Biochemical Characterization of Amandin, the Major Storage Protein in Almond (*Prunus dulcis* L.)

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The almond major storage protein, amandin, was prepared by column chromatography (amandin-1), cryoprecipitation (amandin-2), and isoelectric precipitation (amandin-3) methods. Amandin is a legumin type protein characterized by a sedimentation value of 14S. Amandin is composed of two major types of polypeptides with estimated molecular weights of 42–46 and 20–22 kDa linked via disulfide bonds. Several additional minor polypeptides were also present in amandin. Amandin is a storage protein with an estimated molecular weight of 427,300 ± 47,600 Da ($n = 7$) and a Stokes radius of 65.88 ± 3.21 Å ($n = 7$). Amandin is not a glycoprotein. Amandin-1, amandin-2, and amandin-3 are antigenically related and have similar biochemical properties. Amandin-3 is more negatively charged than either amandin-1 or amandin-2. Methionine is the first essential limiting amino acid in amandin followed by lysine and threonine.

KEYWORDS: Amandin; storage protein; almond; ELISA

INTRODUCTION

Tree nuts are energy-rich foods due to their high lipid (45–70% w/w) and protein (20–25% w/w) content. On a global basis, almonds rank first in tree nut production, and the United States is the largest almond producer. On average, over the past 10 years (1990–1999), U.S. almond production has accounted for ~33% of global almond production. In 1999, U.S. almond production was 360,000 metric tons, of which 210,000 metric tons ($500,000,000 value) were exported (*1*). In California, the major site of U.S. almond production, the Nonpareil variety thrives and accounts for about half of total domestic almond production. Carmel, Mission, Neplus, and Peerless are the other major commercial varieties, which together with Nonpareil account for >90% of the total almond production in the United States.

Almonds belong to the Rosaceae family, which also includes apples, pears, peaches, prunes, and raspberries (*2*). Although the exact origin of almonds has been difficult to determine, it

has been suggested that almonds are native to the temperate, desert areas of western Asia, from where they gradually spread to other regions of the world (*3*). Domesticated almonds have been documented from Bronze Age sites in Greece and Cyprus and were common in Palestine by 1700 BC (*4*). In addition to cultivated almond, *Prunus dulcis*, >30 wild or minor cultivated almond species are known to exist.

Osborne and Campbell (*5*) investigated almond meal protein solubility in water and salt solutions and isolated the major globulin (amandin), which contained 19.2–19.5% nitrogen. Subsequent studies on almond meal proteins by several investigators (*6–11*) have clearly established that almond proteins are highly soluble in aqueous media and that a single storage protein dominates almond protein composition. In the literature, this major storage protein in almonds has been described as amandin (*5*) or simply as almond major protein (AMP) (*12*). Reported sedimentation values of amandin have varied in a narrow range [11 S (*13*), 11.4 S (*10*), 13.0 S (*9*), and 14 S (*11*)]. Steenkamp and Joubert (*9*), on the basis of ultracentrifuge and N-terminal amino acid analyses, recognized that the major storage protein in almonds had an oligomeric nature (12 polypeptides per mole) and belonged to the legumin class of seed proteins (*14*). Garcia-Mas et al. (*15*) isolated and sequenced two cDNA clones from almonds and concluded that the cDNAs corresponded to two storage protein polypeptides of 61.0 and

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4334 *J. Agric. Food Chem.,* Vol. 50, No. 15, 2002

Sathe et al.

55.9 kDa, which they designated prunin-1 and prunin-2, respectively. Garcia-Mas et al. (*15*) also found that prunin-1 and prunin-2 were each composed of two polypeptides (41.8 and 38.6 kDa and 22.8 and 20.4 kDa). Our earlier studies have shown that these polypeptides correspond to amandin polypeptides (*11*).

Tree nut induced allergies are frequently permanent and are sometimes fatal (*16, 17*). Although the fatal food allergic reactions that occur in the United States are difficult to determine accurately, several estimates suggest the number to exceed 100 fatalities per year (*17, 18*). Increased almond consumption increases the potential for unintended exposure of almond-sensitive individuals to almonds and almond products. Thus, characterization of almond seed storage proteins is warranted. Sathe (*8, 24*) had earlier reported that amandin is the dominant protein in all U.S. marketing almond varieties. Subsequent studies (*11*) using ultracentrifugation and gel densitometry have shown that amandin accounted for ~70% of total soluble proteins in Nonpareil almonds. More recently, amandin has been shown to be recognized by human serum IgE from almond allergic patients (*20*). Here we describe methods to prepare amandin and some of the protein's biochemical properties.

MATERIALS AND METHODS

Materials. Whole Nonpareil almonds were a gift from Blue Diamond Growers, Sacramento, CA. Sources of electrophoresis chemicals have been reported earlier (*8*). DEAE DE-53 was from Whatman, Hillsboro, OR. Molecular weight standards, Con A Sepharose 4B, Sephacryl S 200, Sepharose 6B, Sepharose CL 6B, and Sephacryl S 300 HR were from Pharmacia Inc., Piscataway, NJ. Hydroxylapatite, Tris [tris-(hydroxymethyl)aminomethane], and DEAE-Sephadex were from Bio-Rad Laboratories, Richmond, CA. All other chemicals were from Sigma Chemical Co., St. Louis, MO, or Fisher Chemical Co., Orlando, FL, and were of reagent or better grade.

Methods. *Preparation of Flour.* Defatted Nonpareil almond flour was prepared as described earlier (*8*).

Amandin Purification. Amandin was prepared according to three methods. *(1) Column Chromatography (Amandin-1).* Defatted Nonpareil almond flour was extracted for 1 h with 0.02 M Tris-HCl buffer (pH 8.1) (flour/buffer ratio 1:10 w/v), with constant magnetic stirring provided. The slurry was filtered through glass wool and Whatman filter paper no. 4 and centrifuged (12000*g*, 20 min, 4°C), and the supernatant was loaded onto a DEAE DE-53 anion exchange column (5 × 50.6 cm) previously equilibrated with the extraction buffer. The column was flushed with the equilibrium buffer until the absorbance at 280 nm reached the baseline and then developed with 0−0.4 M NaCl linear gradient (3000 mL each) in the equilibrium buffer. Fractions (15 min per fraction, column flow rate = 79.2 mL/h) containing amandin were pooled, concentrated using an Amicon concentrator and a YM 10 membrane under nitrogen gas pressure, and loaded onto a Sephacryl S 300 HR column (5 × 52.6 cm). The gel filtration column was equilibrated with 0.02 M Tris-HCl buffer (pH 8.1) containing 0.1 M NaCl and 0.001 M NaN$_3$ prior to loading the protein and was eluted with the equilibrium buffer. Fractions (15 min per fraction, column flow rate = 58.5 mL/h) containing amandin were collected, concentrated on a YM 10 membrane, dialyzed against distilled water (48 h, 6 changes, 5 L each), lyophilized, and stored in airtight plastic bottles at −20°C until further use. All purification steps were done at 4°C.

