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ARTICLES

Comparison of the Protein-Bound and Free Amino Acid Contents of Two Northern Adapted Soybean Cultivars[†]

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The total protein and the protein-bound and free amino acid contents of two northern adapted soybean cultivars, Maple Arrow and AC Proteus, were compared as potentially useful and practical indices for evaluating their protein quality. As the content of total protein was increased by 10.1% in AC Proteus by breeding, the concentration of protein-bound arginine, aspartic acid, and histidine increased, while the levels of threonine, tryptophan, and methionine decreased. The free amino acid content of AC Proteus increased from 2.4 to 3.7% of the dry weight, and glutamic and aspartic acids and arginine represented 52.8% of the total. Both cultivars contained an excellent balance of essential amino acids (EAA) limited only in methionine, followed by tryptophan. Compared to the FAO/WHO reference value of 33.9%, mean values for total EAA ranged from 46.1 to 46.5%, and both cultivars had mean protein efficiency ratio values of 2.7. The 4-hydroxyproline-rich glycoproteins found in the extracellular matrices of soybean seeds ranged from 2.12 to 2.36 g/kg of total protein in Maple Arrow and AC Proteus, respectively.

INTRODUCTION

There is an increasing interest in the development of high-protein soybean [*Glycine max* (L.) Merr.] cultivars adapted to the northern growing areas of Canada and other countries. Soybean is a relatively new agricultural crop in Canada, and until recently all of the Canadian production of soybeans was confined to the more temperate regions of southwestern Ontario (Agriculture Canada, 1991). However, genetic improvements of soybean cultivars have led to the development of early maturing, cold tolerant soybean genotypes (Voldeng and Saindon, 1991a–c), with improved yields, pest resistance, seed quality, and high protein content (Buzzell and Voldeng, 1980; Saindon et al., 1989a,b, 1990). Partly as a result of these changes, the yields and total production of soybeans in Canada in 1991 increased 8.8% over the previous year to reach 1.4 million tons (Agriculture Canada, 1991).

Extensive field trials have been carried out in Canada to identify the most productive soybean cultivars which will grow in areas with longer daylengths (>16 h) and northern latitudes (latitude >45° N) (Loiselle et al., 1990; Saindon et al., 1989a,b, 1990). These studies have been concerned primarily with the agronomic characteristics and seed quality aspects of this crop. A comparison of the average protein content and amino acid composition of two northern adapted cultivars has been reported by Zarkadas et al. (1993a). These two cultivars include Maple Arrow, a widely grown variety, and AC Proteus, a newly

released high-protein genotype developed by back crossing to Maple Arrow (Voldeng and Saindon, 1991a). Analyses showed that both contained an excellent balance of essential amino acids required for both humans and animals and were limited only in methionine, followed by tryptophan (Zarkadas et al., 1993a). Lysine concentration was particularly high in both cultivars. Their amino acid scores, adjusted for digestibility, were almost as high as those of milk and egg proteins, and their calculated average protein efficiency ratio (PER) was 2.7 (Zarkadas et al., 1993a,b). Kakade et al. (1973) and Liener (1979) have reported lower nutritive values for unheated soybeans than the present study, which may be attributed to the deleterious effects of protease inhibitors. Nutritional studies with humans have shown values for protein quality of adequately processed soybean protein ranging from 62 to 92% of casein (Torun et al., 1981; Fomon and Ziegler, 1979; Erdman and Fordyce, 1989).

Like many species of the Leguminosae, soybeans contain three major groups of proteins. The first includes the enzymes involved in metabolism, the second the structural proteins, i.e., ribosomal, chromosomal, and membrane proteins, and the third the larger and more homogeneous fraction known as storage proteins and lectins, which include glycinin, β-conglycinin, etc. (Liener, 1979; Koshiyama, 1983; Nielsen, 1984; Spencer and Higgins, 1982; Chrispeels, 1984; Wilson, 1987; Harada et al., 1989; de Lumen, 1990; George and de Lumen, 1991; Wolf, 1993). In addition, soybeans accumulate unique nonprotein and free amino acids (Van Etten et al., 1959; Krober and Gibbons, 1962; Bell, 1976; Fowden, 1990). However, there is no quantitative information available on the protein-bound and free amino acid contents of soybeans. It has been postulated that the accumulation of one or more of these unique components in the free amino acid pool is a

genetically controlled characteristic of a particular species or group of species (Bell, 1976; Evans and Bell, 1978; Fowden, 1972; 1990; Davis et al., 1991). The actual composition of the free amino acid pool fraction varies, depending on maturity, nutrient supply from the soil and from fertilizer treatment, climatic conditions during growth, storage conditions, and genetic factors (Krober and Gibbons, 1962; Brandt, 1976; Kappor and Gupta, 1977; Macnicol, 1977; Goldberg, 1986; Di Martino-Rigano et al., 1989). Spencer and Higgins (1982) have indicated that the levels of free amino acids in the pool of the developing seeds, which may be an important element in the synthesis of storage proteins, are correlated with the composition of the major storage proteins being synthesized (Krober and Gibbons, 1962; Brandt, 1976; Kappor and Gupta, 1977). Because of the metabolic importance of the free amino acid pools in cereals and legumes, several methods have been developed for their extraction from defatted meal by perchloric acid or with a chloroform–ethanol–water mixture (Bligh and Dyer, 1959; Newell et al., 1967). These extractions are usually followed by a cleanup procedure and then evaporation or lyophilization. The simplest and most effective solvent for the extraction of free amino acids from full fat meal was 60–90% cold or hot aqueous ethanol (Bowman, 1946; Mustakas et al., 1962; Kappor and Gupta, 1977; Wright, 1981; Magne and Larher, 1992). Extraction of full fat meal, with a lithium citrate buffer (pH 1.55) for 15 min, offers an alternative simple procedure for the isolation of free amino acids (Marshall et al., 1989). An accurate assessment of the free amino acid contents of soybeans is essential not only as a data base on soybean free amino acid pool composition but also for the nutritional evaluation of soybean diets supplemented with free amino acids.

The two objectives of the present study were, first, to compare the levels and variation of the protein-bound and free amino acid pools of the two northern adapted soybean cultivars Maple Arrow and a newly released high-protein genotype, AC Proteus, and, second, to establish whether differences in the amino acid composition and protein contents of the ethanol-soluble and ethanol-insoluble fractions of these two varieties could be correlated with their protein quality.

MATERIALS AND METHODS

Materials. Type DC-5A (lot 746) cation-exchange spherical resin, sized to 6.0 ± 0.5 mm, was purchased from Dionex Chemical Co., Sunnyvale, CA. The amino acid standards were obtained as follows: 4-hydroxyproline from Calbiochem-Behring Corp., La Jolla, CA; norleucine from Pierce Chemical Co., Rockford, IL; 3-nitrotyrosine from Aldrich Chemical Co., Milwaukee, WI; and the standard amino acid calibration mixture from Beckman Instruments, Inc., Palo Alto, CA. Highly purified ninhydrin and hydrindantin (Nin-Sol AF) dissolved in sequenal grade dimethyl sulfoxide was purchased from Pierce. Octanoic acid was obtained from Eastman Kodak Co., Rochester, NY, and phenol was a product of J. T. Baker Chemical Co., Phillipsburg, NJ. Hydrochloric acid (Analar), hydrobromic acid (Aristar), formic acid (88.0%), and hydrogen peroxide (30.0%) were purchased from BDH Inc., Poole, England. High-purity sodium hydroxide (50.0% w/w), which was used to prepare all buffers and reagents, was a product of Allied Fisher Scientific, Fair Lawn, NJ. The three highly purified microcolumn citrate buffers (pH 3.295, 0.20 M; pH 4.10, 0.20 M; pH 6.40, 1.0 M) and sample dilution buffer (pH 2.2, 0.20 M) recommended for high-sensitivity single-microcolumn analysis were used as described previously (Zarkadas et al., 1987). All other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Experimental Procedures. *Selection of Plant Materials and Sample Preparation.* The two soybean genotypes selected for this investigation were cv. Maple Arrow and AC Proteus. Maple Arrow has been widely grown in central and eastern Ontario (USDA Maturity Group 00). The high-protein line AC Proteus was developed by three cycles of crossing and back crossing to Maple Arrow with selection of the highest protein F_3 bulks in each cycle as the nonrecurrent parent. After the second back cross, bulk selection in the F_3 for protein was followed by pedigree selection and yield evaluation of F_5 derived bulks. The high-protein line used for the first cross to Maple Arrow (DU-41) was selected from the cross of PI 189950 to a high-protein selection from cv. Merit × PI 153293 as described previously by Voldeng and Saindon (1991a).

