al.* 1982). However, little has been undertaken to determine the
31 effects of selection for high protein on amino acid composition. Serretti *et al.* (1994)
32 found breeding for high protein induced significant variations in amino acid
33 concentrations in four high protein lines (BARC-6, BARC-7, BARC-8 and BARC-9)

1 compared to two normal protein controls ('Essex' and 'Manokin') but did not produce
2 a consistent increase or decrease in all concentrations in these lines (Fig 2).

3
4 (Figure 2 about here)

5
6 Cysteine plus methionine account for 30 to 45g/kg of the amino acid residues
7 of glycinin (Nielson *et al.* 1989). Thus the sulphur amino acid content of glycinin is
8 similar to that of other high quality dietary proteins. Conversely, sulphur amino acids
9 account for less than 10g/kg of the amino acids residues of β -conglycinin (Harada *et al.*
10 *al.* 1989). The mature β -subunit of β -conglycinin is composed of approximately 416
11 amino acid residues and lacks both methionine and cysteine (Coates *et al.* 1985).
12 Hence the β -subunit of β -conglycinin is primarily responsible for the low sulphur
13 amino acids content of the combined soya bean storage proteins.

14 Imsande (1998) recently has shown that nitrogen (protein) concentration of
15 soya bean seed is significantly affected by nitrogen source supplied to the plant
16 during seed growth. Plants subsisting solely on N₂ fixation produced low protein
17 seed. For those grown on mineral N, the seed contained increasingly greater protein
18 concentration with increasing proportion of nitrogen supplied in the reduced form.
19 Paek *et al.* (1997) analyzed the seed produced from these treatments to investigate
20 how change in seed protein concentration, independent of genotype, changes seed
21 protein composition. The results of two experiments were consistent in showing that
22 raising soya bean seed protein concentration through enhanced plant N simulation
23 results in poorer protein quality (Fig 3).

24
25 (Figure 3 about here)

26
27 It was demonstrated that the sulphur-poor β -subunit of 7S protein is more
28 strongly expressed under conditions of nitrogen application whereas S-containing
29 subunits are influenced less, or not at all, thereby deteriorating the 11S/7S ratio. The
30 failure to increase the glycinin concentration in parallel with total protein
31 concentration could possibly be explained by a limitation of sulphur uptake and/or
32 assimilation.

33 Gayler & Sykes (1985) examined the effects of sulphur deficiency on the

1 complement of proteins laid down in developing seeds of soya bean. Sulphur
2 deficiency caused a 40% decrease in the level of glycinins and a contrasting
3 elevation in the level of β -conglycinins. The subunit composition of these proteins
4 was also affected. There was in particular a three fold increase in the β -subunit of
5 β -conglycinins in the sulphur deficient seeds, and this accumulated largely as the B₆
6 isomer of β -conglycinins, a protein which, while virtually devoid of methionine and
7 cysteine, retains the physical properties of a normal 7S storage protein. This
8 demonstrates that a high degree of selectivity can be exerted over the accumulation
9 of proteins in developing seeds. Studies into the effects of nitrogen source and
10 timing of sulphur deficiency on seed yield and expression of both 11S and 7S
11 storage proteins demonstrated that sulphur assimilated during seed filling appears to
12 be the main source of S-containing amino acids for synthesis of seed proteins. This
13 is confirmed by the relatively weak effect of vegetative sulphur status on the 11S-7S
14 ratio.

15 *Free amino acids.*

16
17
18 An alternative approach to altering the nutritionally essential amino acid profile
19 through changing the relative contents of storage protein is to increase the levels of
20 free amino acids in the seed. Typically the pool of any free amino acid is less than
21 0.05 of the protein-bound quantity. The sizes of the free amino acid pools are usually
22 tightly regulated. The amino acid biosynthetic pathways are controlled at the enzyme
23 level by the concentration of the amino acid products of the pathways. The enzymes
24 at the beginning or at branch points of pathways are susceptible to inhibition or
25 enhancement of activity by binding of amino acids to regulatory sites that affect
26 catalytic activity. Possible approaches to increasing biosynthesis of the free amino
27 acids includes altering the regulatory site of the native regulated enzymes or
28 shunting around the regulated step by the introduction of an unregulated enzyme
29 with the same catalytic specificity. Biosynthesis is then no longer inhibited by amino
30 acid pool size. In the absence of high levels of catabolism the amino acid can
31 accumulate to significant levels.

