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11S and 7S Globulins of Coconut (*Cocos nucifera* L.): Purification and Characterization†

ROBERTA N. GARCIA, RAMBO V. AROCENA, ANTONIO C. LAURENA, AND EVELYN MAE TECSON-MENDOZA*

Institute of Plant Breeding, College of Agriculture, University of the Philippines Los Baños, College, Laguna 4031, Philippines

Total globulins extracted with 0.4 M NaCl in buffer from coconut endosperm separated into two peaks on gel filtration: peak I corresponding to 11S globulin or cocosin and peak II to 7S globulin with native molecular weights of 326 000 and 156 000, respectively. The percent composition of total globulins was estimated to be 11S, 86% and 7S, 14%. On SDS−PAGE, cocosin resolved into two closely migrating bands at approximately 34 000 (acidic polypeptide) and another set of 2 bands at 24 000 (basic polypeptide). Each set consisted of one darkly stained band and one lightly stained band. The 7S globulin consisted of three bands of 16 000, 22 000, and 24 000. Three isoforms of cocosin were identified after anion exchange chromatography. Cocosin, but not the 7S, was found to have disulfide bonds. Using periodic acid-Schiff's reagent, all of the bands of cocosin on SDS−PAGE were positive for carbohydrate. However, when con A-peroxidase was used, only the basic polypeptide stained positively for carbohydrate. For the 7S globulin, no carbohydrate group was detected using the PAS and con A-peroxidase tests. The 7S globulin was easily extracted with 0.10−0.15 M NaCl, whereas cocosin was extracted with 0.35 M NaCl. The N-terminal amino acid sequences of the 34 k band and 24 k band of cocosin were SVRSVNEFRXE and GLEETQ, respectively, and that of the 7S was EQEDPELQK.

KEYWORDS: Globulins; coconut proteins; cocosin; storage proteins

INTRODUCTION

Coconut, *Cocos nucifera*, is one of the most economically important palm species and is cultivated mainly for the endosperm. The majority of the coconut globulins is the 11S storage globulin called cocosin with a molecular weight of 300 000−360 000 (*1*). Under reduced conditions of electrophoresis, two sets of bands of cocosin were noted, a set at about 30 200 and another at 22 000. On anion exchange chromatography under dissociating conditions, Carr et al. (*1*) further observed at least eight peaks. They also crystallized cocosin and obtained hexagonal as well as octahedral crystals. More recently, Balasundaresan et al. (*2*) reported the crystallization of cocosin producing rhombohedral crystals.

DeMason and Chandra Sekhar (*3*) reported that reduced coconut proteins fractionated into 7 major bands ranging from 55 000 to 17 000 and that these bands were glycosylated. Kwan et al. (*4*) reported also 7 major bands with similar molecular weights ranging from 14 000 to 52 000. Earlier Wallace and Dieckert (*5*) purified a coconut globulin having a similar banding pattern with cocosin. They called the globulin fraction S2-45-45. This fraction was dominated by two molecular weight classes of polypeptides with molecular weight of 30 000 and 19 000 on SDS−PAGE. In addition, a small amount of polypeptide with molecular weight of 51 000 was present. DeMason and Chandra Sekhar (*3*) observed that reduced proteins from the coconut endosperm showed prominent bands at 55 000 (doublet), 34 700, 31 600, 25 700, 22 400 (doublet), 20 900, and 17 000.

The 7S−9S globulins are trimeric proteins of MW∼ 150 000 to 190 000 that lack sulfur-containing amino acids and hence cannot form disulfide bonds. They are the major storage proteins in mungbean (*6*), French bean and winged bean (*7*) and in the embryos of oil palm (*8*) and date palm (*9*). For soybean, it accounts for 30% of the total globulins (*10*). On the other hand, the 7S globulins are minor components in peas (*11*).

To understand better and obtain more definitive information on the physicochemical nature of coconut proteins, we undertook this study of coconut globulins to provide baseline information for future researches. This paper thus reports on the isolation and purification of the 11S globulins or cocosin and the 7S globulin from the mature endosperm of coconut and their characterization as to molecular weight, number and molecular weight of subunits, N-terminal amino acid sequences, presence of disulfide linkage and carbohydrate moiety, and solubility in salt solutions. The isolation and characterization of the coconut 7S globulin is reported for the first time.

* To whom correspondence should be addressed. E-mail: emtm@laguna.net.