Representative samples of seed of the two cultivars were taken from each of the four replicates of the Ontario soybean variety trial grown at four different sites at Agriculture Canada's Central Experimental Farm, Ottawa, in 1989. The dried seed samples were then pulverized in a standard electrically driven end runner mill (Cyclone Sample Mill, U. D. Corp., Fort Collins, CO), passed through a 0.5-mm mesh sieve, lyophilized, and then stored at –20 °C in polypropylene bottles until used.

Extraction Procedure and Preparation of Ethanol-Soluble (F1) and Ethanol-Insoluble Protein (F2) Fractions from Soybean Seeds. The alcohol-soluble free amino acids and peptides of soybean seeds, including free proline and 4-hydroxyproline, known to be present in certain plant tissues (Khanizadeh et al., 1989; Minero-Amador et al., 1992), were extracted as follows. Samples (2.0 g) of the pulverized soybean seeds were extracted with 50 mL of 70% (v/v) ethyl alcohol, as described previously for plant materials (Minero-Amador et al., 1992), in a VirTis homogenizer (Model 32; VirTis, Gardiner, NY) for 10 s (full speed) at 2 °C. The supernatant, designated ethanol-soluble fraction F1, was recovered by decantation through eight layers of cheesecloth to trap fat particles, and the extraction procedure was repeated two more times. The combined supernatant fractions (F1) were dried under vacuum on a rotary evaporator (Buchi, Rotavapor, Switzerland) at 45 °C, resuspended in double-distilled water, lyophilized, and stored at –70 °C.

The remaining pellet, designated ethanol-insoluble protein fraction F2, was suspended in the same extraction solvent and rehomogenized for 10 s, and the extraction procedure was repeated twice. The combined pellets were then suspended in distilled water and lyophilized. The dried pellets were finally ground to pass through a 0.5-mm screen and were stored at –70 °C until needed.

Preparation of Tissue Hydrolysates. Duplicate samples (0.05 g) were hydrolyzed in Pyrex (No. 9860) test tubes (18 × 150 mm) under vacuum (below 10 mmHg) with triple-glass-distilled constant-boiling HCl (6.0 M) containing 0.2% (v/v) phenol at 110 ± 0.5 °C for periods of 24, 48, 72, and 96 h with the usual precautions described by Zarkadas et al. (1988c). Analyses of individual acid hydrolysates were performed on the clear filtrate in duplicate by methods described previously (Zarkadas et al., 1986, 1988b,c).

Procedures for Amino Acid Analyses. Amino acid analyses were carried out on a Beckman Spinco Model 121 MB fully automated amino acid analyzer using single-column methodology (Zarkadas et al., 1986, 1987, 1990). The automated instrument was equipped with a Beckman Model 406 analog interface module, a system Gold (Beckman Instrument, Inc., Altex Division, San Ramon, CA) chromatographic data reduction system, and an IBM (AT series) compatible personal computer, which was obtained from Microcom AL Computer, Ottawa, ON. The incorporation of these components to the system increased the sensitivity of the analysis and enabled quantitation of amino acids at the picomole level as described previously (Zarkadas et al., 1987).

Complete amino acid analyses were carried out on each fraction (F1 and F2) isolated from each of the four replicate soybean samples (50.0 mg) according to the standard procedures described previously (Zarkadas et al., 1986, 1987). Each of the four replicates was divided into two subsamples, i.e., A and B, which were then hydrolyzed in duplicate for 24, 48, 72, and 96 h as described previously (Zarkadas et al., 1988a–c). Analyses of individual acid hydrolysates were performed in duplicate. The data reported for serine and threonine in Tables II and III represent the average values of 72 determinations extrapolated to zero time of hydrolysis

by linear regression analysis of the results. The values for valine, isoleucine, leucine, and phenylalanine are the average of 48 values obtained from the 48, 72, and 96 h of hydrolysis. All others are reported as the average values of 72 determinations from 24, 48, 72, and 96 h of hydrolysis.

Methionine and cyst(e)ine were determined separately in each fraction (50.0-mg samples) according to the performic acid procedure of Moore (1963). Norleucine was added in the hydrolysate as an internal standard. Recoveries of cyst(e)ine as cysteic acid and methionine as methionine S,S-dioxide were calculated relative to alanine, valine, leucine, and isoleucine present in the sample and represent the average of 24 determinations.

Tryptophan in soybean samples (50.0 mg) was also determined separately after alkaline hydrolysis (Hugli and Moore, 1972) on a single column as described previously (Zarkadas et al., 1986), using 3-nitrotyrosine as the internal standard, and the data presented in Tables II and III represent the average of 24 determinations.

4-Hydroxyproline (Berg, 1982) was determined separately from a concentrated 24-h hydrolysate (equivalent to 50.0 mg of protein/ analysis) using a single column (21 × 0.6 cm) packed with Dionex DC-6A resin (Zarkadas et al., 1986). Recoveries of Pro(4-OH) were calculated relative to alanine, isoleucine, and leucine. The Pro(4-OH) data represents the average values of 24 determinations.

Protein Determination. Precise quantitation of the protein mass in each soybean acid hydrolysate was carried out according to the methods described by Horstmann (1979), Nguyen et al. (1986), and Zarkadas et al. (1988a,c) as

$$WE = \sum_{i=1}^{19}(a_i b_i) \qquad (1)$$

where a is the mole fraction of an amino acid i found in the analyzed aliquot and b is the molecular weight of amino acid residue i (in micrograms). The mean residue weight, WE (in micrograms per nanomole), and conversion factor, CF (in micrograms per nanomole), for determining the protein mass in each sample analyzed in the absence of tryptophan and cyst(e)ine was calculated as described previously (Horstmann, 1979; Zarkadas et al., 1988a). A conversion factor CF′ (in micrograms per nanomole) was also calculated according to the method of Horstmann (1979) for determining protein mass in the absence of tryptophan, cyst(e)ine, proline, and/or Pro(4-OH) as described previously (Zarkadas et al., 1988a,b).

The protein content of each sample calculated by multiplying CF or CF′ by the nanomoles of total amino acids in each acid hydrolysate was calculated as follows:

$$P = CF' \sum_{i=1}^{15} x_i \qquad (2)$$

Determination of Total Protein and 4-Hydroxyproline-Rich Glycoproteins. In this study, an attempt was also made to relate the amounts of protein-bound 4-hydroxyproline, which occurs exclusively in the 4-hydroxyproline-rich glycoproteins of the primary cell walls of the angiosperms, i.e., extensin, arabinogalactan protein, and salt-extractable glycoproteins (Lamport, 1977; Fincher et al., 1983; Wilson and Fry, 1986; Cooper et al., 1987; Cassab and Varner, 1988), to the contents of these extracellular matrix proteins in soybean seeds.

Zarkadas et al. (1988c, 1990, 1993a) have shown that a simple method to calculate the amount of a specific protein j present in plant tissue from the quantitative determination of a given unique amino acid i known to occur exclusively in the specific protein (j) was

$$P_i = C_i \frac{[1000]}{n'_i} \frac{WE(P_i)}{M_{r(i)}} \qquad (3a)$$

where P is the concentration of a specific primary cell wall glycoprotein j (i.e., extensin, expressed in grams per kilogram of total protein), C_i is the mean concentration of a unique protein-bound amino acid, i [i.e., Pro (4-OH), in grams per kilogram of total protein], WE(P_i), is the weight equivalent of a specific protein j (i.e., extensin, WE = 0.1095 mg/nmol) determined from its known amino acid composition according to the method of Horstmann (1979), and n'_i is the number of residues of a unique

amino acid residue i per 1000 amino acid residues (n'_i = 455). The anhydrous molecular weight ($M_{r(i)}$) of Pro(4-OH) is 113.12.

