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Biochemical and Functional Characterization of an Albumin Protein Belonging to the Hemopexin Superfamily from *Lens culinaris* Seeds

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ABSTRACT: The present paper reports the purification and biochemical characterization of an albumin identified in mature lentil seeds with high sequence similarity to pea PA2 . These proteins are found in many edible seeds and are considered potentially detrimental for human health due to the potential allergenicity and lectin-like activity. Thus, the description of their possible presence in food and the assessment of the molecular properties are relevant. The M_r, p*I*, and N-terminal sequence of this protein have been determined. The work included the study of (i) the binding properties to hemine to assess the presence of hemopexin structural domains and (ii) the binding properties of the protein to thiamin. In addition, the structural changes induced by heating have been evaluated by means of spectroscopic techniques. Denaturation temperature has also been determined. The present work provides new insights about the structural molecular features and the ligand-binding properties and dynamics of this kind of seed albumin.

KEYWORDS: seed proteins, PA2, thiamin-binding protein, hemopexin domains

INTRODUCTION

Legume plants constitute one of humanity's most important crops for food and feed. Lentil is one of the worldwide-cultivated oldest grain legumes, mainly produced for human consumption, which is becoming increasingly popular in developed countries, where it is perceived as a healthy component of the diet.[1] Lentil (*Lens culinaris* M.) seed contains about 24% (on dry weight basis) of proteins, some of which have been extensively characterized at the molecular level.[2] However, information for many of them is still incomplete. The aim of this work is to increase the knowledge of the lentil proteins that may exert biological activities. In particular, we focused on the albumin fraction, where nonstorage proteins such as protease inhibitors, amylase inhibitors, lectins, and other functional proteins, including the "housekeeping" enzymes, are mainly present.[3] Whereas storage globulins are typically stored in protein bodies,[4] albumins are mainly found in the cytoplasm and account for about 8% of the total seed proteins in lentil seed.[2] The first albumin proteins that have been described and characterized are the so-called PA2 and PA2-like proteins in pea (*Pisum sativum*) and chickpea (*Cicer arietinum*) seeds,[5,6] respectively, where they represent the most abundant albumin protein.[7] In pea, PA2 was found to be present in two forms of 48 and 53 kDa. Each of the two proteins is made up of two different polypeptides of molecular weights of 24 and 25 kDa, respectively.[5] PA2-like has an apparent molecular weight between 44 and 46.4 kDa and is made up of two ~23 kDa subunits.[7]

Structural studies reveal that pea PA2 and homologous proteins[8,9] are composed of four copies of a conserved repeat, described as a hemopexin-type repeat, which is structurally related to a group of mammalian regulatory proteins that includes vitronectin.[8] Vitronectin is a secretory protein found in mammalian serum and the extracellular matrix that promotes cell adhesion and spreading, inhibits membrane-damaging effects, and binds to several serpin serine protease inhibitors. Hemopexin behaves as an extracellular antioxidant, being involved in the prevention of hemin-mediated oxidative damage during intravascular hemolysis related to hemoglobin turnover.[10] Chickpea PA2 albumin is also able to in vitro bind hemin, indicating common structural features with pea PA2 and other similar proteins.[9,11]

Although PA2 from pea and chickpea seeds has been purified and widely characterized, the physiological role of the protein has been elucidated only in part. In pea, it seems to be involved in regulating polyamine metabolism during seed development,[12] thus playing a role in cell metabolism regulation. Plants of a naturally occurring pea mutant that lacks PA2 show reduced contents of spermidine and spermine and increased amounts of organic acids and amino acids.[12] Polyamines and their biosynthetic enzymes have been implicated in a wide range of metabolic processes in plants, ranging from organogenesis to protection against oxidative[13] and environmental[14] stresses and against infection by fungi and viruses.[14] A protein with close homology to PA2 from chickling vetch (*Lathyrus sativus*) has been shown to interact with both spermine and hemin.[9] Binding of heme resulted in the dissociation of the dimer into monomers with concomitant release of bound spermine, suggesting a role for this protein in sensing oxidative stress through a ligand-regulated monomer−dimer transition switch.[9]

The PA2-like albumin from chickpea was found to bind thiamin and, thus, a vitamin storage role has been proposed.[15]

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Resting seeds usually contain high amounts of thiamin, mainly in the metabolically inactive unphosphorylated form, which is tightly bound to different kinds of proteins, including PA2 albumin.[16]

Despite this nutritionally relevant positive issue, a number of negative effects of PA2 proteins have been reported, so that their presence has been considered to be potentially detrimental for human and animal health.[12] Lectin-like activity has been described for either pea or chickpea PA2 because they are able to agglutinate human erythrocytes.[7,17] However, these proteins cannot be considered typical lectins because they are not glycoproteins and they do not require Mn^{2+} or Ca^{2+} ions for agglutination activity.[7] It has been postulated that the possible physiological role of lectins is in host−pathogen interactions and cell−cell communications.[18,19] PA2 proteins are resistant to proteolysis during germination[5,6] and to digestion in the gastrointestinal tract of chickens.[20] PA2 and PA2-like proteins may have some potential to act as allergens as the chickpea PA2 protein is able to bind IgE from the sera of chickpea-allergic individuals.[7] However, the possible allergenicity of these proteins requires further investigation.[21]