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EXPERIMENTAL PROCEDURES

Materials. The endosperm of mature (11−12 mo) coconut var Laguna Tall was grated, ground in liquid nitrogen, defatted twice by stirring for 1 h with *n*-hexane (1:10 w/v) and dried under the hood.

Extraction of Proteins. Total proteins were extracted from 5 g of defatted coconut meal with 90 mL of extraction buffer (0.4 M NaCl in 35 mM potassium phosphate buffer, pH 7.6 with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol and 0.02% sodium azide). The mixture was stirred for 1 h on an ice bath. The homogenate was passed through four layers of cheesecloth and then centrifuged at 23 500*g* for 15 min. The supernatant was collected and dialyzed against distilled water containing 10 mM β-mercaptoethanol for 48 h. The dialysate was centrifuged for 15 min at 23 500*g* to separate the globulins from the albumins. The globulin precipitate was washed three times with distilled water containing 10 mM β-mercaptoethanol.

Gel Filtration Chromatography. Thirty mg of globulin was loaded onto a HiLoad 26/60 Superdex 200 (Amersham Pharmacia) connected to a Fast Protein Liquid Chromatography (FPLC) system with 35 mM potassium phosphate buffer pH 7.6 containing 0.4 M NaCl, 10 mM β-mercaptoethanol and 1 mM EDTA as elution buffer. Flow rate was controlled at 1.0 mL/min. Fractions were routinely analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE).

The standard curve for the Superdex 200 column was prepared using the following protein standards and their respective molecular weights: bovine serum albumin, 66 000; proglycinin A1aB1b homotrimer, 160 000; and glycinin A3B4 monohexamer, 333 000. The progylcinin, glycinin and BSA standards were courtesy of Dr. S. Utsumi and Dr. M. Adachi of Kyoto University, Japan.

Anion Exchange Chromatography. The cocosin fraction obtained from FPLC-gel filtration was pooled, concentrated using Ultra-Free Cell (Millipore), and applied on a RESOURCE Q anion exchange column (1 mL). The sample was eluted using a gradient of 0−0.5 M NaCl in 20 mM Tris-HCl, pH 8.0 with the FPLC system at a flow rate of 1.0 mL/min. Fractions were analyzed by SDS−PAGE.

Subunit Molecular Weight Determination by SDS-Polyacrylamide Gel Electrophoresis (SDS−PAGE). Protein fractions were run on an 11% discontinuous denaturing gel (*12*) at 110 V for 1 h and 40 min and stained with 0.25% Coomassie blue R250 solution using a minigel electrophoresis apparatus (ATTO Corp., Japan). The gels were destained with methanol:acetic acid:water (5:5:1 v/v). The molecular weight of the subunits was measured by using the low molecular weight calibration kit (Pharmacia Biotech) consisting of the following proteins: phosphorylase b (94 000); bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

Gel patterns were scanned using the QuantiScan software (Biosoft) to determine the percent composition of the different proteins.

Detection of Presence of Carbohydrate Moiety. The presence of the carbohydrate moiety of cocosin and of the 7S globulin was tested using two methods: (1) the general PAS (periodic acid-Schiff) staining method (*13*) and (2) by peroxidase-conjugated Con A. Samples were run on SDS−PAGE. For the first test, the gels were directly stained with PAS. For the second test, the electrophoresed bands on the gels were transferred onto a nitrocellulose membrane and detected using peroxidase-conjugated Con A (Seikagaku Kogyo) as described by Katsube et al. (*14*).

Detection of Presence of Disulfide Linkage. Cocosin and 7S globulin samples were dialyzed against the elution buffer without β-mercaptoethanol for 24 h and run on SDS−PAGE in the absence of β-mercaptoethanol.

Protein Measurement. The protein content of samples was determined using the Bradford method (*15*) with bovine serum albumin as standard.

Solubility in Different Concentrations of NaCl. Total globulins were sequentially treated with varying concentrations of sodium chloride (NaCl) from 0 to 0.4 M, by gently pipetting the protein solution 20 times, followed by centrifugation at 23 500*g* for 10 min. The dissolved protein was analyzed using SDS−PAGE.