Substituting the computed parameters for extensin in eq 3a, the total 4-hydroxyproline-rich glycoproteins in grams per kilogram of total protein in soybean seeds was calculated according to the method of Khanizadeh et al. (1989) by the following convention:

$$amt\ of\ extensin\ (P_{ext-1}) = amt\ of\ Pro(4\text{-}OH) \times 2.128$$

$$(3b)$$

Predicting Properties of Proteins from Amino Acid Compositions. Previous studies have shown that amino acid compositions represent a large body of easily accessible data not clearly related in any simple way to useful structural properties of proteins (Khanizadeh et al., 1989). It would therefore be useful if there were unequivocal ways of grouping amino acids into classes with distinct properties in the hope that such classes correlate to some extent with the rather general properties of the proteins in mixtures. One feature of protein structures that is fairly reliable is the tendency of the side chain of charged or very polar amino acid residues to be external, to interact strongly with water, and to have high solubility in water. At the opposite end of the polarity scale are the apolar or hydrophobic side chains, which tend to have low solubility in water and therefore will be internal (Bigelow, 1967; Nozaki and Tanford, 1971). Barrantes (1973, 1975) has grouped the amino acids into four classes, total charged, hydrophilic, hydrophobic, and apolar, and simply compared the ratio (R) of the frequencies of occurrence (χ) of whatever particular side chains of proteins one wishes to stress, e.g.

$$R = \sum_k \chi_k / \sum_j \chi_j \qquad (4)$$

where k can be hydrophilic and j hydrophobic side chains or k polar and j nonpolar as defined by Barrantes (1973). Basic residues: histidine + lysine + arginine. Acidic residues: aspartic acid + glutamic acid + asparagine + glutamine. Total charged residues: basic + acidic. Hydrophilic residues: total charged + threonine + serine. Hydrophobic residues: valine + methionine + isoleucine + leucine + tyrosine + phenylalanine + tryptophan. Apolar residues: hydrophobic − tyrosine. Ratio 1 (R_1): hydrophilic/hydrophobic. Ratio 2 (R_2): hydrophilic/apolar. Ratio 3 (R_3): total charged/hydrophobic. Ratio 4 (R_4): total charged/ apolar.

Although the choice of residues used to construct these ratios is somewhat arbitrary (Barrantes, 1973, 1975), one particular ratio scale that reliably weighs the tendency of charged or very polar residues to be external is R_3. This ratio is convenient because it spreads out different proteins over a wide scale range, from 0.36 to 2.03, and gives a measure with more information about the system.

Statistical Analysis. Data processing of the results was carried out by a FORTRAN computer program developed for this purpose. Analysis of variance, conducted on the amino acid data, for a completely randomized block design (factorial) was done by the general linear model procedure (SAS, 1991) and represents the average values from eight subsamples per genotype.

RESULTS AND DISCUSSION

To quantitatively establish the occurrence and variation of protein-bound and free amino acid contents of the two northern adapted cultivars, Maple Arrow and AC Proteus, pulverized soybean seeds were extracted with a mixture of 70% (v/v) ethanol, which effectively separated the ethanol-soluble fraction (F1) from the ethanol-insoluble protein fraction (F2). The yields obtained for these fractions, expressed on a dry weight basis, are summarized in Table I. The ethanol-soluble fraction (F1) in Maple Arrow constituted 21.2% of the sample on a dry weight basis compared to 19.0% in the newly released high-protein genotype AC Proteus. These differences were statistically highly significant ($P < 0.01$). Similar differences were found in the yields obtained for the ethanol-insoluble protein fractions (F2) between these two cultivars (Table I).

Table I. Recoveries of the Ethanol-Soluble (F1) and Ethanol-Insoluble (F2) Soybean Protein Fractions Isolated from a Widely Grown Cultivar, Maple Arrow, and a Newly Released High-Protein Genotype, AC Proteus, after Solvent Extraction with an Ethanol-Water Mixture

| | soybean genotype | | | | | |
| | Maple Arrow | | AC Proteus | | | |
	mean ± SEM[a]	CV	mean ± SEM[a]	CV	CV	F
	Ethanol-Soluble Soybean Fraction F1					
g of dry matter/2.0 g of meal	0.3821 ± 0.003	1.93	0.3393 ± 0.002	1.40	1.75	91.65**
% recovery on dry wt basis (DWB)	21.17 ± 0.28	2.69	18.98 ± 0.25	2.60	1.86	68.72**
g of protein/100 g of dry soybean fraction FI	2.42 ± 1.57	15.67	3.73 ± 0.31	16.7	22.56	7.09[ns]
	Ethanol-Insoluble Soybean Fraction F2					
g of dry matter/2.0 g of meal	1.4234 ± 0.010	1.48	1.4492 ± 0.017	2.35	1.24	4.21[ns]
% recovery on dry wt basis	78.83 ± 0.28	0.72	81.02 ± 0.24	0.61	0.47	68.78**
g of protein/100 g of dry soybean fraction F2	42.70 ± 1.27	5.96	52.81 ± 1.02	3.86	4.55	43.07**

[a] Mean ± standard error of measurements (SEM) for four replicates. Significance; F, values from analysis of variance between genotypes; **, $P < 0.01$; ns, not significant; CV, coefficient of variation. [b] Protein content was determined by amino acid analysis according to the method of Horstmann (1979).

Protein Determination. To establish whether the amino acid composition or protein contents of soybeans could be used as potentially useful indices for assessing their protein quality, both F1 and F2 fractions were subjected to accurate and detailed amino acid analysis at the picomole range by the single-column methodology described previously (Zarkadas et al., 1986, 1987). The results of the amino acid analyses of F1 and F2 fractions from Maple Arrow and AC Proteus, along with the levels of statistical significance obtained from analysis of variance, are presented in Tables II and III, expressed as grams of anhydrous amino acid per kilogram of anhydrous fat- and ash-free tissue protein. The data represent the average values of four replicates ($N = 4$). The results show deviations of less than 2.5% from the average values obtained among the four replicates of each cultivar. These data allow comparisons to be made between the present results and those recommended by FAO/WHO/UNU (1985) and FAO/WHO (1990) reference amino acid patterns for humans. The Joint FAO/WHO Expert Consultation Group (FAO/WHO, 1990) have suggested that amino acid data be reported as milligrams of amino acids per gram of protein or as grams of amino acids per gram of nitrogen. For purposes of comparison the data from this study have also been calculated in this way, as grams of amino acid per 16 g of total nitrogen, and are presented in Table IV.

The data on the total nitrogen content of both soybean cultivars and their separated fractions (F1 and F2) reported in Table IV have been calculated by the method recommended by Heidelbaugh et al. (1975). The total nitrogen of these samples ranged from 16.62 to 18.10%, which is considerably higher than the 16.0% value frequently assumed for proteins and which serves as the basis for the factor of 6.25 used to convert total nitrogen to crude protein. The protein conversion factors among these samples varied from 5.90 in Maple Arrow to 5.94 in AC Proteus, as did the F2 (5.99–6.01) ethanol-insoluble fractions and the F1 (5.82–5.52) fractions. These results give further support to the recommendations of Benedict (1987) and Khanizadeh et al. (1992) that the protein conversion factor of 6.25 be used only for calculating the crude protein content of different foods.

Quantitative amino acid analysis of the ethanol-soluble fraction (F1) indicated that the free amino acids were comparatively small in amount and that their protein contents averaged 2.42 and 3.73% of the total dry mass of this fraction for Maple Arrow and AC Proteus, respectively. Brandt (1976) and Bright et al. (1982) reported that free amino acids in cereals account for approximately 2.0% of the total amino acid content. It has been reported

that very small amounts of hydrophobic proteins and peptides are also extracted in the ethanolic fraction. Bowman (1946) and Frattali (1969) isolated a protease inhibitor from soybean by extraction with 60% aqueous ethanol which was shown to be different from the soybean ethanol-insoluble trypsin inhibitor of Kunitz (1947) and had a molecular weight of 8000 (Birk, 1985). In the present study recoveries for both fractions, F1 and F2, were calculated on total protein determined from their respective amino acid composition, as presented in Tables II–IV. The average protein recoveries reported in Table I represent accurate determinations of the absolute amount of protein present.

Several investigators have indicated that the major ethanolic fraction of soybean seeds is composed primarily of lipids and carbohydrates (Smith, 1981; Wright, 1981; Magne and Larher, 1992). The oil content of soybeans ranges from 18.0 to 21.0% and varies inversely with the amount of protein present. The average total sugar was 8 g/100 g of seed (Smith, 1981). Hymowitz et al. (1972) analyzed soybeans from 60 selected lines from maturity groups 00–IV for individual and total sugars and found that sucrose, raffinose, and stachyose represented on the average 60, 4, and 36%, respectively.