The detection of the possible presence of proteins similar to PA2 in food and the assessment of their molecular properties are therefore relevant. The screening of a variety of seed albumin extracts obtained from different grain legume species showed that proteins similar to PA2 are present in many other species, including sweet pea (*Lathyrus oderatus*) and lentil.[5] For this latter plant the identification and preliminary crystallographic studies of a 2S albumin similar to PA2 have recently been described,[22] but no further information is available. In the present paper we report the biochemical and functional characterization of the albumin protein belonging to the hemopexin superfamily, which we purified from mature lentil seeds. The work included the study of (i) the binding properties to hemine for assessing the conserved presence of hemopexin structural domains and (ii) the binding properties of the protein to thiamin. In addition, we evaluated its denaturation midpoint (T_m) and the heat-induced structural changes of the protein as such and in the presence of thiamin as a ligand, by means of spectroscopic techniques. This novel characterization is relevant for applicative purposes, in particular for food processing and safety. The information obtained on thiamin binding and dissociation, together with thermal stability studies, provides new insights on the ligand-binding dynamics through conformational changes.

MATERIALS AND METHODS

Materials. Lentil seeds (*Lens culinaris* L. var. Macrosperma) were of commercial origin. All reagent grade chemicals were from Sigma-Aldrich (Milano, Italy) unless otherwise indicated. Chromatography media were from GE Healthcare (Milan, Italy).

Methods. *Protein Quantification.* Protein concentrations were determined according to the dye-binding method of Bradford,[23] using bovine serum albumin as standard.

Protein Purification. Lentil flour (5 g) was extracted with distilled water (1:10, w/v) at 4 °C for 3 h under mild stirring, in the presence of a protease inhibitor cocktail (Sigma-Aldrich, catalog no. P9599). The suspension was then centrifuged at 10000*g* at 4 °C for 20 min. The supernatant (total albumin extract) was fractionated by ammonium sulfate precipitation. The proteins precipitated between 35 and 70% saturation were resuspended in 20 mL of 30 mM ammonium bicarbonate and extensively dialyzed against the same buffer. The solution was then concentrated about 5 times by ultrafiltration using a Centricon Ultracell device (cutoff 10000) and loaded onto a Superdex 75 column (10 × 300 mm) equilibrated with 30 mM ammonium bicarbonate buffer containing 100 mM NaCl. Elution was performed with the same buffer (flow rate = 0.5 mL/min). The fractions containing PA2-like were collected, diluted with Milli-Q water (1:20), and loaded onto a Mono-Q HR5/5 column equilibrated with 50 mM Tris-HCl, pH 8.0 (flow rate: = 0.5 mL/min). After the unbound proteins had been washed with the equilibration buffer, a linear gradient of NaCl from 0 to 500 mM in the same buffer was applied over a period of 50 min with a flow rate of 1 mL/min. Albumin was eluted at 80 mM NaCl concentration.

Electrophoretic Techniques. SDS-PAGE was carried out as described by Laemmli[24] on 12% polyacrylamide gels. For runs under nonreducing conditions, 2-mercaptoethanol was omitted in the denaturing buffer. Polypeptide bands were stained with Coomassie blue R-250. The M_r of the polypeptides was determined by comparison with a standard protein solution (GE Healthcare, Milan, Italy) containing phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa). Nonreducing/reducing double SDS-PAGE was performed as follows. After SDS-PAGE under nonreducing conditions, the lane of interest was excised with a razor blade and incubated in 3 mL of equilibration buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol) with 65 mM DTT for 20 min and then with 245 mM iodoacetamide in the same buffer without DTT for 20 min. The gel lane was then placed horizontally on the top of a 15% polyacrylamide gel and sealed with 0.5% agarose, prepared in Laemmli running buffer. For two-dimensional IEF/SDS-PAGE, isoelectric focusing (IEF) was performed on 7 cm, pH 3−10 nonlinear IPG strips (GE Healthcare). The strips were rehydrated overnight in a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer pH 3−10 (GE Healthcare) containing 10 μg of the protein sample. Strips were focused at 8000 Vh, with a maximum of 2500 V, at 20 °C using a Multiphor II electrophoresis unit (GE Healthcare). Prior to the second dimension, strips were incubated in equilibration buffer with 65 mM DTT for 10 min and then with 245 mM iodoacetamide in the same buffer without DTT for 10 min.

Size Exclusion (SE) Chromatography. For the determination of M_r under native conditions, purified PA2-like protein was submitted to gel filtration chromatography, performed with a Superdex 75 column (10 × 300 mm) equilibrated with 30 mM ammonium bicarbonate buffer containing 100 mM NaCl. Elution was performed with the same buffer at a flow rate of 0.5 mL/min. For column calibration, the following protein standards have been used: aprotinin (6.5 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa), and glucose oxidase (180 kDa).

