Isolation and Characterization of Chenopodin, the 11S Seed Storage Protein of Quinoa (*Chenopodium quinoa*)

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Quinoa (*Chenopodium quinoa*), a seed crop from the Andes region of South America, has been reported to have an exceptional seed protein amino acid composition. The purpose of this study was to isolate and characterize a major seed storage protein of quinoa, an 11S-type globulin called chenopodin. Extraction of quinoa seed protein was optimized with regard to extraction time, salt concentration, and buffer volumes. Extraction with 0.5 M NaCl solubilized polypeptides having molecular weights of 8000–9000, 22 000–23 000, 32 000–39 000, and 50 000. Enrichment of the chenopodin polypeptides (the A subunit group at 32 000–39 000 and the B subunit group at 22 000–23 000) was achieved by acid precipitation of the extract at pH 5.0. Gel filtration was necessary to purify the native (320 000) chenopodin. The disulfide-bonded A (acidic) and B (basic) polypeptides were separated by denaturation, reduction, and alkylation followed by ion-exchange chromatography. The amino acid compositions of the A and B polypeptides were similar to those of the acidic and basic subunits from other 11S seed globulins. The N-terminal sequence of one of the B polypeptides (GLEETICSARLSENIDDPSKA) was highly homologous to the basic subunits of several other 11S storage proteins, especially to rapeseed cruciferin.

INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd) is a dicotyledonous plant indigenous to the Andes region of South America, where it has been used as a staple food crop for hundreds of years by the native population (Risi and Galwey, 1984). Commercial quinoa production in the United States has been successful (Johnson and Croissant, 1989) because of quinoa's popularity in the health food market (Sokolov, 1992). Quinoa seeds have approximately 5.0% fat, are relatively high in protein (13.8%), and compare favorably in other nutritional aspects to the major grains and legumes (Becker and Hanners, 1990; Gonzalez et al., 1989; Gross et al., 1989; Risi and Galwey, 1984). Amino acid analysis of whole quinoa seeds has shown them to meet or exceed the FAO standards for essential amino acid content (Wood, 1985).

Quinoa seeds are considered anatomically to be "fruits" and mainly consist of a starchy perisperm with an attached oil and protein-rich embryo. Ultrastructural studies have demonstrated the presence of protein bodies in the embryo (Varriano-Marston and DeFrancisco, 1984). Electrophoretic comparisons of quinoa seed proteins from different cultivars and accessions have been used for genetic analysis (Fairbanks et al., 1990). There have been no studies, however, on the individual storage proteins of quinoa seeds. In this paper we present our work on the isolation and characterization of one of the major seed storage proteins of quinoa, an 11S-type globulin which we have named chenopodin.

MATERIALS AND METHODS

Materials. Quinoa seeds (cv. Ancient Harvest from the Quinoa Corp., Boulder, CO) were purchased from a local health food store. All materials for electrophoresis were from Bio-Rad Laboratories (Richmond, CA). High-purity urea was obtained from Pierce Chemical Co. (Rockford, IL). Chromatographic media and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970) using a 12.5% acrylamide separating gel with a 4% acrylamide stacking gel. Acrylamide monomer to bis(acrylamide) ratio was 37.5:1. Samples were prepared in buffer containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01 mg/mL bromophenol blue in 62.5 mM tris(hydroxymethyl)-aminomethane (Tris-HCl), pH 6.8, and then heated at 95 °C for 3 min prior to loading. Electrophoresis was run at 25 mA/gel (0.75-mm thickness). Gels were stained with 0.125% Coomassie Blue R-250 in 50% methanol/10% acetic acid and destained in 20% methanol/10% acetic acid.

Extraction and Acid Precipitation of Quinoa Seed Protein. Quinoa seeds were ground to a coarse flour in a coffee mill, defatted with cold acetone, vacuum filtered, and then dried at 37 °C. Protein extraction conditions were optimized by gently stirring 1-g lots of defatted flour at room temperature for various times (0.5–2.0 h), using a range of NaCl concentrations (0.0–1.0 M in 50 mM Tris-HCl, pH 8.0), at buffer to flour ratios ranging from 10 to 100 mL/g. Extracts were centrifuged at 5 °C for 10 min at 10000*g* and the supernatants assayed for protein according to the method of Bradford (1976) using bovine serum albumin as a standard.