Protein determinations in each of the ethanol-insoluble protein fractions (F2) showed that in three cycles of crossing and back crossing of Maple Arrow (Voldeng and Saidon, 1991a) there was a highly significant ($P < 0.01$) increase of protein content in the new cultivar, AC Proteus, from 42.7 to 52.8%. This represents an increase of 10.11 g of protein/100 g of dry ethanol-insoluble proteins (F2). These results are in accord with those reported recently by Zarkadas et al. (1993a) for the soybean seeds for these two cultivars but are considerably higher than the 3.3% protein increase reported by Brim and Burton (1979) and Burton et al. (1982) in five cycles of recurrent selection for high protein in their soybean varieties. The mean residue weight (WE, micrograms per nanomole) and conversion factors CF and CF' (micrograms per nanomole) given in Tables II and III can be used in all subsequent protein quantitations as described previously by Horstmann (1979) and Zarkadas et al. (1988a).

Free Amino Acid Profiles of Soybeans. A comparison of the free amino acid profiles of the ethanol-soluble soybean fractions (F1) investigated showed that the levels of many of the individual amino acids were similar. However, cultivar to cultivar variations in free amino acid content were significant with respect to five amino acids. The variation noted for aspartic acid between these two cultivars was statistically significant ($P < 0.05$), with the AC Proteus being consistently higher in this amino acid

Amino Acid Composition of Soybeans

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Table II. Amino Acid Contents of the Ethanol-Soluble Fraction (F1) Isolated from Two Northern Adapted Soybean Cultivars after Ethanol–Water Extraction (Grams of Amino Acid per Kilogram of Total Protein)

	soybean genotype					
	Maple Arrow		AC Proteus		between genotypes[a]	
amino acid	mean ± SEM[a]	CV[a]	mean ± SEM[a]	CV[a]	CV	F
aspartic acid	134.12 ± 5.97	8.90	160.96 ± 3.73	4.64	8.48	9.21*
threonine	20.88 ± 0.45	4.30	17.59 ± 1.16	13.22	6.44	14.08*
serine	26.65 ± 1.27	9.56	22.12 ± 1.31	11.81	14.53	3.28[ns]
glutamic acid	236.18 ± 8.44	7.15	198.46 ± 5.72	5.76	8.45	8.41[ns]
proline	30.18 ± 2.07	13.69	29.29 ± 2.66	18.14	15.89	0.07[ns]
glycine	25.66 ± 1.11	8.67	22.82 ± 0.84	7.41	4.54	13.29*
alanine	27.42 ± 1.92	14.02	20.33 ± 1.10	10.83	10.56	15.80*
cyst(e)ine	89.97 ± 3.23	7.19	68.95 ± 6.29	18.27	15.82	5.59[ns]
valine	20.23 ± 2.00	9.91	17.96 ± 1.62	18.03	7.38	5.20[ns]
methionine	7.70 ± 0.55	14.46	5.97 ± 0.39	13.25	15.48	5.40[ns]
isoleucine	23.49 ± 0.99	8.43	20.93 ± 1.75	16.72	8.44	3.76[ns]
leucine	31.8 ± 1.41	8.85	30.49 ± 2.56	16.81	10.63	0.33[ns]
tyrosine	63.20 ± 1.30	4.13	55.07 ± 4.10	14.91	12.80	2.34[ns]
phenylalanine	65.21 ± 2.22	6.82	54.44 ± 4.10	13.86	13.10	3.78[ns]
histidine	15.88 ± 1.61	20.28	29.61 ± 3.47	23.49	30.15	8.02[ns]
lysine	20.55 ± 1.81	17.58	19.00 ± 1.86	19.56	8.53	1.68[ns]
arginine	87.88 ± 2.01	4.58	169.27 ± 21.61	25.53	24.84	12.99*
tryptophan	55.71 ± 5.98	21.48	42.69 ± 1.93	9.07	18.73	4.00[ns]
4-hydroxyproline	17.23 ± 1.71	19.82	14.10 ± 1.98	28.03	22.33	1.60[ns]
ammonia	29.99 ± 5.27	35.18	14.81 ± 1.55	20.94	36.27	6.99[ns]
WE,[b] μg/nmol	0.123325 ± 0.0007	1.25	0.125025 ± 0.0008	1.38	0.53	13.04*
CF,[b] μg/nmol	0.128050 ± 0.0013	2.47	0.128900 ± 0.008	1.30	0.98	0.89[ns]
CF',[b] μg/nmol	0.136150 ± 0.0012	1.78	0.136375 ± 0.008	0.79	0.91	0.07[ns]
basic[c]	124.30 ± 4.28	6.88	217.89 ± 23.92	21.95	20.88	13.71*
acidic[c]	370.30 ± 4.66	2.52	359.42 ± 5.10	2.84	3.47	1.47[ns]
charged[c]	494.59 ± 4.49	1.82	577.32 ± 20.88	7.23	5.93	13.54*
hydrophobic[c]	267.42 ± 5.19	3.88	227.47 ± 10.78	9.48	8.86	6.63[ns]
hydrophilic[c]	542.13 ± 4.57	1.68	617.03 ± 19.67	6.38	5.31	11.86*
apolar[c]	204.22 ± 4.20	4.11	172.46 ± 7.65	8.88	8.66	7.59[ns]
R1[c]	0.494 ± 0.012	5.11	0.371 ± 0.029	15.72	13.09	9.29*
R2[c]	2.659 ± 0.071	5.36	3.614 ± 0.027	15.11	14.88	8.27[ns]
R3[c]	1.852 ± 0.048	5.22	2.568 ± 0.21	16.74	16.15	8.05[ns]
R4[c]	2.43 ± 0.067	5.53	3.383 ± 0.27	15.98	15.67	8.84*
total protein[b] g/kg of dry sample	24.25 ± 1.90	15.67	37.33 ± 3.13	16.79	22.56	7.09[ns]

[a] Mean values and standard error of measurements (SEM) for 4 replicates and 64 determinations. The values for valine, isoleucine, leucine, phenylalanine, tryptophan, and 4-hydroxyproline are the average of 32 determinations. Significance: F, values from analysis of variance between genotypes; *, $P < 0.05$; ns, not significant; CV, coefficient of variation. [b] The total protein, WE, CF, and CF' constants were calculated according to the methods of Horstmann (1979) and Zarkadas et al. (1988a–c), where CF is the apparent average residue molecular weight increased in proportion to the missing tryptophan and cyst(e)ine values, while CF' was also calculated for determining protein mass in the absence of tryptophan, cyst(e)ine, proline, and 4-hydroxyproline. [c] Calculated according to the method of Barrantes (1973, 1975) using eq 4: ratio 1 (R_1) hydrophilic/hydrophobic; ratio 2 (R_2), hydrophilic/apolar; ratio 3 (R_3), total charged/hydrophobic, and ratio 4 (R_4), total changed/apolar.

than Maple Arrow. Mean arginine values ranged from a low of 87.9 g/kg of protein in Maple Arrow to 169.3 g/kg of protein in AC Proteus, reflecting the increased levels of totally charged and hydrophilic amino acids in the ethanol soluble-fraction of AC Proteus (Tables II–IV).

During the evolution of plants, the predominant amino acids used for the transport and storage of nitrogen have been asparagine and glutamine, with arginine present primarily for storage (Pate, 1980; Miflin and Lea, 1977, 1982). The ureides allantoin and allantoic acid have also been reported to be sources of nitrogen for the synthesis of seed proteins and during seed germination (Schubert and Boland, 1990). These authors reported that in soybean seedlings ureides accounted for 0.7 and 6.0% of the total nitrogen of cotyledons and axes, respectively, at the peak of ureide accumulation. The possible explanation for such a limited number of amino acids being involved as nitrogen transport and storage compounds may be related to their involvement in all six key pathways in the biosynthesis of amino acids in the developing seeds of a number of species (Lea and Miflin, 1974; Lea et al., 1976, 1990, 1992; Miflin and Lea, 1977, 1982; Blevis, 1989). Their other common feature is that they are closely related to glutamate and aspartate; thus, their carbon skeletons can easily be derived

from, or give rise to, metabolites of the citric acid cycle (Miflin and Lea, 1977, 1982). Nitrogen is incorporated into plant tissues in its reduced form as ammonia during the synthesis of glutamine and glutamate by the reductive amination of α-ketoglutarate. Glutamic acid is the precursor of arginine, proline, and other amino acids including aspartic acid. Asparagine is formed by the transfer of the amide proup of glutamine to aspartic acid (Lea et al., 1990) by glutamine–ATP-dependent asparagine synthetase (Streeter, 1973; Miflin and Lea, 1977). Pate (1980) showed that 60–70% of the total nitrogen present in the xylem and phloem of lupins and other legumes was present as asparagine and suggested that only asparagine might act as a temporary store of reduced nitrogen to prevent the buildup of toxic levels of ammonia in plant tissues. The data presented in Table II strongly support the concept that a limited number of amino acids are used for the transport and storage of nitrogen into the soybean seeds. The predominant free amino acids present in the ethanol-soluble fraction (F1) from both cultivars were, in fact, glutamic acid, aspartic acid, and arginine, the sum of which accounted for 45.8 and 52.8% of the free amino acids present in Maple Arrow and AC Proteus, respectively.