(2) Cryoprecipitation (Amandin-2). Amandin-2 was prepared by cryoprecipitation as previously described (*11*). Briefly, defatted Non-pareil almond flour (10 g) was extracted for 0.5 h (constant magnetic stirring provided) with 50 mL of water containing 0.02% NaN$_3$ (the extractant) and centrifuged (at room temperature); the residue was similarly re-extracted with 50 mL of the extractant and centrifuged. The combined supernatant, which was turbid and yellow, was pressure filtered through 0.45 µm filters (Millex, Millipore Corp., Bedford, MA), the clear filtrate was refrigerated (4°C) overnight (12−14 h), and the milky sticky precipitate was recovered after centrifugation (12000*g*, 4

°C, 20 min). The precipitate (amandin-2) was dispersed in water, lyophilized, and stored in an airtight plastic bottle at −20°C until further use.

(3) Isoelectric Precipitation (Amandin-3). Amandin-3 was prepared using isoelectric precipitation at pH 5.0 as previously described (*11*). Lyophilized amandin was stored in an airtight plastic bottle at −20°C until further use.

Additional low-pressure column procedures used to check amandin purity included Sephacryl S 200, Sepharose 6B, and Sepharose CL 6B gel filtration, Con A Sepharose affinity, DEAE-Sephadex anion exchange, and hydroxylapatite surface adsorption chromatography.

All low-pressure column chromatographies were done at 4°C.

Separation of Amandin-1 Polypeptides (Fractions Amandin-1A and Amandin-1B). All steps were carried out at 4 °C. Crude amandin-1 was prepared by loading defatted Nonpareil whole almond flour extract onto a Sepharose CL-6B gel filtration column (5.0 × 44.5 cm) using 20 mM Tris-HCl buffer (pH 8.1) containing 0.1 M NaCl and 1 mM NaN$_3$ as equilibrium and elution buffer. Fractions containing amandin-1 were pooled, concentrated (YM 10 membrane and Amicon concentrator), dialyzed against distilled deionized water, and lyophilized. The lyophilized amandin-1 was reconstituted in 20 mM Tris-HCl (pH 8.1) buffer (equilibrium buffer), and was loaded onto an anion exchange column (DEAE DE-53, 2.6 × 36.5 cm) previously equilibrated with the same buffer. The column was eluted with a 0−0.350 M NaCl linear gradient, 500 mL each in equilibrium buffer (tubes 20−77), and flushed with 2 M NaCl in equilibrium buffer (tubes 78−120). Fractions were collected every 20 min, and absorbance was read against equilibrium buffer. Amandin-1A (tubes 30−35) and amandin-1B (tubes 36−52) were pooled, concentrated (~5-fold, each) on an Amicon concentrator using a YM 10 membrane, dialyzed against distilled deionized water (4°C, at least six to eight changes, 5 L each), lyophilized, and stored at −20 °C until further use.

Reduction/Alkylation of Amandin-1A and Amandin-1B and Separation of Polypeptides by High-Pressure Liquid Chromatography (HPLC). Thirty microliters of amandin (A or B) at 1 mg/mL in borate saline buffer (BSB; 0.1 M H$_3$BO$_3$, 0.025 M Na$_2$B$_4$O$_7$, and 0.075 M NaCl, pH 8.2) solution was reduced by the addition of 70 µL of 6 N guanidine-HCl, 0.2 M Tris-HCl, and 10 mM DTT and incubation for 90 min at 37 °C. Iodoacetic acid was added (final concentration = 20 mM) and alkylation allowed to proceed for 60 min at 37°C. One hundred microliters of solution was then injected onto reversed phase HPLC. Reversed phase HPLC was performed on a Beckman System Gold (Beckman Coulter, Fullerton, CA) equipped with a 126 programmable solvent delivery system, a 210A manual sample injection valve, and a model 168 diode array detector. Reduced/alkylated protein samples were injected onto a Phenomenex (Torrance, CA) Jupiter C4 analytical column (250 × 4.6 mm) equilibrated with 30% acetonitrile/0.1% trifluoroacetic acid (TFA), and the column was subjected to an acetonitrile gradient from 30 to 50% over 130 min. Column effluent was monitored at 214 and 280 nm, and peak fractions were collected manually.

Reduced and alkylated amandin HPLC fractions were analyzed by gel electrophoresis and visualized by Coomassie staining. SDS-PAGE (12%) was carried out in the presence and absence of a reducing agent dithiothreitol (DTT). Aliquots of previously dried HPLC fractions were dissolved in 0.1% TFA, redried, and dissolved in 15 µL of SDS-PAGE sample buffer with or without DTT. Ten microliters of each peak fraction was loaded on the gel and electrophoresed. Gels were stained with Coomassie Brilliant blue R and dried.

Analytical Methods. *Protein Determination.* Soluble protein was determined according to the method of Lowry et al. (*21*). Bovine serum albumin (BSA) in appropriate buffer was used to prepare standard protein curves simultaneously. Concentrations for measurement of extinction coefficients of amandin-2 (cryoprecipitated and subsequently eluted off Sephacryl S 200 column) were determined by evaporation of the solutions at 80°C followed by drying at 100°C in a vacuum oven.

Electrophoresis, Ultraviolet Spectra, Ultracentrifugal Analysis, Glycoprotein Staining, and Isoelectric Focusing. These were done as described earlier (*11, 19*). Isoelectric focusing was done in the absence of 6 M urea. In addition, the Gelcode Glycoprotein Staining (Pierce

Almond Major Storage Protein

J. Agric. Food Chem., Vol. 50, No. 15, 2002   **4335**

Chemical Co., Rockford, IL) procedure was used according to the manufacturer's instructions.

Amino Acid Composition. Amino acid composition was determined using the Waters Pico-Tag column amino acid analyzer (Waters Chromatography Division, Milford, MA). Typically 0.5 mg of protein was hydrolyzed in 600 μL of 6 N HCl in the presence of nitrogen (18 h, 110°C), and 20 μL of the hydrolysate was injected for analysis. γ-Aminobutyric acid (250 pmol) was used as an internal standard. Tryptophan content was determined according to colorimetric method B of Spies and Chambers (*22*). Prior to tryptophan determination, it was necessary to adjust the pH of distilled deionized water to 9.5 with dilute alkali (0.1 N NaOH) to enable protein solubilization. All amino acid data were corrected for 100% recovery.