N-Terminal Amino Acid Sequencing. Purified proteins were run on SDS−PAGE in the presence of β-mercaptoethanol and the separated protein bands transferred onto PVDF membrane by the semi-dry blotting method. Blotting was done for 2 h at 45 mA. The membrane was stained with 0.25% Coomassie R250 for 5 min followed by destaining with methanol:acetic acid:water (45:10:45) or with 0.1% Ponceau S in 0.1% acetic acid followed by destaining with 0.1% acetic acid. N-terminal amino acid sequence of the 7S was determined using a protein sequencer Procise 492 (Applied Biosystems Inc) either gas-phase or liquid-phase system at Kyoto University Graduate School of Agriculture, Uji, Kyoto, Japan and that of cocosin at Newcastle Protein, University of Newcastle, Callaghan, NSW Australia, with a Perkin-Elmer ABI model 494 Procise Sequencer with an on-line 140C microgradient HPLC.

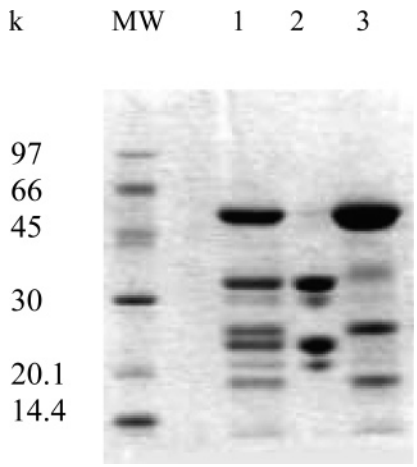

Figure 1. SDS–PAGE profile of proteins extracted from coconut meal. Lanes (MW) molecular weight markers; (1) total protein; (2) globulins; (3); albumins.

Sequence Analysis. Comparison of the N-terminal sequences of the acidic and basic subunits of the 11S globulin and the 7S globulin was done using the FASTA database version 3.0t74 December 1996 (*16*) and the BLAST database (*17*).

RESULTS AND DISCUSSION

Storage Proteins of Coconut Endosperm. *Fractionation.* Albumin and globulins were fractionated and isolated from mature coconut endosperm based on their solubility in water and salt solution. SDS−PAGE profiles of the total proteins, globulins, and albumins are shown in **Figure 1**. Based on the relative intensities of the bands, the globulins were estimated to comprise 75% of the total proteins, and albumins, 25%. The previously reported composition of coconut proteins was 62−67% for globulins and 21−31% for albumins (*18*). Kwan et al. (*4*) reported a similar value for albumins (21%) and a lower value for globulins (40%).

Purification of the 7S and 11S Globulins. Application of total globulins on Superdex 200 or Sephacryl S200 resulted in the separation of two major peaks with molecular weight of 326 000 and 156 000, (**Figure 2**) corresponding to 11S or cocosin and 7S globulin, respectively. This is the first report on the isolation of the smaller 7S component of coconut globulins. Based on the relative peaks, the composition of total globulins is estimated to be 11S, 86% and 7S, 14%.

On SDS−PAGE, peak I or cocosin, resolved into two sets of two bands, one set at approximately 34 000 and another set of two bands at 24 000 (**Figure 3A**). These are termed the acidic and basic polypeptides, respectively and affirm previous results (*1*). A band with molecular weight of 55 000 was observed, and it was noted that its amount was greater in some gel filtration runs as evidenced by a thicker band compared with the 34 000 and 24 000 bands. This 55 000 band was earlier observed by Kwan et al. (*4*) and DeMason and Chandra Sekhar (*3*). As is

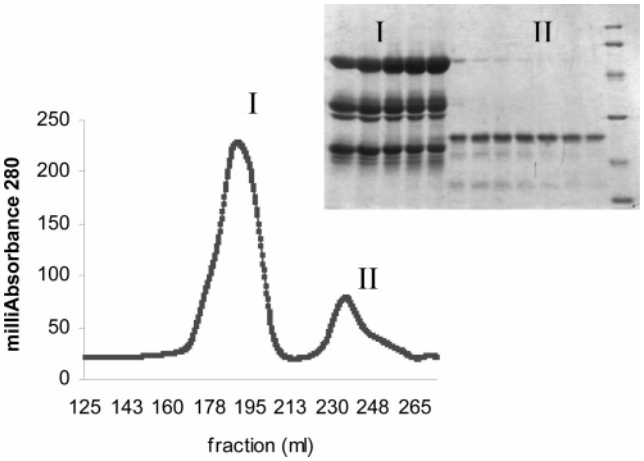

Figure 2. Gel filtration profile of coconut globulins on HiLoad 26/60 Superdex 200 column. Peak I, 11S; peak II, 7S.