Amino acid biosynthesis in plants is regulated by end-

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Table III. Comparison of the Amino Acid Composition of the Ethanol-Insoluble Fraction (F2) Isolated from Two Northern Adapted Soybean Cultivars (Grams of Amino Acid per Kilogram of Total Protein) after Ethanol–Water Extraction

| amino acid | soybean genotype | | | | significance levels between genotypes[a] | | Griffith's soybean protein concentrate |
| | Maple Arrow | | AC Proteus | | | | |
	mean ± SEM[a]	CV[a]	mean ± SEM[a]	CV[a]	CV	F	
aspartic acid	91.93 ± 1.36	2.96	97.65 ± 0.72	1.88	2.20	15.14*	
threonine	44.17 ± 0.82	3.70	38.31 ± 0.51	2.65	1.56	166.13***	
serine	52.25 ± 1.34	5.11	47.10 ± 1.36	5.77	7.21	4.13ns	
glutamic acid	183.27 ± 5.30	5.79	186.96 ± 1.37	1.47	3.30	0.73ns	
proline	59.84 ± 4.94	16.50	58.17 ± 3.03	10.43	13.15	0.09ns	
glycine	36.62 ± 0.84	4.63	36.52 ± 1.30	7.14	6.11	0.00ns	
alanine	35.98 ± 1.54	8.55	34.81 ± 0.29	1.69	6.08	0.58ns	
cyst(e)ine	21.98 ± 1.20	10.93	20.87 ± 1.24	11.93	12.98	0.32ns	
valine	49.09 ± 1.33	9.69	48.07 ± 2.33	9.68	7.42	0.16ns	
methionine	12.06 ± 1.16	1.56	10.24 ± 0.47	9.11	8.78	6.86ns	
isoleucine	49.59 ± 1.38	5.15	48.41 ± 1.55	6.70	5.52	0.38ns	
leucine	83.65 ± 0.65	1.56	81.43 ± 1.10	2.72	1.64	5.37ns	
tyrosine	44.00 ± 1.25	5.69	41.86 ± 0.76	3.65	4.68	2.28ns	
phenylalanine	59.69 ± 2.09	7.01	59.04 ± 1.01	3.43	6.01	0.07ns	
histidine	22.09 ± 2.07	18.77	26.42 ± 1.20	9.11	11.03	5.22ns	
lysine	64.99 ± 1.15	3.55	65.56 ± 0.97	2.97	2.77	0.20ns	
arginine	74.69 ± 1.37	3.67	85.51 ± 0.77	1.80	3.15	36.64**	
tryptophan	13.01 ± 0.26	4.06	12.04 ± 0.34	5.66	2.03	28.62**	
4-hydroxyproline	1.108 ± 0.073	13.16	0.977 ± 0.068	13.71	18.38	0.65ns	
ammonia	12.22 ± 1.28	20.95	9.44 ± 2.13	45.26	20.38	3.18ns	
WE,[b] μg/nmol	0.113500 ± 0.0002	0.367	0.114125 ± 0.0002	0.35	0.41	3.48ns	
CF,[b] μg/nmol	0.122950 ± 0.008	14.27	0.114500 ± 0.0002	0.39	10.32	0.84ns	
CF',[b] μg/nmol	0.123250 ± 0.0008	1.32	0.123650 ± 0.0002	0.36	0.92	0.24ns	
basic[c]	161.77 ± 1.68	2.08	177.49 ± 2.35	2.64	2.32	31.79**	
acidic[c]	275.19 ± 6.37	4.63	284.62 ± 1.24	0.87	2.82	2.86ns	
charged[c]	436.96 ± 4.79	2.19	462.11 ± 3.13	1.35	1.48	28.42*	
hydrophobic[c]	311.09 ± 4.32	2.78	301.11 ± 4.71	3.13	2.20	4.39ns	
hydrophilic[c]	533.5.41	2.03	547.52 ± 2.55	0.93	1.66	4.94ns	
apolar[c]	267.08 ± 3.74	2.80	259.24 ± 4.93	3.81	2.59	2.67ns	
R_1[c]	0.58 ± 0.01	4.51	0.55 ± 0.01	3.89	3.57	5.47ns	
R_2[c]	1.99 ± 0.04	4.19	2.12 ± 0.04	4.49	3.73	4.57ns	
R_3[c]	1.41 ± 0.03	4.69	1.53 ± 0.03	3.98	3.15	15.78*	
R_4[c]	1.64 ± 0.036	4.45	1.78 ± 0.04	4.49	3.19	14.50*	
total protein[b]							
g/kg of dry sample	427.08 ± 12.74	5.96	528.09 ± 10.18	3.86	4.55	43.07**	573.20
extracell matrix glycoprotein (eq 2)[d]							
g/kg of total protein	2.357 ± 0.15	13.16	2.122 ± 0.14	13.71	18.38	0.65ns	
g/kg of dry sample	1.006 ± 0.06		1.120 ± 0.073				

[a] Mean values and standard error of measurements (SEM) for 4 replicates $[N = (2 \times 4) - 1 = 7]$ and 64 determinations. The values for valine, isoleucine, leucine, phenylalanine, tryptophan, and 4-hydroxyproline are the average of 32 determinations. Significance: F, values from analysis of variance between genotypes; ***, $P < 0.01$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant; CV, coefficient of variation. [b] Computed according to the methods of Horstmann (1979) and Zarkadas et al. (1988a–c). [c] Calculated according to the method of Barrantes (1973, 1975) using eq 4. [c] Data for 4-hydroxyproline-rich glycoproteins were calculated from the amounts of 4-hydroxyproline found in the acid hydrolysates of the ethanol-insoluble protein fraction (F2) of soybean according to eq 2 and represent the mean values for 32 determinations from 4 replicates.

product inhibition of key biosynthetic enzymes, and in this way cellular concentrations of particular amino acids normally are maintained within closely defined levels (Miflin and Lea, 1977; Lea et al., 1990; Fowden, 1990). The levels of free amino acids of the aspartate family of amino acids, which includes lysine, methionine, threonine, and isoleucine reported in Table II, appear to illustrate this concept. The synthesis of isoleucine is, in turn, closely related to the synthesis of the other branched-chain amino acids valine and leucine (Bryan, 1990). The concentrations of all these free essential amino acids are very similar between cultivars and are low in the ethanol-soluble fraction compared to the concentration of their precursor, aspartic acid. If the aspartate family of amino acids is present in excess, they inhibit the initial biosynthetic enzyme, aspartate kinase, which catalyzes the first step in the overall pathway for the conversion of aspartic acid to β-aspartyl phosphate (Bryan, 1990). This end-product inhibition has been successfully utilized in cereals by Bright et al. (1982), who, by selecting the appropriate mutant lines of barley that inhibited the first step of the aspartate pathway, were able to increase dramatically the total levels of free threonine and methionine in the barley grain by

6.0% without effect on the composition of the barley proteins. Similar isoenzymes of aspartate kinase sensitive to lysine or threonine have been partially separated from soybeans (Matthews and Widholm, 1979).

