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Crystallographic Characterization and Molecular Symmetry of Edestin, a Legumin from Hemp

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Edestin, a legumin class reserve protein from hemp seeds having six identical subunits was crystallized from ammonium phosphate at pH 5 and subsequently characterized by X-ray diffraction. The crystals are of space group $R\bar{3}2$ with $a=127$ Å and $\gamma=116^\circ$ having an equivalent triply centered hexagonal cell of $a=b=215$ Å, $c=80$ Å. There is one hexameric protein in the rhombohedral unit cell, hence the subunits of the Edestin molecule must be arranged with 32 point group symmetry.

Keywords: crystallization; edestin; legumin; diffraction

There are two major classes of storage proteins in leguminous plants, vicilins and legumins. The former are about M_r 150,000 having three identical subunits, with each monomer in turn displaying two genetically and structurally homologous domains related by pseudo 2-fold symmetry. Thus, vicilin molecules have exact 3-fold, but pseudo 32, point group symmetry (Lawrence *et al.*, 1990; Ko *et al.*, 1993a). Two vicilin proteins, phaseolin (Lawrence *et al.*, 1990) and canavalin (Ko *et al.*, 1993a) have now been solved to moderately high resolution by X-ray diffraction analysis and found to be extremely similar. Their subunits contain homologous amino and carboxyl terminal domains that include an eight-stranded "Swiss Roll" β -barrel and a looping extension having some short α -helices.

Legumins were among the first proteins of any sort to be crystallized (for a review see McPherson, 1991), and were characterized in particular by Osborne (1892) in the late nineteenth century. Later work showed legumins to be about twice the molecular mass of the vicilins, or $M_r \approx 300,000$ to 340,000 and to be composed of six independent subunits (Bewley & Black, 1978; Derbyshire *et al.*, 1976). While legumins contain disulfide bridges, vicilins do not, and it is otherwise clear that legumins are not simply aggregates of vicilin subunits or their close homologs. Legumins are quite distinct from vicilins (Bewley & Black, 1978; Derbyshire *et al.*, 1976), though recent studies indicate some regions of sequence homology suggesting possible common ancestors (Gibbs *et al.*, 1989; Borroto & Dure, 1987).

Although legumins have been crystallized in abundant quantities from many plants, no high-resolution X-ray diffraction analysis of a specific legumin protein has yet been carried out. This may be attributed primarily to the generally small size to which these crystals grow and the inherent disorder they usually display. The most thorough characterization of any legumin crystals was by Drenth & Wiebenga (1955), who analyzed edestin from hemp seed, tobacco seed globulin, and excelsin from Brazil nuts. For most of their crystals, the investigators were able to show crystallographic symmetry approximately consistent with pseudo cubic, rhombohedral unit cells although the true symmetry was, they concluded, probably lower. In the end it appeared that all of the crystals contained some disorder and their pursuit by X-ray crystallography was abandoned.

We have now obtained large crystals of edestin, the major legumin protein of hemp seed. Although the extent of their diffraction pattern is still limited, they nonetheless show exact symmetry and allow us to deduce the molecular symmetry and probable overall shape of the hexamer.

Because of the Marijuana Tax Act, hemp seed is no longer available in the United States. Edestin, extracted from hemp seed is, however, and was purchased from Sigma Co. of St Louis, MO. This was dissolved in water at 50°C in the presence of trace amounts of NH_4OH , sufficient to maintain the pH above 9.0, to a final protein concentration of 30 mg/ml. The solution was clarified by centrifugation and filtration through 0.22 µm Millipore filters.