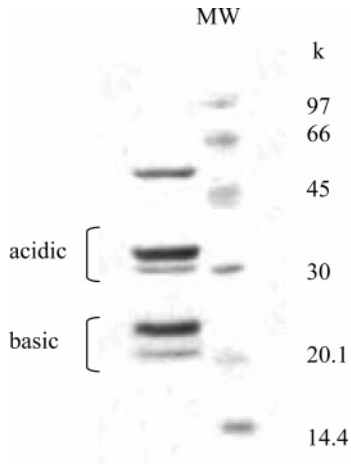

Figure 3. SDS–PAGE profile of cocosin purified by FPLC-gel filtration using HiLoad 26/60 Superdex 200 column.

shown later, this band at 55 000 refers to the recombined acidic and basic polypeptides of the 11S globulin.

The observed molecular weight of cocosin of 326 000 with subunit molecular weight of 24 000 and 34 000 compares well with those of other 11S globulins. Soybean glycinin has a native molecular weight of 300 000−380 000 and subunit molecular weight of 22 000 and 35 000 (*19*), whereas mungbean legumin has a native molecular weight of 360 000 and subunit molecular weight of 24 000 and 40 000 (*6*). The highly polymerized amaranth globulin also showed the characteristic SDS−PAGE pattern of 11S globulins: having two bands, 30 000 and 20 000 (*20*, *21*). A minor band at 55 000 was also reported for amaranth (*21*). Similar banding patterns and values were also obtained for the 11S globulin of sesame seeds (*22*). Marcone et al. (*23*) described a major 11S-like globulin in pili nut which was composed of two main polypeptides of 22 600 and 31 600. In oats, the major storage protein was found to be an 11S globulin (*24*), whereas in rice, the major storage protein, glutelin, was found to have 11S globulin-like properties (*25*).

On the other hand, peak II corresponding to 7S globulin with native molecular weight of 156 000 exhibited four bands on SDS−PAGE with molecular weights of 55 000, 24 000, 22 000, and 16 000 (**Figure 4**). The 24 000 as the major band and the two smaller ones may be fragments, while the 55 000 band would constitute the monomer. This minor component was detected only when 100 mg of the sample was applied on the HiLoad 26/60 Superdex 200 column. It was also observed that

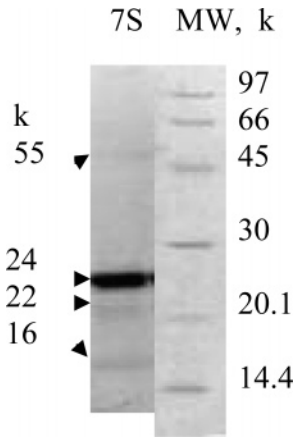

Figure 4. SDS–PAGE profile of coconut 7S globulin.

Table 1. Summary of Molecular Weight, Number of Polypeptides/Subunits, and Percent of Globulins from Coconut Endosperm

	mol wt × 10^3		
globulin	native	subunit/peptide	% of total globulins
7S	156 000	16, 22, 24	14
11S (cocosin)	326 000	24, 34, 55	86

the 55 000 polypeptide was easily degraded as evidenced by faint bands on succeeding SDS−PAGE analyses. **Table 1** summarizes the native and subunit molecular weights of the coconut 11S and 7S and their percent composition.

The 7S coconut globulin belongs to the 7S-9S globulins grouped as vicilins and which are generally characterized by a trimeric organization with an oligomeric molecular mass of 150 000−200 000. The soybean β-conglycinin is a trimer having a molecular mass of 150 000−200 000 and consists of four types of subunits: α' (72 000), α (68 000), β (52 000), and a minor subunit γ (52 000) (*19*, *26*). The mungbean 8S has a molecular mass of 200 000 and consists of four subunits of 64 000, 48 000, 32 000, and 26 000 (*6*). Immunoblot analysis of coconut endosperm proteins revealed a minor band at 67 000 and two minor bands at 22 000 which were recognized by antibodies to soybean 7S conglycinin (*3*). This indicates the presence of the vicilin-type of protein in coconut endosperm. The present study showed the 16 000 to 24 000 bands of 7S but none of the 67 000 observed by DeMason and Chandra Sekhar (*3*). Tecson-Mendoza et al. (*6*) showed that antibodies to β-conglycinin cross-reacted with the major bands of mungbean vicilin and the 28 000 band of basic 7S but not with 11S globulins.

