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ISOLATION, PURIFICATION AND BIOPHYSICAL CHARACTERIZATION OF BASIC 7S GLOBULIN FROM *COCOS NUCIFERA*

Alpana Kumari*

*Ram Nirikshan Atma Ram College, S K Prui Samastipur-848101, Bihar, India.

ABSTRACT

Elucidation of structure-function relationships of seed storage proteins is a prerequisite for developing theoretically new food and/or food materials based on seed storage proteins. Furthermore, recent findings suggest that the 7S globulin, among all storage proteins in the coconut seeds, is responsible for the up-regulation of LDL receptors and that this activation is induced by the α and αβ- subunits of 7S globulin. The molecular mechanism underlying this biological response is currently under investigation. Here we report the purification and biophysical characterization of 7S globulin protein from the coconut endosperm. The total protein was separated by centrifugation at 13500g for 15 min at 4 ºC. The total precipitated protein of the 60% ammonium sulfate was subjected to column chromatography on a Hi-Prep 1.5/20 DEAE Sephadex A-50 HR column at a flow rate of 1 mL/min followed by purification using gel filtration chromatography. The purified fractions will be analyzed by SDS-PAGE. The band of SDS PAGE was excised and identified by mass spectrometry. These fragments are analyzed through MASCOT and identified through BLAST searches. We further perform circular dichroism (CD) analysis to observe the effect of temperature on secondary structure of the protein and thermal denaturation studies were carried out at 222 nm. This study will provide a sound basis for understanding in the structure-function relationship of coconut protein especially basic 7S globulin.

Keywords: Basic 7S Globulin, cocosin, Coconut Endosperm, Circular Dichroism, Mass Spectrometry, Cholesterol metabolism, Dietary Proteins and Seed Storage Proteins.

INTRODUCTION

The wide applications of coconut water can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones. Apart from these useful substances in the coconut endosperm, it contains several globulin proteins which act as a storage protein [1, 2]. The majority of the coconut globulins is the 11S storage globulin called cocosin with a molecular weight of 300 000−360 000. Annotation, the process by which structural or functional information is inferred for genes or proteins, is crucial for obtaining value from genome sequences. In this study one of the goals is to annotate the protein sequence of 7S globulin, in order to strengthen the functional information from the protein sequence itself. The 7S globulin protein has many remarkable features however a very small number of literatures are available for its structural properties. One of the earliest impacts of structural genomics on the structural biology field was the ability to identify overall success rates for the major steps in structure determination. A more sophisticated approach has been developed as well in which success rates, both overall and for individual steps, is analyzed as functions of the physical properties of proteins.

In the recent studies [3] it has been suggested that (i) consumption of soy protein isolate results in substantial inhibition of the progression of atherosclerosis in mice

Corresponding Author:-Alpanakumari Email:- alpanakumari24@gmail.com

with genetically engineered susceptibility; (ii) this effect is diminished in mice fed isoflavone-deficient soy protein; and (iii) it is independent of plasma lipoprotein concentrations and the presence or absence of LDL receptors. These findings indicate the probable importance of direct inhibitory effects of soy isoflavones and their metabolites on the initiation and progression of atherosclerosis. Furthermore, it has been suggested that the contribution of the protein component of soybean protein isolates to the inhibitory effect of soybean-protein products on Non-heme-inon absorption. Non-heme-iron absorption from liquid-formula meals containing a series of soybean-protein derivatives was measured in human volunteers by using an extrinsic radioisotopic tag method. Soybean proteins are widely accepted as an effective dietary tool in improving the lipid profile in hypercholesterolemic subjects [4].

The basic 7S globulin accounts for about 3% of the total protein [5]. Furthermore, recent findings suggest that the 7S globulin, among all storage proteins, is responsible for the up-regulation of LDL receptors [6] and that this activation is induced by the α and α'- subunits of 7S gluobulin [7]. The molecular mechanism underlying this biological response is currently under investigation. Recent data concerning the effect of 7S globulin subunits on the up-regulation of LDL receptors indirectly identify the subunit as the candidate responsible for this biological effect [7]. All these findings suggest that this protein is very important however, a few literatures are available for the structural and biophysical properties of this protein. There is a need to purify this protein in bulk amount in order to develop a structure-function insight of this protein. With a recently developed separation technique it became possible to purify the 7S globulin and thus allowing us to evaluate its structural and biophysical properties in detail.

