Quaternary Structure and Model for the Oligomeric Seed Globulin from *Amaranthus hypochondriacus* K343

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With the use of electron microscopy and computer image analysis techniques, it was demonstrated that the seed storage globulin from *Amaranthus hypochondriacus* exists as a dodecameric structure with its constituent subunits arranged into two annular hexagonal rings placed parallel to one another, resulting in a hollow oblate cylinder. These findings would suggest that seed storage proteins from leguminous and nonleguminous dicotyledonous plant classes such as from the *Amaranthus* family have similar overall quaternary structures.

Keywords: *Amaranth; globulin; amaranthin; electron microscopy; image analysis*

INTRODUCTION

Directly or indirectly plants comprise up to 95% of the present world's food supply (Walsh, 1984) with grains providing in excess of 50% of man's basic protein requirement (Johnson, 1984). Seed proteins from these grains have historically represented the major source of proteins for both human and animal nutrition. The nutritional quality of these seed proteins, predominantly storage proteins, depends largely on their amino acid composition. While the nutritional value of a protein is related both to its essential amino acid content and to its digestibility, the utilization of these proteins as food ingredients is largely determined by their functional properties such as emulsification and foaming abilities/stabilities (Kinsella, 1976). Although much is known about the primary, secondary, and tertiary structures of many storage proteins, little is known regarding their quaternary structures and how these hierarchical-type structures influence each other. Knowledge of these structures would ultimately lead to a clearer understanding of the structure/function relationships found in these quaternary structured proteins and how they affect the above functional properties.

The most significant advancements made in the study of the quaternary structure of seed storage proteins have been made in the study of various legume proteins. The 11S soybean globulin, commonly referred to as glycinin, was first observed to be composed of 12 subunits of various molecular weights and physicochemical properties (Catsimpoolas et al., 1967). It was not until 1975 that Badley et al. (1975) attempted to experimentally determine the geometric arrangement and association of these 12 subunits. Using electron microscopy and X-ray scattering methods, they were successful in demonstrating that these subunits were packed into two identical hexagonal rings packed one on top of the other, yielding an oblate cylinder of 55 Å radius and 75 Å thickness.

Various studies have also been undertaken in attempts to determine the types of relationships and features common to other legumin globulin proteins. Such studies have revealed that the peanut globulin (arachin) (Yamada et al., 1979), the 12S *Pisum sativum* (pea) globulin (Gatehouse et al., 1981), and the *Vicia fava* broad bean globulin (Mori and Utsumi, 1979), although having diverse genetic origins, were all composed of 12 polypeptide chains and associated in a glycinin-like structure.

Work in our laboratory has been performed on the dicotyledonous family Amaranthaceae to establish if the glycinin-type model is also valid for globulins from nonleguminous dicotyledonous plants. The molecular architecture of the amaranth seed storage globulin (amaranthin) has been speculated upon by Marcone and Yada (1991, 1992). The present work outlines a study that provides information concerning the subunit arrangement within the oligomer.

MATERIALS AND METHODS

Sample Preparation. Non-heat-treated milled flour of *Amaranthus hypochondriacus* K343 (lot 1010) was purchased from American Amaranth Inc. (Briceyln, MI). The amaranth flour was defatted with cold HPLC grade acetone in the ratio of 1:10 (w/v) (flour/acetone). The mixture was mechanically stirred in a sealed 2 L container for 36 h at −20 °C. This was followed by three additional extractions with cold acetone. The pooled extracts were dried under a stream of pure nitrogen gas or alternatively under reduced pressure.

Protein Fractionation. The globulin protein was extracted from the defatted meal according to the method of Konishi et al. (1985), modified as follows. A 200 g sample of meal was extracted at room temperature for 18 h with 4.0 L of a 32.5/2.6mM K_2HPO_4/KH_2PO_4 buffer (pH 7.50) containing 0.4 M NaCl and 0.02% NaN_3. The meal/buffer mixture was centrifuged at 10000*g* for 20 min in a L8-70M Ultracentrifuge (Beckman Instruments, Mississauga, ON). The supernatant was dialyzed (cutoff 12 000−14 000 Da) for 72 h against 60 L of denionized water followed by 60 L of double-distilled water at 4 °C.