32 Early work in this area used cell culture selection approaches to identify
33 mutants with deregulated biosynthetic pathways (Bright & Shewry 1983). The

1 standard approach is to grow cells in the presence of an amino acid analogue that
2 inhibits growth by shutting down a biosynthetic pathway and starving the cells for the
3 end product of the pathway. Most of the cell culture work has been done on the
4 aspartate biosynthetic pathway which produces lysine, threonine and methionine,
5 and on the aromatic pathway which produces tryptophan, tyrosine and
6 phenylalanine.

7 Soya beans accumulate unique non-protein and free amino acids (Krober &
8 Gibbon 1962). However, there is little quantitative information available on the free
9 and protein-bound amino acid contents of soya beans. It has been postulated that
10 one or more of these unique components in the free amino acid pool is a genetically
11 controlled characteristic of a particular species or group of species (Davis *et al.*
12 1991). The actual composition of the free amino acid pool varies, depending on
13 maturity, nutrient supply from the soil and from fertilizer treatment, climatic conditions
14 during growth, storage conditions and genetic factors (Krobor & Gibbons 1962).
15 Spencer & Higgins (1982) have indicated that the levels of free amino acids in the
16 pool of the developing seeds, which may be an important element in the synthesis of
17 storage proteins, are correlated with the composition of the major storage proteins
18 being synthesized. Accurate assessment of the free amino acid pool is essential as
19 it affects the nutritional evaluation of non-ruminant diets based on soya bean
20 supplemented with free amino acids.

21 A comparison of two Northern adapted soya bean cultivars made by Zarkadas
22 *et al.* (1994), studying free amino acid profiles of the ethanol-soluble fractions
23 showed that levels of many of the individual amino acids were similar in a high
24 protein cultivar (AC Proteus) and a commercial strain (Maple Arrow) (See Fig 4).
25 However, cultivar to cultivar variations in free amino acid content were significant in
26 respect to five amino acids; aspartic acid, threonine, glycine, alanine and arginine.
27 Further work is required to establish a database of soya bean free amino acid
28 composition.

29
30 (Figure 4 about here)

31 32 2.7. Altering methionine content.

33
34 Soya bean protein quality has traditionally been lower than the Food and

1 Agricultural Organization (FAO) nutritional recommendations for methionine,
2 cysteine and threonine (Wilson 1987). The average concentration of these amino
3 acids in soya bean seed range from 10.7 to 12.6, 12.4 to 13.7 and 40.0 to 42.0 g/kg
4 crude protein for methionine, cysteine and threonine, respectively (Serretti *et al.*
5 1994). Methionine is the primary limiting amino acid in soya beans which
6 compromises nutritional quality. Expressed as a proportion of total protein, the soya
7 bean contains only 0.56 as much methionine and cysteine as the nutritionally
8 complete FAO reference protein, egg protein.

9 Although this problem can be overcome by supplementation with free
10 methionine and complementation with other protein-containing raw materials and
11 cereals, these solutions are less satisfactory than improving the amino acid profile of
12 soya beans. Leaching of methionine during processing and bacterial degradation
13 leading to formation of undesirable volatile sulphides are associated with
14 supplementation. The poultry and pig industry would derive considerable savings if
15 an improved variety of soya bean overcame the requirement for supplementation of
16 cereal-soya bean meal based animal feed with exogenous methionine.

