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Characterisation and Crystallisation of an 11S Seed Storage Globulin from Coconut (*Cocos nucifera*)

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ABSTRACT

Cocosin, an 11S seed storage globulin from coconut, has been purified and characterised by HPLC gel filtration and anion-exchange chromatography. At least eight different subunit species have been identified. Despite this heterogeneity, cocosin crystallises very readily into forms typical of this class of protein. The molecular weight of the protein was in the range 250 000–300 000, higher than the value of 208 000 previously ascribed. A subunit molecular weight of approximately 54 000, together with the crystal symmetry, suggests that cocosin is a typical hexameric 11S globulin, and not a tetramer as was previously postulated.

INTRODUCTION

Coconuts are an important commodity in many tropical countries, particularly India and the Philippines. Although mostly valued for their oil, interest in extracting the protein component for human consumption is increasing (Rhee & Lusas, 1979). As well as being highly nutritious, the protein also has excellent functional properties with foaming and emulsifying capacities greater than many legume and oilseed proteins, including soya which is already used extensively as a food ingredient (Chakraborty *et al.*, 1987).

The majority of the protein in the coconut endosperm is cocosin, one of a

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large class of seed storage proteins known as 11S globulins. These proteins are generally hexamers (MWt 300 000–360 000), with each subunit comprising an acidic and basic chain linked by a disulphide bond (for review see Wright, 1987). Although the subunits are similar they are not identical, being derived from a multigene family, and molecules of these globulins exhibit considerable heterogeneity. Ultracentrifugation studies of cocosin show it to have a somewhat lower molecular weight of 208 000 (Sjögren & Spychalski, 1930), which has led to the suggestion that it is a tetramer rather than a hexamer (Dieckert & Dieckert, 1976).

The sequences of many 11S globulin subunits have been determined (Wright, 1987) and indicate very close homologies. Secondary and quaternary structures have also been predicted (I'Anson *et al.*, 1987; Wright, 1987). However, little is known about the detailed tertiary structure of these proteins, an essential prerequisite for any comprehensive understanding of their food functionalities. The prime reason for this lack of structural evaluation is because most 11S proteins do not crystallise readily, and those crystals that have been reported were not suitable for X-ray diffraction studies (Wright, 1987). Preliminary work, however, undertaken in this laboratory revealed that cocosin crystallises quite easily, meriting a closer investigation.

This investigation was performed to (1) characterise the coconut protein in greater detail than in previous studies, and to explain some of the apparent differences between it and other 11S globulins; and (2) to look more closely at the protein's different crystal forms.

MATERIALS AND METHODS

Preparation of cocosin

The coconuts used were of a commercial variety grown in the Dominican Republic. Meat from the nuts was ground in a coffee grinder and the fat removed by extraction with petroleum ether. Cocosin was extracted and purified from the dried meal using a modified method for the extraction and purification of excelsin, the 11S globulin from brazil nut (Drenth & Wiebenga, 1955). Approximately 20 g of dried meal was extracted overnight in 300 ml 0·2M Na_2HPO_4, 0·2M KH_2PO_4, 10% NaCl, pH 6·8, at room temperature, and for a further 30 min at 40°C. The resulting slurry was filtered successively through Whatman No. 1 filter paper, 0·8, 0·45 and 0·22 μm Millipore filters. Butanol (150 ml) was then stirred with the filtrate for 1 h at room temperature. After centrifugation at 16 000*g* for 30 min the butanol was decanted off and the remaining protein solution was dialysed

against several changes of distilled water at 1°C for 2 days. The resulting precipitate was centrifuged at 16 000*g* for 30 min, resuspended in 50 ml 4% ammonium sulphate solution and centrifuged again at 38 000*g* for 1 h. Ammonium sulphate was added to the clarified solution to 33% saturation and centrifuged as before. The resulting supernatant was then increased to 50% saturation with respect to ammonium sulphate and the precipitate collected by centrifugation. Ammonium sulphate was removed from the pellet by dialysis against distilled water and the material stored as a slurry at +1°C until required for crystallisation and gel filtration experiments.