The retentate was again centrifuged at 10000*g* for 20 min at 4 °C. The resultant crude globulin pellet was resuspended in 200 mL of Milli-Q water and centrifuged at 10000*g* for 20 min at 4 °C. The pellet was frozen in liquid nitrogen (−196 °C) and immediately lyophilized.

Protein Purification. The globulin was purified to homogeneity by gel filtration and anion-exchange chromatographies and stored desiccated at −77 °C under a dry argon blanket until further study, as previously described (Marcone and Yada, 1991). Purity was evaluated by sodium dodecyl

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sulfate and native polyacrylamide gel electrophoresis as previously described (Marcone and Yada, 1991).

Electron Microscopy. The purified globulin was diluted in 10 mM phosphate buffer, pH 7.0, to a concentration of 250 μg/mL, adsorbed to an ultrathin carbon film for 1 min, and negatively contrasted in 1% (w/v) phosphotungstic acid for 1 min as described by Sommerville and Scheer (1987). Phosphotungstic acid was selected after a systematic evaluation of other common negative stains (buffered uranyl acetate, uranyl oxalate, ammonium molybdate) because it is readily soluble at neutral pH and was shown to yield the greatest stability for this particular oligomer. Specimens were imaged in a Phillips EM400T transmission electron microscope (TEM) operating at 80 kV and at a nominal instrument magnification of 100000×. Each specimen area was not preilluminated prior to being micrographed, to minimize the total electron dose.

Electron micrographs of negatively stained molecules imaged in the bright field TEM mode were visually scanned to select distinct particles of recurring appearance and size. Selected particles were digitized individually using a Hitachi KP-113 solid state camera mounted on a dissecting microscope (Wild Leitz M3B) and interfaced to a Vision8 frame grabber (Everex) in an IBM-compatible personal computer. During digitization all images were acquired with the emulsion facing the camera to preserve handedness in the digitized image. The digitized globulin images were stored in arrays of 128 × 128 pixels (picture elements), where one pixel corresponded to an area of 0.125 × 0.125 nm at the object level. A total of 730 distinct particles were selected.

Single particle analysis of digitized images was performed in the framework of the IMAGIC image processing system as previously described and summarized here (Harauz and Boekema, 1991). The single macromolecular images were pretreated by band-pass filtering to suppress the very low and very high spatial frequencies. The very high spatial frequencies represented mostly noise, while the very low spatial frequencies represented unwanted information, e.g., gradual fluctuations in the average densities that depended largely on the amount and uniformity of specimen staining. After band-pass filtering, the particles were surrounded by a circular mask to cut away unnecessary background. The images were then standardized by floating within this mask to a zero average density and by multiplication of each pixel by a factor to normalize the variance. In the negatively stained amaranth subunit images, larger values (positive and white) represented mainly the biological structure and smaller values (negative and black) were more representative of stain embedding and penetration.

Isolated macromolecules exhibited a full range of rotational orientations in the plane of the support film which had to be corrected for, and a translation in the plane was also required to bring the particle into the center of its image. A computerized double-alignment algorithm based on cross-correlation functions was used to bring all of the particles into register with one another and was performed repeatedly with successive improvements in choice and quality of reference image. Following the alignment process, multivariate statistical analysis (MSA) and hierarchical ascendant classification (HAC) were performed to select the most homogeneous subset of aligned images for another averaging. These average images represented the two-dimensional constructions of particular recurring projections of the macromolecular complex (Harauz and Boekema, 1991).