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 report the isolation and purification of the 7S globulin from the mature endosperm of coconut and their characterization as to molecular weight, number and molecular weight of subunits and secondary structure. We further performed the heat-induced denaturation studies to know the stability of the protein.

MATERIALS AND METHODS

Materials

Fresh coconuts were obtained from local vendors in Darbhanga, India. The coconut water was collected and centrifuged at 10,000 rpm to remove the debris. Supernatant was stored at -86 ºC until further use. All reagents of highest purity grade were purchased from Sigma-Aldrich (St Louis, MO, USA), Merck (Darmstadt, Germany) and GE health care (USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration molecular mass standard markers (116 to 14 kD) were purchased from BioRad Laboratories (U.S.A.). Sepharose 6B and Blue Dextran 2000 were obtained from GE health care (USA). Molecular masses of coconut proteins were estimated using a protein standard curve of known molecular masses (18.5 to 106 kDa). Sodium cacodylatetrihydrate and Sephadex G-150 matrix were obtained from Sigma-Aldrich (St Louis, USA) and GE Healthcare UK Limited, respectively. Syringe filter of 0.22 µ used to filter the protein sample, was purchased from Millipore Corporation (USA). NaCl and EDTA were bought from Merck (India). Other chemicals were purchased from local suppliers. All chemicals used were of molecular biology research grade.

Protein Extraction

Routine extraction of globulins from coconut endosperm was done with 0.4 M NaCl in 35mM potassium phosphate buffer, pH 7.6, 0.1 mM p-aminodino Phenyl methanesulfonyl fluoride hydrochloride (APMSF, Wako Chemicals), 10-7 M pepstatin A, 10 mM β-mercaptoethanol, and 0.02% sodium azide at a ratio of 1g to 18 mL by stirring for 4 h on ice. The slurry was filtered through glass wool, the filtrate was centrifuged (12000g, 20 min, 4 °C), and to the supernatant was added dry sodium bisulfite (0.98 g/L). The pH of the mixture was adjusted to 6.4 with 2.0 M HCl, kept in a cold room (4 °C)) overnight (14 h). The globulins started precipitating after 24 h, and precipitation was complete after another 18-24 h. The total globulins were separated from albumin by centrifugation at 13500g for 15 min and were washed three times with distilled water containing 10 mM β-mercaptoethanol. The precipitate was washed five times with distilled water, suspended in a minimum amount of distilled water, dialyzed (72 h, 5 lit each, six changes), and freeze dried. All subsequent steps were performed at 4 °C.

Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. The supernatant (500ml) was collected. For 30% precipitation, we have added 88g of ammonium sulfate, kept it over a magnetic stirrer for six hours at 4ºC and centrifuged it 10,000 rpm for 15 minutes at 4ºC. A saturated solution of ammonium sulfate was slowly added to the crude extract to 60% saturation with stirring on an ice bath. The precipitate and supernatant were collected and dissolved in 10 mMTris-HCl buffer, pH 8.0, (buffer A) and dialyzed against 10 mMTris-HCl buffer, pH 8.0. The globulins started precipitating after 24 h, and precipitation was complete after another 18-24 h. The total protein was separated by centrifugation at 13500g for 15 min at 4 ºC. The total precipitated protein of the 60% ammonium sulfate was dissolved in 10 mM sodium acetate buffer, at pH 5.0 collected and used it for further process dialysis.

The supernatant was discarded and precipitate solubilized and dialyzed for further purification.

Anion Exchange Chromatography

Dialysed samples were loaded on an anion-exchange resin Amberlite IRA 900Cl, the resin was rinsed with phosphate buffer, and active components were eluted by stepwise increasing NaCl concentrations: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2 mol/l NaCl. Total protein in collected fractions was monitored by measuring the absorbance at 280 nm. For each fraction protein concentration was determined. Fractions were analyzed by SDS-PAGE.