Gel filtration by HPLC

All HPLC analyses were performed using a fast protein liquid chromatography (FPLC) system (Pharmacia) with P-500 pumps and a UV-1 detection unit monitoring the absorbance at 280 nm. All solutions for FPLC were filtered through 0·22 μm Millipore membranes. Prior to FPLC analysis, the sample was centrifuged in an MSE Micro Centaur at full speed for 2 min.

Gel filtration analyses were performed on a Superose 6 HR 10/30 column equilibrated in 50 mM Tris buffer, pH 8·2, containing 0·5M NaCl. The cocosin slurry was dissolved in the above column buffer to a concentration of 10 mg/ml. An aliquot of 200 ml (2 mg cocosin) was then injected onto the column and a flow rate of 0·4 ml/min used throughout. Material eluted in the main peak from this column (see Fig. 1(a)) was used as the starting material for anion-exchange chromatography and gel electrophoresis analysis.

FPLC anion-exchange chromatography

The subunit composition of cocosin was assessed by the method of Lambert *et al.* (1987). The sample was denatured by the addition of urea to 6M and excess salt was removed by application to a PD-10 (Pharmacia) column packed with Sephadex G-25 equilibrated with 50 mM Tris, pH 8·0, containing 6M urea. Cocosin (0·5 mg) in 0·5 ml buffer was injected onto a Mono-Q 5/5 (Pharmacia) anion-exchange column equilibrated in the sample buffer. Unbound material was eluted and the column subsequently developed with a linear gradient of sodium chloride (0–500 mM in 60 min). The flow rate used was 1·0 ml/min throughout. All urea-containing solutions were made fresh daily and all analyses performed at room temperature.

SDS gel electrophoresis

SDS-PAGE in a discontinuous buffer system was performed according to the method of Laemmli (1970). Sample preparation and electrophoresis conditions were as described by Lambert *et al.* (1987).

Protein assays

The concentrations of coconut 11S globulin were evaluated by the method of Lowry *et al.* (1951).

Crystallisation

One millilitre of 10% NaCl was saturated with cocosin and the resulting solution filtered through a 0·22 μm Millipore filter. The filtrate contained 10–15 mg of protein per millilitre. Some 0·2 ml of the filtered solution was added to 0·4 ml distilled water and the precipitate redissolved by warming to 60°C. Crystals formed readily on cooling back to room temperature. Photomicrographs of crystals were taken with a range of magnifications—see Fig. 5.

RESULTS AND DISCUSSION

Figure 1(a) shows the gel filtration profile of cocosin after ammonium sulphate precipitation. In addition to the main peak, some higher molecular

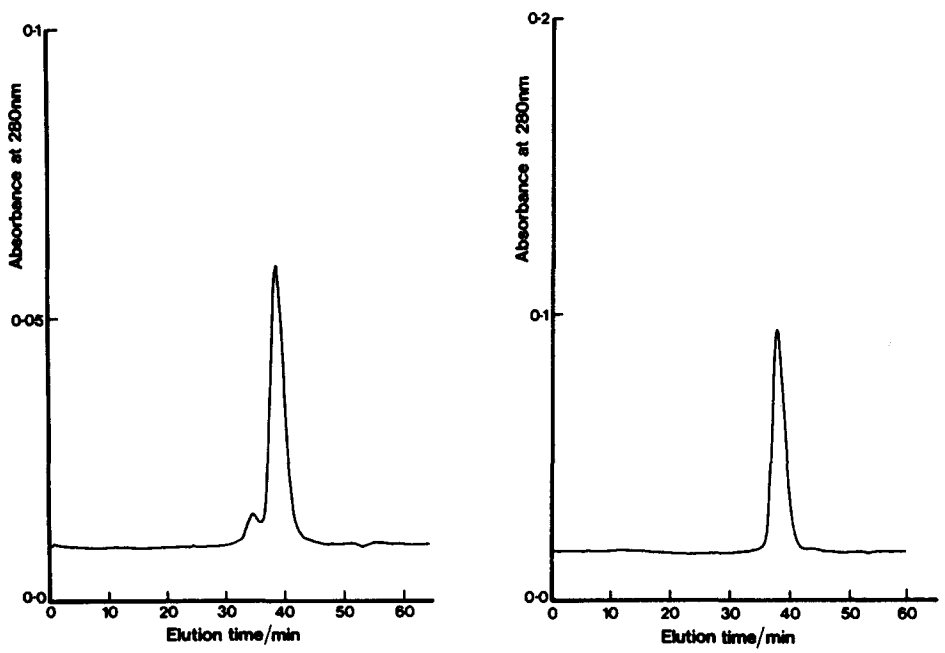

Fig. 1. HPLC gel filtration profiles of cocosin (a) after ammonium sulphate precipitation and (b) after gel filtration. Samples were prepared and analysed as described in the Methods section.