A 10000*g* supernatant from a 0.5 M NaCl/50 mM Tris-HCl, pH 8.0, extract was acidified to various pH values (3.0–6.0) with 10% acetic acid at room temperature with gentle stirring to determine the optimal pH for the isoelectric precipitation of chenopodin. The precipitated protein was collected by centrifugation as above and then resuspended in the same buffer. Insoluble material was removed by centrifugation. The supernatants before and after acid treatment and the resolubilized protein were assayed according to the Bradford method to estimate recoveries. Resolubilized acid-precipitated protein was examined by SDS–PAGE to determine the enrichment in chenopodin polypeptides.

Purification of Chenopodin by Gel Filtration Chromatography. A 1.5 × 92.5 cm column of Sepharose CL-6B (Pharmacia, Piscataway, NJ) was prepared and equilibrated at 5 °C with extraction buffer. Crude chenopodin was prepared by acid precipitation at pH 6.0 and redissolved as described above. After centrifugation to remove insoluble material, approximately 25 mg of protein (1.5 mL) was loaded onto the column and eluted

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with 0.5 M NaCl/50 mM Tris-HCl, pH 8.0, at 25 mL/h with 280-nm monitoring. Fractions of 4 mL were collected, assayed for protein, and analyzed by SDS-PAGE. Fractions containing pure chenopodin were pooled, dialyzed against distilled water at 5 °C, and lyophilized. The column was calibrated with standard proteins (cytochrome *c*, bovine serum albumin, alcohol dehydrogenase, ferritin, and thyroglobulin) for native molecular weight estimations.

Alkylation. Lyophilized chenopodin (6 mg) was reduced and denatured by dissolving in 1 mL of 8 M urea/1 mM disodium ethylenediaminetetraacetic acid/10 mM dithiothreitol/50 mM Tris-HCl, pH 8.6. After reduction at room temperature for 2 h, 0.1 mL of 0.2 M iodoacetic acid (in the same buffer without dithiothreitol) was added and the sample allowed to incubate for 30 min in the dark at room temperature. The carboxymethylated protein was dialyzed against distilled water at 5 °C and lyophilized.

Separation of Chenopodin Subunits by Ion-Exchange Chromatography. A 0.5 × 8 cm column of DEAE-Sepharose CL-6B (Pharmacia) was prepared and equilibrated at room temperature with 6 M urea/20 mM Tris-HCl, pH 7.0. Carboxymethylated chenopodin (6 mg) was dissolved in 0.3 mL of the same buffer and applied to the column. After elution of unbound protein, the column was step eluted at 15 mL/h with approximately 5 mL each of the same buffer containing 0.05, 0.10, 0.15, and 0.20 M NaCl. The eluate was monitored at 280 nm, and fractions of 1 mL were collected. Fractions were analyzed by SDS-PAGE for the presence of chenopodin A- and B-subunit polypeptides. Selected fractions were dialyzed against distilled water and lyophilized.

Amino Analysis and Protein Sequencing. The amino acid compositions of the carboxymethylated chenopodin and its purified A- and B-subunit polypeptides were determined after acid hydrolysis for 1 h at 150 °C. N-Terminal Edman sequencing was performed on the B subunit.

RESULTS AND DISCUSSION

Extraction of Quinoa Seed Protein. Under optimal extraction conditions (1 h of stirring in 0.5 M NaCl/50 mM Tris-HCl, pH 8.0, at 10 mL/g of flour) the yield of protein averaged approximately 65 mg/g of defatted flour. Extending the extraction time beyond 1 h did not increase the protein yield further (data not shown). The amount of protein extracted was independent of buffer to flour ratios in the range tested (10–100 mL/g), so 10 mL/g was chosen to minimize the extraction volume. Even in the absence of NaCl, approximately 40 mg of protein/g of flour could be recovered. Yield increased steadily up to 0.5 M NaCl, but NaCl concentrations as high as 1.0 M did not significantly increase the yield of extractable protein.

Electrophoretic (SDS-PAGE) analysis of protein extracted at various NaCl concentrations (data not shown) demonstrated that the patterns and relative abundance of polypeptides were nearly identical, indicating that all of the major albumin- and globulin-type proteins were represented under optimal extraction conditions (i.e., 0.5 M NaCl).

