Gel Electrophoresis and Amino Acid Analysis of the Nonprotein Nitrogen Fractions of Defatted Soybean and Almond Meals

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ABSTRACT

Defatted soybean and almond meals were extracted with 0-5*M* trichloroacetic acid (TCA), and the extracts were analyzed for Kjeldahl nitrogen. Minimum extractability of nitrogenous compounds occurred at 0.4-1.0*M* TCA and was assumed to represent the nonprotein nitrogen (NPN). Above 3*M* TCA, the nitrogen solubility increased rapidly as a result of solubilization of the bulk of the storage proteins. Analysis of 0.6*M* TCA extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the NPN fraction contained polypeptides of 12 kDa for soybean meal and 28, 12, 10, and 7 kDa for almond meal. These polypeptides were retained by 3.5 kDa cutoff dialysis membranes when the TCA extracts were dialyzed against water to remove the TCA and low molecular weight constituents. The polypeptides represent about 1-2% of the meal nitrogen. The NPN fractions were also characterized by amino acid analysis. The predominant free amino acids were arginine, aspartic, and glutamic acids for soybean meal, and aspartic acid, glutamic acid, proline, arginine, and alanine for almond meal. The polypeptides of almond meal NPN contained 65 mol% glycine, suggesting the presence of glycine-rich proteins that are structural elements of plant cell wall proteins.

The nonprotein nitrogen (NPN) fraction of biological materials is generally considered to consist of free amino acids, amides, peptides, polypeptides, and other low molecular weight compounds containing nitrogen, such as alkaloids and cyanoglycosides. The distinction between NPN and proteins is not clear-cut. Synge (1955) and Pirie (1955) arbitrarily defined proteins as molecules of 10 kDa and larger. According to this definition, NPN may therefore contain polypeptides up to 10 kDa. There are well-known exceptions to this definition, including insulin (5.7 kDa) (Ryle et al 1955), Bowman-Birk soybean trypsin inhibitor (7.9 kDa) (Odani and Ikenaka 1973), and crambin (4.7 kDa) (Teeter et al 1981); these are less than 10 kDa but are considered to be proteins. Nonetheless, this arbitrary division between proteins and NPN has been a useful approximation. Little is known, however, about the molecular size of the largest polypeptides that are found in the NPN fraction of biological materials as determined by a variety of methods (Bell 1963). Extraction of NPN with trichloroacetic acid (TCA) or precipitation of proteins with TCA are two of the most widely used methods. Becker et al (1940) conducted a study with defatted soybean meal in which they extracted the meal with TCA in the range of 0-4*M*. They found that a minimum in the extractability of nitrogenous compounds occurred in the range of 0.5-1.2*M* TCA. They assumed that this minimum in solubility represented the NPN fraction and recommended that 0.8*M* TCA be used to extract the NPN. This classical method has been used for many years. Krober and Gibbons (1962) surveyed 381 soybean seed samples over a six-year period and found NPN values ranging from 2.5 to 13% of total seed nitrogen; most samples, however, contained 4-5% NPN. Except for amino acid composition data (Bhatty and Finlayson 1973, Bhatty et al 1973), little has been reported about this fraction of soybeans. Limited information is available about the molecular size of any TCA-soluble polypeptides that may be present. Becker et al (1940) reported that upon electrodialysis of 0.8*M* TCA extracts of defatted meal, only 0.2% of the meal nitrogen failed to dialyze (nondialyzable fraction). The molecular cutoff value for the cellophane dialysis membrane used was not given, but presumably the NPN fraction of soybeans contains a small amount of polypeptides of 10-15 kDa.

In the course of developing a TCA extraction procedure for determining the NPN content of jojoba meal (Wolf et al 1994), we also examined the NPN fractions of soybean and almond meals. We characterized the fractions by amino acid analysis and gel electrophoresis and the results are described here.

MATERIALS AND METHODS

Materials

Solvents and chemicals were of reagent grade. Defatted soybean meal was prepared from Raiden soybeans by dehulling and extracting with hexane. Defatted almond meal was obtained from S. K. Sathe, Department of Nutrition, Food, and Movement Sciences, Florida State University, Tallahassee, FL.

Extraction of Meals with TCA

The method of Becker et al (1940) was used with minor changes. Portions (10 ml) of TCA solution (0-5*M*) were added to 200 mg

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of meal in screw-capped centrifuge tubes. The samples were shaken for 1 hr at room temperature, centrifuged, and filtered through glass wool. The supernatants were analyzed for nitrogen (AOAC 1984). Results are expressed as percent of the total nitrogen extracted by the various concentrations of TCA. For gel electrophoretic analysis of the high molecular weight polypeptides extracted by various concentrations of TCA, the extracts were dialyzed against distilled water in dialysis tubing (Spectra/Por, Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cutoff of 3.5 kDa, and then freeze-dried.

Isolation of NPN Fractions

The extraction procedure was scaled up using 2–10 g of meal as necessary to provide sufficient material for analysis. After the initial extraction with 0.6*M* TCA and centrifugation, the meal residue was reextracted with one-half of the original volume of TCA. The extracted meal was placed into a Buchner funnel, washed with diethyl ether to remove the excess TCA, and then freeze-dried. The two TCA extracts were combined, extracted repeatedly with water-saturated diethyl ether to remove the TCA, and then freeze-dried.

Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the Fling and Gregerson (1986) modification of the Laemmli (1970) procedure using a model SE 600 cell (Hoefer Scientific Instruments, San Francisco, CA). AcrylAide (FMC BioProducts, Rockland, ME) was substituted for N, N′-methylenebisacrylamide as the crosslinking agent. Gel compositions were 13.5% T and 0.4% C. Proteins were stained with Coomassie Blue (Fling and Gregerson 1986). The molecular weight standards (Bio-Rad Laboratories, Hercules, CA) included myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin.

Carbohydrate Analysis

The phenol-sulfuric acid method of Dubois et al (1956) was employed using D-glucose as a standard.

Amino Acid Analysis

Analyses were performed on freeze-dried NPN fractions and meals by the University of Illinois Biotechnology Center, Genetic Engineering Facility (Urbana, IL) using an HP1090 liquid chromatograph with AminoQuant (o-phthalaldehyde) derivatization and fluoresence detection, according to the manufacturer's instructions (Hewlett Packard, Palo Alto, CA). The hydrolyzate column and program were used for the unhydrolyzed samples as well as for the hydrolyzates; the chromatograms for the unhydrolyzed samples revealed no unknown peaks that obscured the free amino acid peaks. The samples were hydrolyzed with 6*M* HCL at 110°C for 24 hr in sealed tubes after vacuum removal of air.

RESULTS

Effect of TCA Concentration on Extractability of Nitrogen

Figure 1 shows extractability of nitrogenous compounds of soybean and almond meals over the concentration range of 0–5*M* TCA. Results with soybean meal (Fig. 1A) indicate that the minimum in extractability is in the range of 0.4–1.0*M* TCA. The discontinuity in the curve is the region (~0.01–0.1*M*) in which the proteins precipitate at the isoelectric point, redissolve, and reprecipitate as the TCA concentration increases (Becker et al 1940). The minimum in solubility represents 4.1 ± 0.1% (standard deviation, $n = 4$) of the total meal nitrogen and is assumed to correspond to the NPN fraction as originally proposed by Becker et al (1940). At TCA concentrations above 1*M*, the nitrogen solubility increased and reached a value of 97% at 4–5*M*. In a separate experiment, the ratio of meal to TCA was varied in 50-mg increments from 50 to 300 mg in 10 ml of 0.6*M* TCA. The NPN value for the six samples was constant at 3.7 ± 0.2% (standard deviation) and in good agreement with the value obtained from the data in Fig. 1A. The ratio of meal to TCA volume is not critical in the range used.

Almond meal (Fig. 1B) yielded a curve similar to that of soybean meal. The nitrogen solubility was at a minimum in the range of 0.4–1.0*M* TCA corresponding to a NPN value of 4.8 ± 0.3% (standard deviation, $n = 4$) of the total nitrogen. Above 1*M*, TCA solubility increased and reached a maximum of 69% at 4–5*M* TCA. As with soybean meal, varying the meal to TCA ratio in 50-mg increments from 50 to 300 mg in 10 ml of 0.6*M* TCA had no effect on the NPN value. The mean value for the six meal-TCA ratios was 4.2 ± 0.2 (standard deviation).

Characterization of Materials Extracted by TCA

Chemical composition. Two batches of soybean meal were extracted with 0.6*M* TCA, followed by washing of the extracts with diethyl ether to remove the TCA, and freeze-drying. Yields and analytical results are shown in Table I. Recovery of meal solids was 41–54%, and the nitrogen recovered was 4.0–4.7% of the meal nitrogen, which is in reasonable agreement with the value of 4.1% for the NPN obtained above (Fig. 1A). The low nitrogen content of 0.8–0.9% suggested the presence of large amounts of other materials. The oligosaccharides sucrose,

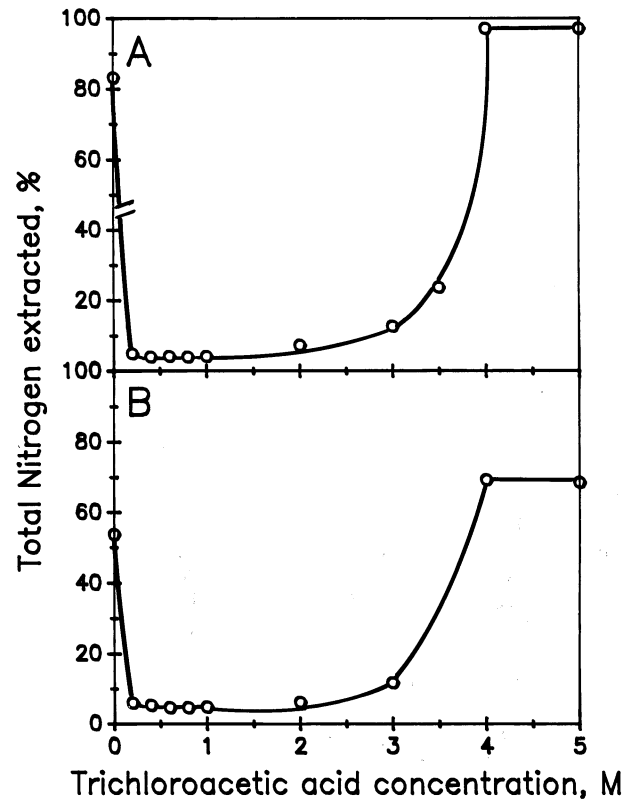

Fig. 1. Extractability of total meal nitrogen as a function of trichloroacetic acid concentration for soybean **(A)** and almond **(B)** meals.