weight material, possibly cocosin aggregates, is also present. We did not, however, observe a third low molecular weight peak seen by Hagenmeier *et al.* (1972), nor did we observe any evidence of breakdown which has hampered previous investigations (Sjögren & Spychalski, 1930). Possibly the more rigorous purification procedure used in this study has eliminated any such material. Figure 1(b) shows a profile of a sample from the main peak rechromatographed, illustrating the purity of the starting material used in subsequent anion-exchange chromatography studies. The relative molecular weight of this material was adjudged to be 250–300 000 when compared with proteins of known molecular weight (Fig. 2). This value is considerably higher than the 208 000 reported previously by Sjögren and Spychalski (1930).

SDS-PAGE analysis of cocosin was performed under reducing conditions to cleave the acidic and basic chains which comprise each subunit. Figure 3

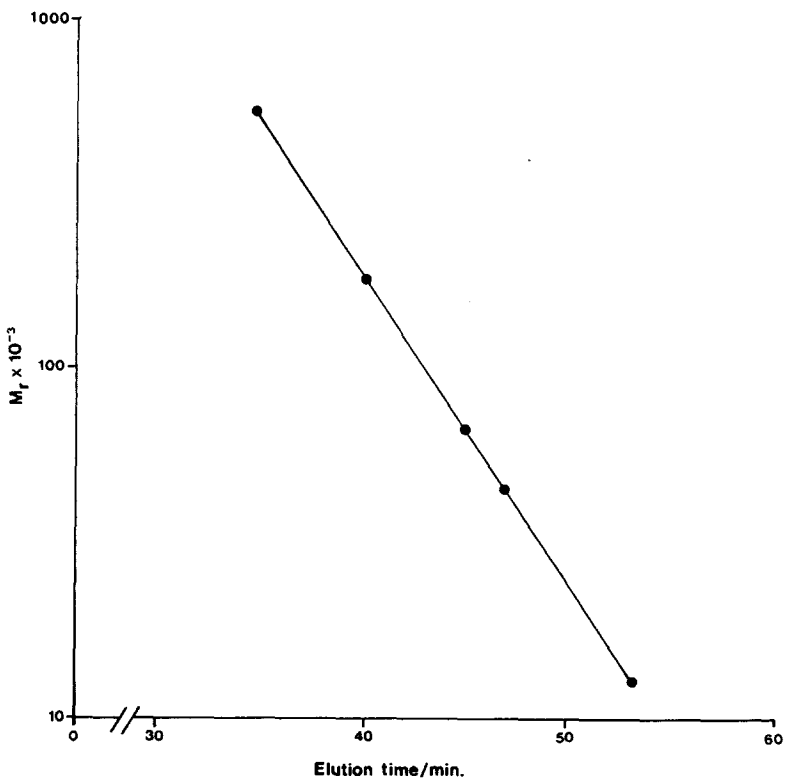

Fig. 2. Calibration curve for the determination of molecular weight using a Superose 6 gel filtration column. The standard proteins used were: porcine thyroglobulin, MWt = 520 000; *Aspergillus niger* glucose oxidase, MWt = 180 000; bovine serum albumin, MWt = 67 000; hen ovalbumin, MWt = 45 000; horse heart cytochrome c, MWt = 12 500. Samples were prepared and analysed as described in the Methods section.