17 18 *Increasing content of high methionine protein in soya beans*

19
20 One of the possible approaches to improving the nutritional quality of soya bean
21 seed protein would be to identify endogenous methionine-rich proteins and increase
22 their biosynthesis. George & de Lumen (1991) combined the powerful resolution
23 available with two dimensional gel electrophoresis and an *in vitro* labeling method
24 which allowed them to identify a 10.8kDa protein in soya bean with an exceptionally
25 high methionine content of 121 mg/g. Determination of the N-terminal sequence of
26 the first 20 amino acids of this protein has revealed it to be identical to a sequence in
27 sunflower seed 2S albumin protein that contains 150 mg/g protein. Introduction of a
28 homologous gene encoding for an endogenous protein could lead to a more stable
29 integration into the genome and would therefore be less likely to interfere with the
30 normal biology of the seed compared with the introduction of a heterologous gene
31 coding for a foreign protein. However, a 17 fold increase in the level of the
32 methionine-rich protein identified by George & de Lumen (1991) would be required to
33 bring the methionine content up to that of the FAO reference protein.

34 The production of high methionine polypeptides has several drawbacks

1 besides the fact that most alterations affect either:

2
3 a) the ability of proteins to be properly transported, processed and deposited
4 in the storage tissue cells of developing seeds

5
6 b) this ability to be reactivated upon germination (Muntz *et al.* 1997).

7
8 Taken together, the results achieved so far by introducing high methionine subunits
9 from naturally occurring globulins indicate that it will not be possible to achieve
10 significant increases in the methionine content of the seed protein via this strategy in
11 the near future.

12 Currently the most promising approach to improving methionine content is to
13 introduce genes that have been isolated from foreign plants which code for proteins
14 with extremely high methionine levels. The favoured subjects for such experiments
15 are the genes encoding 2S albumins from either Brazil nut (*Bertholettia excelsa*,
16 H.B.K.) (Altenbach *et al.* 1987) or sunflower (*Helianthus annuus*) (Kortt & Caldwell
17 1990). The feasibility of this strategy was initially shown by using the Brazil nut 2S
18 albumin gene (BNA) to transform tobacco and rape (Altenbach *et al.* 1989). The
19 Brazil nut albumin gene has also been successfully used to transform soya bean
20 (Townsend & Thomas 1994).

21 Townsend & Thomas (1994) also reported one transgenic soya bean line in
22 which 10mg/g of the salt-extractable seed protein was Brazil nut albumin. However,
23 flour from this line showed only a 30% increase in the methionine content as
24 compared to the wild-type. Here it appears that the formation of some endogenous
25 methionine-containing proteins are suppressed by the metabolic demands imposed
26 by expressing the high levels of Brazil nut albumin. The Brazil nut albumin acts as a
27 strong methionine sink which, in soya bean, out-competes the formation of at least
28 some of the other methionine-containing endogenous proteins. In addition, Muntz *et al.*
29 *(1997)* reported that the increased methionine levels occurred at the cost of other
30 non-protein sulphur compounds. It is not yet known whether the total amount of
31 sulphur-containing compounds that can be formed in transgenic seeds is limited by
32 available sulphur. Future experiments using cotyledon culture or whole plants are
33 necessary to determine whether an additional sulphur supply could prevent the
34 reallocation of sulphur among the endogenous sulphur-containing compartment of

1 the seed.

2 Horta & Sodek (1997) report that the *in vitro* culture of isolated, immature
3 soya bean cotyledons (in direct contact with the medium) on a defined medium
4 containing glutamine and methionine as sole sources of nitrogen and sulphur for 7
5 days led to rates of growth and reserve protein accumulation close to, or greater
6 than, those occurring *in situ*. It was also noted that methionine suppressed synthesis
7 of the 7S subunit and the free amino acid pool was increased more than fifty-fold
8 (see Table 4). However, many other amino acids also showed large increases. It
9 was concluded that the data showed that an abnormal situation arises on culturing
10 with a methionine-containing medium, a fact which must be considered before
11 attributing the effects on growth and protein synthesis directly to methionine.

12
13 (Table 4 about here)

14
15 Following the discovery that Brazil nut albumin was the major allergen of
16 Brazil nut, there have been new arguments against employing genetic engineering
17 for improvement of nutritional value.

18 19 *Mutants*

20
21 Mutations are part of the basic phenomena of life. Natural mutations have allowed
22 living material to adapt and develop to different ecological conditions. Changes in the
23 genetic code are translated into changes in the proteins synthesized. Hence
24 beneficial changes to the amino acid profile of soya bean storage proteins caused by
25 mutation may be used to develop new cultivars.