The ethanol-soluble fraction (F1) of both soybean cultivars (Table II) was found to contain levels of phenylalanine, tyrosine, and tryptophan higher than the amounts reported in Tables III and IV for the ethanol-insoluble protein fraction (F2). These high levels of aromatic amino acids are rather unexpected since studies with labeled precursors have establishd that the biosynthesis and levels of aromatic amino acids in plants are regulated by a sequential feedback control mechanism (Besler et al., 1971; Miflin and Lea, 1977). The first divergent steps in the synthesis of phenylalanine, tyrosine, and tryptophan are inhibited by the final products (Gilchrist and Kosuge, 1980). Tryptophan synthesis is controlled by feedback inhibition of anthranilate synthetase (Besler et al., 1971; Widholm, 1973) and synthesis of phenylalanine and tyrosine by chorismate mutase inhibition (Woodin and Nishioka, 1973; Gilchrist and Kosuge, 1980). The possible explanation for such a high accumulation of aromatic amino acids in the F1 fraction

Table IV. Comparison of the Amino Acid Composition and Nitrogen Contents of Two Northern Adapted Soybean Cultivars and the Isolated Ethanol-Soluble (F1) and Ethanol-Insoluble (F2) Protein Fractions ((Grams of Amino Acids per 16 g of Nitrogen)

	untreated soybean meal				soybean genotypes[a] (ethanol-water extraction)									
					F1 ethanol-solubilized soybean fraction				F2 ethanol-insoluble protein fraction					
			significance level between genotypes				significance level between genotypes				significance level between genotypes	
amino acid (AA)	Maple Arrow (mean ± SEM)	AC Proteus (mean ± SEM)	CF	F	Maple Arrow (mean ± SEM)	AC Proteus (mean ± SEM)	CV	F	Maple Arrow (mean ± SEM)	AC Proteus (mean ± SEM)	CF	F
aspartic acid	6.499 ± 0.337	7.778 ± 0.416	14.75	2.95[ns]	12.533 ± 0.709	14.246 ± 0.322	6.06	8.91*	8.845 ± 0.109	9.355 ± 0.092	2.468	10.34*
threonine	3.954 ± 0.151	4.000 ± 0.109	5.89	0.08[ns]	1.951 ± 0.075	1.564 ± 0.133	5.63	30.58**	4.251 ± 0.091	3.671 ± 0.078	2.406	73.98**
serine	5.098 ± 0.143	5.530 ± 0.191	6.46	3.16[ns]	2.488 ± 0.131	1.964 ± 0.150	14.84	5.02[ns]	5.026 ± 0.099	4.517 ± 0.175	7.54	4.00[ns]
glutamic acid	17.947 ± 0.427	19.036 ± 0.302	4.07	4.19[ns]	22.018 ± 0.691	17.634 ± 1.014	9.14	11.69*	17.632 ± 0.415	17.913 ± 0.224	3.57	0.39[ns]
proline	4.997 ± 0.262	4.925 ± 0.111	8.69	0.06[ns]	2.829 ± 0.248	2.607 ± 0.266	17.54	0.43[ns]	5.746 ± 0.505	5.580 ± 0.334	14.20	0.10[ns]
glycine	3.486 ± 0.040	3.422 ± 0.110	5.67	0.11[ns]	2.399 ± 0.140	2.029 ± 0.133	4.96	22.75**	3.524 ± 0.087	3.478 ± 0.143	7.45	0.06[ns]
alanine	3.795 ± 0.106	3.619 ± 0.197	10.34	0.42[ns]	2.358 ± 0.273	1.804 ± 0.125	11.55	10.61*	3.459 ± 0.128	3.335 ± 0.020	5.90	0.77[ns]
cysteine	2.360 ± 0.079	2.213 ± 0.103	8.24	1.22[ns]	8.412 ± 0.450	6.146 ± 0.712	18.49	5.66[ns]	2.115 ± 0.117	1.996 ± 0.102	11.80	0.48[ns]
valine	5.121 ± 0.060	4.861 ± 0.020	1.55	2.79**	1.890 ± 0.114	1.598 ± 0.173	5.57	18.13*	4.723 ± 0.117	4.602 ± 0.197	5.43	0.33[ns]
methionine	1.009 ± 0.035	0.917 ± 0.049	8.45	2.58[ns]	0.722 ± 0.065	0.577 ± 0.048	9.54	2.59[ns]	1.160 ± 0.057	0.980 ± 0.036	7.96	8.93*
isoleucine	4.868 ± 1.082	4.764 ± 0.141	4.79	0.41[ns]	2.193 ± 0.105	1.868 ± 0.208	8.87	6.52[ns]	4.771 ± 0.115	4.636 ± 0.131	4.53	0.80[ns]
leucine	7.709 ± 0.128	7.503 ± 0.270	4.59	0.70[ns]	2.974 ± 0.162	2.723 ± 0.304	11.72	1.14[ns]	8.051 ± 0.099	7.082 ± 0.136	1.79	6.11[ns]
tyrosine	3.922 ± 0.089	3.695 ± 0.143	4.47	3.51[ns]	5.897 ± 0.153	4.902 ± 0.488	14.53	3.21[ns]	4.236 ± 0.145	4.014 ± 0.168	5.54	1.89[ns]
phenylalanine	5.312 ± 0.085	5.008 ± 0.191	3.55	5.48[ns]	6.092 ± 0.281	4.852 ± 0.467	15.53	4.25[ns]	5.747 ± 0.234	5.859 ± 0.149	6.68	0.11[ns]
histidine	3.232 ± 0.503	3.069 ± 0.463	15.30	0.23[ns]	1.479 ± 0.149	2.613 ± 0.298	30.11	6.77[ns]	2.129 ± 0.212	2.529 ± 0.110	10.82	5.05[ns]
lysine	6.449 ± 0.081	5.906 ± 0.212	5.73	4.71[ns]	1.909 ± 0.138	1.679 ± 0.162	7.02	6.68[ns]	6.256 ± 0.145	6.280 ± 0.074	1.98	0.08[ns]
arginine	7.280 ± 0.228	7.636 ± 0.458	9.65	0.49[ns]	8.211 ± 0.332	14.833 ± 1.453	20.72	15.39*	7.185 ± 0.096	8.216 ± 0.057	2.65	50.92**
tryptophan	1.202 ± 0.049	1.162 ± 0.048	8.95	0.28[ns]	5.186 ± 0.522	3.744 ± 0.134	20.17	4.89[ns]	1.252 ± 0.032	1.155 ± 0.039	2.57	19.96*
4-hydroxyproline	0.132 ± 0.002	0.107 ± 0.007	7.11	17.72**	1.601 ± 0.143	1.263 ± 0.208	20.53	2.65[ns]	0.107 ± 0.007	0.096 ± 0.007	18.58	0.70[ns]
ammonia	1.129 ± 0.072	1.009 ± 0.085	20.03	0.63[ns]	2.767 ± 0.412	1.065 ± 0.369	43.81	8.22[ns]	1.17 ± 0.116	0.898 ± 0.191	19.23	3.84[ns]
total AAN[b]												
g of AAN/kg of protein	169.61 ± 1.465	168.35 ± 0.65	0.499	0.74[ns]	171.68 ± 4.078	181.02 ± 5.556	3.03	6.11[ns]	166.289 ± 0.99	167.05 ± 1.85	1.193	0.30[ns]
g of AAN/kg of dry mass	56.683 ± 1.349	70.289 ± 2.62	6.02	25.31**	4.013 ± 0.33	6.607 ± 0.722	26.08	7.02[ns]	70.441 ± 1.951	87.53 ± 1.308	3.61	71.88**
g of AAN/16 g of N	94.355 ± 0.809	95.154 ± 0.281	1.41	0.72[ns]	93.348 ± 2.129	88.632 ± 2.670	3.00	5.95[ns]	95.975 ± 0.537	95.813 ± 1.042	1.37	0.03[ns]

[a] Mean values and standard error of measurements (SEM) for 4 replicates [N (2 × 4) − 1 = 7] and 64 determinations. The values for valine, isoleucine, leucine, tryptophan, 4-hydroxyproline, and phenylalanine are the average of 32 determinations. Significance: F, values from analysis of variance between genotypes; **, $P < 0.01$; *$P < 0.05$; ns, not significant; CV, coefficient of variation between genotypes. [b] Total amino acid nitrogen (AAN) was determined according to the methods of Heidelbaugh et al. (1975), Horstmann (1979), and Zarkadas et al. (1988a–c).

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of both soybean cultivars may be related to the presence of mutant isoenzymes which alter the feedback inhibition. There is evidence that cultured *Nicotiana tabacum* and *Daucus carota* cells have an anthranilate synthetase, which is much less sensitive to tryptophan feedback inhibition, and accumulate tryptophan to 20–30-fold higher levels (Widholm, 1973). Further evidence for such an occurrence is that when anthranilate was supplied externally to the resistant soybean cells, the levels of tryptophan increased 20-fold (Widholm, 1974). These observations suggest that the high levels of tryptophan found in the ethanol-soluble fraction (F1) of soybean seeds may be compartmentalized *in situ* away from the site of synthesis. Similar isoenzymes for chorismate mutase have been identified in several plants with altered feedback inhibition by phenylalanine and tyrosine (Woodin and Nishioka, 1973; Gilchrist and Kosuge, 1980), and the high levels of these amino acids may be related to the presence of such isoenzymes.