Isoforms of Cocosin. When the cocosin sample was resolved on an anion chromatography column, at least three isoforms were observed (**Figure 5**). Peaks 1, 2, and 3 had very similar SDS−PAGE patterns characteristic of the cocosin. Carr et al. (*1*) reported that at least eight peaks were obtained on anion exchange chromatography. Our results also indicate that the single protein peak obtained from gel filtration is composed of several polypeptides of different charges. Tai et al. (*22*) reported three 11S globulin isoforms in sesame seed of molecular masses 60 000, 55 000, and 50 000, respectively. On the other hand, Bernardo et al. (*27*) identified and characterized three isoforms of the 8S globulins isolated from a mungbean cDNA library.

Detection of Disulfide Linkage in Cocosin and 7S Globulin. In the presence of β-mercaptoethanol, the acidic (34 000) and basic polypeptides (24 000) of cocosin were evident (**Figure 6**). In the absence of β-mercaptoethanol, the two bands combined to form the 55 000 band as shown by a more intense

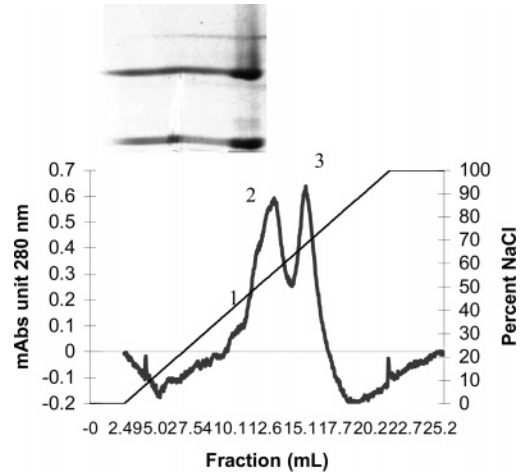

Figure 5. Anion exchange chromatography of cocosin on Resource Q column. At least three isoforms were separated (1, 2, and 3). A similar SDS – PAGE pattern was observed for all 3 isoforms. Inset shows the SDS–PAGE pattern of isoform 2.

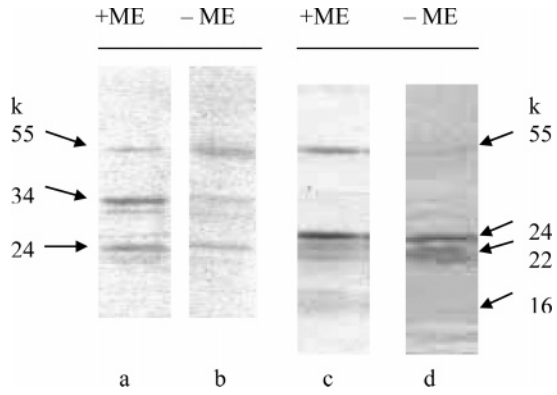

Figure 6. Cocosin (**a** and **b**) and 7S globulin (**c** and **d**) in the presence and absence of β-mercaptoethanol. For cocosin, arrows show acidic and basic subunits and their complex. The protein samples were dialyzed against phosphate buffer pH 7.6 without β-mercaptoethanol (β-ME) for 24 h and then electrophoresed under denaturing conditions.

55 000 band compared with the other bands and the electrophoretic profile in the presence of the reducing agent. These results indicate the presence of a disulfide bridge that links the acidic and basic polypeptides. This explains the presence of a 55 000 band in the cocosin band profiles from gel filtration runs in our study and in other reports (*1, 3, 4*).

However, the coconut 7S globulin showed no disulfide bonds as indicated by similar band intensities in the presence, as well as, in the absence of β-mercaptoethanol (**Figure 6**). Thus, the 7S in coconut is also comparable to pea vicilin (*11*), mungbean vicilin (*6*), alfin (*28*), and the 7S globulin of oil palm embryo (*8*), which lacked disulfide bonds. The 7S globulins have no disulfide linkages mostly due to a deficiency in sulfur-containing amino acids such as methionine and cysteine.