RESULTS

A field of view of a purified globulin negatively contrasted with phosphotungstic acid is shown in Figure 1. Individual complexes appear roughly 9 nm in size, and the primary recognizable projections show a round structure with a central stain-filled depression or hole. A single globulin complex exhibiting six major domains in projection with roughly 6-fold rotational symmetry is shown in Figure 2. This particular image was chosen to be the initial reference to align the other 730 digitized images in preparation for multivariate statistical analysis (MSA), sorting by hierarchical ascendant classification (HAC), and averaging of the most homogeneous subsets. Two more cycles of multireference alignment with respect to selected class averages, MSA/HAC, and reaveraging were then applied. The "best" averages of characteristic views finally obtained were those comprising a set of well-aligned, homogeneous globulin images (Figure 3). Each of these averages had either six (a, b, e) or four density peaks, which by their arrangement and separation suggested either 6- or 2-fold rotational symmetrization (Figure 4), followed by mirror symmetrization (Figure 5) to improve their appearance. (It should be noted, however, that the class in Figure 3e did not lend itself to mirror symmetrization.) Figure 6 shows the symmetrized characteristic views with isodensity contour lines superimposed to depict more clearly the density variations.

To understand the results of the single particle analysis, a three-dimensional model comprising 12 spheres arranged in two parallel layers, each with 6 spheres centered on the vertices of a hexagon, was constructed in the computer. Two-dimensional projec-

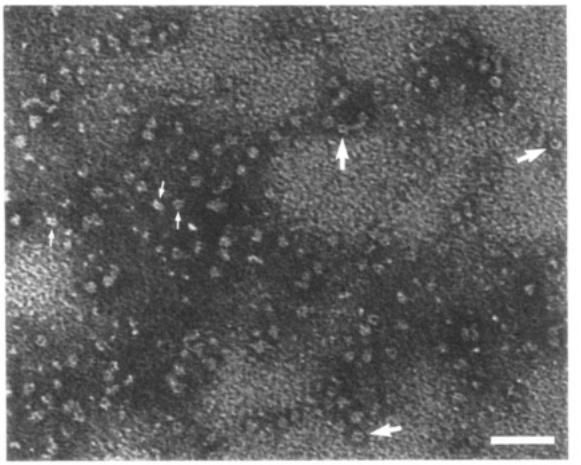

Figure 1. Electron micrograph of a purified preparation of globulin, negatively contrasted with phosphotungstic acid. Some individual complexes are indicated by small arrows (side views) and others by large arrows (top views). Scale bar represents 50 nm.

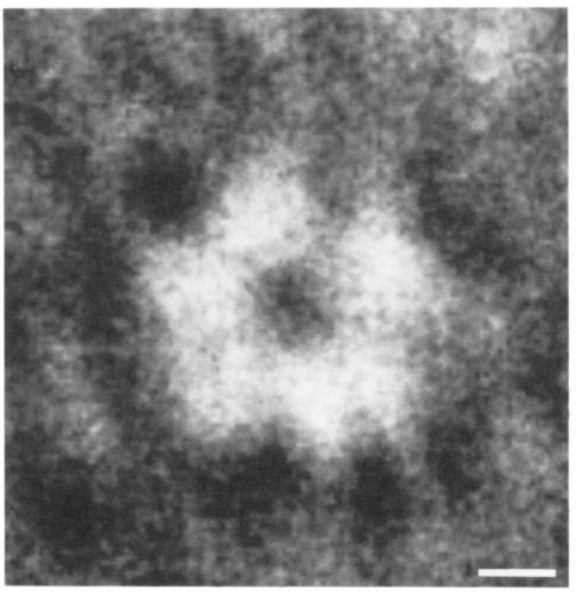

Figure 2. Electron micrograph of a single globulin complex exhibiting six stain-excluding regions and a central stain-filled depression or hole (protein is white, stain is dark). Scale bar represents 2.5 nm.