N-Terminal Amino Acid Sequencing. SDS-PAGE was carried out as described earlier, on reduced and carboxymethylated amandin-1 polypeptides, and the proteins were transferred to a Trans-Blot PVDF membrane (0.2 μm). The N-terminal amino acid sequences were determined using blotted protein on an ABI 477A sequencer with an on-line 120A HPLC system (Applied Biosystems, Inc., Foster City, CA). Sequence data were collected utilizing the ABI 610 software (Applied Biosystems, Inc.) and analyzed with FASTA programming (European Bioinformatics, http://www2.ebi.ac.uk/fasta3/).

Stokes Radius. A Sephacryl S 300 HR column (1.6 × 93.5 cm) was used to estimate the Stokes radius of amandin. Equilibrium and elution buffers were 0.02 M Tris-HCl (pH 8.1) containing 0.1 M NaCl and 0.001 M NaN$_3$. Fractions were collected every 15 min, and protein elution was monitored by UV absorbance at 280 nm. The column was calibrated using Pharmacia standard proteins (both high and low molecular weight kits). Each standard protein was eluted at least twice to calibrate the column. Column flow rate was set at 20 mL/h.

Enzyme-Linked Immunosorbent Assay (ELISA). Inhibition ELISAs were done as described earlier (*20*). Rabbit polyclonal antibodies raised against amandin-1 were used in these assays. Amandin-1 was used to coat the ELISA plates.

Statistics. Where appropriate, data were analyzed for significance using Fisher's protected LSD test at $p = 0.05$ (*23*) using SPSS software (SPSS, Chicago, IL, 2000). Linear regressions and correlation coefficients were determined when appropriate.

RESULTS AND DISCUSSION

Amandin Purification. Amandin was prepared according to three different methods. Typical column chromatography profiles for amandin-1 preparation are shown in **Figure 1**. Defatted Nonpareil almond flour protein extract was typically resolved into five to seven fractions by the DEAE DE-53 anion exchange column. Amandin typically eluted off the DEAE DE-53 anion exchange column at an NaCl concentration range of 170−210 mM (**Figure 1A**) (range for multiple preparations was 140−225 mM), depending on the type of gradient used. High-resolution gel filtration column chromatography of amandin-1 off the anion exchange column (**Figure 1B**) typically gave two peaks. The second peak off the gel filtration column contained amandin-1. Using other gel filtration media such as Sephacryl S 200, Sepharose 6B, and Sepharose CL 6B in an effort to improve peak resolution for amandin-1 (post DEAE DE-3) proved to be unsuccessful (data not shown). Attempts to clean up minor impurities in amandin-1 using additional chromatographies (post anion exchange and gel filtration columns) such as Con A Sepharose and hydroxylapatite were also unsuccessful (data not shown). Non-denaturing non-dissociating polyacrylamide gel electrophoresis (NDND-PAGE) was used to monitor amandin-1 elution off the columns (see the gel pictures in **Figure 1**). Typical yield data from a representative preparation are summarized in **Table 1**. Amandin accounts for ~70% of total soluble proteins in almond meal (*11*). Amandin is a multimeric complex protein with a sedimentation velocity of 14 S. From ultracentrifugal analysis of amandin-1 (**Figure 2**) it can be seen
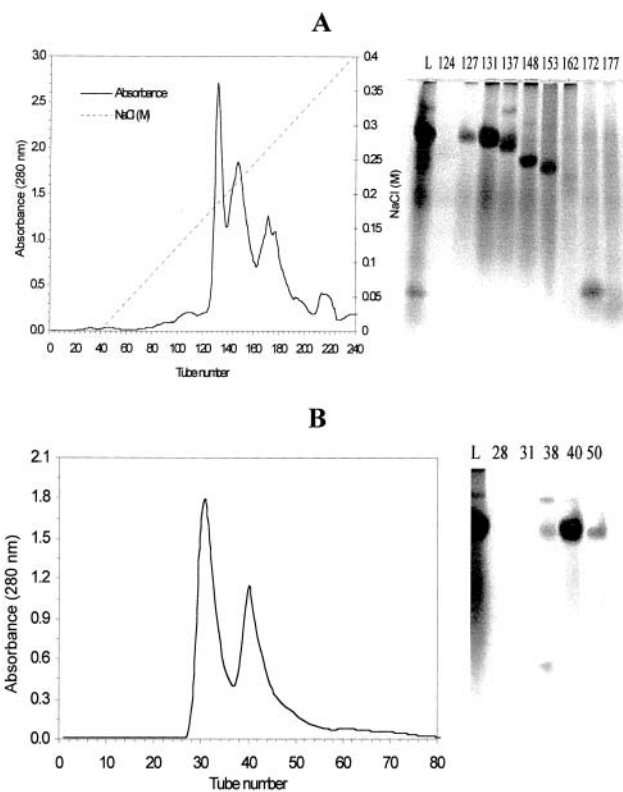

Figure 1. (A) Elution profile for 0.02 M Tris-HCl buffer (pH 8.1) extract of defatted Nonpareil almond flour off DEAE DE-53 anion exchange column (5.0 × 50.6 cm). Fractions containing amandin (tubes 128–137, 198 mL) were pooled. (B) Elution profile for amandin fractions pooled in (A) off Sephacryl S 300 HR (5 × 52.6 cm) column. Tubes 38–50 were pooled to yield amandin-1. Insets: (A) and (B) are NDND-PAGE analysis of fractions eluting off the corresponding column indicated by number on top of the gel lane. L = protein loaded on to the column.

Table 1. Summary of Amandin-1 Purification by Column Chromatography[a]

purification step	total vol (mL)	protein (mg/mL)	total protein (mg)
20 mM Tris-HCl (pH 8.1) extract	164	13.49	2212
DEAE DE-53, pooled fractions	17[b]	64.55	1097
Sephacryl S300 HR, pooled fractions	11[b]	82.68	909

[a] Data are for a typical preparation starting with 20 g of defatted almond (Nonpareil) flour extracted with 200 mL of 20 mM Tris-HCl (pH 8.1) buffer. [b] Final volume after concentrating the pooled fractions, under nitrogen pressure, using an Amicon concentrator fitted with a YM 10 membrane.

that the protein is essentially pure. Earlier, we (*11*) have shown that cryoprecipitated amandin (amandin-2) is 90% pure by ultracentrifugation. In that same study, we reported that the amandin prepared using isoelectric precipitation (amandin-3) was less pure than amandin-2 (*11*). Typically, the main impurities in amandin-3 are 9S and 19S (respectively, 6.7 and 6.0%) components. The 19S component is an amandin polymer (*11*). These data illustrate that amandin can be easily prepared in several ways and that such preparations have comparable biochemical purity.