Similarly, the two bands of mungbean 11S (40 000 and 24 000), combined to form ~60 000 band in the absence of β-mercaptoethanol (*6*). The acidic and basic polypeptides of soybean glycinin were joined together by a disulfide bond (*29*). The hexameric quaternary structure of cocosin as suggested by Carr et al. (*1*) is the most thermodynamically favorable in which the disulfide linkages contribute to strengthen the molecular stability. Current results indicate that cocosin consists of six monomers of 55 000 and a native molecular weight of 326 000.

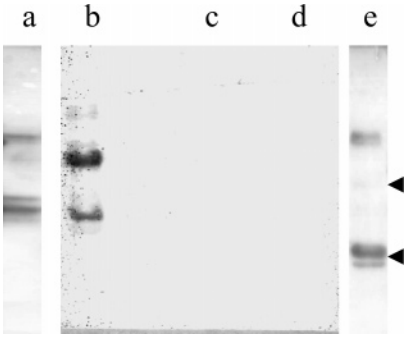

Figure 7. Detection of carbohydrates in coconut cocosin by peroxidase-conjugated con A. **a**, mungbean basic 7S; **b**, mungbean 8S; **c**, coconut 7S; **d**, soybean glycinin; **e**, cocosin. Arrowheads on lane e denote the acidic and basic polypeptides.

The 11S globulins have two pairs of highly conserved cysteine residues (*30*). One is involved in an intrachain disulfide bond in the acidic chain, whereas the other forms an interchain disulfide bond that links the acidic and basic polypeptides (*29*). Using an in vitro assembly system involving faba bean 11S globulin and tobacco transformed with mutant legumin genes, Jung et al. (*31*) demonstrated that the interchain disulfide bond is particularly important in the assembly of the 11S globulins. In soybean glycinin, the highly conserved interchain disulfide bond is buried in the interface (*32*).

Detection of Carbohydrate Moiety in Cocosin and 7S Globulin. Treatment of SDS−PAGE gels with PAS resulted in the staining of all of the bands of cocosin (data not shown), similar to the report of DeMason and Chandra Sekhar (*3*). However, when peroxidase-conjugated con A was used, only the basic band (24 000) of cocosin and the recombined protein (presumably also with the basic band) were stained (**Figure 7**) indicating the presence of carbohydrate moiety. The control samples used were glycinin (negative control) and mungbean vicilin and basic 7S (positive control). The latter were shown to react positively to peroxidase-con A (*6*). Glycosylation is not commonly observed among 11S, but it was reported to be present in the 12S globulin of lupin (*33*). Con-A conjugated to peroxidase gave higher detection sensitivity to the basic cocosin polypeptide.

The 7S globulin of coconut was observed to have no carbohydrate moiety since no band was formed using Con A-HRP staining (**Figure 7**) and PAS staining for carbohydrates (data not shown). This is a very interesting finding, since the 7S globulins usually contain mannose and glucosamine attached by an *N*-glycosidic linkage between *N*-acetyl-D-glucosamine and asparagines. However, this could be a first reported case of a 7S globulin having no carbohydrate moiety attached to the protein. In the same manner, the uniqueness of the coconut globulins is described by the presence of a carbohydrate group in the basic polypeptide of cocosin, a property that is rarely observed among 11S globulins.

Ericson and Chrispeels (*34*) obtained 1% glucosamine in the 11S legumin of *Phaseolus aureus*. However, Tecson-Mendoza et al. (*6*) showed that only vicilin and the basic 7S but not the 11S legumin of mungbean was positive for carbohydrate moiety. The results of Ericson and Chrispeels (34) were explained as being due to the difficulty in separating the 8S from the 11S globulin (*6*).

Solubility in Different Concentrations of Sodium Chloride. Globulins started to dissolve in as low as 0.01 M NaCl (**Figure 8**). Maximum dissolution was observed at 0.35 and 0.40 M NaCl. At 0.40 M NaCl, the entire globulin pellet was observed

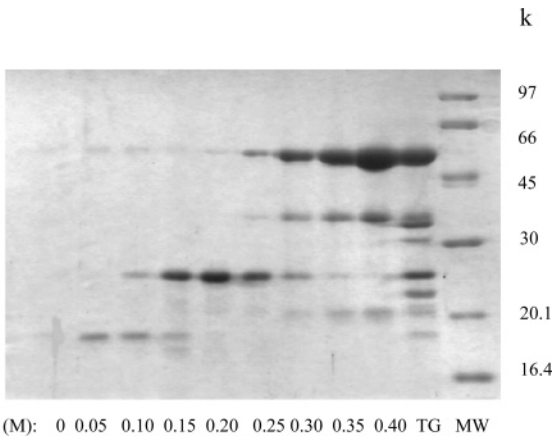

Figure 8. SDS–PAGE patterns of coconut globulins extracted sequentially with 0–0.40 M NaCl. TG, total globulins; MW, molecular weight markers.