Amino Acid Sequencing. The N-terminal sequence of the protein was determined using a pulse liquid sequencer (Procise model 491, Applied Biosystems, Foster City, CA). Sequence analysis was performed following SDS-PAGE and blotting on polyvinylidene difluoride membranes (Millipore) essentially according to the method of Matsudaira.[25]

Hemin Binding Activity Assay. Albumin-2 binding to hemin (Sigma-Aldrich, catalog no. H9039) was carried out essentially as described by Pedroche et al.[11] Hemin stock solution was freshly prepared by dissolving 1 mg of bovine hemin chloride in 500 μL of 100 mM NaOH, kept on ice, and protected from light until used. Albumin-2 protein, dissolved in PBS (final concentration = 4 μM), was mixed with increasing concentrations of hemin solution (from 0 to 150 μM). The final volume was adjusted to 1 mL with PBS. The samples were incubated for 40 min at room temperature. For each experimental point, carried out in triplicate, identical amounts of hemin were added to reference tubes containing buffer alone. The formation of the complex hemin−protein was determined by recording the absorbance at 401 nm. The concentration of free hemin was calculated by subtraction of the concentration of bound hemin from the concentration of total hemin. The concentration of bound hemin was calculated from the ratio between the absorbance at 401 nm at

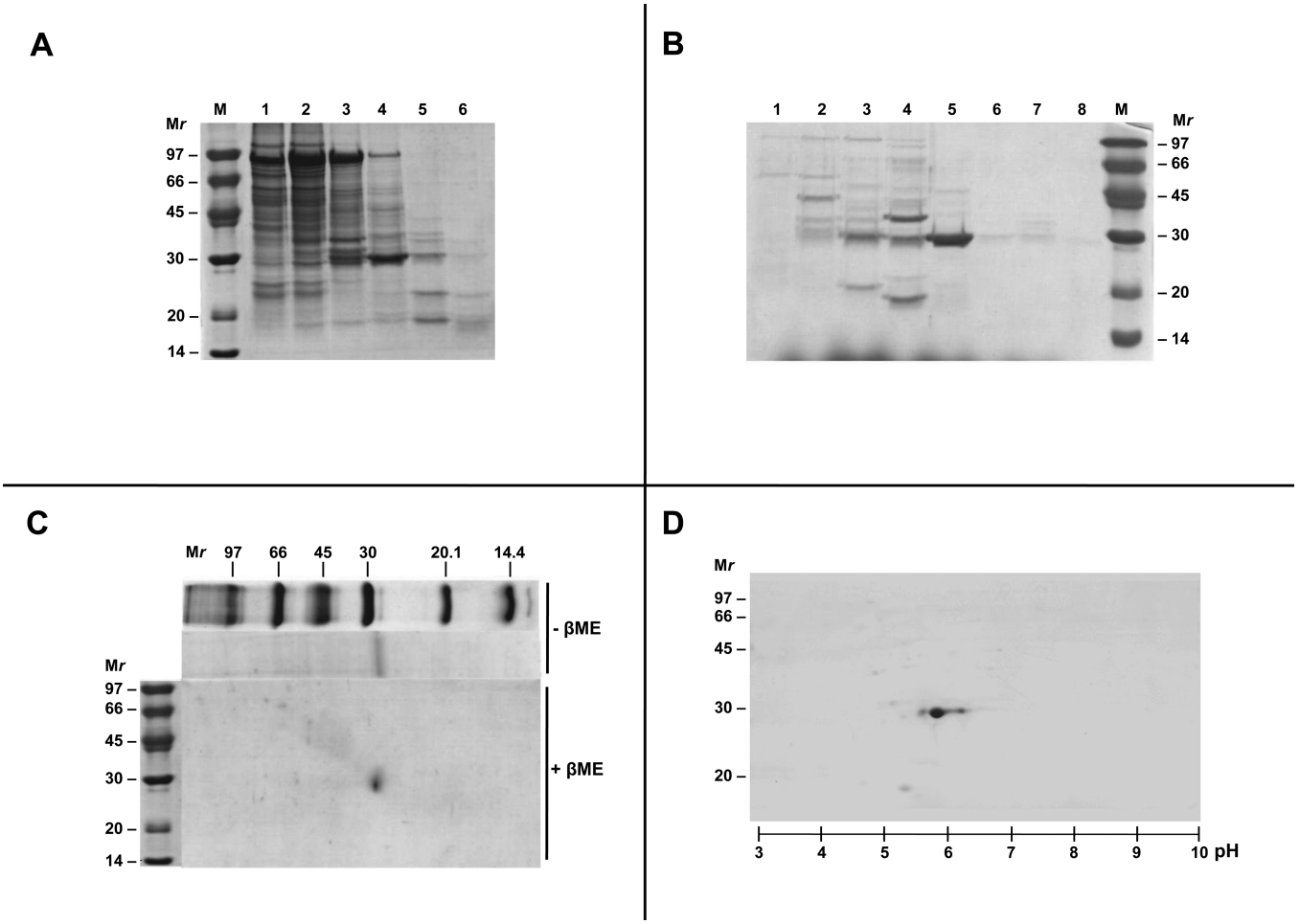

Figure 1. Albumin-2 purification and characterization. (A) SDS-PAGE showing the composition of the fractions separated by SE chromatography of the seed water extract. Numbers at the top of the gel refer to the collected fractions. Fraction 4 (F4) was selected for further analysis. The gel was run under nonreducing condition. (B) SDS-PAGE of the peaks obtained following fractionation of F4 by ion-exchange chromatography. Albumin-2 was found in the fifth fraction. Numbers at the top of the gel refer to the separated fractions. The gel was run under nonreducing condition. (C) Nonreducing/reducing double SDS-PAGE of the purified protein. The lane from the first SDS-PAGE run under nonreducing conditions was treated with β-mercaptoethanol (β-ME)-containing loading buffer and loaded horizontally on the second SDS-PAGE. (D) Two-dimensional electrophoresis (IEF/SDS-PAGE) of the purified protein. Ten microliters of each fraction (in panels A and B) or 3 μg of protein (in panels C and D) was loaded on the gels. All gels were stained with Coomassie blue. See text for experimental details. M, molecular markers. M_r values are expressed as kDa.

any experimental point and the maximum observed absorbance.[11] This ratio multiplied by the protein concentration yields the amount of bound hemin at each experimental point. The binding was analyzed by Scatchard analysis[26] by plotting the ratio of bound/free hemin versus the bound hemin concentration.