The total extractable protein (Figure 1, lane P) contained polypeptides ranging in size from 8000 to 100 000. The major polypeptides were estimated to have molecular weights of 8000–9000, 22 000–23 000, 32 000–39 000, and 50 000. The 8000–9000 group of polypeptides is similar in size to the 2S-type of proteins found in many seeds. The 11S-type of seed proteins is characterized by having two heterogeneous sets of polypeptides in the size ranges 30 000–40 000 (acidic subunits) and 20 000–25 000 (basic subunits) which are joined by disulfide bonds in the native protein. The corresponding quinoa seed polypeptides are indicated in the figure by A and B, respectively. If unreduced prior to electrophoresis, a major band appears at approximately 55 000 while the A- and B-band intensities decrease significantly (data not shown). Since the

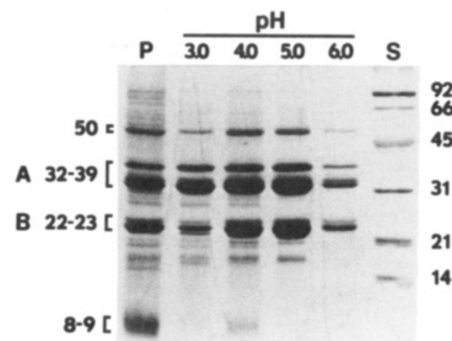

Figure 1. Acid precipitation of quinoa seed protein. Supernatants of defatted quinoa flour extracts were prepared as described under Materials and Methods. The pH was adjusted to the indicated values with 10% acetic acid. Precipitated protein was redissolved in extraction buffer (pH 8.0) and analyzed by SDS-PAGE. S, molecular weight standards. P, protein before acid precipitation. Numbers at left are estimated molecular weights ($\times 10^{-3}$) of the major extractable proteins. A and B refer to the chenopodin subunit groups. Numbers at right are molecular weights ($\times 10^{-3}$) of standard proteins.

Table I. Recovery of Acid-Precipitated Quinoa Seed Protein

pH	total protein precipd,[a] mg	amt of precipd protein resolubilized,[b] mg	% recovery from precipd protein	% recovery from original extract
3.0	99.2	4.0	4.0	2.7
4.0	111.0	24.0	21.6	16.2
5.0	100.1	60.1	60.0	40.5
6.0	53.0	39.2	74.0	26.4

[a] From an extract of 1 g of defatted flour containing 148.5 mg of protein. [b] Precipitated protein was suspended in extraction buffer (pH 8.0), and the resolubilized protein was measured after removal of insoluble material by centrifugation.

acidic and basic polypeptides of 11S-type proteins are synthesized as one long precursor, which is later separated by proteolytic cleavage (Brown et al., 1982), the 50 000 polypeptide appearing in reduced samples may be a chenopodin A–B species that escaped postsynthetic processing.

Chenopodin Enrichment by Acid Precipitation. Isoelectric precipitation of extracted proteins was performed at various pH values in an attempt to partially purify and concentrate chenopodin. SDS-PAGE analysis of redissolved proteins after precipitation at four pH values is shown in Figure 1. An obvious enrichment of chenopodin polypeptides was obtained by precipitation at pH 5.0 and pH 6.0. At lower pH values (3.0 and 4.0) many minor polypeptides over a wide size range were also precipitated. One interesting observation is that less of the B-chenopodin polypeptides were precipitated at pH 3.0 relative to the A polypeptides. Presumably, these are normally disulfide bonded and should exist in equimolar amounts. The data suggest that there may be some acid-induced disulfide disruption, resulting in a higher solubility of the B polypeptides at pH 3.0.

Quantitation of the recovery of protein by acid precipitation is summarized in Table I. Treatment at pH 4.0 gave the most protein precipitation, but the best resolubilization occurred with protein that was precipitated at pH 6.0. The best overall recovery of protein was obtained at pH 5.0. Very low recoveries of the pH 3.0 precipitated protein were probably due to irreversible acid denaturation. In consideration of these data, pH 5.0 was selected as the optimal pH for the enrichment and recovery of chenopodin.

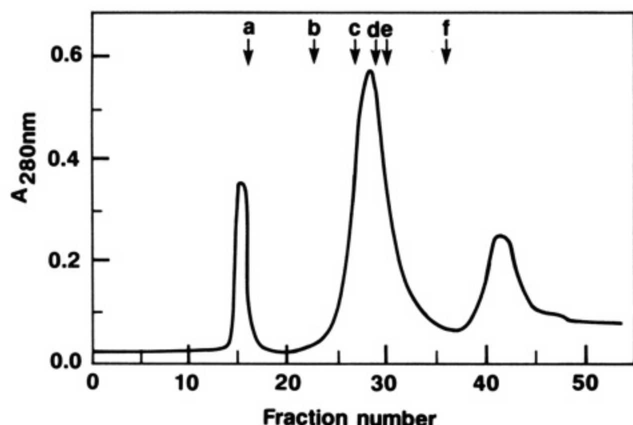

Figure 2. Gel filtration chromatography of acid-precipitated quinoa seed protein. Protein precipitated by pH 5 treatment was redissolved in extraction buffer, applied to a Sepharose CL-6B column, and eluted as described under Materials and Methods. Elution positions of Blue Dextran and standard proteins are indicated by the arrows: a, Blue Dextran (M_r 2 000 000); b, thyroglobulin (M_r 669 000); c, ferritin (M_r 440 000); d, alcohol dehydrogenase (M_r 150 000); e, bovine serum albumin (M_r 66 000); f, cytochrome *c* (M_r 12 400).