TABLE I
Yield (%) and Composition (%) of 0.6*M* Trichloroacetic Acid (TCA) Extracts of Soybean and Almond Meals[a]

	Yield		Composition	
Meal	Of Meal Mass	Of Meal Nitrogen	Nitrogen Content	Carbohydrate Content
Soybean	54	4.7	0.79	72
Soybean	41	4.0	0.91	83
Almond	30	4.9	1.13	36
Almond	26	4.7	1.28	39

[a]TCA was removed by diethyl ether extraction prior to freeze-drying. Data expressed on a dry basis.

raffinose, and stachyose, plus other TCA-soluble materials, such as ash, would be expected to be extracted. Carbohydrate analysis by the phenol-sulfuric acid method confirmed the presence of high concentrations of carbohydrates; values of 72–83% were obtained.

Similar experiments with almond meal yielded results also shown in Table I. A lower recovery of meal solids was obtained (26–30%) than with soybean meal, but the recovery of nitrogen (4.7–4.9%) agreed well with the NPN value of 4.8% (Fig. 1B). The nitrogen content of the solids (1.1–1.3%) was somewhat higher, and carbohydrate content (36–39%) was considerably lower than for soybean meal.

SDS-PAGE analyses of the NPN fractions obtained by 0.6*M* TCA extraction of the two meals indicated the presence of polypeptides in the range of 7–28 kDa; hence, 0–5*M* TCA extracts of the two meals were prepared on a preparative scale, dialyzed against distilled water in 3.5 kDa cutoff dialysis tubing, and freeze-dried to determine whether polypeptides could be recovered, particularly in the region of minimum extractability. Nondialyzable materials were recovered from all of the TCA extracts as summarized in Tables II and III. Discrepancies between the nitrogen yield data in Tables II and III, as compared to Figure 1, probably result from losses during dialysis and freeze-drying plus errors introduced by the additional step of moisture analysis in the preparative scale procedure.

With soybean meal, only 4–6% of the meal was recovered in the 0.6–2.0*M* TCA extracts. The nitrogen isolated accounted for about 1% of the total meal nitrogen, as compared to 4.1% for the NPN value. Thus, only about 25% of the NPN is in the form of polypeptides, assuming that all of the nitrogen in the dialyzed fraction is polypeptide in nature. The 0 and 4.0*M* TCA extracts yielded almost pure protein (91–99%), but the materials isolated from the 0.6 and 2.0*M* TCA extracts were very low in nitrogen content. Calculation of crude protein contents suggested the presence of large quantities of nonprotein materials. Analysis for carbohydrates revealed that about 30% of the 0.6 and 2.0*M*

TABLE II
Yield (%) and Composition (%) of Nondialyzable Fractions Extracted from Soybean Meal with Trichloroacetic Acid (TCA)[a]

TCA (*M*)	Yield		Composition		
	Of Meal Mass	Of Meal Nitrogen	Nitrogen	Crude Protein[b]	Carbohydrate
0	28.0	47.1	15.8	98.8	2.6
0.6	4.1	0.9	1.3	8.1	34.0
2	6.4	1.4	2.1	13.1	28.9
3.5	17.2	18.5	10.0	62.5	12.6
4.0	42.1	65.5	14.6	91.3	5.0

[a]Values expressed on a dry basis.
[b]N × 6.25 (AOCS 1976).

TABLE III
Yield (%) and Composition (%) of Nondialyzable Fractions Extracted from Almond Meal by Trichloroacetic Acid (TCA)[a]

TCA (*M*)	Yield		Composition		
	Of Meal Mass	Of Meal Nitrogen	Nitrogen	Crude Protein[b]	Carbohydrate
0	33.4	59.8	16.7	86.5	5.2
0.6	7.2	1.9	2.4	12.4	25.0
1	5.9	1.4	2.2	11.4	24.6
2	6.0	1.9	2.9	15.0	25.7
3	8.6	6.0	6.5	33.7	18.7
4	25.0	31.6	11.8	61.1	8.2
5	51.7	79.0	14.2	73.6	4.9

[a]Values expressed on dry basis.
[b]N × 5.18 (AOAC 1984).