Electrophoresis. NDND-PAGE analysis of the three amandins (**Figure 3A**) indicated that the dominant polypeptides in all three preparations had similar electrical charges (indicated by arrow in the right-hand margin). In **Figure 3A** the indicated
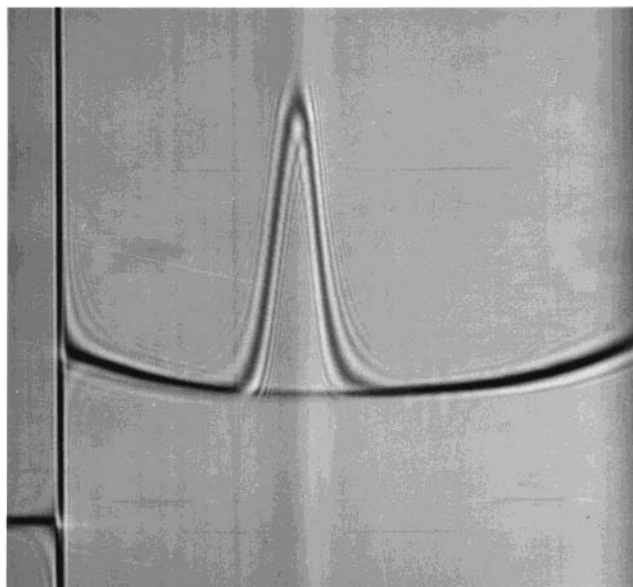
4336 *J. Agric. Food Chem.,* Vol. 50, No. 15, 2002

Sathe et al.

Figure 2. Ultracentrifuge pattern for amandin-1 in the standard phosphate buffer [potassium phosphate–sodium chloride buffer (0.033 M K$_2$HPO$_4$, 0.0026 M KH$_2$PO$_4$, and 0.4 M NaCl), pH 7.6, $\mu = 0.5$]. Sedimentation is from left to right.

positions of marker proteins [identified by their molecular weights (MWs)] are to be used for that purpose only, and therefore the reader is cautioned not to interpret them to indicate MWs. When compared with amandin-1 and amandin-2, amandin-3 (lane 3) contained several additional prominent bands (shown by −) with more negative charge indicated by their higher mobility. The major band in amandin-3 had a slightly lower mobility than the ones in amandin-1 and amandin-2 (*R$_f$* values for amandin-1, amandin-2, and amandin-3 were 0.141, 0.141, and 0.133, respectively), suggesting a slightly different electrical charge. Ultracentrifugal analysis of both amandin-1 and amandin-2 (data not shown) also showed that amandin-1 and amandin-2 were indistinguishable. SDS-PAGE analyses of the three protein preparations in the absence and presence of a reducing agent are shown in parts B and C of **Figure 3**, respectively. In the absence of a reducing agent, all three amandin preparations were characterized by two major polypeptides with estimated MWs of 61 and 63 kDa (indicated by arrows in the extreme right margin, **Figure 3B**). Several (16–25) minor polypeptides of varying MWs (range = 12–166 kDa) were noted in all amandin preparations. Polypeptides that distinguish amandin-1 from amandin-2 and amandin-3 are marked by ← and ∋ on the right-hand side of the amandin-1 gel track (**Figure 3B**, lane 1). SDS-PAGE analysis of the three amandins in the presence of reducing agent (**Figure 3C**) revealed that the amandins were mainly composed of two major classes of polypeptides with estimated MWs in the range 42–46 kDa (acidic polypeptides) and 20–22 kDa (basic polypeptides) linked by disulfide bonds. Several minor polypeptides were also noted in all three amandins (MWs indicated in the extreme right margin). The number (range of 14–20 polypeptides) and relative proportion (subjectively judged on the basis of staining intensity and bandwidth) of the minor polypeptides varied considerably. SDS-PAGE analysis in absence/presence of a reducing agent clearly showed amandin-3 to be distinct from amandin-1 and amandin-2. As stated earlier, the main difference between amandin-3 and amandin-1 and amandin-2, as revealed by ultracentrifugal analyses, is that the amandin-3 preparations typically contain 9S and 19S compo-

nents (*11*). The charge heterogeneity (as revealed by the NDND-PAGE), the polypeptide composition, and the estimated MWs of polypeptides of amandins are consistent with the earlier findings on almond proteins (*8, 20, 24*).

On the basis of the polypeptide composition and electrophoretic mobility of amandin polypeptides (**Figure 3B,C**) all amandins were similar with respect to the major polypeptides but distinctly different with respect to several minor polypeptides. Earlier it was shown that amandin is not sensitive to ionic strength but is sensitive to pH (*11*). The differences in polypeptide composition of the three amandins are therefore unlikely to result from a change in the ionic strength of different buffers used in the protocols followed and are more likely due to the differences in pH encountered during extraction and subsequent preparation steps. Another potential source of amandin polypeptide microheterogeneity is proteolysis. Isoelectric focusing of amandins (**Figure 4**) confirmed the charge heterogeneity of the protein as illustrated by the presence of several bands (p*I* range = 4.55–6.3). Isoelectric focusing data also indicated the presence of at least two additional bands in the acidic p*I* range (indicated by arrows in the right margin). Exposing amandin to acid pH during its preparation may have caused deamidation of some of the amide groups, leading to generation of the acidic polypeptides observed in the isoelectric focusing and to charge heterogeneity. Indeed, one can see more negatively charged species in amandin-3 compared to amandin-1 and amandin-2 in NDND-PAGE gels (**Figure 3A**). However, exposing amandin-1 to acid pH (up to 0.1 M HCl) for extended periods (up to 24 h) failed to generate the additional acidic polypeptides observed in amandin-3, indicating the origin of these additional polypeptides to be other than amandin-1 polypeptides (data not shown). Although one cannot completely rule out proteolysis, proteolysis was unlikely to occur because very long incubations (>10 days) at high temperature (37°C) are required to produce noticeable proteolysis in almond protein extracts (*11*) and because low temperature (4°C) and short durations (<10 days) were used in all amandin preparations. To prevent microbial growth, 1 mM NaN$_3$ was added to all buffers used, and therefore microbial proteolysis is unlikely to produce the observed polypeptide heterogeneity. These data, taken together with those from NDND-PAGE and SDS-PAGE, suggest that amandin is composed of two major types of polypeptides, termed acidic and basic, linked by disulfide bonds.