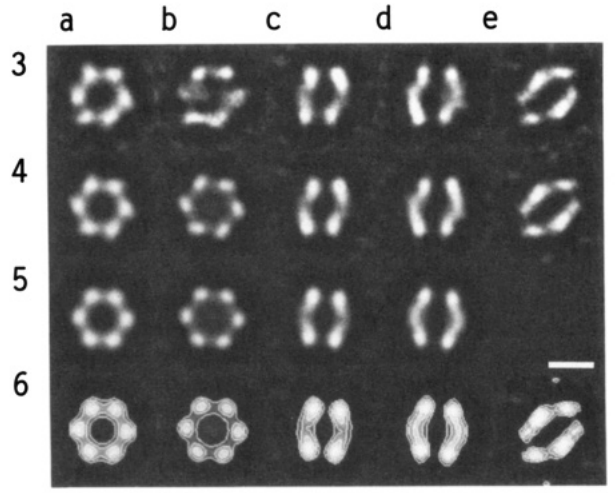

Figure 3–6. (3) Class averages of (a) 77, (b) 54, (c) 71, (d) 91, and (e) 44 images, derived by a single particle electron image analysis. These images represent characteristic views or projections of the globulin complex as they lie in preferred orientations on the carbon support. There are six density maxima in (a) and (b), four in (c) and (d), and six in (e). The arrangement of maxima and their separation suggest 6-fold rotational symmetry in (a) and (b) and 2-fold rotational symmetry in (c), (d), and (e). (4) Class averages of Figure 3 after application of appropriate rotational symmetry. (5) Class averages of Figure 3 after application of appropriate rotational and mirror symmetry. Mirror symmetrization was not appropriate for the image in column e. (6) Symmetrized characteristic views form Figure 5 with isodensity contour lines superimposed. Scale bar represents 5 nm.

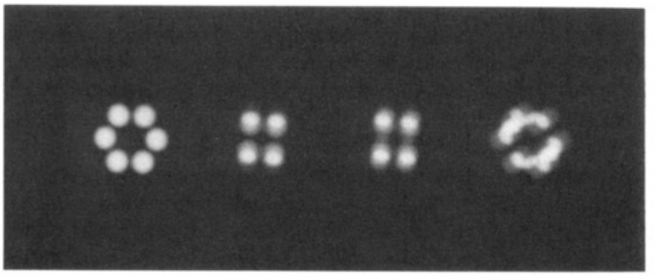

Figure 7. Two-dimensional projections of a three-dimensional computational model comprising 12 spheres of equal size and density arranged in two parallel layers with a central 6-fold axis of rotational symmetry. These projections correspond to the symmetrized characteristic views of globulin derived by single particle analysis.

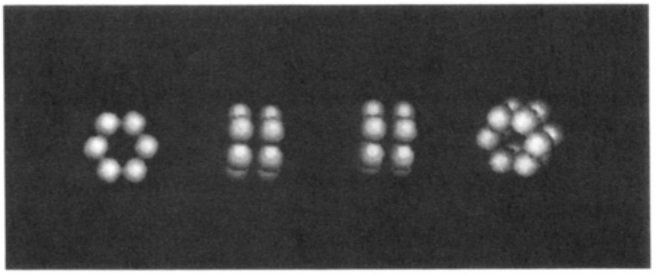

Figure 8. Shaded surface representations of the three-dimensional model from viewing angles corresponding to those in Figure 7.

tions of this model at 1000 different orientations were calculated and visually compared with the characteristic projections of the globulin. The best matching projection images are shown in Figure 7, and corresponding representations of the surfaces of the three-dimensional model are shown in Figure 8.

The projections in columns a and b of Figures 3–6 represent a view from the "top" of the complex down its central axis, while those in columns c and d represent a view from the "side". The top views differ from each other primarily in the amount of central stain accumulation; the separation of the six density maxima in (b) is the same as in (a), even though in the course of the analysis (b) was thought potentially to represent a distinct orientation. There are two potential side projections when one considers the three-dimensional model as in Figure 4a,b: either through a central subunit or between two central subunits. The projections in columns c and d are the latter type, which would be expected to be more stable. Finally, the projection in column e appears also to be a view from the top, but at an angle of roughly 32° with respect to the central axis. This appearance is consistent with the phenomenon of "rocking" of the complex around the top preferred orientation because of unevenness of the underlying carbon support. The distance between density peaks in the 6-fold rotationally symmetric plane is 2.8 nm, while the distance between planes is 3.2 nm.

