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RESEARCH ARTICLE

Purification and Spectroscopic Analysis of 11S Globulins from Seeds of *Cucumis sativus* L.

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ABSTRACT

This study describes the purification and spectroscopic analysis of a trimeric (~ 180 kDa) 11S globulin protein from the seeds of *Cucumis* (*C.*) *sativus* L. The predominant seeds storage protein of *C. sativus* L. is a salt soluble globulin (~ 50 kDa), which is composed of large (28-31 kDa) and small (19-22 kDa) subunits linked together through disulfide linkage. The N-terminal amino acid sequence of small subunit showed high homology (83%) to that of many monocotyledonous and dicotyledonous plant 11S globulins (also commonly known as Cruciferins). Further random amino acids sequences were obtained from MALDI-Q-TOF MS/MS and multiple sequence alignment exhibited 72% sequence similarity with cruciferin of *Cucurbita maxima*. *C. sativus* Cruciferin (*Cs*Cr) from seed was first partially purified by ammonium sulfate precipitation (40% saturation constant) and further by gel filtration chromatography. Purified *Cs*Cr exhibited a typical single polypeptide of approximately 50 kDa monomeric protein under non-reduced condition of SDS-PAGE while produces two bands of major molecular weights of 28 kDa (also called α-polypeptides) and 19 kDa (β-polypeptides) under non-reduced conditions which is very much typical of 11S globulins and confirming the presence of disulfide linkages between two sub-units. Highly purified *Cs*Cr was produced in 25 mM Phosphate buffer of pH 7.0 and subjected to Dynamic Light Scattering (DLS) measurement which showed the monodispersive nature of the 11S globulins with hydrodynamic radius of approx. 5.7 nm confirming the trimeric nature (~ 180 kDa) of the protein. *Cs*Cr was further subjected to Circular Dichroism (CD) spectra which indicated the presence of α-helices and β-sheets in the native conformation of approximately 180 kDa proteins. This is a first report describing the purification and spectroscopic study of an 11S Globulin/Cruciferin protein from seeds of *C. sativus.*

INTRODUCTION

Cucumis sativus L. (commonly known as Cucumber) is a tropical plant belonging to the family *Cucurbitaceae* commonly known as gourd family (Mukherjee et al., 2013). This plant is fairly important from medicinal, economical and food values (Kocyan et al., 2007). The fleshy fruits of *C. sativus* are edible and are consumed fresh in salads or as fermented pickles (Sotiroudis et al., 2010). Their seeds are having a high content of proteins ranging from 25-30% while fat contents range from 40-45%. They are also having a high amount of vitamins and minerals (Younis et al., 2000).

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Storage proteins form the major fraction of the seeds proteins (Singh and Matta, 2008) and are the transient depots for reduced nitrogen for developing seedlings (Miernyk and Hajduch, 2011). The globulins are part of the super family Cupin (Dunwell et al., 2004). During seed development; the storage proteins are synthesized on the rough-endoplasmic reticulum (ER) and targeted to the lumen of ER where these are sorted from main protein traffic through the secretory pathway to their place of deposition; the Protein Storage Vacuoles (PSV) (Frigerio et al., 2008). Studies have shown that the storage proteins are of very much importance in different pharmaceutical and nutraceutical applications. They are being used as molecular markers for genetic variations and biodegradable natural herbicides (Witherspoon, 1998). The hydrolyzates of wheat gluten and corn rice prolamins are producing peptic hydrolyzates called exorphins, having morphine like nature and have pharmaceutical importance (Zioudrou et al., 1979). These peptides are also inhibitors of angiotensin converting enzymes, thus preventing hypertension (Kawamura, 1997). Additionally, there have been reports which indicated the anti-oxidative and antithrombotic activities of cruciferins (Wanasundara, 2011). The globulins are generally characterized as having a high molecular weight with sedimentation coefficient ranging from 7S to 11S (Wright, 1988). Since cucumber is used as fresh salad all over the world, it is of interest to characterize the edible proteins of this plant. Therefore, in this study, the 11S globulin was purified from the mature seeds of *Cucumis sativus*. The protein was subjected to different analytical instruments in order to understand their processes of oligomerization and further characterization at molecular level.

MATERIALS AND METHODS

The initial characterization of *Cucumis sativus* Cruciferin (Voucher number: BZBOT00115245) *Cs*Cr was performed in the Department of Botany, Bahauddin Zakariya University, Multan, Pakistan. Identification through N-terminal sequencing and mass spectrometry were done in Institute of Clinical Chemistry, University Hospital Hamburg-Eppendorf, Hamburg, Germany. Further Molecular characterization regarding the purification and spectroscopic analysis were performed in Laboratory for Structural Biology of Infection and Inflammation, University of Hamburg, Hamburg, Germany.