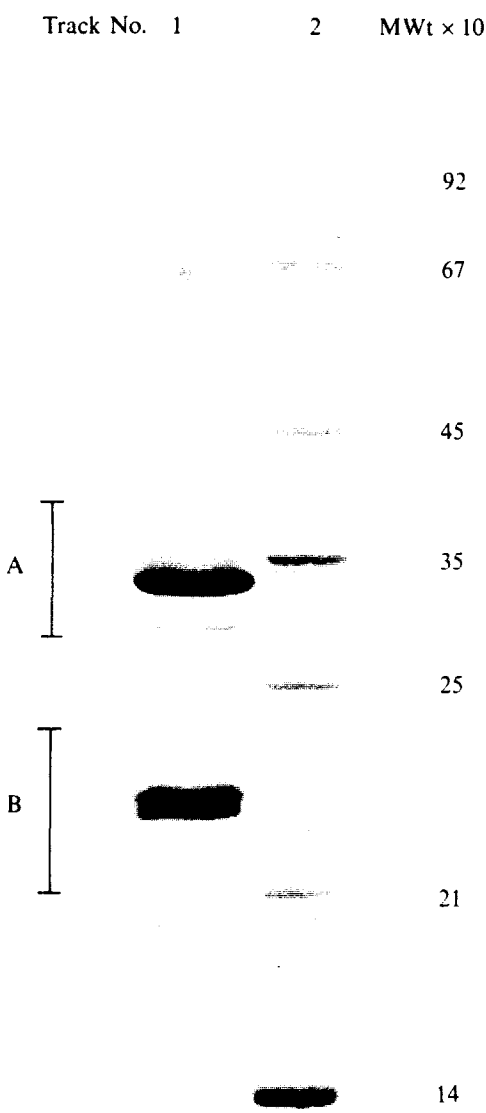

Fig. 3. SDS polyacrylamide gel electrophoresis of cocosin under reducing conditions. Acidic and basic polypeptide chains are indicated by letters A and B respectively. The right-hand track contains MWt standard proteins: rabbit muscle phosphorylase b, MWt = 92 000; bovine serum albumin, MWt = 67 000; hen ovalbumin, MWt = 45 000; rabbit muscle lactate dehydrogenase, MWt = 35 000; bovine pancreas chymotrypsinogen, MWt = 25 000; soya bean trypsin inhibitor, MWt = 21 000; turkey lysozyme, MWt = 14 000. Samples were prepared and analysed as described in the Methods section.

reveals a set of bands at about 32 000 MWt (acidic polypeptides) and another set at approximately 22 000 MWt (basic polypeptides), which is consistent with other 11S globulins (Lambert *et al.*, 1987). A subunit MWt of approximately 54 000 can therefore be deduced, indicating that there are five or six subunits per multimer, as opposed to the tetrameric structure originally proposed by Dieckert and Dieckert (1976). From the above data and the shape of the crystals described below it seems improbable that cocosin has anything other than a hexameric configuration.

We have recently described a rapid and sensitive method for the assessment of subunit heterogeneity in 11S globulins (Lambert *et al.*, 1987). Using FPLC anion-exchange chromatography under dissociating conditions, the subunit composition of several 11S globulins was determined. Figure 4 shows the profile obtained in the present study for cocosin. It can be seen that the monodisperse material revealed by gel filtration is, in fact, composed of several subunits each carrying slightly different charges. At least eight different species can be identified. Compared with other 11S globulins (Lambert *et al.*, 1987) the cocosin profile resembles most closely that of sesame 11S, being typified by significant amounts of unbound material and the majority of subunits eluting prior to 30 min, suggesting that cocosin and sesame subunits are more positively charged compared with other 11S species.

Figure 5 shows examples of typical crystals obtained from cocosin. Figure

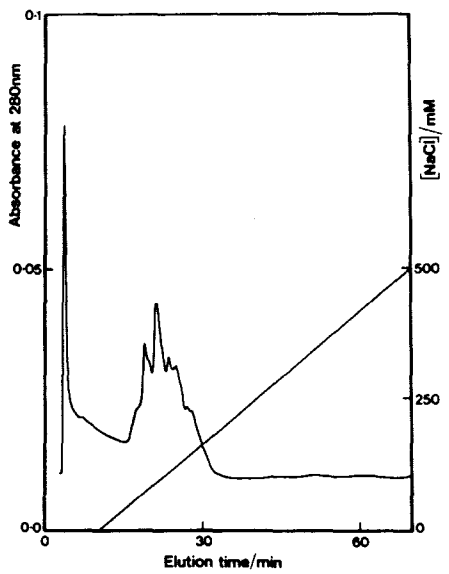

Fig. 4. FPLC analysis of cocosin on a Mono-Q anion-exchange column. The straight line indicates the sodium chloride gradient. The sample was prepared and analysed as described in the Methods section.