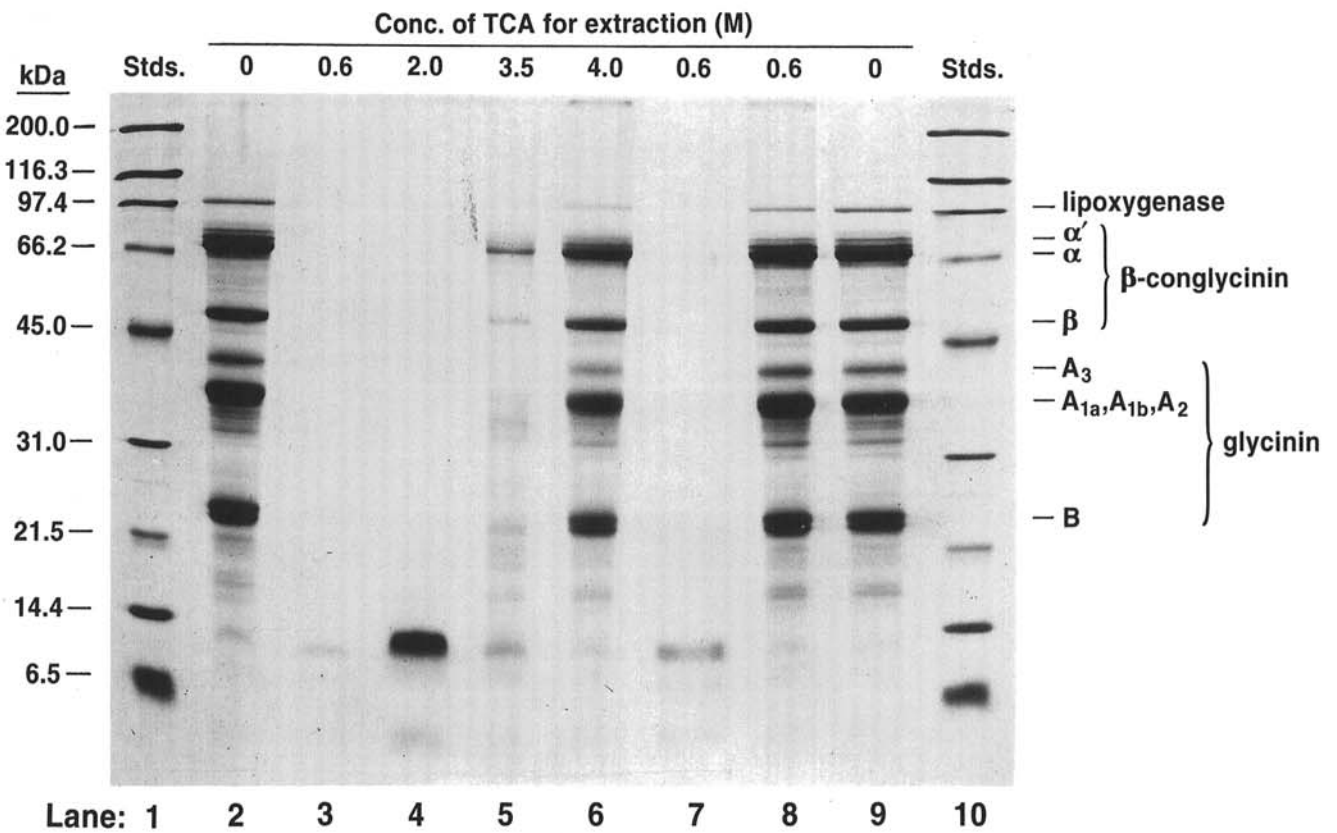

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern for soybean meal fractions extracted by various concentrations of trichloroacetic acid (TCA). Lanes 1 and 10, molecular weight standards (2.5 μg/band); lane 2, 0*M* TCA extract (50 μg); lane 3, 0.6*M* TCA extract (100 μg); lane 4, 2*M* TCA extract (160 μg); lanes 5 and 6, 3.5 and 4*M* TCA extracts, respectively (50 μg); lane 7, diethyl ether extracted 0.6*M* TCA extract (4800 μg); lane 8, proteins from 100 μg of 0.6*M* TCA extracted meal; and lane 9, proteins from 100 μg of defatted soybean meal. TCA concentrations used to make the extracts are indicated across top of gel.

TCA extracted fractions consisted of polysaccharides. Nonetheless, the sum of protein plus carbohydrates only accounted for about 42% of the polymeric materials.

As with soybean meal, the 0.6–1*M* TCA almond meal extracts (Table III) yielded low recoveries of the meal (6–7%), and nitrogen recovery amounted to 1–2% of the total meal nitrogen. The recovered nitrogen accounts for about 40% of the NPN. The nitrogen content of the polymeric materials was 2–3% for the 0.6–2.0*M* TCA extracts. The carbohydrate content of these fractions was 25%, but the sum of protein plus carbohydrate was only about 40%, essentially the same as noted with soybean meal.

SDS-PAGE analyses. Analysis of the TCA-extractable fractions by SDS-PAGE revealed the presence of polypeptides, thus confirming the polymeric nature of the dialyzed fractions. The freeze-dried extractables from soybean meal are shown in Figure 2. The water-extractable soybean proteins (0*M* TCA extract) in lane 2 gave a typical pattern with major bands corresponding to lipoxygenase (100 kDa), α'-subunit (78 kDa), α-subunit (71 kDa), and β-subunit (50 kDa) of β-conglycinin plus the acidic (37–42 kDa) and basic (25 kDa) polypeptides of glycinin. The fraction extracted by 0.6*M* TCA gave only a weak band at 12 kDa (lane 3), despite being loaded at 100 μg as compared to only 50 μg for the more complex mixture in the water-extractable proteins (lane 2). With 2*M* TCA, a major 13 kDa band and weak band of about 3 kDa near the bottom of the gel were extracted and retained on dialysis (lane 4). Variations in sample loading and carbohydrate content (Table III) may account for the differences between the 12 kDa and 13 kDa bands in lanes 3 and 4, respectively. The 3.5*M* TCA extractable materials (lane 5) exhibited a series of minor bands that generally corresponded to the bands in the water-extractable proteins in lane 2. The 4*M* TCA extract (lane 6) was essentially the same as that in lane 2; high TCA concentrations obviously extracted the bulk of the proteins. Lane 7 is the NPN fraction that was isolated with 0.6*M* TCA and then extracted with diethyl ether to remove the TCA (rather than by dialysis), followed by freeze-drying. The pattern is the same as that in lanes 3 and 4, indicating that the fractions in lanes 3 and 4 did not incur loss of high molecular weight peptides as a result of dialysis. Analysis of the proteins solubilized from meal that has been extracted with 0.6*M* TCA (lane 8) gave a pattern identical to that for the proteins extracted from the starting meal (lane 9). This result shows that the polypeptides found in the NPN fraction (lanes 3 and 7) are a minor portion of the total soybean meal polypeptides.