Thiamin-Binding Studies. The interaction between albumin-2 and thiamin (Sigma-Aldrich, catalog no. T4625) was carried out essentially as described by Adamek-Świerczyńska and Kozik.[15] Each binding assay was prepared in triplicate. Aliquots of albumin-2 protein dissolved in 50 mM Tris-HCl, pH 8.0 (protein final concentration = 9 μM), were mixed with increasing amounts of thiamin (10 mg/mL, prepared in 50 mM Tris-HCl, pH 8.0) and the volume was adjusted to 500 μL with the same buffer. After 2 h of incubation at 4 °C, separation of the unbound thiamin was performed by spun chromatography. Glass chromatographic columns (10 × 60 mm) were filled with Sephadex G25 Fine swelled with water and dried by spinning for 5 min at 1800*g*. After sample loading, columns were centrifuged again for 1 min at 1800*g*. The eluate contained the protein−thiamin complexes, whereas the unbound thiamin was retained by the resin. Protein recovery was determined for each sample. The thiamin content in the eluates was essentially determined by fluorometric detection according to the method of Mikowska et al.[27] Samples (350 μL) were mixed with 22 μL of 1% potassium hexacyanoferrate (III) and 188 μL of 15% NaOH. Fluorescence intensities were monitored at an excitation wavelength of 365 nm and an emission wavelength of 430 nm and correlated to thiamin content by means of a calibration curve. The binding property was analyzed by Scatchard analysis by plotting the ratio of bound/free thiamin versus the bound thiamin concentration. The equation of the best fitting regression line allowed the calculation of the dissociation constant ($K_d = -1/\text{slope}$) and the apparent thiamin−protein molar ratio, calculated as the *x*-intercept value divided by the protein concentration used in the binding tests.

Spectroscopic Techniques. Circular dichroism (CD) spectra were recorded on a J-810 Jasco spectropolarimeter, equipped with a Peltier-based computer-driven temperature control, and analyzed by means of the Jasco dedicated software. Measurements have been carried out in the 190−260 nm region (far-UV), with a cell path of 0.1 cm. The protein was dissolved in 50 mM Tris-HCl, pH 8.0, at a concentration of 0.3 mg/mL. Time progressive heating of protein solutions was carried out at 0.5 °C/min from 20 to 95 °C while ellipticity changes at 220 nm were monitored continuously. T_m values were calculated from the first

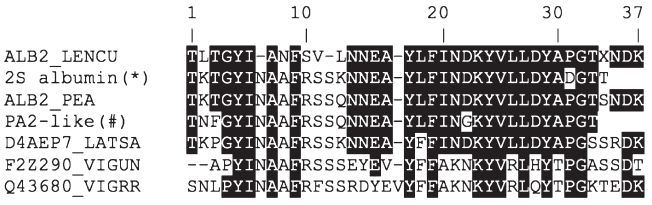

Figure 2. N-Terminal amino acid sequence of lentil albumin-2 and alignment with similar seed proteins. Proteins are indicated with the UniProtKB database accession numbers (ALB2_LENCU, albumin-2; ALB2_PEA, PA2; D4AEP7_LATSA, LS-24; F2Z290_VIGUN, CP4; Q43680_VIGRR, albumin). The sequences tagged with asterisk (*) and hash (#) symbols are taken from refs 21 and 7 , respectively. The numbering reported on the top of the alignment concerns the first sequence. Conserved amino acids with respect to albumin-2 are black-shaded. Gaps (-) have been inserted to maximize the global alignment.

derivative of the recorded traces. Deconvolution of the spectra was carried out by using the tool "protein secondary structure estimation" of the Jasco CD analysis software, approximating the mean residue weight (MRW) at 110 Da.[28] Fluorescence spectra were recorded using an LS50 spectrometer (Perkin-Elmer) coupled to a Peltier-based computer-driven temperature control and analyzed with the FLWinlab (Perkin-Elmer) dedicated software. Progressive heating of protein solutions was carried out at 0.5 °C/min from 20 to 95 °C while the intrinsic fluorescence changes were monitored continuously at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Protein (0.3 mg/mL) was dissolved in 50 mM Tris-HCl, pH 8.0.

RESULTS AND DISCUSSION

Albumin-2 Purification and Molecular Characterization. As detailed under Methods, the total seed albumin extract was first fractionated by ammonium sulfate precipitations. A protein of about 29 kDa precipitated mainly in the fraction obtained between 35 and 70% saturation. It has been possible to monitor the presence of the protein only by SDS-PAGE, carried out under nonreducing conditions, assuming that the searched lentil albumin protein had an expected M_r of about 26−30 kDa.[5,22,29] The lack of any suitable and easy to perform assays to test the presence of a PA2-like albumin through the steps of the purification procedure prevented the quantitative analysis at each purification step and, as consequence, the compilation of a purification table.