Gel Filtration. Gel filtration (Sephacryl S 300 HR, Sephacryl S 200, Sepharose 6B, and Sepharose CL-6B) of amandins, regardless of preparation method used, typically yielded one major peak (i.e., minor impurities could not be removed). A typical elution pattern for amandin-2 (after it was eluted from the Con A Sepharose column as shown in **Figure 5A**) off Sephacryl S 300 is shown in **Figure 5B**. Ultracentrifugal analyses (**Table 2**) as well as SDS-PAGE electrophoresis of the peak clearly illustrate the purity and the polypeptide microheterogeneity of amandin. Rechromatography of the peak containing the amandin on the same column did not change the elution profile, indicating the stability of protein preparations (data not shown). Subjecting amandin-1 preparations to hydroxylapatite column chromatography essentially yielded one peak and failed to remove any of the minor polypeptides in the amandin-1 preparations (data not shown). Thus, subjecting amandin-1, amandin-2, and amandin-3 to additional chromatography using a variety of packing media such as Sephacryl S 200, Sepharose 6B, Sepharose CL-6B, hydroxylapatite, Con A Sepharose, or Sephadex anion exchange did not remove minor impurities present in amndin preparations (data not shown).
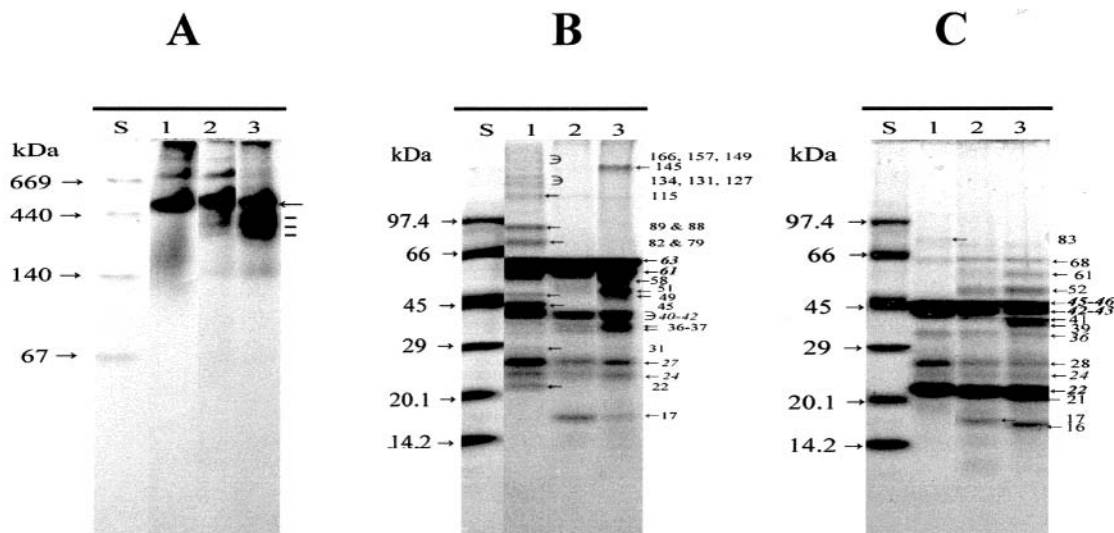
Figure 3. Electrophoretic analyses of amandins: (A) NDND-PAGE; (B) SDS-PAGE in the absence of 2% (v/v) β-ME; (C) SDS-PAGE in the presence of (2% v/v) β-ME. Protein load in lanes 1–3 (amandin-1, amandin-2, and amandin-3, respectively), 50 μg each. All gels were 1.5 mm, 3–30% (A) and 8–25% (B,C) linear acrylamide gradient gels. Major bands are italicized in bold. Other common bands are italicized. Normal font indicates some difference between amandins (such as difference in band intensity or absence of band). Note that the MWs in part A are shown only for the purposes of locating the standard proteins and *do not* indicate MWs.
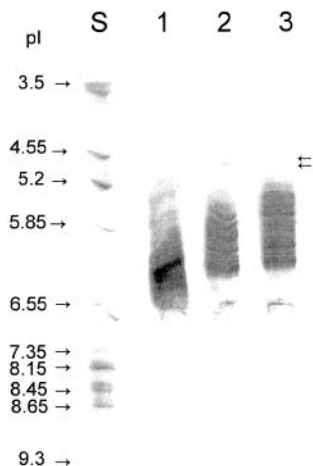

Figure 4. Isoelectric focusing of amandins, in the absence of urea, in 5% monomer acrylamide gels. S = standards. Lanes 1–3 are amandin-1, amandin-2, and amndin-3, respectively.

Stokes radius for amandin-1 using a Sephacryl S 300 HR column [1.6 × 93.5 cm; $y = 0.29311 + 0.00924x$, where $x =$ Stokes radius, $y = (-\log K_{av})^{-1/2}$, $K_{av} = (V_e - V_0)/(V_t - V_0)$, $V_e =$ elution volume in mL, $V_t =$ total gel bed volume in mL, $V_0 =$ void volume in mL], and $r = 0.993$] and 20 mM Tris-HCl (pH 8.1) buffer containing 0.1 M NaCl and 1mM NaN$_3$ was determined to be 65.88 ± 3.21 Å ($n = 7$). The elution volumes (V_e) for amandin-2 and amandin-3 were identical to that of the amandin-1 ($n = 2$ each for amandin-2 and amandin-3), indicating no major differences in the hydrodynamic radii of the three protein preparations. On the basis of the gel filtration data (Sephacryl S 300 HR), we *estimate* the MW of amandin-1 to be 427,300 ± 47,600 Da ($n = 7$, regression equation for standard proteins was $y = -0.352589x + 2.138683$ and $r = 0.987$). It should be mentioned here that MW determination by gel filtration must be viewed with caution because the relationship between MW and hydrodynamic radius is typically valid only for globulins of spherical shape (*25*). Indeed, a wide range of MW for amandin-2 preparations (range 310,000−480,000 Da, $n = 9$, with an overall mean ± standard deviation of
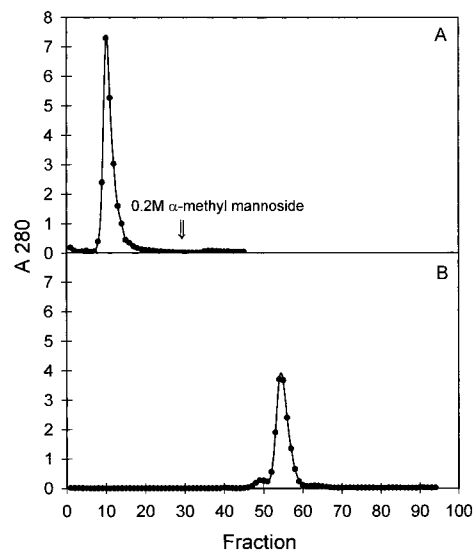

Figure 5. Elution profiles for amandin-2 off (A) Con A Sepharose 4 B (2.6 × 12.5 cm) column and (B) two Sephacryl S 300 columns in tandem (2.6 × 93 cm each).