26 27 *Glycinin*

28
29 Screening of USA and Japanese germplasm collections has resulted in the recovery
30 of a number of mutants that either lack individual 7S or 11S subunits or which
31 contained aberrant subunits. One of the earliest mutants that was discovered was
32 found in the Japanese variety 'Raiden'. It was first noticed because there was a
33 discrepancy between the number of glycinin units described by research groups
34 (Kitamura & Shabasaki 1975; Moreira *et al.* 1981). Staswick & Nielson (1983)

1 demonstrated that the G4 subunit (A₄A₅B₃) in the CX635-1-1-1) cultivar was absent
2 in Raiden. A partial screening of the USDA-ARS (USA) germplasm collection
3 revealed that this mutation is fairly commonly encountered (Nielson, unpublished
4 data, reported by Nielson and Nam, (in press). Later it was shown that the allele that
5 caused this subunit to be absent from the seed was inherited as a genetic recessive
6 (Scallan *et al.* 1987). Recently, a mutant that apparently lacks the Gy5 gene product
7 in a collection of *Glycine soja* was described by Kitamura (1993). Subunits from Gy5
8 play an important role in determining gel hardness and transparency in tofu
9 (Nakamura *et al.* 1986). Because *G. soja* has cross-compatibility with the
10 domesticated soya bean *Glycine max*), it should be possible to introduce this trait
11 into cultivated varieties. Many other glycinin mutations are also described in the
12 literature (reviewed by Nielson and Nam, in press).

13 14 *Conglycinin*

15
16 A number of mutations have been discovered that effect the \exists -conglycinin subunit
17 genes. The Japanese variety 'Kiburi' is characterized by the absence of the
18 \forall ' subunit, a recessive genetic allele (Kitamura *et al.* 1984) that appears to be the
19 result of a small deletion. One report describes independent, single recessive genes
20 that strongly reduce the amount of \forall - and \exists -subunits accumulated in seeds
21 (Tsukada *et al.* 1986). Because the \exists -subunits accumulated in the seed are the
22 products of multiple genes (Harada *et al.* 1989), it is difficult to rationalize this result
23 unless the mutation is in a gene that in some manner regulates the accumulation of
24 these subunits. Hayashi *et al.* (1998) recently characterized a soya bean mutant
25 lacking the 7S subunits, \forall , $\forall N$ and \exists in an attempt to elucidate the complexities of
26 regulation of 7S subunit expression. The results indicated that the deficiency is not
27 caused by a lack of, or structural defects in, the 7S globulin subunit genes, but rather
28 arises at the mRNA level.

29 It has become possible to breed soya bean varieties with a markedly modified
30 protein composition ranging from extremely high to extremely low 7S to 11s ratios
31 using mutant genes for the two soya bean storage globulins. Kitamura (1995)
32 described a modification of the storage seed protein composition using mutant genes
33 that produced a low 7S line where the 7S globulin content was only about half that of

1 ordinary varieties, whereas the 11S globulin content was approximately 15% higher
2 than that of ordinary varieties. Consequently, the total protein content remained
3 about the same in both mutant and ordinary varieties. By manipulating the variant
4 alleles identified so far, it is possible to develop varieties with extremely high 11S:7S
5 ratios which may improve nutritional value. Genotypic variation for 11S:7S is low
6 among cultivated soya bean (*Glycine max.* (L) Merrill) germplasm but ratios ranging
7 from 1.7-4.9 have been observed among accessions of the wild ancestor of
8 cultivated soya bean *Glycine soja*. Thus the wild soya bean germplasm was
9 evaluated as a source of genes that govern protein synthesis that may have been
10 lost during the domestication of *Glycine max.* It was concluded that *Glycine soja* may
11 have a different complement of genes that influence the expression of 11S and 7S
12 proteins than *Glycine max.* germplasm and suggested wild soya beans may be a
13 useful genetic resource for the further improvement of protein quality in cultivated
14 soya beans (Kwanyen *et al.* 1997).