Figure 3 shows results of SDS-PAGE analysis of the freeze-dried TCA extractables obtained from almond meal. Lane 2 contains the water-extractable almond meal proteins (extracted with 0*M* TCA). Bands ranging from 67 kDA to about 6 kDa were observed, but major bands occurred at 44, 42, 37, and 28 kDa. The 44, 42, and 28 kDa bands constitute the major polypeptides of amandin, the 14S globulin that makes up about 70% of the almond proteins (unpublished data). My estimates of the molecular weights of the polypeptides of amandin compare with values of 41, 39, 22, and 21 kDa reported by Sathe (1993). The

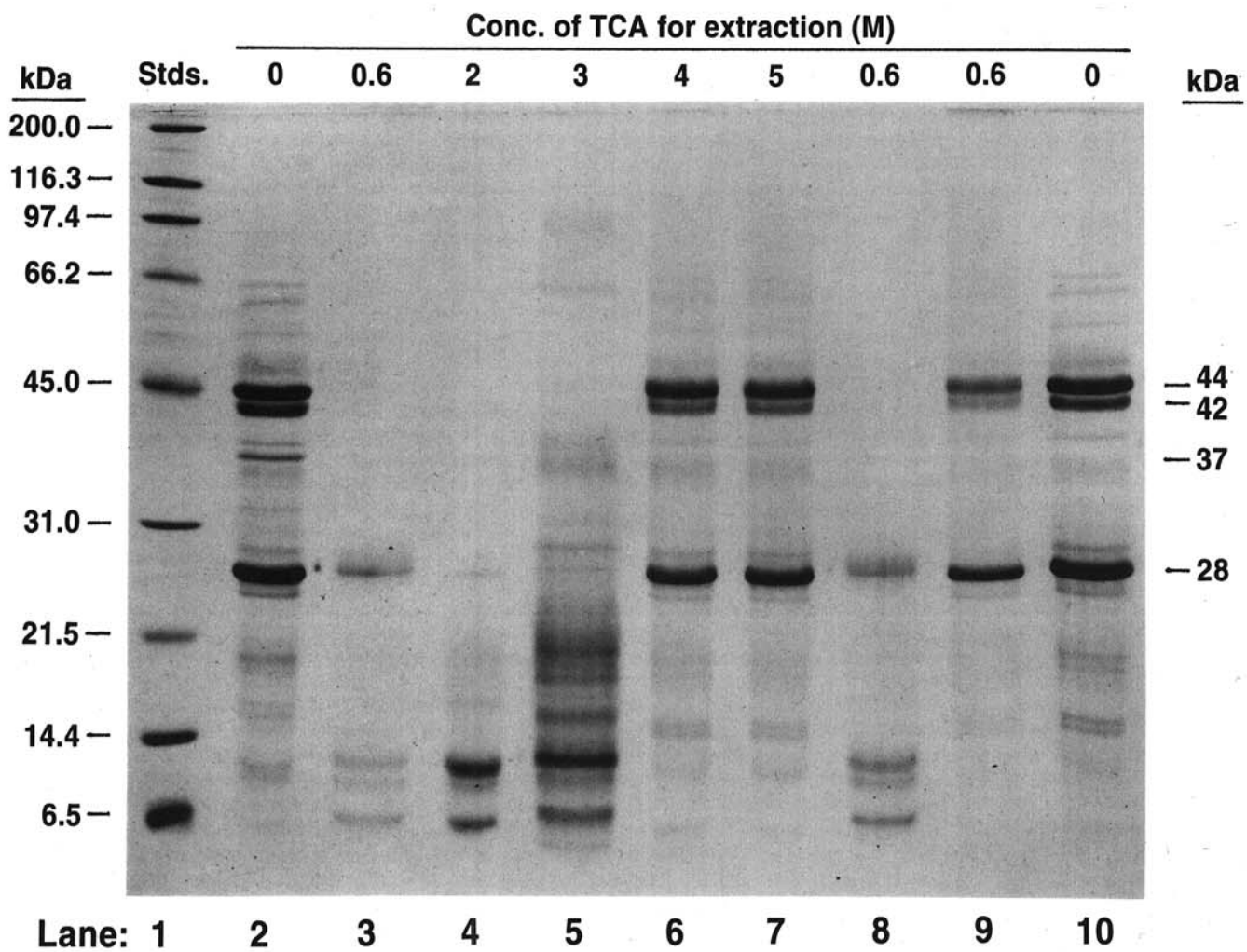

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern for almond meal fractions extracted by various concentrations of trichloroacetic acid (TCA). Lane 1 molecular weight standards (2.5 μg/band); lane 2, 0*M* TCA extract (50 μg); lane 3, 0.6*M* TCA extract (120 μg); lane 4, 2*M* TCA extract (200 μg); lane 5, 3*M* TCA extract (200 μg); lane 6, 4*M* TCA extract (60 μg); lane 7, 5*M* TCA extract (50 μg); lane 8, diethyl ether extracted 0.6*M* TCA extract (1,000 μg); lane 9, proteins from 60 μg of 0.6*M* TCA extracted meal; and lane 10, proteins from 140 μg of defatted almond meal. TCA concentrations used to make the extracts are indicated across top of gel.

discrepancy between my value of 28 kDa and Sathe's values of 21–22 is larger than expected, but may be attributed to differences in conditions used in the SDS-PAGE analyses. I used a non-gradient gel of 13.5% polyacrylamide, whereas Sathe used a linear gradient of 8–25% polyacrylamide.

The 0.6*M* TCA extract of almond meal (lane 3) contained four major bands: a diffuse band at 28 kDa and three more distinct bands of 12, 10, and 7 kDa. The 2*M* TCA extract in lane 4 was similar to lane 3, except that the 12 and 7 kDa bands were higher in concentration relative to the other bands. Extraction with 3*M* TCA yielded a more complex pattern (lane 5) with many minor bands; the 12 and 7 kDa fractions were major bands, and a 5 kDa fraction was also observed. The 4 and 5*M* TCA extracts (lanes 6 and 7, respectively) closely resembled the water-extractable proteins in lane 2 and confirm that high TCA concentrations extract the bulk of the proteins, as suggested in Figure 1B. Lane 8 represents the solids obtained when a 0.6*M* TCA meal extract was extracted with diethyl ether to remove the TCA, followed by freeze-drying. The pattern is identical to that of the 0.6*M* TCA extract that was freed of TCA, sugars, and other