The purification of the 29 kDa protein was then achieved by combining two different chromatographic steps. The gel filtration separation allowed the collection of six fractions (Figure 1A). The 29 kDa protein was essentially found in the fourth peak (Figure 1A, lane 4). Ion exchange chromatography separated eight different fractions: the 29 kDa protein eluted in the fifth, when the NaCl concentration in the elution buffer was 80 mM. At the end of the procedure SDS-PAGE analysis showed the presence of a major band with an M_r of 29 kDa (Figure 1B, lane 5). Densitometric analysis of the Coomassie blue-stained gel indicated a purity grade of 95%.

The N-terminal amino acid sequencing revealed a single sequence, thus indicating that only one protein was present in the 29 kDa band, and allowed the first 37 residues of the polypeptide to be determined (residue 34 remained undetermined). This sequence is reported as the first sequence in Figure 2, and it has been deposited in the UniProtKB/Swiss-Prot sequence databank under the accession number ALB2_LENCU (P86782). The name assigned to the protein is albumin-2.

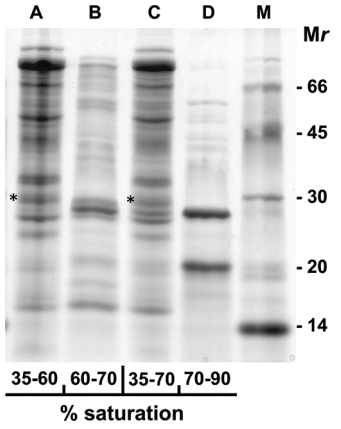

Figure 3. Fractionation of lentil seed water extract with ammonium sulfate (AS). A preliminary precipitation of the water extract at 35% AS saturation was carried out. The resulting supernatant was then subjected to two different experimental settings. In the first, AS concentration was raised to 60% and then to 70% (lanes A and B); in the second, AS at 70 and 90% of saturations has been used (lanes C and D). Only the proteins collected in the precipitate have been analyzed by SDS-PAGE (10 μg/lane). Albumin-2 is indicated with an asterisk (*). The gel was stained with Coomassie blue. M, molecular markers. M_r values are expressed as kDa.

A BLAST search[30] against UniProtKB Swiss-Prot/TrEMBL database (release 2011_2) indicated that the protein is clearly structurally related to pea PA2. The search retrieved very few sequences deriving from seeds, namely *Pisum sativum*, *Lathyrus sativus*, *Vigna unguiculata*, and *Vigna radiata*, besides to an *Oryza sativa* putative protein, a pea anther-specific protein, and a *Medicago truncatula* putative hemopexin. These three latter sequences have been omitted in the alignment shown in Figure 2. Instead, sequences taken from the literature have been included for the sake of completeness, these sequences being of albumins from lentil[22] and chickpea[7] seeds. The strong homology shared by the proteins included in the alignment is evident. It is worth noting that the albumin-2 sequence of this work differs from the albumin previously described in lentil seeds[22] for 6 amino acids of the 34 aligned (positions 2, 8, 10, 11, 12, and 31) and for two gaps.

To verify if the protein we isolated is a natural variation of the albumin previously identified[22] or a novel gene product, a series of selective ammonium sulfate precipitations have been carried out (Figure 3). In our case, the majority of albumin-2 was precipitated from the aqueous seed extract using ammonium sulfate between 35 and 70% of saturation, as described under Methods and shown in Figure 3, lane C. The 2S albumin was instead yielded with a higher amount of precipitant.[22] Actually, the precipitation of albumin-2 was complete already at 60% saturation (Figure 3, lane A), because between 60 and 70% no significant amount of protein was evident at the 30 kDa region of the SDS-PAGE (Figure 3, lane B). By increasing the percentage of ammonium sulfate to 90%, no precipitation of proteins with the expected electrophoresis mobility was observed (Figure 3, lane D). These results indicate that albumin-2 and 2S albumin are isoforms and that only one version of the protein accumulated in lentil seeds.

The total yield of albumin-2 was 134 ± 11 μg/g of seed (average of three purification runs). Gupta et al.[22] reported a yield of 120 μg/g of seed for the 2S lentil albumin.