Gel Filtration Chromatography

Around 50 mg of globulin was loaded onto a HiLoad 26/60 Superdex 200 (GE health care, USA) connected to a Fast Protein Liquid Chromatography (FPLC) system with 50 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 0.5 mL/min. Fractions were routinely analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel Electrophoresis

Appropriate protein samples (typically 50-100 µg of protein) were loaded on 12% arcylamide separating gel and 12% arcylamide stacking gel. The gel was run on typically at 80-120V, until the tracing dye migrated to the gel edge. Finally when the process was completed the protein band was stained with coomassie brilliant blue R-250 and destained with destaining solution (45% methanol and 10% glacial acetic acid).

Mass Spectrometry

Accurate molecular mass and identification of the purified protein was carried out on MALDITOF (Kratos analytical Ltd. Shimadzu group company Japan), equipped with a 337 nm pulsed UV laser, a 1.7m length flight tube and a curved field reflectron.The gel pieces were crushed in a 0.5 mL eppendorf tube, dehydrated with ACN for 10 min, and vacuum dried. Gel pieces were then rehydrated with 50 µL of ammonium bicarbonate: ACN (25 mM: 10% v/v) supplemented digestion with 1 pmol of trypsin (MASS grade from Sigma) overnight at 378 °C. Peptides were extracted from the gel with 50% v/v ACN/0.1% v/v TFA for 30 min at 378C. The extract was removed from the gel and vacuum-concentrated to about 10 µL. Then, 2 µL of digested peptides were mixed with 1 µL of matrix solution (10 mg/mL of 3-cyano-4-hydroxycinnamic acid in 0.1% v/v TFA/33% v/v ACN/33% v/v ethanol) and spotted on a MALDI plate in aliquots of about 1 µL. MS-MALDI was performed on Perkin Elmer/Perceptive Biosystems Voyager-DE-RP MALDI-TOF mass spectrometer (USA) using reflector mode to obtain monoisotopic peptide masses. These fragments are analyzed through MASCOT and identification will be done through BLAST searches.

CD measurements

Circular dichroism studies were carried out to determine the secondary and tertiary structure of 7S globulin in the solution form. CD studies were carried out in Jasco Spectropolarimeter (model J-715) equipped with peltier-type temperature controller (PTC-348)and inter faced with personal computer. Protein concentration used for CD measurement was in the range of 18-20µM and 0.1cm path length cell was used for far-UVCD. CD instrument was routinely calibrated withd-10 camphor sulphonicacid. Baseline correction was always carried with buffer in question and data stored. The spectrum of the native protein and protein in the denatured solution were stored and data acquisition was carried out using the J700 software provided by Jasco. The baseline of the buffer solution was subtracted from the native and denatured spectra. Atleast 6 accumulations of the scanning were carried to average out the spectrum to improve upon signal to noise ratio in each case including the baseline. N_2 was flushed continuously through the machine at the rate of 2-2.5lit/min and higher below 200nm to minimize the noise level.

Measurement of thermal transition curves

To observe the effect of temperature on secondary structure of the proteins in the presence and absence of denaturants, thermalde naturation studies were carried out at 222nm,themeasureofsecondary structure index [8] in the Jasco-715 spectropolarimeter with peltier-type (PTC-348WI) temperature controller. The rate of heating was 1^{O}C per minute from 20-85^{O}C with response time of 4sec, band width of 2nm and stepre solution of 0.1^{O}C.

RESULTS AND DISCUSSION

Purification of Basic 7S globulin

Ammonium sulfate fractions (60%) were extensively dialyzed and loaded on to the anion-exchange column packed with Amberlite IRA 900Cl resin, preequilibrated with phosphate buffer. The bound proteins were eluted in a stepwise increasing NaCl concentrations: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2 mol/l NaCl. The eultion profile of ion exchange chromatography is shown in Figure 1.