low molecular weight materials by dialysis, as depicted in lane 3. Lane 9 contains the proteins from meal that was extracted with 0.6*M* TCA to remove the NPN fraction, while lane 10 contains the proteins from untreated meal. Extraction with TCA apparently caused some aggregation of protein because light streaking was noted from the top of the gel to the bottom of lane 9. There are no major differences between lanes 9 and 10, thus only minor polypeptides were extracted by 0.6*M* TCA (lane 8), as noted with soybean meal (Fig. 2).

Amino acid analyses. The NPN fractions from the two meals were also characterized by amino acid analysis. Table IV shows data for the soybean NPN fraction. Arginine constituted almost 40 mol% of the free amino acids, followed by aspartic and glutamic acids. These three made up 73 mol% of the total amino acids. After acid hydrolysis, these three amino acids still constituted 65 mol% of the total, but arginine was only the third highest in concentration instead of first. The dialyzed NPN fraction approximated the composition of the undialyzed preparation except for arginine, which decreased dramatically. Free arginine was removed by dialysis, and apparently there was relatively little polypeptide-bound arginine present. Comparison of the amino acid compositions of the TCA-extracted and unextracted soybean meals revealed very minor differences. Removal of the NPN fraction thus had an inconsequential effect on the amino acid composition of the meal, as would be expected if the NPN were a small percentage of the total meal nitrogen as noted earlier.

Table V gives results for the comparable NPN fractions of almond meal. Aspartic and glutamic acids were the major free

TABLE IV
Amino Acid Composition (mol %) of Nonprotein Nitrogen Fractions of Soybean Meal Prepared by 0.6*M* Trichloroacetic Acid (TCA) Extraction Plus Extracted and Unextracted Meals

	0.6*M* TCA Extracts			Meals	
Amino Acid	Diethyl Ether-Extracted		Dialyzed	TCA-Extracted[a]	Unextracted
Asx	17.3[b]		24.1	19.8	12.7	13.3
Glx	15.3		24.2	23.7	17.3	17.6
Ser	2.4		3.5	10.0	6.8	6.2
His	1.0		1.1	1.8	2.0	1.7
Gly	1.8		3.8	6.5	8.0	7.0
Thr	5.3		2.9	3.8	4.8	4.9
Ala	5.6		4.6	4.9	6.9	6.6
Arg	39.9		17.1	2.1	5.2	5.6
Tyr	0.5		2.5	0.9	2.1	2.5
Val	1.6		1.9	2.4	5.6	5.5
Met	2.0		0.6	1.0	1.1	0.8
Phe	1.5		2.9	1.6	4.6	4.5
Ile	1.4		2.1	4.8	4.9	4.9
Leu	1.2		2.1	6.6	8.5	8.4
Lys	1.6		2.4	5.1	6.4	5.6
Pro	1.6		4.4	5.0	3.2	5.2

[a]Extracted with 0.6*M* TCA.
[b]Values in this column were obtained on unhydrolyzed sample to determine free amino acids.