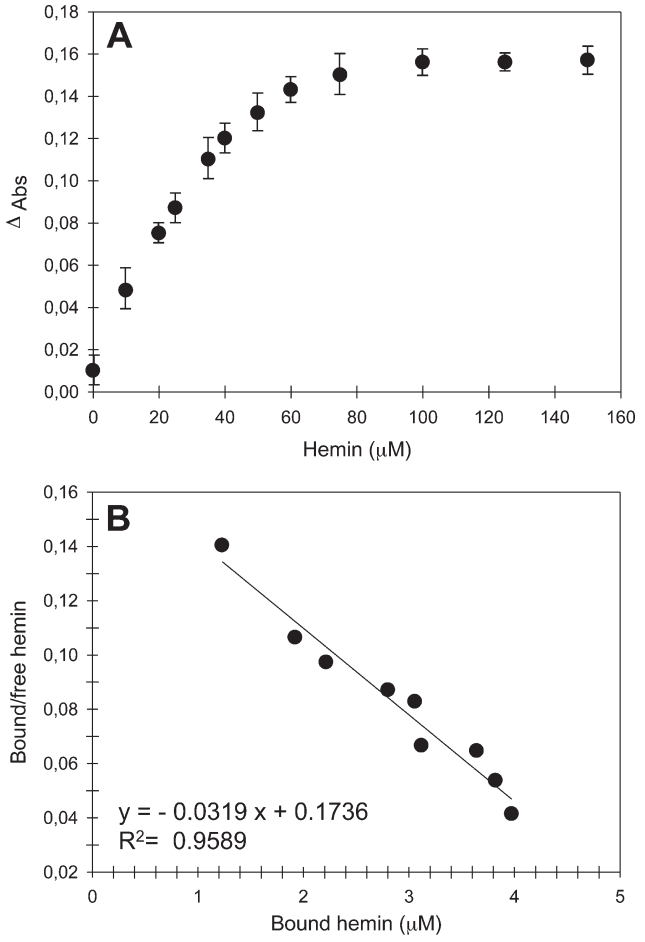

Figure 4. Binding of albumin-2 to hemin (A) and respective Scatchard analysis (B). Increasing amounts of hemin were added to a 4 μM protein solution. Each experimental point was carried out in triplicate. See text for experimental details.

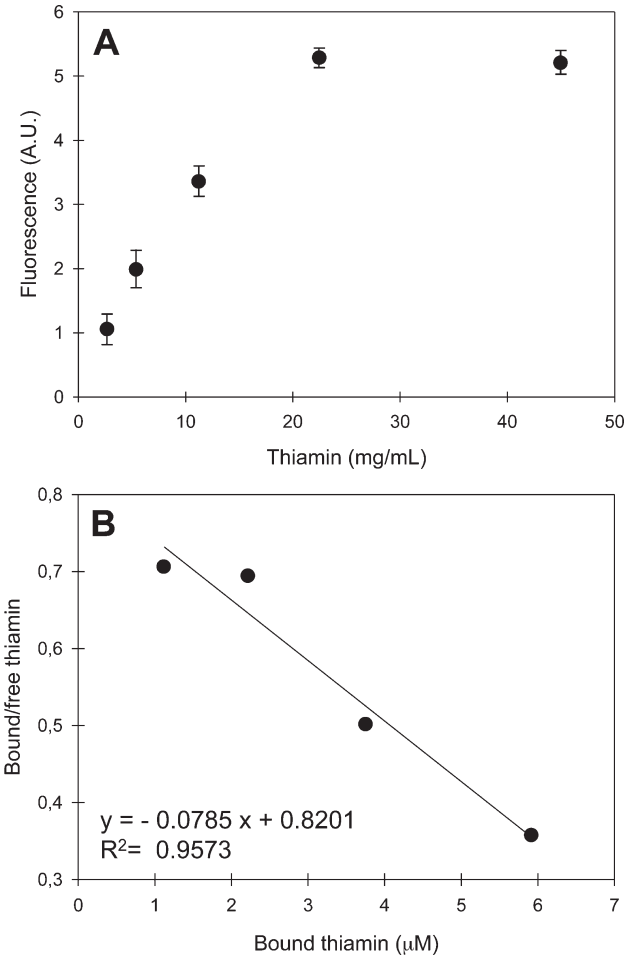

Figure 5. Binding of albumin-2 to thiamin (A) and respective Scatchard analysis (B). Increasing amounts of thiamin were added to a 9 μM protein solution. Fluorescence was monitored at an excitation wavelength of 365 nm and an emission wavelength of 430 nm. Each experimental point was carried out in triplicate. See text for experimental details.

Following SE chromatography carried out under native conditions, albumin-2 eluted as a protein with M_r of about 60 kDa, indicating a dimeric status of the protein. The two subunits are not linked by disulfide bridges, as has been shown by SDS-PAGE run under nonreducing conditions (Figure 1B). Moreover, each monomer consists of one only polypeptide chain, as shown by two-dimensional nonreducing/reducing SDS-PAGE (Figure 1C), indicating that no proteolytic events occurred post-translationally or during the purification procedure. IEF/SDS-PAGE analysis indicated a p*I* of 5.9 (Figure 1D). This value is consistent with that calculated with ProtParam online tool (available at www.expasy.org) for chickling vetch homologous protein (p*I* 6.29), but differs from that of pea PA2 (p*I* 5.16). On the 2D gels two minor spots with the same M_r, but different p*I* values (5.7 and 6.4, respectively) are also visible. The nature of these two polypeptides has not been further investigated.

Presence of Hemopexin Structural Domains. Several studies have shown that PA2s are composed by four hemopexin-like repeats, the first of them located at the N-terminal region.[9,12] Consequently, the protein has been included in the hemopexin superfamily. Hemopexin is a mammalian plasma glycoprotein with two structurally related domains that combine to bind a single heme molecule with great affinity. Heme is essential to all living beings as the prosthetic group of essential molecules such as cytochromes. The antioxidant activity of the heme−protein complexes has been extensively documented and depends on the particular nature of the heme−protein interaction, heme−protein dissociation constant, and amino acids in the vicinity of hemin.[10,31,32]

As part of the structural characterization, and in light of the differences evidenced by the N-terminal amino acid sequencing, we have verified if hemopexin domains are functionally present also in lentil albumin-2. To this purpose, assessments of the binding properties of the protein to hemin have been carried out. A fixed amount of albumin-2 was titrated with increasing amounts of hemin. The hemin bound to the protein increased in a saturable manner (Figure 4A). The binding was studied by Scatchard analysis (Figure 4B). The apparent dissociation constant (K_d) of the hemin−protein complex was determined as 32 μM, whereas the hemine−protein molar ratio was 3:1, assuming the dimeric status of the protein. These results are in good agreement with those (36 μM and 2.4:1, respectively) obtained for chickpea PA2 elsewhere[11] and confirm the presence of functional hemopexin-like domains in the amino acid sequence of lentil albumin. Not all of the seed albumin proteins possessing these structural domains are able to bind hemin, as in

the case of CP4 protein from *Vigna unguiculata*.[33] For this, a functional diversity of hemopexins in plants has been postulated.[33] The alignment of Figure 2 suggests a higher sequence similarity of albumin-2 with chickling vetch LA24[9] and chickpea PA2-like[7] proteins, which do bind hemin, rather than with CP4, which does not. Thus, albumin-2 should likely play physiological roles similar to those of PA2 and LA24.