Preliminary crystallization conditions were determined using the Crystal Screen crystallization reagent kit (Hampton Research-Riverside, CA). Edestin was crystallized by vapor diffusion (McPherson, 1982, 1990) in CRYSCHEM sitting drop plates (Morris, *et al.*, 1989) at room temperature in 24 to 48 hours. The reservoirs of the optimized crystallization conditions contained 0.75 ml of 0.5 M ammonium phosphate with 0.05 M sodium citrate adjusted to pH 5.0. To prepare the protein microdrops, equal volumes, generally 250 μ l, of the protein solution and the reservoir were rapidly mixed prior to dispensing. A heavy precipitate formed almost at once and after 5 minutes this was removed by centrifugation. The clear supernatant, containing protein and reservoir components, was then deployed as 15 μ l droplets on the posts of the CRYSCHEM plate and the plates sealed with clear tape.

Crystals were mounted in quartz capillaries by conventional methods and an extensive series of precession photographs were recorded at 17°C using Buerger precession cameras on an Enraf-Nonius Diffractis 583 generator operated at 32 mA and 40 kV. The radiation was nickel-filtered, CuK α .

Photographs showed the intensities could be indexed on a rhombohedral lattice consistent with a real unit cell of $a=127$ Å and $\gamma=116^\circ$. The equivalent, triply centered hexagonal unit cell is $a=b=215$ Å and $c=80$ Å. The reciprocal lattice demonstrated 3 mm symmetry, based on both zero and upper level reflections, consistent only with 32 point group crystallographic symmetry. The crystals are, therefore, of space group $R32$.

The volume of the hexagonal unit cell containing three lattice points is 3.21×10^6 Å³ or 1.07×10^6 Å³ per rhombohedral lattice point. If a hexamer having molecular mass of 340,000 is assumed for one edestin molecule, and one of these is in turn taken to be the contents of the rhombohedral unit cell, then the volume to mass ratio, V_m , is found to be 3.14 Å³/dalton. This is consistent with values found for most other proteins (Matthews, 1968; Gilliland, 1988) and is almost certainly correct. Because the rhombohedral unit cell exhibits 32 point group symmetry, its contents must as well. Thus, the edestin molecule itself must possess this same 32 symmetry. This means that the six subunits of edestin must be related to one another by an exact 3-fold axis of symmetry and three dyad axes perpendicular to it.

While edestin crystals show reasonable stability in the X-ray beam, and respectable lifetime, the limit of resolution of the present crystals is not more than about 3.5 Å. We are, however, making progress in extending this limit, principally by refinement of crystal growth conditions and increased purification of the protein.

The edestin described here is quite characteristic of most, if not all, of the legumin family (Derbyshire *et al.*, 1976) and, therefore, most members of the group probably have their subunits related by the 32 symmetry elements found here. It is likely that

differential glycosylation of subunits, and subunits derived from different gene alleles, may result in the symmetry being only quasi-symmetry in some cases. It will, however, probably hold in most every other sense for other legumins. It is interesting that vicilin proteins, though half the size, also form pseudo hexamers of quasi 32 symmetry, when the internal structural repeats are taken into account. Perhaps this is a remnant of some common ancestral origin or, equally, it may result from some shared structural and/or functional necessity, such as constrained packing in the protein bodies of the seeds. In any case, it appears that the two classes of molecules share gross quaternary structure characteristics.

The vicilin trimer (or pseudo hexamer) is a disk-shaped molecule of diameter about 90 Å and thickness of 45 Å (Lawrence *et al.*, 1990; Ko *et al.*, 1993a). This molecule can be packed, in the case of canavalin, with pseudo 32 symmetry into an $R3$ rhombohedral unit cell of $a=81$ Å and $\gamma=113^\circ$ or an equivalent hexagonal cell of $a=b=135$ Å, $c=75$ Å (McPherson & Spencer, 1975; Ko *et al.*, 1993b). It might be concluded, given the edestin unit cell above, that the legumin subunits must also be arranged in an even larger disk of diameter approximately 145 Å but having about the same thickness. Thus, one would expect it to be a fairly open ring structure of subunits alternately oriented "up" and "down", and exhibiting a large channel through its center, even broader than the nearly 20 Å diameter hole seen in the vicilins.

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