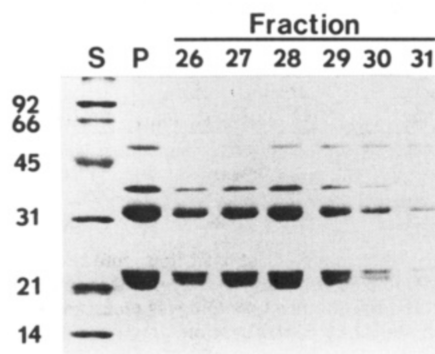

Figure 3. Electrophoretic analysis of gel filtration fractions containing chenopodin. The indicated fractions from the second peak of the Sepharose CL-6B run (Figure 2) were analyzed by SDS-PAGE as described under Materials and Methods. S, molecular weight standards. P, protein from the pH 5 precipitation loaded on the column.

Purification of Chenopodin by Gel Filtration Chromatography. An enriched chenopodin preparation (from a pH 5.0 precipitation) was fractionated further by Sepharose CL-6B chromatography (Figure 2). Three major 280-nm-absorbing peaks were resolved; however, the first and third peaks did not contain significant amounts of protein according to the Bradford assay, suggesting that they probably consisted of nucleic acids and low molecular weight components, respectively. Chenopodin was located in the second peak with an estimated native molecular weight of 320 000. This estimate is within 8% of a calculated molecular weight of 348 000, which is based on average molecular weights of the A and B polypeptides of 35 500 and 22 500, respectively, assuming a typical 11S quaternary structure of $(A-B)_6$ (Brown et al., 1982).

SDS-PAGE analysis of the proteins in the second peak (Figure 3) showed that the chenopodin A- and B-polypeptide groups were present in all fractions. Peak fractions and fractions from the descending part of the peak also contained the 50 000 polypeptide, but fractions from the ascending part of the peak did not. Therefore, only the fractions in the ascending part of the peak were pooled and used as purified chenopodin.

Separation and Analysis of Chenopodin Subunits. After reduction and alkylation of purified chenopodin, its

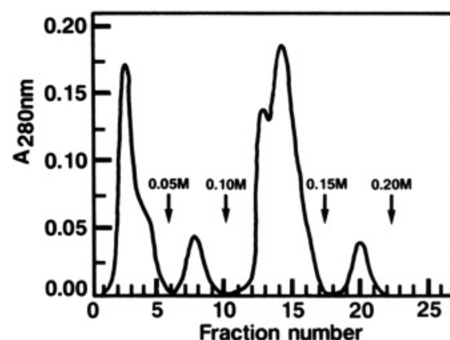

Figure 4. Separation of chenopodin subunits by ion-exchange chromatography. Purified chenopodin was denatured, reduced, and carboxymethylated as described under Materials and Methods and then dissolved in 6 M urea, 25 mM Tris-HCl (pH 7.0). After application to a DEAE-Sepharose CL-6B column, a step elution was performed with the same buffer containing increasing concentrations of NaCl (arrows).

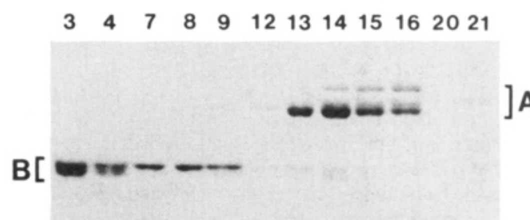

Figure 5. Electrophoretic analysis of ion-exchange fractions containing the A and B subunits of chenopodin. The indicated fractions from the DEAE-Sepharose CL-6B chromatography (Figure 4) were analyzed by SDS-PAGE as described under Materials and Methods.

A and B polypeptides were fractionated by ion-exchange chromatography under denaturing conditions (Figure 4) and analyzed by SDS-PAGE (Figure 5). Some of the B polypeptides were unbound at pH 7.0, while others required step elution with buffer containing 0.05 M NaCl. One of the B polypeptides (fraction 7) appeared to be very pure. Its 280-nm absorbance was relatively low, but its mass was consistent with the protein in the other fractions as judged by staining of the gel (Figure 5). The A polypeptides were eluted with NaCl concentrations of 0.10-0.15 M. One A polypeptide (fraction 13) was resolved from the other A polypeptides, but a trace amount of B polypeptides was present, probably due to disulfide bonding to the A polypeptide (i.e., incomplete alkylation). The relative binding affinities of the A and B polypeptides to the DEAE column are evidence that the A polypeptides are more acidic than the B polypeptides.