TABLE V
Amino Acid Composition (mol %) of Nonprotein Nitrogen Fractions of Almond Meal Prepared by 0.6*M* Trichloroacetic Acid (TCA) Extraction Plus Extracted and Unextracted Meals

	0.6*M* TCA Extracts			Meal	
Amino Acid	Diethyl Ether-Extracted		Dialyzed	TCA-Extracted	Unextracted
Asx	20.1[a]		20.6	1.6	11.2	11.6
Glx	20.2		19.5	15.5	23.8	26.6
Ser	5.4		2.9	2.3	5.2	4.9
His	1.8		0.7	0.9	1.9	1.5
Gly	4.4		34.8	64.8	9.7	11.5
Thr	3.5		1.9	1.8	4.0	3.6
Ala	9.0		3.2	1.2	6.9	6.5
Arg	9.3		2.9	1.0	7.0	7.2
Tyr	0.7		0.9	0.8	2.1	1.7
Val	4.3		1.8	0.9	5.1	4.7
Met	0.8		0.3	0.3	0.5	0.3
Phe	2.5		1.0	0.4	4.5	4.3
Ile	2.8		1.7	0.8	4.1	3.8
Leu	1.7		1.0	0.7	7.4	6.9
Lys	2.3		3.5	6.1	2.8	2.5
Pro	11.5		3.5	0.8	3.8	2.5

[a]Values in this column were obtained on unhydrolyzed sample to determine free amino acid composition.

amino acids present, followed by proline, arginine, and alanine. These five amino acids contributed 70 mol% of the total free amino acids. Upon hydrolysis, there was a remarkable increase in glycine to 35 mol%, while aspartic and glutamic acids remained at about 20 mol%, the level found in the free amino acids. The nondialyzable polypeptides contained 65 mol% glycine plus 15 mol% glutamic acid and 6 mol% lysine; each of the other amino acids constituted only 2 mol% or less. As noted with soybean meal (Table IV), extraction of the NPN fraction with 0.6*M* TCA had only a minor effect on the amino acid composition of almond meal.

DISCUSSION

The results on extractability of the nitrogenous constituents of soybean meal as a function of TCA concentration confirm and extend those of Becker et al (1940) and Bhatty and Finlayson (1973). As reported by Becker and coworkers, a minimum in extractability occurred at 0.4–1.0*M* TCA followed by a rapid rise in extractability above 3*M* TCA. They recommended 0.8*M* TCA to extract the NPN fraction, although their data, as well as mine, indicate that lower concentrations of TCA are also suitable; 0.6M TCA was used in most of this study. The solubility curve (Fig. 1A), however, showed a slower increase in extractability above 1*M* TCA. For example, at 3*M* TCA, Becker and coworkers extracted about 28% of the nitrogen, whereas I extracted only 13% of the nitrogen. At 4*M*, Becker et al extracted 95% of the meal nitrogen in agreement with my value of 97% at 4–5 M TCA. My NPN value of 4.1% of the total nitrogen at 0.4–1.0*M* TCA is well within the range of 2.9–7.8% for 12 samples reported by Becker et al (1940). It is also in good agreement with 4–5% NPN as observed for the majority of 381 samples analyzed by Krober and Gibbons (1962).

I found no literature reports of studies on the extractablility of almond meal constituents by TCA. The extractability curve for almond meal (Fig. 1B) is similar to that for soybean meal (Fig. 1A), except at high TCA concentrations where extractability of nitrogen leveled off at about 70% as compared to 97% for soybean meal. From Figure 1B, it is apparent that TCA concentrations of 0.4–1.0*M* can be used to estimate the NPN of almond meal, and I have used 0.6*M* TCA to prepare the NPN fraction for characterization studies. My NPN value of 4.8% of the total nitrogen for almond meal compares well with values of 4.0–5.2% of total nitrogen calculated from data reported for five varieties of almonds by Saura Calixto et al (1982); these workers used 0.6*M* TCA to precipitate the proteins from 0.5*N* NaOH extracts of defatted almond meal.

Although Becker et al (1940) found that almost all of the 0.8*M* TCA extractable material from soybean meal was dialyzable through a cellophane membrane of unspecified porosity, I found that the NPN fraction contained a significant amount of polymeric material that was retained by a 3.5 kDa cutoff membrane (Table II). At 0.6*M* TCA, 4% of the meal and 0.9% of the nitrogen were recovered upon dialysis and freeze-drying. The low nitrogen content (~1%) of the dialyzed extractable fraction suggested the presence of polymers other than polypeptides. In our earlier work on jojoba meal (Wolf et al 1994), we found large amounts of carbohydrate, presumably polysaccharides, present with the NPN polypeptides. Although I found carbohydrates in the dialyzed soybean NPN fraction, the sum of protein plus carbohydrate accounted for only 42% of the sample.

Almond meal yielded a greater proportion of the meal in the nondialyzable NPN fraction (7.2%) (Table III) than did soybean meal, but the nitrogen content was also low (1.9%). As with the corresponding fraction from soybean meal, protein plus carbohydrate accounted for only a fraction (37%) of the total sample. Both samples apparently contain polymers of an unknown nature that do not contain nitrogen or carbohydrate.