15 16 HIGH PROTEIN SOYA BEAN IN DIETS FOR NON-RUMINANTS

17
18 It is crucial that any developments in plant breeding designed to improve the nutrient
19 content of soya beans are accompanied by studies which establish whether a total
20 increase in a nutrient is associated with, at the least, a similar nutritional value (e.g.
21 nutrient utilization which may be assessed through digestibility or performance
22 studies where growth and efficiency of food conversion are evaluated). However
23 there would appear to have been only limited studies in this general area which
24 suggests that there is considerable scope for further investigations particularly with
25 the emergence of an increasing number of novel cultivars.

26 As described earlier, AC Proteus, a new cultivar of soya bean recently
27 developed and registered as germplasm line OT89-16, is primarily intended as an
28 animal feed because it contains more protein (440-480g/kg) and less oil
29 (150-180g/kg) than conventional soya beans that were developed for oil production
30 (Voldeng *et al.* 1996). Zarkadas *et al.* (1993) reported that the content of essential
31 amino acids in AC Proteus was excellent (although, as outlined earlier, the balance
32 of amino acids is not as with conventional cultivars). Growth performance of birds fed
33 diets containing full fat high protein soya beans was similar to that obtained with
34 diets containing conventional soya beans when the diets were isonitrogenous and

1 isoenergetic (see Table 5).

2
3 (Table 5 about here)

4
5 For pig diets, the combination of higher protein and lower oil concentrations in
6 AC Proteus compared to conventional soya bean has the potential benefit of a more
7 optimal protein-to-energy ratio in the diet as well as a reduction in the quantity of
8 soya bean required to reach the recommended levels of amino acids in the diet.

9 McNiven & Castell (1996) demonstrated AC Proteus could replace soya bean
10 meal or full fat soya bean on an equivalent protein basis, potentially reducing the
11 cost of protein supplementation in pig starter diets (Table 6).

12
13 (Table 6 about here)

14 15 CONCLUSIONS

16
17 No one raw material contains all the nutrients and energy-yielding components in the
18 correct proportions necessary to support optimum growth and performance of
19 non-ruminant farm animals which is the reason why compound diets are formulated
20 with the objective of correcting individual imbalances. However, there is considerable
21 scope for increasing concentrations of those nutrients known to be deficient. In the
22 case of soya beans, which are the most important protein-containing crop worldwide,
23 relative deficiencies of nutritionally essential amino acids have been recognized as
24 limitation to their increased use. Knowledge of the biosynthetic pathways responsible
25 for the synthesis of the major proteins contained within the seed and the inherent
26 genetic variability amongst cultivars has potential for generating improved lines. A
27 major future research effort will be to confirm that any such developments are
28 associated with improved nutritional value.

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1 **Table 1. Globulin-type protein composition of soya bean seed.**

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Sv Units	Nomenclature	Content (mg/g globulin)	Molecular Weight kDa	Amino Acid	
				Methionine (mg/g)	Cysteine (mg/g)
2S	∇-Conglycinin.	127	18-33	50	48
7S	Ξ- Conglycinin	30	104	14	22
7S	ϑ-Conglycinin	350	141-171	14	22
11S	Glycinin	418	317-360	15	30
15S	Poly-glycinin	75	ND	ND	ND

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Sv: Svedberg Units

ND: not determined. (Wilson 1987).

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Table 2. Major soya bean glycinin subunits (Nielson 1985).

Group	Subunit	Subunit Structure	Molecular Weight. kDa.	Number of Methionine Residues
I	G1	A _{1a} B ₂	58	5-6
I	G2	A _{1b} B _{1b}	58	5-6
I	G3	A ₂ B _{1a}	58	7-8
II	G4	A ₃ B ₄	62	3
II	G5	A ₄ A ₅ B ₃	69	3

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Table 3. Effect of increased light induced by mirror reflectors at the soil level on seed development (Schou et al. 1978).