Maple Arrow was significantly ($P < 0.05$) higher in threonine, glycine, and alanine in the F1 fraction than AC Proteus, but both cultivars contained similar amounts of serine and proline. Although serine is a precursor of cysteine, the results (Table II) showed that the amount of cyst(e)ine present in the F1 fraction was more than 3 times higher than the amount of serine. Bowman (1946) and Frattali (1969) have shown that the ethanol-soluble fraction of soybean contains small amounts of various protease inhibitors known to be high in cysteine (Birk, 1985). Sequence studies have shown that these inhibitors contained 14 of 70 amino acid residues (approximately 20%) as cysteine (Odani and Ikenaka, 1972), which might explain the results in the present study. At least 10 protease inhibitors with molecular weights ranging from 6000 to 10 000 have been described in the ethanol soluble Bowman–Birk protease inhibitor class (Tan-Wilson et al., 1986, 1987; Kollipara and Hymowitz, 1992). Of these, the Bowman–Birk is the major inhibitor, which in the soybean cultivar Tracy accounted for 4.0% of the defatted meal corresponding to 4.8% of the total soybean proteins (41.0%). The four low molecular weight protein inhibitors (PI), designated I–IV, accounted for an estimated 2.3% of the defatted meal or about 3.3% of the total soybean proteins. Rackis et al. (1986) by contrast showed that the total amount of protease inhibitors in whole soybean seeds was 2.29 g/100 g of dry sample, which corresponded to 4.96 g of protease inhibitors/100 g of protein. The defatted soybean flour contained 3.24% protease inhibitors or about 5.78 g/100 g of protein compared to soybean protein concentrate, which contained between 0.9 and 2.1 g of protein inhibitors/100 g of protein (Kakade et al., 1973; Rackis et al., 1986). Further studies will be required to establish the levels of all of the protease inhibitors in soybeans.

After acid hydrolysis, the ethanol-soluble fractions (F1) from both cultivars were found to contain relatively high levels of *trans*-4-hydroxy-L-proline (Table II). This unique amino acid is rarely present in the free state in plant tissues. The only reported free 4-hydroxyproline existing in relatively large concentrations has been in the tissue of the sandal (*Sandalum album*), which was found to be the *cis* isomer of 4-hydroxyproline (Lamport, 1977). The form bound to proteins is the *trans* form (Berg, 1982). Cassab et al. (1985) reported that the outermost layer of the soybean seed coat contains 77% of the total 4-hydroxyproline in the seed. It is present as a 4-hydroxyproline-rich glycoprotein which serves as a structural protein in the cell wall (Lamport, 1977; Cooper et al., 1987; Cassab and Varner, 1987, 1988). To date, two other cell wall structural proteins have been characterized, namely, proline-rich proteins (Averyart-Fullard et al., 1988; Kielszewski et al., 1992) and glycine-rich proteins (Keller et al., 1988). The best characterized 4-hydroxyproline-rich glycoprotein from soybeans is extensin, which has been proposed to be the major cell wall protein component primarily localized in two of the external layers of the soybean seed coat (Cassab et al., 1985; Hong et al., 1987; Cassab and Varner, 1987; Ye and Varner, 1991). It has been suggested that the majority of the proline residues in the cell wall glycoproteins are posttranslationally modified to *trans*-4-hydroxyproline. These are then released from the membrane-bound ribosomes as soluble monomers, which when deposited in the cell walls of soybeans become insoluble due to the formation of isodityrosine cross-links (Wilson and Fry, 1986; Cooper et al., 1987; Cassab and Varner, 1988; Varner and Lin, 1989). The ethanol-soluble *trans*-4-hydroxyproline in this study, therefore, might have originated from soluble 4-hydroxyproline-rich glycoproteins in the cell walls, but the reason for such high levels is at present unclear.

Protein-Bound Amino Acid Contents of Soybeans. The protein-bound amino acid composition of the two northern adapted soybean cultivars after extraction were very similar (Tables III and IV). Both cultivars were found to contain high levels of glutamic and aspartic acids, leucine, phenylalanine, lysine, proline, and arginine, and their overall amino acid profiles were very similar to those reported by Zarkadas et al. (1993a) for untreated soybean meals. The most consistent and characteristic feature of the amino acid composition of both cultivars was the very high acidic amino acid content, i.e., glutamic acid and aspartic acid, which together accounted for 27.5–28.5% of the total amino acid residues. The total basic amino acids, i.e., histidine, lysine, and arginine, constituted only 16.2–17.7% of the total. The basic amino acid content of AC Proteus was significantly higher ($P < 0.01$) than that of Maple Arrow, reflecting primarily the variation in the arginine content between these two cultivars.

In soybean seeds, glutamic acid and its amide, glutamine, are central to intermediate nitrogen metabolism and storage of protein nitrogen (Blevis, 1989; Lea et al., 1990). Glutamate is the product of ammonia assimilation via the glutamate synthetase cycle and the acceptor of ammonia in glutamine synthesis (Lea et al., 1990, 1992). Glutamic acid is the immediate donor of the amino group of most amino acids in developing or germinating seeds. In addition, it is the donor of both the nitrogen and carbon atoms in the biosynthesis of arginine and proline (Dashek and Erickson, 1981; Miflin and Lea, 1977, 1982). Aspartic acid, which is derived from oxaloacetate via transamination, serves both as a common precursor for lysine, methionine, threonine, and isoleucine and as an acceptor of the amide group from glutamine in asparagine synthesis for the transport and storage of nitrogen (Lea and Miflin, 1974; Bryan, 1990). Proline also appears to play a role in nitrogen storage in soybean seeds (Dashek and Erickson, 1981) and accounts for an additional 5.8–6.0% of the total amino acid residues in both cultivars. There was, however, a significant variation between cultivars in the content of threonine ($P < 0.01$), tryptophan ($P < 0.01$), and arginine ($P < 0.01$).

The data presented in Table III indicate that, as a result of three cycles of crossing and selection, the arginine content of the ethanol-insoluble fraction (F2) varied from 74.7 g/kg of total protein in Maple Arrow to 85.5 g/kg of total protein in AC Proteus. The 14.5% higher arginine content of AC Proteus, which coincided with a total

Table V. Comparison of the Essential Amino Acid (EAA) Composition of Two Soybean Genotypes and High-Quality Animal Proteins with the Suggested EAA Pattern of Requirements for Humans

EAA	EAA requirements[a]		soybean genotype						animal products		
			untreated soybean meal		F1 ethanol-solubilized protein fraction		F2 ethanol-insoluble protein fraction			cow's	
	preschool child (2–5 years)	adult	Maple Arrow	AC Proteus	Maple Arrow	AC Proteus	Maple Arrow	AC Proteus	egg[a]	milk[a]	beef[b]
	Milligrams of Amino Acid per Gram of Total Protein										
histidine	19	16	26	23	16	29	22	26	22	27	34
isoleucine	28	13	50	48	23	21	50	48	54	47	48
leucine	66	19	79	74	31	30	84	81	86	95	81
lysine	58	16	65	58	20	19	65	65	70	78	89
methionine + cyst(e)ine	25	17	34	30	98	75	34	31	57	33	40
phenylalanine + tyrosine	63	19	95	85	128	109	104	101	93	102	80
threonine	34	9	38	39	21	17	44	38	47	44	46
tryptophan	11	5	11.7	11.1	56	43	13	12	17	14	12
valine	35	13	54	50	20	18	49	48	66	64	50
mg/g nitrogen[c]			2969	2814	2840	2779	3109	3062			
% of total protein											
EAA₁₀ including Arg[d]			46.4	44.9	34.2	40.2	46.1	46.5			
EAA₉ minus Arg	33.9	12.7	45.3	41.8	41.3	36.1	46.5	45.0	51.2	50.4	47.9
EAA₈ minus His Arg	32.0	11.1	42.7	39.5	39.7	33.2	44.3	42.4	49.0	47.7	44.5
protein efficiency ratio[a] egg (PER₁₀)[d,e]			2.77	2.67	2.0	2.39	2.76	2.78			