Thiamin-Binding Capacity. The capacity of albumin-2 to bind thiamin may suggest its possible role in vitamin storage and mobilization and may also have a nutritional relevance for food applications. To test the binding properties of albumin-2 toward unphosphorylated thiamin, a fixed amount of protein has been incubated with increasing concentrations of vitamin (Figure 5). The amount of bound thiamin increased in a saturable manner (Figure 5A). From the Scatchard analysis (Figure 5B), the calculated K_d was 12.8 μM, a value that is 2.7 times lower than that reported for the pea homologue,[15] and indicates a stronger affinity of the lentil protein for this ligand. The apparent thiamin−protein molar ratio was 2.3:1, assuming the dimeric status of the protein. To verify the actual quaternary structure of the protein in the presence of thiamin, a gel filtration chromatography analysis was performed. The complex albumin−thiamin eluted with the same volume of buffer of the free protein, indicating that the dimeric status is not influenced by the presence of thiamin (not shown).

Resting seeds usually contain fair amounts of thiamin, predominantly in the metabolically inactive unphosphorylated form. During germination thiamin is rapidly converted to the active biphosphate form (TPP) by specific kinases.[16] TPP acts as a cofactor of many key enzymes involved in major metabolic pathways such as the citric acid cycle, glycolysis, and the pentose phosphate cycle.[34] Interestingly, a cell protective role of both thiamin and TPP against oxidative damage following abiotic stress has recently been shown in *Arabidopsis thaliana*.[35] It has been postulated that the thiamin-storage function may be exerted by the so-called thiamin-binding proteins (TBPs), which are usually seed globulin with a high affinity for thiamin, the K_d for the ligand being in the 1−2 μM range. TBPs essentially do not bind TPP.[16,36] Contrary to globulin TBPs, which are promptly degraded during the germination of the seeds,[37,38] PA2s are instead quite resistant to proteolysis.[6] It has been suggested that in legume seeds the major thiamin reserve is provided by the thiamin-binding globulin and the associated thiamin-binding albumin may have a backup role in controlling the vitamin bioavailability during germination.[15] Many findings corroborate the hypothesis of a defense role of thiamin, which may confer systemic acquired resistance through priming, or elicitation competency, enhancing the capacity of the plant to express pertinent defense mechanisms to suppress future attacks.[39,40]

Structural Analysis and Thermal Stability. The structural features and the thermal stabilities of the secondary and tertiary structures of the protein were assessed by means of far-UV CD and intrinsic fluorescence, both as such and in the presence of thiamin as the ligand (Figure 6). Spectra obtained at 20 °C in far-UV CD without or with thiamin are not significantly different, suggesting that the secondary structure features of the proteins are not affected by the binding to thiamin (Figure 6A). To quantify the secondary structure elements, deconvolution of the spectra was carried out approximating the mean residue weight (MRW) at 110 Da.[28] In both cases, the resulting composition of secondary structures is 12% α-helix, 34% β-sheet, 21% β-turn, and 33% random coil. These values are in good agreement with those inferred from the three-dimensional model of *Latyrus sativus* albumin, namely, 16% α-helix and 39% β-sheet.[9]