Variable	1973		1974	
	Reflectors	Controls	Reflectors	Controls
Weight of 100 seeds (g)	20.7	20.3	19.8	20.2
Seed (no./pod)	2.0	2.2	2.1	1.9
Fruit weight (g/plant)	24.3	17.1	31.4	25.8
Pods/plant (no.)			48.25	45.75

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1 **Table 4. Dry weight, storage protein and free amino acid accumulation in**
 2 **isolated soya beans cultured on different sulphur sources for 7 days (Horta &**
 3 **Sodek 1997).**

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Treatment	Dry Weight (mg/cotyledon pair)	Protein (mg/cotyledon pair)	Free amino acids (μ mol/cotyledon)
Initial	13.9	2.7	0.8
BM	42.4	10.3	32.5
BM + Met	48.0	11.7	54.9
BM - S	30.6	4.4	34.4
BM - S + Met	49.3	11.6	55.0
<i>In situ</i>	28.6	4.7	1.3

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 6 BM: Basal medium with glutamine as source of N; - S: inorganic sulphur removed; +
 7 Met: methionine (8.4mM) added.

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Table 5. Effect of high & conventional protein soya bean (autoclaved) on growth, feed efficiency and nutrient digestibility in broiler chickens (Chohan et al. 1993).

	Conventional soya bean	High protein soya bean
21 day weight (g)	550.7	567.1
7-21 day gain (g)	422.1	439.5
7-21 day feed intake (g)	660.3	643.2
Feed / gain	1.56	1.47
Coefficient of digestibility of:		
Dry matter	0.933	0.944
Crude protein	0.903	0.916
Gross Energy (metabolizability)	0.942	0.952

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Table 6. Performance effects of replacing a normal cultivar (*Baron*) with a high protein soya bean (*AC Proteus*) on an isonitrogenous, isoenergetic basis (McNiven & Castell 1996).

	Soya bean (g/kg diet)	Average daily gain (g/day)	Average daily feed intake (g/day)	Gain:feed ratio
Baron	177	600	915	0.652
AC Proteus	140	577	882	0.651

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1 **Figure 1. Amino acid profile relative to lysine of a commercial cultivar (Maple**
2 **Arrow) and a high protein cultivar (AC Proteus) of soya bean (Zarkadas et al.**
3 **1994).**

4
5 *A. Essential amino acids (for non-ruminants)*

1 **Figure 1. Amino acid profile relative to lysine of a commercial cultivar (Maple**
2 **Arrow) and a high protein cultivar (AC Proteus) of soya bean (Zarkadas et al.**
3 **1994).**

4
5 *B. Non-Essential amino acids (for non-ruminants)*

- 1 **Figure 2. Amino acid profiles relative to lysine of high-protein soya bean lines**
- 2 **averaged across eight environments.**
- 3 **(Serretti et al. 1994)**
- 4
- 5 *A. Essential amino acids (for non-ruminants)*

1 **Figure 2. Amino acid profiles relative to lysine of high-protein soya bean lines**
2 **averaged across eight environments.**
3 **(Serretti et al. 1994)**

4
5 *B. Non-Essential amino acids (for non-ruminants)*

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1 **Figure 3. Effect of nitrogen source on 11S/7S ratio.**

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3 Key: 0: 4mM KNO₃,
4 2. 2mM NH₄NO₃ (and 1 mM KNO₃ urea + 2mM KNO₃)
5 3 1.5mM urea + 1mM KNO₃
6 4 2mM urea.

7 $Y = +1.6774 - 0.1089x$

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1 **Figure 4** *Amino acid contents of the ethanol-soluble fraction isolated from two*
2 *Northern hemisphere soya bean cultivars after ethanol extraction (Zarkadas et*
3 *al. 1994).*

4
5 *A. Essential amino acid (for non-ruminants)*

1 **Figure 4 Amino acid contents of the ethanol-soluble fraction isolated from two**
2 **Northern hemisphere soya bean cultivars after ethanol extraction (Zarkadas et**
3 **al. 1994).**

4
5 *B. Non-Essential amino acid (for non-ruminants)*

1 Developments in Plant Breeding For Improved Nutritional Quality of Soya Beans
2 I. Protein and Amino Acid Content
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4 E J Clarke and J. Wiseman
5
6 Figure 4B