Table 2. Ultracentrifugal Analyses of Cryoprecipitated Amandin (Amandin-2) Subjected to Con A Sepharose Chromatography Followed by Sephacryl S 300 Gel Filtration[a]

pooled fraction	composition (%)			
	9S	14S	19S	>19S
cryoprecipitated amandin	3.0	88.4	8.6	
Con A fractions 8–23	2.7	94.5	2.8	
Sephacryl S 300 fractions				
46–51	7.7	22.7	61.7	7.9
52–54		97.6	2.4	
55–60		99.8	0.2	
61–68	64.1	35.9		

[a] Analyses in Tris-NaCl (pH 8.1), $\mu = 0.1$

368,000 ± 100,000 Da) using a separate Sephacryl S 300 column was observed. Compared to the major storage protein in cashew nut that has a Stokes radius of 57 Å and MW =
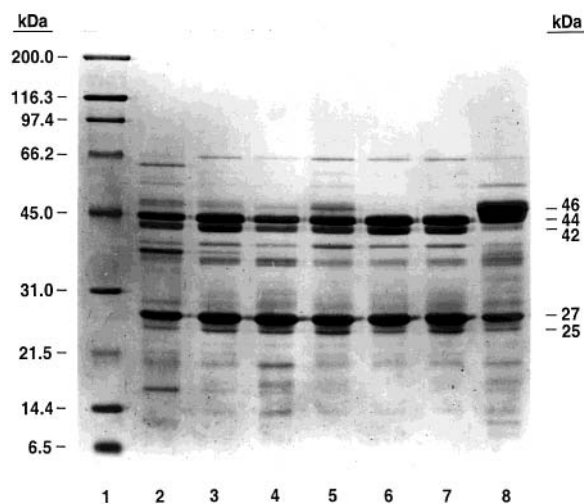
4338 *J. Agric. Food Chem.,* Vol. 50, No. 15, 2002

Sathe et al.

Figure 6. SDS-PAGE (13.5% monomer acrylamide gels) analysis of amandin-2 in the presence of 2% (v/v) β-ME: (lane 1) MW marker standards (MWs indicated in the left margin); (lane 2) defatted Nonpareil flour protein extract using water as the solvent; (lane 3) cryoprecipitated, concentrated amandin; (lane 4) amandin-2 off Con A Sepharose 4B; (lane 5) 19S rich fraction (tubes 46–51) off Sephacryl S 300; (lane 6) amandin-2 off Sephacryl S 300 (tubes 52–54); (lane 7) amandin-2 off Sephacryl S 300 (tubes 55–60); (lane 8) 7S-rich fraction off Sephacryl S 300 (tubes 61–68). Protein load in each lane was 50 μg.

275,590 Da (*19*), amandin appears to have a larger Stokes radius, indicating a less compact structure for amandin (compared to cashew globulin) although both are legumin type proteins.

Ultracentrifugation. Amandin purified by gel filtration (Sephacryl S 300) of an aqueous almond meal extract was adjusted to concentrations of 0.2−0.9% (based on dry weights) in Tris-NaCl (pH 8.1), $\mu = 0.1$, or potassium phosphate−NaCl (pH 7.6), $\mu = 0.5$, and ultracentrifuged to measure $s_{20,w}$ values. Regression analysis of the data as a function of protein concentration yielded $s_{20,w} = 14.10 - 0.16c$, where c = protein concentration in g/100 mL. The extrapolated value of 14.10 S compares with values of 11.4 S reported by Svedberg and Sjögren (*10*) using an oil turbine ultracentrifuge and 13.0 S published by Steenkamp and Joubert (*9*). Although the reasons for discrepancies are not obvious, the S value reported by Svedberg and Sjögren is the mean of six protein concentrations that were run over a pH range of 4.3−9.4 and varying ionic strengths.

Molecular Composition and Properties. Because many seed storage proteins are glycosylated and because glycosylation often leads to polypeptide heterogeneity, learning whether amandin is glycosylated or not was of interest. The ultracentrifugal purity of the cryoprecipitated amandin used in **Figure 5** was 88% (**Table 2**), which compares favorably with a previous value of 91% content of 14S (*11*). On passage through the Con A Sepharose column (**Figure 5A**) there was only a small increase in purity, apparently because of dissociation of the 19S polymer (*11*). The failure of amandin to bind to the Con A Sepharose indicates that amandin is not a typical glycoprotein containing α-D-glucopyranosyl, α-mannopyranosyl, or sterically related sugar residues (*26*). Moreover, elution of the column with α-D-methylmannoside after emergence of the amandin failed to elute any other additional proteins, indicating the absence of glycoprotein contaminants in the cryoprecipitated amandin. On Sephacryl S 300 gel filtration (**Figure 5B**) of pooled fractions (8−23) from the Con A Sepharose column the small leading peak (fractions 46−51, lane 5) consisted primarily of the
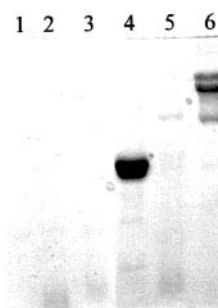

Figure 7. Glycoprotein staining (Gelcode system) of amandins. Lanes 1–6, respectively, are amandin-1, amandin-2, amandin-3, Inca peanut albumin (positive control), soybean 11S (negative control), and soybean 7S (positive control). Protein load in each lane was 40 μg.
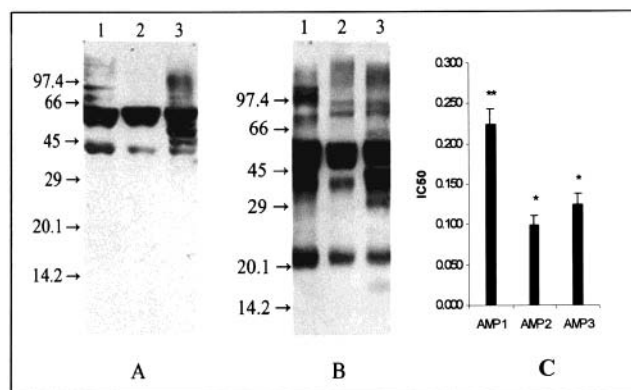

Figure 8. Comparison of antigenicity of amandins (AMP1 = amandin-1, AMP2 = amandin-2, and AMP3 = amandin-3): (A) Western blotting, SDS-PAGE [without 2% (v/v) β-ME, protein load = 15 μg]; (B) Western blotting, SDS-PAGE [with 2% (v/v) β-ME, protein load = 20 μg]; (C) inhibition ELISA using rabbit pAbs raised against amandin-1. (**, significantly different at $p = 0.05$.) Primary Ab dilution used = 10000 (v/v) and secondary Ab dilution used = 1:5000 (v/v).