Table 2. N-terminal Amino Acid Sequence of Coconut 11S (cocosin) and 7S Globulins and Sequence Analysis with Other Storage Proteins

globulin	*k*	N-terminal amino acid sequence	sequence analysis[a]
cocosin acidic subunit	34	SVRSVNEFRXE	71% identity in 7 amino acid (aa) (RSVNEFR) overlap with vicilin GC72-A precursor and C72 precursor (α-globulin A and B, respectively) from cotton, 100% and 75% identity in 4 aa overlap (SVRS) with rice glutelin and *Avena sativa*, respectively
cocosin basic subunit	24	GLEETQ	100% identity in 5 aa overlap (GLEET) with various legumin (11S) precursors from *Vicia faba*, pea, *Brassica napus*, cotton
7S	24	EQEDPELKQ	100% identity in 6 aa overlap (DPELKQ) with sesame seed 7S globulin and 71% identity in 7 aa overlap (EDPELKQ) with oil palm 7S globulin

[a] Using BLASTP 2.2.3 (Altschul et al., 1997); Lawrence et al. (1994).

to dissolve. Cocosin could be extracted optimally with 0.35 M NaCl, whereas the 7S globulin could be extracted with 0.10−0.15 M NaCl. This indicates that the coconut globulins could be purified on the basis of their NaCl solubility at pH 7.6.

In general, globulins are classified as salt soluble storage proteins. At 0.10 and 0.15 M NaCl, bands were observed at the 24 000 and 16 000 with a light band evident at 55 000 (**Figure 8**). These bands correspond to the 7S globulin. The members of the 7S vicilin type of storage proteins are usually very soluble at salts of low concentration. This was also observed with alfin (*28*). The 55 000 band was also observed in the 7S fraction obtained from gel filtration.

On the other hand, cocosin was found to be easily soluble at 0.35 and 0.4 M NaCl solutions. Cocosin has been reported to be rich in a number of charged amino acids (glutamic acid, arginine, aspartic acid, and lysine) which explains its salt solubility (*5*, *35*). Similar results were reported in pigeonpea 11S globulins wherein high amounts of aspartic acid (8.17−11.60%) and glutamic acid (10.57−19.17%) were obtained (*36*). Mungbean 11S could be extracted by 0.35 M NaCl, while 8S could be extracted by a wide range of salt concentrations (*6*).

N-Terminal Amino Acid Sequences of Cocosin and Coconut 7S Globulin. The N-terminal amino acid sequence of both the acidic and basic subunits showed significant homology with storage proteins (**Table 2**). The acidic subunit had 71% identity in 7 amino acid overlap with vicilin from cotton. On the other hand, the N-terminal amino acid sequence of GLEETQ of the basic subunit had 100% identity in 5 amino acid overlap (GLEET) with 19 different storage proteins (*37*). The sequence GLEET is a highly conserved sequence of the basic subunit of 11S globulins. This was also observed in mungbean 11S basic subunit (*6*) and olive seed (*38*).

The coconut 7S showed 100% identity in 6 amino acid overlap (DPELKQ) with sesame seed 7S and a 71% identity with oil palm 7S in a seven amino acid overlap (EDPELKQ).

We have shown that coconut globulins consist of the 11S and 7S at 86 and 14%, respectively. The 11S or cocosin is a hexamer of 326 000 molecular weight with subunits of 55 000. The subunits consist of the acidic (34 000) and basic polypeptides (24 000) linked by disulfide bond. The basic band is *N*-glycosylated which is unusual for legumin type of proteins. This is also the first report on the isolation of 7S coconut globulin with a native molecular weight of 156 000, which resolved into a major band of 24 000 and two minor bands of 22 000 and 16 000, on SDS−PAGE. Thus, it is a trimer with subunit of about 55 000. Moreover, the coconut 7S globulin was shown to have no disulfide linkages and is unglycosylated. The N-terminal sequences of the cocosin and of the 7S globulin have homology with some storage proteins. Using the information derived from this study, we have cloned and characterized the cDNA sequences of the cocosin which we will report in a separate paper.

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