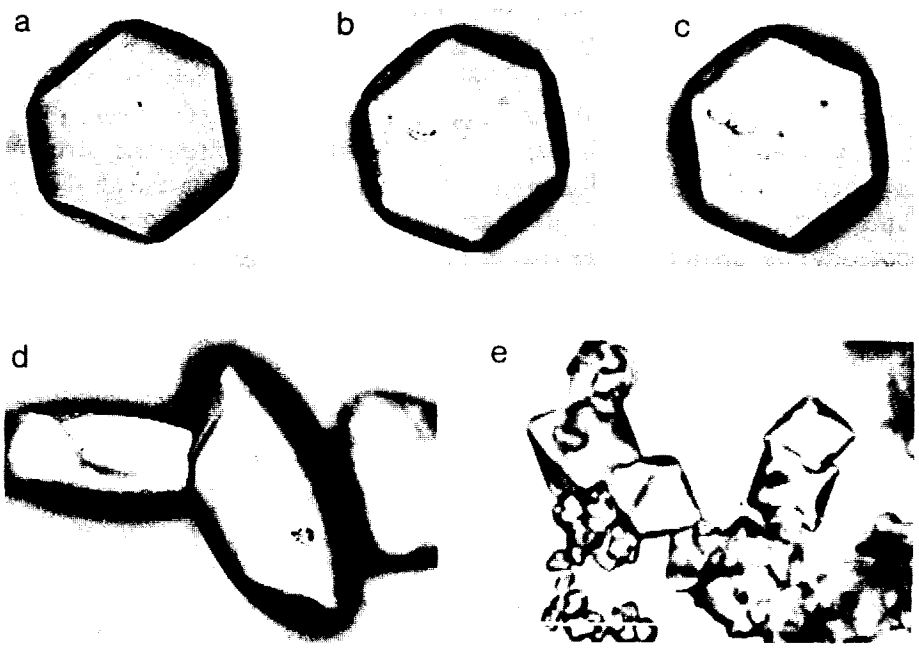

Fig. 5. Crystallised cocosin: (a)–(c) through-focus series of a hexagonal crystal viewed from above; (d) two hexagonal crystals viewed end-on; (e) octahedral crystals. (a)–(d) Scale bar = 0·5 μm; (e) scale bar = 25 μm. Samples were prepared and analysed as described in the Methods section.

5(a), (b) and (c) show a through-focus series of a hexagonal cocosin crystal seen from above and Fig. 5(d) a similar crystal seen sideways on. Such crystals are very similar to those obtained from brazil nut 11S globulin (Osborne, 1892). These were described as being the same shape as the middle one of three pieces formed when an octahedron is cut into three equal parts parallel to one face. This is also true for these crystals, except that the faces of the cocosin crystals are clearly curved rather than planar. As well as hexagonal plates, octahedral crystals with planar faces were also observed (Fig. 5(e)), which are also typical for globulins of this type (see, for example, Osborne (1892)).

To our knowledge this is the first detailed report of cocosin crystals, and thus cocosin can be grouped with the other crystallisable 11S seed globulins, e.g. brazil nut and oat (Osborne, 1892), sesame (Hasegawa *et al.*, 1978), pumpkin (Hara *et al.*, 1976) and edestin and tobacco (Drenth & Wiebenga, 1955). The reason why some 11S proteins crystallise readily while others, like soya and pea, do not is unclear. The composition and packing of component subunits and the resulting surface charge distribution are possible explanations, but other factors could be involved.

Unfortunately, in common with all 11S protein crystals produced to date, those reported herein are of little value for extensive X-ray diffraction studies. It may be feasible to use the cocosin crystals as 'seeds' for growing larger and better quality crystals so at least the 'space grouping' may be determined. Given the great strides that have been made over the last decade in our understanding of seed globulins from many viewpoints (Wright, 1987), it is the lack of a detailed crystal structure which currently is severely hampering the progress of research into this important class of food proteins.

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