The presence of nitrogen in the dialyzed NPN fractions indicated that polypeptides larger than 3.5 kDa were being extracted by 0.6*M* TCA, and this was confirmed by SDS-PAGE (Figs. 2 and 3). The polypeptide material in the soybean NPN fraction was a very minor fraction of about 12 kDa that stained poorly even when applied at 100 μg. This is in agreement with the earlier discussion of the presence of carbohydrate and other nonprotein polymer(s) in this fraction. Similar results were obtained with the NPN fraction that was not dialyzed, except that it was necessary to load 4.8 mg of sample to observe a band in the 12 kDa region. The undialyzed NPN fraction undoubtedly contained large concentrations of the soybean oligosaccharides, sucrose, raffinose, and stachyose (Kuo et al 1988).

Polypeptides were also detected in the NPN fraction of almond meal by SDS-PAGE (Fig. 3). In contrast to the soybean NPN fraction, the almond fraction was more complex with four polypeptide bands ranging from 28 to 7 kDa. The presence of the 28 kDa polypeptide was unexpected because of its high molecular weight as compared to NPN polypeptides from soybean (12 kDa) and jojoba (6–10 kDa) (Wolf et al 1994). The polypeptides obtained in the almond NPN fraction are also unusual in chemical composition as discussed below.

Although the 28 kDa polypeptide has an apparent counterpart in the basic polypeptides of amandin in the water-extractable proteins (lane 2, Fig. 3), close examination of the gel shows the band to be more diffuse, and it migrates slightly slower than the 28 kDa band in lane 2. If the 28 kDa band in the NPN fraction (lanes 3 and 8, Fig. 3) corresponds to the basic polypolypeptides of amandin, one would also expect to see the 42 and 44 kDa acidic polypeptides if they are connected by a disulfide bond, as is suggested by the model for legumin-type proteins of oilseeds and legumes (Derbyshire 1976). An alternative explanation is that the 28 kDa band in the NPN fraction is a minor polypeptide that is soluble in TCA but unrelated to amandin.

My results clearly show that the NPN fractions of soybean and almond meals, as determined by extraction or precipitation with 0.6*M* TCA, contain polypeptides. With soybeans, the polypeptide fraction is only 20% larger than 10 kDa, which was suggested by Pirie (1955) and Synge (1955) as the arbitrary dividing line between proteins and nonproteins. With almond meal, the discrepancy is considerably larger because of the 28 kDa fraction. Since the NPN values for soybean and almond meals are only 4–5% of the total nitrogen, errors in the true protein content introduced by the polypeptides soluble in 0.6*M* TCA will be relatively small. In both soybean (Table II) and almond meal (Table III), the polypeptide fractions represent only 1–2% of the meal nitrogen.

The free amino acids found in the NPN fraction of soybeans (Table IV) are in qualitative agreement with the literature; quantitative differences can be attributed to stage of maturity, environmental conditions during growth, and genetic differences (Krober and Gibbons 1962, Kapoor and Gupta 1977, Zarkadas et al 1994). In decreasing amounts, I found arginine, aspartic, and glutamic acids, whereas Bhatty et al (1973) reported glutamic acid, arginine, and aspartic acid. Bhatty and Finlayson (1973) reported the major free amino acids to be glutamic acid, alanine, and aspartic acid, followed by arginine. Zakardas et al (1994) extracted the NPN from soybeans with 70% ethanol and reported the predominant free amino acids to be glutamic acid, aspartic acid, and arginine. However, they hydrolyzed the samples before analysis. Thus, some of these amino acids likely originated from peptides. On hydrolysis of the undialyzed NPN fraction (Table IV), the order of the major amino acids changed to glutamic acid, aspartic acid, and arginine as observed by Zarkadas et al (1994). The increase in glutamic acid relative to arginine upon hydrolysis can in part be ascribed to the hydrolysis of the dipeptides, γ-glutamyl tyrosine, and γ-glutamyl phenylalanine, which occur in soybeans (Wang et al 1978). The large drop in arginine in the dialyzed NPN fraction, as compared to the undialyzed preparation, indicates that little polypeptide-bound arginine was present.

Analysis of the free amino acids in almond meal (Table V) revealed the presence of predominantly glutamic and aspartic acids followed by proline, arginine, and alanine. Soler et al (1989) reported the major free amino acids in decreasing order as glutamic acid, arginine, proline, and aspartic acid. Two notable changes occurred on hydrolysis of the NPN fractions. The first is the large decrease in aspartic acid when the hydrolysate of the

undialyzed preparation is compared with the dialyzed sample. Apparently, most of the aspartic acid is in the free form and is removed by dialysis. The second major change noted on hydrolysis was the large increase in glycine from 4 mol% in the free form to 35 mol% in the hydrolyzed but undialyzed fraction, to 65 mol% in the hydrolyzed dialyzed sample. Glycine evidently is primarily in the polypeptide form and is suggestive of glycine-rich proteins that are structural proteins of plant cell walls. These proteins have the general structure $(Gly\text{-}X)_n$ where X is frequently Gly but may also be Ala or Ser. The Gly content of the glycine-rich proteins lies between 50 and 70% (Keller 1993, Showalter 1993). The high glycine content of the dialyzed polypeptides also makes it unlikely that the 28 kDa polypeptide observed on SDS-PAGE (lane 3, Fig. 3) is related to amandin as discussed earlier. The 11–14S seed globulins typically contain only 6–9 mol% of glycine (Derbyshire et al 1976).

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