Peak 2 of Figure 1 was collected and concentrated and loaded on a gel filtration column, Sephacryl S-300 HR column (GE Healthcare Bio-science, NJ) at a flow rate of 0.5 mL/min with buffer. The first peak obtained from the S300 column (Figure 2) contains 7S globulin.

The SDS-PAGE profiles of the purified 7S globulin is shown in Figure 3. On the basis of the SDS-PAGE profiles of the purified and/or enriched components, the globulin the basic 7S bands could now be detected and

Fig 1. Elution profile of ion exchange chromatography

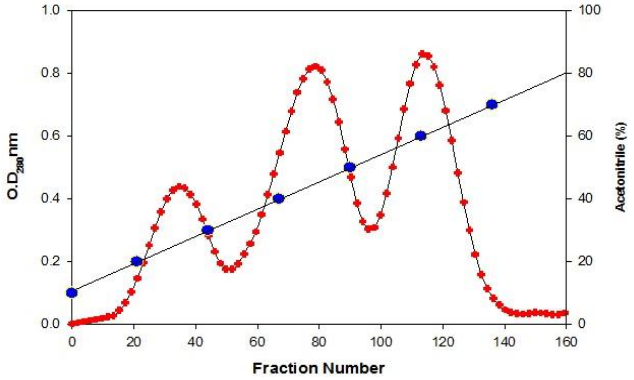


Fig 2. Elution profile of HiPrep 26/60 Sephacryl S-300 column

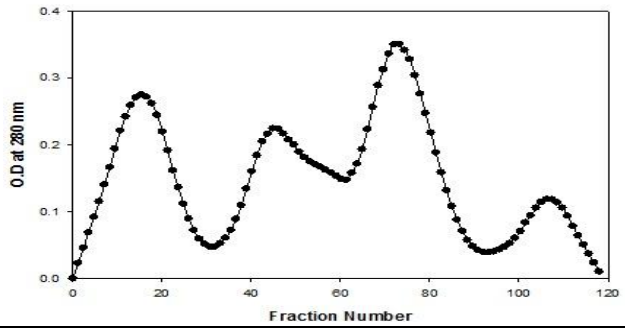
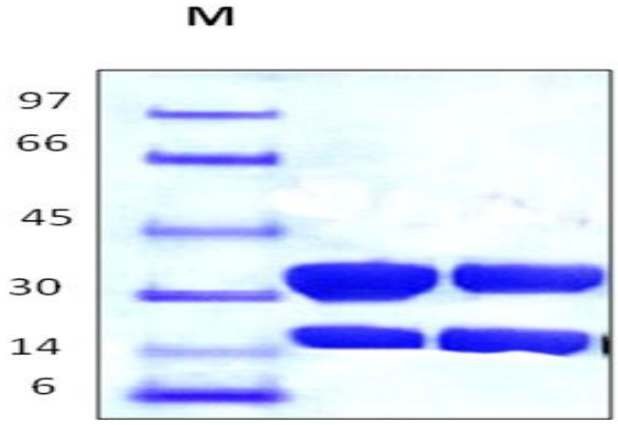


Fig 3. The SDS–PAGE showing purified protein of 34 and 20 kDa bands for 7S globulin.



Lane 1 is standard molecular mass marker.