amandin polymer (**Table 2**). The leading and trailing halves of the main peak (fractions 52−54 and 55−60) were essentially amandin (97.6−99.8%) with traces of the 19S. The small trailing peak (fractions 61−68) was mainly the 9S fraction with some overlapping with amandin from the main peak. SDS-PAGE analyses (**Figure 6**) indicated that the cryoprecipitate represented the majority of the protein in the original water extract used to cryoprecipitate amandin and that there was little change in composition on passage through the Con A Sepharose column. From this figure it is also apparent that the 19S-rich material (fractions 46−51, lane 5) is a polymer of amandin, previously reported to be a dimer (*11*). The 9S-rich protein fraction contained 44 and 27 kDa polypeptides of amandin but lacked the 42 kDa band and exhibited a 46 kDa band instead. These experiments do not rule out the possibility that sugar residues other than glucose or mannose may be involved in the glycosylation of amandin. Therefore, SDS-PAGE in the presence of a reducing agent followed by a chemical staining procedure capable of detecting all sugar residues was used (**Figure 7**). As can be seen from **Figure 7**, amandin is not a glycoprotein.

Ultraviolet spectra of amandin-1, amandin-2, and amandin-3 in several aqueous buffers indicated that all amandins had absorption maxima at 280 nm. Absorbance values for 1% amandin solutions in several aqueous buffers were typically in the range of 5−7 (data not shown).

Recently it has been shown (*20*, *27*) that amandin is an excellent marker for the purpose of detection of trace amounts

Almond Major Storage Protein

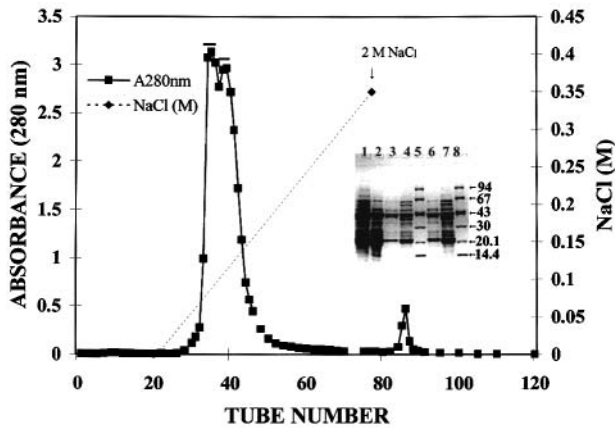*J. Agric. Food Chem.,* Vol. 50, No. 15, 2002 **4339**

Figure 9. DEAE DE-53 anion exchange column (2.6 × 36.5 cm) elution profile for amandin-1 off Sepharose CL 6B. (Inset) SDS-PAGE (8–25% linear gradient, 1.5 mm gels) analysis in the presence of 2% (v/v) β-ME: (lanes 5 and 8) Pharmacia low MW marker (MW indicated in the right margin); (lane 1) protein extract from Nonpareil defatted almond flour (50 μg); (lane 2) amandin-1 (50 μg); (lane 3) amandin-1 A (50 μg); (lane 4) amandin-1 B (50 μg); (lane 6) amandin-1 A (100 μg); (lane 7) amandin-1 B (100 μg).

of almonds in food and that, because human IgE also recognize amandin polypeptides, amandin detection is relevant to human allergies. It is therefore important to know whether amandin-1, amandin-2, and amandin-3 are antigenically similar or different. Amandin-1, amandin-2, and amandin-3 were therefore evaluated by using rabbit polyconal antibodies and Western blotting

Table 3. Amino Acid Analysis of Amandin Fractions off DEAE DE-53[a]

amino acid	amandin-A	amandin-B	amino acid	amandin-A	amandin-B
Asx	9.97	10.42	Tyr	2.24	2.60
Glx	24.06	19.08	Val	4.80	5.17
Ser	4.94	4.80	Met	1.09	1.34
Gly	9.19	8.63	Cys	0.27	0.27
His	1.42	1.51	Ile	3.14	3.06
Arg	7.83	7.65	Leu	7.11	8.13
Thr	2.21	3.24	Phe	4.39	4.60
Ala	7.60	7.39	Lys	5.11	6.76
Pro	4.67	5.39			

[a] Data are expressed as g of amino/100 g of protein and are corrected for 100% recovery. Each value represents an average of two determinations.

(**Figure 8**). As can be seen from this figure, all three amandin preparations are antigenically similar. These data suggest that for the purpose of detecting trace amounts of almonds in food, one may use amandin prepared according to any one of three methods outlined in this paper. Corylyn (a major storage protein in hazelnuts) has been similarly reported to be a useful marker protein for hazelnut detection in foods using ELISA (*28*).

Un-denatured amandin-1 was subjected to anion exchange (DEAE DE-53) column chromatography to separate individual polypeptides for the purpose of amino acid sequencing. These experiments resolved amandin-1 into two populations, termed amandin-1A and amandin-1B (**Figure 9**), that were different with respect to polypeptide composition (see the inset) but similar with respect to amino acid composition (**Table 3**) and ultracentrifugal analysis (data not shown). Similar results were
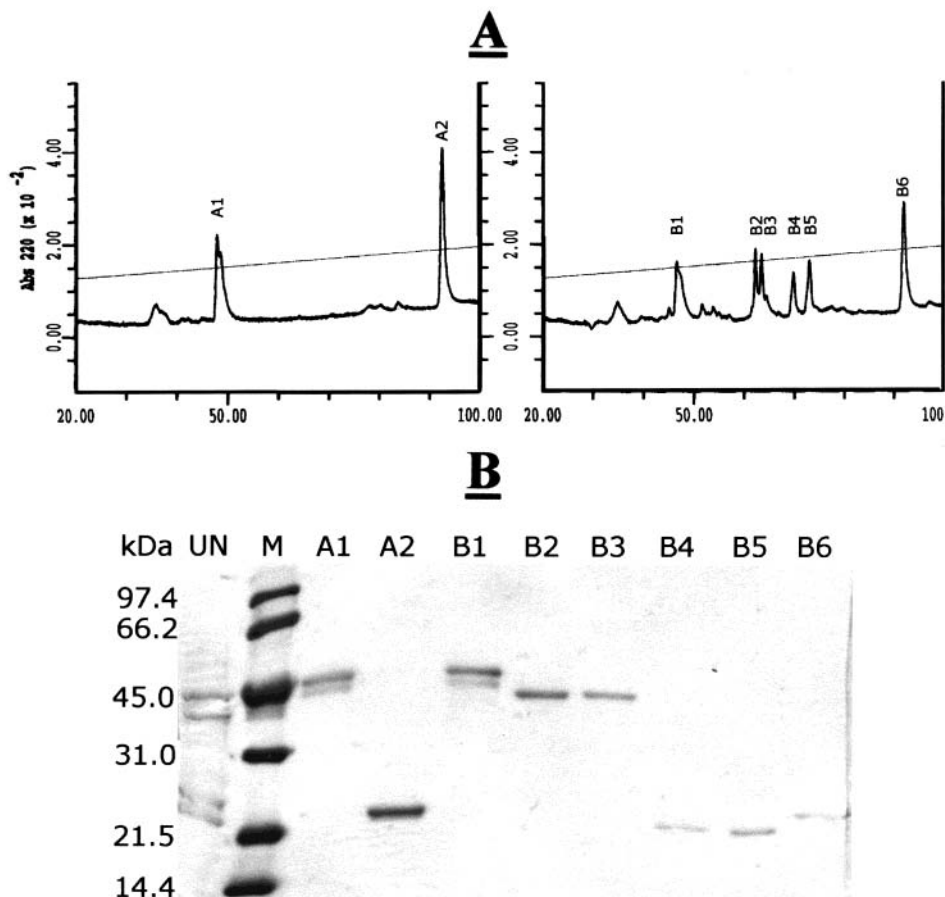