DISCUSSION

The hexagonal ring structure of purified globulin combined with its inner stained channel (Figures 1 and 2) has also been observed in the structure of various studied storage proteins, in particular the 11S soybean globulin, as well as in such oligomeric enzymes such as glutamine synthetase (Valentine et al., 1968). Although the overall apparent molecular weights of both the amaranth globulin and the 11S soybean globulin oligomers were purported to be in very close agreement, i.e., 337 500 (Marcone and Yada, 1991) and 350 000 (Kitamura and Shibasaki, 1975; Kitamura et al., 1976; Marcone et al., 1994), respectively, this observed conservation of quaternary structure between the two globulins was a significant finding in light of the different types of subunits making up their ensembles. Although both proteins could be classified as hetero-oligomers (i.e., composed of more than one dissimilar subunit), the soybean globulin was shown to be composed of three dissimilar subunits [i.e., 47 000, 34 000, and 18 000 (Kitamura and Shibasaki, 1975; Kitamura et al., 1976; Marcone et al., 1994)], whereas the amaranth oligomer was composed of five dissimilar subunits [i.e., 37 000, 31 500, 26 500, 20 500, and 14 500 (Marcone and Yada, 1991)]. Although these major differences in the number of distinct molecular weight subunits were found to exist between the two oligomers, previous densitometric investigation by Marcone and Yada (1991, 1992) into the relative molar ratio of amaranth subunits did suggest that the amaranth globulin was composed of between 11 and 12 subunits, thereby analogous to the number of subunits found in the 11S soybean globulin.

Single particle analysis of the images in Figure 1 yielded averages of three predominant characteristic views of the globulin, one considered a top view and the other side views (Figure 3). Rotational (6- or 2-fold) and mirror symmetrization improved further the signal-to-noise ratio of the averaged images (Figure 6). What these results suggested was that the amaranth subunits were packed into two hexagonal rings, placed one on top of the other, thereby yielding the structure of a hollow oblate cylinder.

A three-dimensional model was constructed computationally, comprising 12 equivalent spheres arranged into two parallel hexameric rings. Projections (Figure 7) and surface (Figure 8) representations were consistent with the electron image results.

Examination of Figure 2 reveals that the PTA stain used in this study did not appear to have penetrated to

any significant degree in between the subunits forming the individual hexagonal ring structures. Badley et al. (1975) noticed that PTA has an affinity primarily for hydrophilic areas, so that the lack of stain between the subunits of the 11S soybean globulin would suggest the existence of mainly hydrophobic-type interactions in that region. Similar types of forces may also exist between those subunits of the amaranth oligomer. Since such hydrophobic-type forces are known to increase in strength with increase in temperature up to 70 °C, the predominance of hydrophobic-type interaction between the subunits of soybean and amaranth globulins would serve to partially explain their observed exceptional thermal stabilities when compared to other similar proteins, e.g., wheat gluten (Konishi and Yoshimoto, 1989; Biliaderis, 1983).

The presence of stain between the amaranth's two hexagonal rings (Figure 3) would suggest that they, like the rings of the 11S soybean globulin, are held together by electrostatic and/or hydrogen bonding forces. Marcone and Yada (1991) had shown that the globulin was composed of two moieties held together by weak secondary forces which were readily dissociable under alkaline pH conditions, suggesting the involvement of electrostatic and/or hydrogen bonding forces.

On the basis of the above results it was concluded the major species of the *A. hypochondriacus* globulin is a dodecameric structure with *D*6 symmetry and dimensions of 2.8 nm from the center of the complex to the center of the subunit, 2.8 nm between subunits in a ring, and 3.2 nm between subunits in opposite rings. The individual rings were 9 nm in outer diameter. In addition, the data would tend to indicate that the glycinin-type model is similar for both leguminous and nonleguminous dicotyledonous plants.

For a long time plant taxonomists have shown great interest in the use of protein investigations as a means of classification and identification of unknown plant specimens. Since in classical taxonomic studies it is critical for the morphologist to compare plant organs at the same stage of development, the study of seeds and their storage proteins has the added advantage of being little affected by the environment, geographic origin, seasonal fluctuation, and chromosomal rearrangements. It is interesting to speculate that the study of the structure of such secondary metabolites as proteins may serve one day as an additional future technique available to chemical taxonomists in their investigation.

NOMENCLATURE

In keeping with the present method of nomenclature for seed storage globulins, which uses either the genus or family name of origin followed by the suffix "in", for example 11S soybean (glycinin), pea (legumin), sunflower (helianthin), oil seed rape (cruciferin), and peanut (arachin), the amaranth seed globulin should be named amaranthin.