Fig 4. Far-UV CD spectra of basic 7S globulin on increasing temperature

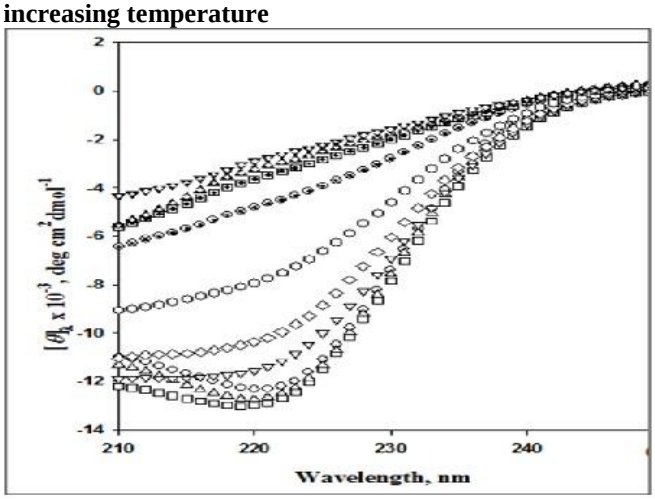
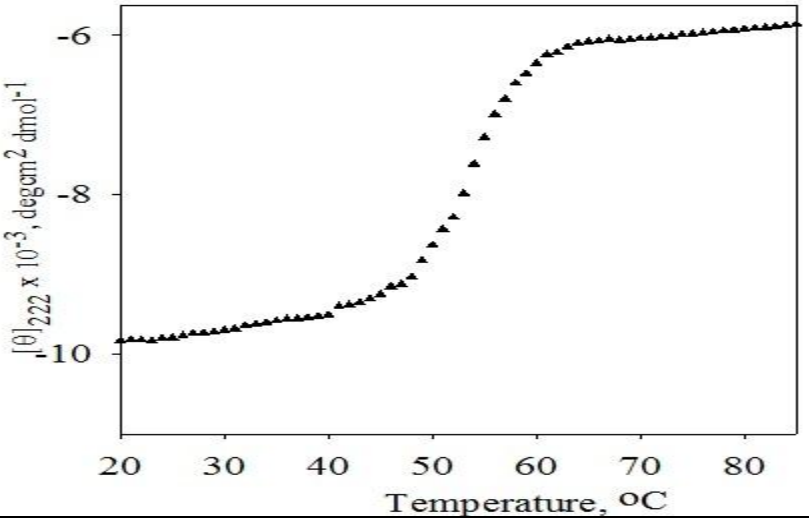


Fig 5. Thermal denaturation of basic 7S globulin in the absence of the denaturant at pH



differentiated in the total globulin lane. In the presence of β-mercaptoethanol, basic 7S exhibited two bands, 28000 and 16000 (Figure 3). In the absence of β-mercaptoethanol, these two bands combined to form 42000 kDa. Such bands but very lightly stained were also observed in the purified or enriched fractions of basic 7S globulin.

Thermal denaturation of 7S globulin

Thermal denaturation curve of 7S globulin at pH 7.2 shown in Figure 4 was measured by monitoring the changes in $[\theta]_{222}$ in the temperature range 20-85 °C. The reversibility of thermal denaturation was completely

established as revealed by the identical CD spectra before and after heating the sample solutions.

Figure 5 shows the heat-induced denaturation of 7 S globulin measured by monitoring changes in $[\theta]_{222}$. It has been observed that the denaturation of the protein is reversible at different temperature. It can be seen in the Figure 5 that the heat-induced denaturation followed by monitoring change in $[\theta]_{222}$ follows a single step transition from 25 to 85 °C. However, heat-induced denaturation of 7 S globulin seems to be complete in the measurable temperature range.

CD data was obtained at 222nm to see the structural changes. We carried out thermal denaturation of 7S globulin protein monitored at $[\theta]_{222}$ and analyzed for ΔH_m&T_m values on increasing temperature. The values obtained for $\Delta H_m(0)$ and $T_m(0)$ are 69 ± 3 kcal mol^{-1} and 59.8 ± 0.2 ^oC, respectively for 7S globulin protein.

CONCLUSION

In order to understand why the individual subunit of 7S globulin exhibited different properties, its structure was elucidated (*9*). Several studies have been reported on glycinin heat denaturation. Koshiyama *et al*., [10] found that at pH 7.6 the heat denaturation temperature of glycinin was influenced by ionic strength. Moreover, found no indications for a correlation between the denaturation temperature and the protein structure at ambient temperatures. Furthermore, according to another report at 0.5 and pH 7.6 the acidic polypeptides were present in the soluble fraction after heat treatment, whereas the basic polypeptides were found in the precipitate. However, these studies do not describe the effect of heat treatment of soy glycinin at low pH, whereas, as demonstrated in the preceding paper, pH has a dominant influence on the structural properties of glycinin [11].

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