Figure 10. Reversed phase HPLC (C₄) column separation profiles for reduced and carboxymethylated amandin-1 A and amandin-1 B (A) and SDS-PAGE (12% monomer acrylamide, 1.5 mm gels) analysis of separated polypeptides in the presence of 2% (v/v) β-ME (B). UN = unalkylated amandin-1 (control); M = MW markers (MW indicated in the left margin).

Table 4. Amino Acid Composition of Amandins[a]

amino acid	g/100 g of protein					
	amandin-1	amandin-2	amandin-3	CMP[c]	11S[d]	FAO/WHO[b]
Asx	8.82	8.89	9.45	7.95	13.9	
Glx	26.96	25.11	21.15	17.35	25.1	
Ser	3.58	4.09	4.39	6.19	6.5	
Gly	5.91	7.50	5.89	7.84	5.0	
His	2.59 (1.3626)	2.88 (1.5158)	2.87 (1.5102)	1.48	2.6	1.9
Arg	14.93	10.67	13.03	9.90	8.9	
Thr	1.72 (0.5056)	3.04 (0.8937)	2.76(0.8130)	3.01	4.1	3.4
Ala	4.65	4.95	5.17	5.00	4.0	
Pro	4.46	4.37	4.78	5.29	6.9	
Tyr	2.83	2.61	3.35	2.48	4.5	
Val	4.00(1.1444)	4.17 (1.1903)	4.54 (1.2945)	6.52	4.9	3.5
Met	0.05 (0.0665)	0.83 (0.3871)	0.60 (0.2917)	0.80	1.3	
Cys/2	0.23	0.30	0.28	0.36	1.7	2.5[e]
Ile	3.31 (1.1820)	3.07 (1.0936)	3.69 (1.3188)	4.34	4.9	2.8
Leu	7.00 (1.0598)	6.85 (1.0370)	7.63 (1.1554)	8.02	8.1	6.6
Phe	5.59 (1.3351)	4.81 (1.1773)	5.78 (1.4492)	4.13	5.5	6.3[f]
Lys	1.23 (0.2113)	3.03 (0.5216)	2.27 (0.3903)	7.02	5.7	5.8
Trp	2.17 (1.9699)	2.89 (2.6245)	2.42 (2.1993)	2.30	1.5	1.1
E/T[g] (%)	27.65	31.54	32.54	40.50	44.80	

[a] Data are corrected for 100% recovery. Figures in parentheses are the amino acid scores for the corresponding amino acid. [b] Recommended for children (2–5 years), although recommended for dietary protein quality evaluation for all age groups (except infants) by the joint FAO/WHO Expert Consultation (1990). [c] Cashew major protein (CMP) data are from Sathe et al. (*19*). [d] Soybean 11S globulin data are from Fukushima (*31*). [e] Met + Cys/2. [f] Phe + Tyr. [g] Essential/total amino acid ratio.

obtained when amandin-2 was subjected to a different anion exchange (DEAE Sephadex) column chromatography followed by ultracentrifugal and SDS-PAGE analyses of the separated fractions (data not shown). When amandin-1A and amandin-1B were reduced, carboxymethylated, and subjected to reversed phase HPLC (C₄ column) separation, amandin-1A was resolved into two polypeptide populations (indicated by A1 and A2 in **Figure 10A**) and amandin-1B was separated into six polypeptide fractions (B1−B6, **Figure 10A**). SDS-PAGE analysis of separated polypeptides (**Figure 10B**) suggested certain fractions to be single polypeptides. N-Terminal sequencing of the separated polypeptides by HPLC afforded unambiguous assignments for only A1, B1, and B3 polypeptides with N-terminal sequences of, respectively, RQSQLS, RQSQLSPQNQC, and GVEETFCSARLSQN. These N-terminal sequences had 100% identity with prunin precursor cDNA derived amino acid sequence (Pru2 protein precursor, Pfam PF00190, EMBL X78120.1) for a hexameric 11S (legumin family) seed storage protein from almond (*Prunus amygdalus* Batsch) seeds (*7*). Attempts to further purify separated polypeptides by repeated HPLC analysis and subsequent attempts to obtain N-terminal sequences were unsuccessful. The complex nature of amandin polypeptide composition observed in our study is consistent with similar observations on several legumin seed storage proteins (*29, 30*).

Amino acid analysis of amandins is summarized in **Table 4**. Compared to the FAO/WHO pattern, methionine is the first limiting amino acid in amandin followed by lysine and threonine. The essential/total amino acid ratio (E/T %) of amandin was lower than the E/T % ratios for major storage globulins in cashew (CMP) and soybeans (11S). These results suggest that although the seed storage proteins may belong to the same grouping based on certain criteria (such as ultracentrifugation), individual seed proteins must be carefully evaluated with respect to polypeptide composition, polypeptide microheterogeneity, and other molecular properties.

Conclusions. Amandin, the major storage protein in almonds, can be isolated and purified by column chromatography, cryoprecipitation, and isoelectric precipitation methods. Amandins prepared according to three methods have similar polypep-

tide compositions and amino acid compositions. The isoelectric precipitation method resulted in amandin with more negative electrical charge than amandin prepared by chromatographic and cryoprecipitation methods. Regardless of preparation method, all amandins are antigenically related and therefore are suitable for the purpose of detecting almonds using our ELISA procedure.

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Almond Major Storage Protein

J. Agric. Food Chem., Vol. 50, No. 15, 2002 **4341**

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