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LITERATURE CITED

Badley, R. A.; Atkinson, D.; Hauser, H.; Oldani, D.; Green, J. P.; Stubbs, J. M. The structure, physical and chemical properties of the soy bean protein glycinin. *Biochim. Biophys. Acta* **1975**, *412*, 214–228.

Bhushan, R.; Reddy, K. Reconstitution of conarchin from isolated subunits. *Int. J. Pept. Protein Res.* **1989**, *33*, 313–316.

Biliaderis, C. G. Differential scanning calorimetry in food research. A review. *Food Chem.* **1983**, *10*, 239–265.

Catsimpoolas, N.; Rogers, D. A.; Circle, S. J.; Meyers, E. W. Purification and structural studies of the 11S component of soybean. *Cereal Chem.* **1967**, *44*, 631–637.

Gatehouse, J. A.; Croy, R. R. D.; Morton, H.; Tyler, M.; Boulter, D. Characterisation and subunit structures of the vicilin storage proteins of pea (Pisum sativum L.) *Eur. J. Biochem.* **1981**, *118*, 627–633.

Harauz, G.; Boekema, E. J. Processing and analysis of electron images of biomolecules. In *Image Analysis in Biology*; Donat Hader Ed.; CRC Press: Boca Raton, FL, 1991; Chapter 11.

Johnson, V. A. New horizons in cereal production. In *Cereal Production*; Gallagher, E. J., Ed.; Butterworth: London, 1984; Chapter 1, pp 1–14.

Kinsella, J. E. Functional properties of proteins in foods: a survey. *CRC Crit. Rev. Food Sci. Nutr.* **1976**, *7*, 219–280.

Kitamura, K.; Shibasaki, K. Isolation and some physicochemical properties of the acidic subunits of soybean 11S globulin. *Agric. Biol. Chem.* **1975**, *39*, 945–951.

Kitamura, K.; Takagi, T.; Shibasaki, K. Subunit structure of soybean 11S globulin. *Agric. Biol. Chem.* **1976**, *40*, 1837–1844.

Konishi, Y.; Yoshimoto, N. Amaranth globulin as a heat-stable emulsifying agent. *Agric. Biol. Chem.* **1989**, *53*, 3327–3328.

Konishi, Y.; Fumita, Y.; Ikeda, K.; Okuno, K.; Fuwa, H. Isolation and characterization of globulin from seeds of *Amaranthus hypochondriacus L. Agric. Biol. Chem.* **1985**, *49*, 1453–1459.

Marcone, M. F.; Yada, R. Y. Isolation, purification and characterization of the oligomeric seed globulin from *Amaranthus hypochondriacus. Agric. Biol. Chem.* **1991**, *55*, 2281–2289.

Marcone, M. F.; Yada, R. Y. Study of the charge profile and covalent subunit association of the oligomeric seed globulin from *Amaranthus hypochondriacus. J. Agric. Food Chem.* **1992**, *40*, 385–389.

Marcone, M. F.; Bondi, M. C.; Yada, R. Y. Isolation of the soybean 11S globulin by isoelectric precipitation and sephacryl S-300 gel filtration chromatography: a new purification technique. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 413–415.

Mori, T.; Utsumi, S. Purification and properties of storage proteins of broad bean. *Agric. Biol. Chem.* **1979**, *43*, 577–583.

Sommerville, J.; Scheer, U. *Electron microscopy in molecular biology: a practical approach*; IRL Press: Oxford, U.K., 1987.

Valentine, R. C.; Shapiro, B. M.; Stadtman, E. R. Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli. Biochemistry* **1968**, *7*, 2143–2152.

Walsh, E. I. Developing yield potential of cereals. In *Cereal Production*; Gallagher, E. J., Ed.; Butterworth: London, 1984; Chapter 5, pp 69–93.

Yamada, T.; Aibara, S.; Morita, Y. Dissociation-association behaviour of arachin between dimeric and monomeric forms. *Agric. Biol. Chem.* **1979**, *43*, 2549–2556.

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