The amino acid compositions of the A and B polypeptides from these two fractions were compared with the composition of the native chenopodin (Table II). Typical of 11S storage globulins (Derbyshire et al., 1976), chenopodin was found to be high in glutamine/glutamic acid, asparagine/aspartic acid, arginine, serine, leucine, and glycine. If compared to the FAO reference protein (FAO, 1973), chenopodin meets or exceeds the requirements for leucine, isoleucine, and phenylalanine plus tyrosine. The A subunit was significantly higher in glycine, methionione, and histidine and lower in alanine, cysteine, leucine, and tyrosine than the B subunit.

The N-terminal sequence of the B subunit is very homologous to basic polypeptide sequences of 11S-type storage proteins from other dicotyledenous seeds (Table III). The highest degree of homology (76%) is with cruciferin, a major rapeseed (*Brassica napus*) storage protein. A high background of glycine in the first few sequencing cycles made the conclusive identification of

Table II. Amino Acid Composition[a] of Chenopodin and Its A and B Subunits

amino acid	chenopodin	A subunit	B subunit
Asx	10.5	10.1	11.0
Thr[b]	3.9	3.8	4.2
Ser[b]	8.9	9.5	8.8
Glx	14.8	16.0	13.2
Pro	4.9	5.0	4.0
Gly	8.7	10.2	6.8
Ala	5.3	5.1	8.0
Val	6.8	5.0	5.9
Cys[c]	0.8	0.4	0.7
Met	1.7	1.9	1.1
Ile	4.9	5.0	5.7
Leu	7.4	6.7	8.6
Tyr	2.9	2.5	3.6
Phe	4.0	3.6	4.3
Lys	3.2	3.2	2.9
His	3.0	3.7	1.4
Arg	9.7	8.7	9.8
Trp[d]			

[a] Reported as mol %. [b] Corrected for partial degradation during acid hydrolysis. [c] Determined as (carboxymethyl)cysteine. [d] Destroyed by acid hydrolysis.

Table III. N-Terminal Sequence of the Chenopodin Basic Subunit Compared to Homologous Sequences[a] from Other 11S Storage Proteins

sequence																					species
1				5					10					15					20		
G	L	E	E	T	I	C	S	A	R	L	S	E	N	I	D	D	P	S	K	A	quinoa
G	L	E	E	T	I	C	S	A	R	C	T	D	N	L	D	D	P	S	N	A	rapeseed[b]
G	L	E	E	T	V	C	T	A	K	L	R	L	N	I	A	P	S	S	A	P	pea[c]
G	L	E	E	T	V	C	T	V	K	L	R	L	N	I	A	Q	P	A	R	P	fava bean[c]
G	V	D	E	N	I	C	T	M	K	L	R	E	N	I	G	Q	P	S	R	P	soybean[c]

[a] Boxed residues indicate homology. Underlined residues are uncertain. In the comparison sequences, where two residues were reported in the same cycle, only the most abundant one is given. [b] From Simon et al. (1985). [c] From Casey et al. (1981).

glycine as the N-terminal amino acid difficult, but homology with the other proteins strengthens that identification. Also, less than 10% of the mass of the B polypeptide was sequencible, indicating that over 90% of this particular B polypeptide is blocked at the N terminus or that the preparation was composed primarily of another B-subunit species that is N-blocked. Two-dimensional electrophoresis will be necessary to determine such heterogeneity. Sequencing of the A-subunit group was not attempted because there is evidence of N-terminal blockage in the acidic polypeptides of some 11S-type proteins (Simon et al., 1985).

Conclusions. From the data presented, it is obvious that chenopodin is a member of the highly conserved 11S storage globulin family. Fairbanks et al. (1990) concluded that three quinoa seed globulin polypeptides (34 300, 35 600, and 36 200) were encoded by at least two separate loci and could be used as variable markers for the identification and classification of quinoa germplasm. These polypeptides are most likely the chenopodin A subunits identified in our study. The fact that only four of chenopodin's essential amino acids exceed the FAO standards suggests that other proteins must contribute to the reported high nutritional quality of quinoa seed protein. The other major protein of quinoa seeds, the 8000–9000 globulin, is currently under investigation.

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