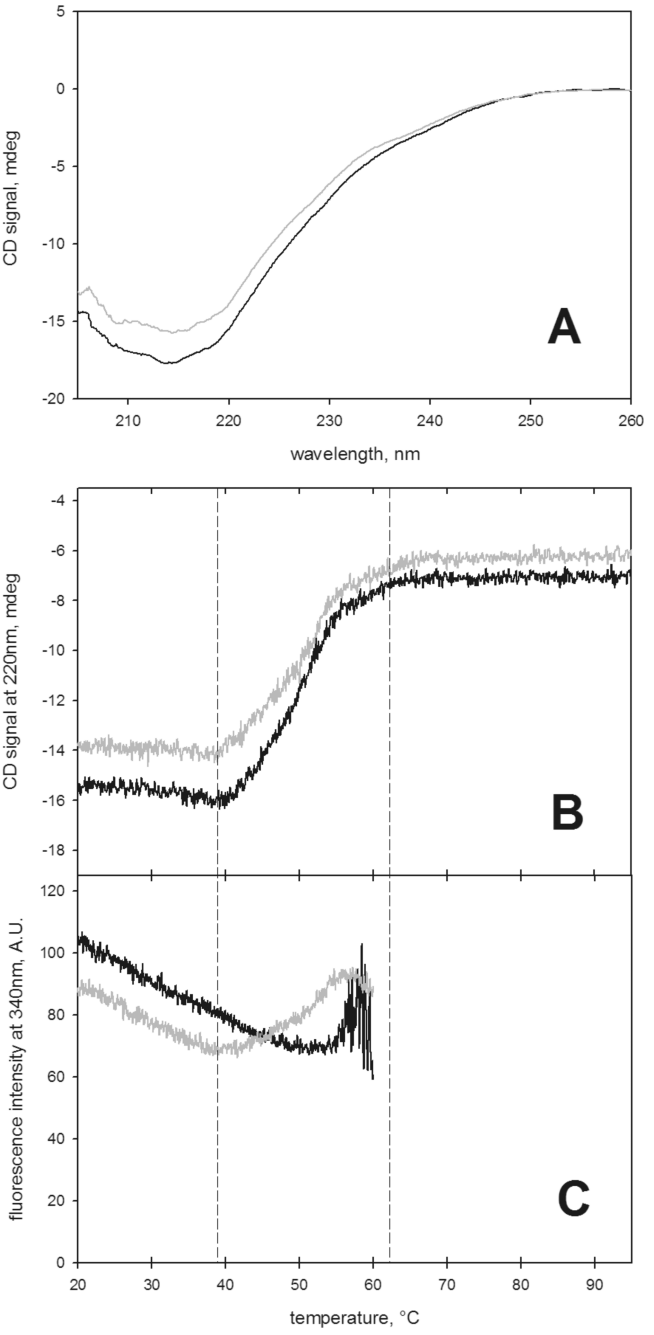

Figure 6. Spectroscopic structural analysis and thermal stability of albumin-2 as such (black lines) and in the presence of thiamin (gray lines): (A) CD spectra recorded in the far-UV at 20 °C; (B, C) spectra recorded in the course of a progressive heating, carried out at 0.5 °C/min from 20 to 95 °C. In panel B are reported far-UV CD spectra recorded at an excitation wavelength of 220 nm, whereas in panel C are shown intrinsic fluorescence spectra recorded at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. The left vertical dashed line points out the temperature at which structural variations due to thermal treatment became evident, whereas the right one points out the T_m of the protein, valid only for panel B.

The CD signal recorded in the course of a progressive heating indicates that albumin-2 is stable up to 38−40 °C (Figure 6B,

black line), and then structural variations became evident. The T_m was estimated at 63 °C. The CD trace recorded in the presence of thiamin is de facto coincident (Figure 6B, gray line). This indicates that even the thermal stability of the secondary structures is not influenced by the presence of thiamin.

Fluorescence spectra taken when albumin-2 was bound to thiamin showed a general lowering of the recorded signal intensities compared to the ligand free protein (not shown). This reduction of intensity was in all probability caused as the result of the quenching effects of thiamin on the tryptophan fluorescence, as already shown elsewhere.[41]

Intrinsic fluorescence has been used to assess the thermal stability of the tertiary structures (Figure 6C). Under the experimental conditions adopted, albumin-2 showed a high propensity to aggregate and precipitate during the transition from native to unfolded form. This event did start before the temperature reached the T_m (Figure 6C, black line). Whereas CD is quite insensitive to this kind of event,[42] tryptophan fluorescence is highly affected by the change in the aggregation state.[43] Generally speaking, it is not possible to predict the effect of solvent exposure on the total intensity of emission upon unfolding.[43] In our case, during the temperature ramps a progressive decrease of fluorescence was observable. For the protein without ligand this decrease was linear and observed until the T_m was reached, without any significant variation having reference to the unfolding process (Figure 6C, black line). Instead, in the presence of thiamin, fluorescence started to increase at 38−40 °C (with a shape of the fluorescence trace that resembled the unfolding observed by CD) (Figure 6C, gray line). Clearly, this increase in fluorescence intensity cannot be attributed to the unfolding process, but to the release of the thiamin from the protein with a consequent disappearance of the quenching effects. This behavior strongly suggests that thiamin is released as soon as minimal modifications of the protein tertiary conformation occur.

The present work provides new insights about the structural molecular features of this kind of seed albumins. Our results complement what has been shown for homologous proteins and confirm a fine-tuning of the ligand-binding dynamics through conformational changes.[9] Various interesting hypotheses have been suggested to depict the molecular mechanisms at the basis of their biological activity.[9,12,15] This kind of albumin might act as a temporary thiamin deposit during germination,[15] but also they may be involved in sensing oxidative stress through a regulated monomer−dimer transition switch upon spermine and heme binding.[9] Both spermine and thiamin have been shown to play important roles in alleviating oxidative stress during different abiotic stress conditions, including metal toxicity, drought, salinity, and chilling,[13,35] and in defense against biotic attacks.[40] Taken all together, these remarks lead to the conjecture of a possible role of albumin-2 proteins in plant defense. Studies in this direction have not been undertaken because it was out of the scope of the present work.

From an applicative point of view, it has been suggested that a reduced content of PA2 in pea seeds would lead to a number of improved seed quality characteristics,[12] because the protein displays a number of characteristics that are considered to be undesirable for human nutrition and health. This may be also the case of lentil albumin-2. However, most of the potential negative effects may not be problematic, considering its high sensitivity to thermal treatments. It cannot be excluded that, thanks to the binding characteristics and dynamics properties, this protein may be used in pharmacological and medical applications. Indeed, proteins traditionally considered as antinutritional factors have recently been reappraised and are now thought to be favorable for their potential bioactivities as described for several molecules, as, for example, the Bowman−Birk trypsin/chymotrypsin inhibitors.[44,45]

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