Percent Protein Digestibility in Man[a]

			86	86			86	86	95	97	98

Percent Amino Acid Score Adjusted for Digestibility

			95	93			95	94	119	119	94

[a] Data from FAO/WHO/UNU (1985) and FAO/WHO (1990). [b] Data taken from Bodwell (1987). [c] Computed from reference protein standards (FAO/WHO, 1965). [d] Calculated according to the methods of Lee et al. (1978) and Pellet and Young (1984). EAA₁₀: threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, tryptophan, histidine, and arginine. PER₁₀ values were calculated from egg [PER = 0.06320 (EAA₁₀) − 0.1534]. [e] Calculation of protein rating was carried out by comparison of the amino acid composition of the two soybean cultivars with that of the reference pattern established by FAO/WHO/UNV (1985) from eq 6 [100 × concn of AA in product (mg/g of protein)/concn of AA in FAO/WHO/UNV (1985) pattern (mg/g of protein)] for preschool child (2–5 years) and adult.

increase of 10.1% in protein content, suggests a possible shift in the proportions of the various storage proteins present in this cultivar. The methionine contents of the F2 fractions of Maple Arrow and AC Proteus were 12.1 and 10.2 g/kg of total protein, respectively, which corresponded to 15.7% less methionine in AC Proteus. These results suggest that the methionine levels vary inversely with total protein and that the amino acid composition of the major types of storage proteins including glycinins and β-conglycinins is not constant. Both glycinins and β-conglycinins are families of proteins assembled from a number of different subunits which differ in their contents of sulfur-containing amino acids from 0.0 to 3.0% (w/w) (Thanh and Shibasaki, 1978; Koshiyama, 1983; Nielsen, 1984; Wolf, 1993).

Several studies have shown that the composition of soybean storage proteins is also affected by nutrient availability and enviromental conditions (Thomson et al., 1981; Holowach et al., 1984; Gayler and Sykes, 1985; Grabeau et al., 1986). Methionine supplementation of an *in vitro* soybean cotyledon or of intact soybean plants was reported to affect the amounts of several amino acids including a 21.9% increase in methionine and a decrease of 11.4% in arginine content. The authors suggested that these changes were due to a dramatic decrease in the synthesis of the methionine-devoid β-subunit of the β-conglycinin storage protein (Thomson et al., 1981; Grabau et al., 1986) and an increase in the ratio of glycinins to β-conglycinins. Gayler and Sykes (1985), by contrast, showed that sulfur deficiency in developing seeds of the cultivar Wayne caused a 40.0% decrease in the level of glycinins and an elevation in the level of β-conglycinins. The proportion of the β-subunit of β-conglycinins was also increased 3-fold in the sulfur-deficient seeds.

Improvements in the nutritional quality of soybean proteins, therefore, will necessitate an increase in me-

thionine-containing storage proteins or a reduction in the β-subunit of β-conglycinin devoid of methionine or a combination of the two (de Lumen, 1990; George and de Lumen, 1991). Recurrent selection of soybeans has been successfully used by Brim and Burton (1979) as a plant breeding procedure for increasing the percent protein in soybeans without significantly decreasing yields. This procedure, however, has not increased methionine levels (Burton et al., 1981).

Although the amino acid profiles of both cultivars were similar, there appeared to be a preferential accumulation of certain amino acids in the seeds of these two cultivars. To simplify a discussion of the changes occurring in such plant tissues, Barrantes (1973, 1975) has suggested grouping the amino acids into four classes, namely, totally charged, hydrophilic, hydrophobic, and apolar. He then compared the ratio (R) of the frequencies of occurrence of these particular groups, especially in terms of the proportion of total charged and hydrophobic residues (ratio 3). This method of amino acid classification was used in this study, and the results obtained are summarized in Tables II and III. These data indicated a small but significant increase ($P < 0.05$) in total charged amino acids and in the R_3 ratio and a decrease in hydrophobicity of the storage proteins in AC Proteus compared to Maple Arrow. This suggests that it may be both part of the changes that occur in the subunit composition of the storage proteins and the differential gene expressions that determine protein transformation patterns in the seeds of this new cultivar (Chrispeels, 1984; Goldberg, 1986).

The extracellular matrices of soybean seeds from both cultivars contained small amounts of protein-bound 4-hydroxyproline (Tables III and IV). From the known amino acid composition and distribution of 4-hydroxyproline in the primary sequence of these glycoproteins, the content of 4-hydroxyproline-rich glycoproteins of soybean seeds

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was calculated by multiplying the amount of 4-hydroxyproline found in their acid hydrolysates by 2.128 (eq 3b), as described previously (Khanizadeh et al., 1989; Zarkadas et al., 1993a). These results show that the levels of 4-hydroxyproline-rich glycoproteins in soybean seeds were low, ranging from a low of 2.12 g/kg of total protein in AC Proteus to a high of 2.36 g/kg of protein in Maple Arrow, which corresponds to about 0.10–0.11% on a dry weight basis. These values are in close agreement with those reported previously for the entire seeds (Zarkadas et al., 1993a) but are lower than those reported by Cassab et al. (1985), who have shown that this glycoprotein is approximately 2.0% of the total dry weight of the seed coat or about 0.34% of the weight of the entire seed.

Evaluation of Protein Quality of Soybeans. Comparison of the essential amino acid (EAA) patterns (milligrams per gram of dietary nitrogen) of the two new soybean cultivars, and of the isolated fractions F1 and F2 (Table V), indicates that both soybean cultivars contain significant amounts of all EAA required for both human and animal nutrition (Block and Mitchell, 1946; Oser, 1951; FAO/WHO, 1965), with methionine and tryptophan as the major limiting amino acids. These results are in close agreement with earlier findings by Zarkadas et al. (1993a) for the untreated soybean meal.

An even more accurate assessment of the protein quality of foods was recommended by the U.S. Department of Agriculture (Expert Work Group, 1984), Lee et al. (1978), and Pellett and Young (1984). It involves the use of the determination of the complete amino acid composition, EAA content, and calculated protein efficiency ratio (PER) as indices of protein quality. Lee et al. (1978) defined the 10 EAA (EAA_{10}) as being threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine, and tryptophan. The Joint FAO/WHO Expert Consultation Group (FAO/WHO/UNU, 1985; FAO/WHO, 1990) have recommended that, in conjuction with *in vivo* protein digestibility data, the use of the reference amino acid pattern for the 2-5-year-old child be used as the reference pattern (Table V) in the evaluation of foods for all persons except infants. The nine essential amino acids (EAA_9) included all of the above except arginine (FAO/WHO/UNU, 1985). Since cystine and tyrosine can replace methionine and phenylalanine, respectively, the two sulfur-containing (methionine plus cystine) and two aromatic amino acids (phenylalanine plus tyrosine) are usually considered together.

The F2 fraction of soybeans had a mean value for total EAA_{10} that ranged from 46.1 to 46.4% and a calculated protein efficiency value (Lee et al., 1978; Pellett and Young, 1984) close to 2.7 (Table V). These values are in close agreement with those reported previously for the entire soybean seeds (Zarkadas, et al., 1993a) but are considerably higher than the average rat bioassay PER value of 2.3 for soybeans reported by others (Torun et al., 1981; Bodwell et al., 1980). Mean values for corrected amino acid scores ranged from 95% in Maple Arrow to 93% in AC Proteus. The F2 fractions from soybean contained all of the EAA_9 (FAO/WHO/UNU, 1985) ranging from 45.0 to 46.5%, which is considerably higher than the 33.9% reference pattern value given by FAO/WHO (1990). These results correspond closely with the mean essential amino acid values (Table V) calculated according to the methods of Lee et al. (1978) and Pellett and Young (1984).

The data presented in this paper show the variations that exist between the total protein and the protein-bound and free amino acid contents of two northern adapted soybean cultivars. As the content of total proteins was increased in AC Proteus by breeding, the concentration of protein-bound arginine, aspartic acid, and histidine increased, while the levels of threonine, tryptophan, and methionine decreased by comparison with the parent Maple Arrow. From these results, it became evident that a potentially useful means for evaluating the protein quality of different soybean cultivars would be based on accurate quantitation of their amino acid composition, corrected for protein digestibility, as recommended by FAO/WHO/UNU (1985), FAO/WHO (1990), Pellett and Young (1984), and Zarkadas et al. (1993a,b).

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