Protein quantifications

All the protein quantifications for the crude extract or the purified globulins were calculated using Bradford reagent (Bradford, 1976). For microbial growth inhibition, 0.02% sodium azide (NaN_3) was added into purified globulins.

SDS-PAGE analysis

Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (E-VS10-SYS, Germany) was used for electrophoretic analysis of the purified globulin. The procedure was carried out under the dissociating conditions formulated according to the method of Laemmli, (1970). The 12% resolving gel was prepared and the samples were loaded in non-reduced and reduced conditions. For reduction of the sample, β-mercaptoethanol was added and samples were heated prior to loading. The electrophoresed gel bands were stained with coomassie dye and destained using acetic acid and methanol. The protein ladder Catalog# 22610 (Thermo Scientific) was used for the molecular weight estimation.

N-terminal amino acid sequencing by Edman degradation

Polyvinylidene fluoride (PVDF) membrane (Standard Millipore Immobilon 0.45 micrometer membrane) blot was prepared according to the standard protocol (Towbin et al., 1979). The transfer of protein bands was performed at 200 mA for 4 h. The N-terminal sequencing (Applied Biosystems Edman Sequencer 476) was performed according to Edman degradation methodology (Hewick et al., 1981). The sequence was searched with Protein BLAST (Altschul et al., 1997) (UniProtKB) to get homologous sequence information.

Extraction and purification of *Cs*Cr

Seeds of *C. sativus* were bought from the local market of Multan, Pakistan. The dry seeds of *C. sativus* were ground in pestle and mortar using liquid nitrogen as grinding medium. For each extraction, 5 g of finely ground powder was blended with 50 ml of the 100 mM phosphate-buffer of pH 7.0. The slurry obtained was stirred continuously for 4 hours. The fine homogenate was centrifuged (Ogawa 6470, Japan) at 10,000 rpm for 20 minutes. The supernatant was filtered through Whatman filter paper no. 42 (11µm) to obtain a clear extract while the pellet was discarded. For the purification of globulins, 5 ml crude extract was subjected to 40% (w/v) Ammonium sulfate saturation constant. The precipitates were pelletized down at low centrifugation of 5000 rpm for five minutes and supernatant was discarded. The pellet was re-dissolved in 5 ml of respective buffer and dialyzed (Spectra/pore 3, Cat no. 132725; MWCO; 3.5 kDa). For further purification of *Cs*Cr, an ÄKTA system (GE Healthcare Life Sciences, UK) was applied while the software UNICORN^{TM} 4.12 was used for designing and performing the experiment. The desalted globulins were purified by loading onto a pre-equilibrated Superdex 200 column (Hi-Load 16/60). The protein was eluted with 25 mM phosphate-buffer of pH 7.0 containing 0.15 M NaCl at a flow rate of 1ml/min. The fractions that showed high A_{280} absorbance were pooled and analyzed on 12% SDS-PAGE.

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MALDI-Q-TOF MS/MS analysis

In-gel digestion of *Cs*Cr was done using mass spectrometric grade Trypsin as per manufacturer's instructions. The protein band was excised out of the Coomassie stained SDS-PAGE and was destained for 45 min with 100 mM NH_4HCO_3, 50% acetonitrile (ACN). After drying, the gel was pre-incubated for 1 h in 10-20 ml of trypsin solution (10 ng/µl). Then sufficient amount of digestion buffer was added and gel was incubated with mild shaking overnight at 37 °C (12–14 h). Tryptic digests were solubilized in Milli-Q water followed by double extraction with 50% ACN, 5% TFA for sixty minutes each time. The pooled extracts were dried in a SpeedVac™ concentrator (Thermo Scientific) at 48 °C. The samples were then processed for mass spectrometry analysis.

Dynamic Light Scattering (DLS)

*Cs*Cr was analyzed by *in situ* DLS measurements (SpectroLight 300; Xtal Concept, Germany) to confirm the monodispersity of the protein solution in 25 mM phosphate buffer. The hydrodynamic radius of native globulin was calculated as well. Pure transparent globulin was used to take a series of measurements with sampling time of 45 s and a laser light of 659 nm was used. A fixed angle of 90º was used to collect the scattered light. Analysis of the autocorrelation function in terms of particle size distribution was done by program CONTIN (Provencher, 1982).

Circular Dichroism (CD) Spectroscopy

The CD measurements of *Cs*Cr were performed on Jasco J-815 Spectropolarimeter (Japan). The purified *Cs*Cr (1 mg/ml) was prepared in 25 mM phosphate buffer of pH 7.0 and data were recorded in the far-UV range from 195 to 260 nm in a cuvette of 1 mm path length. Spectra manager™ software was used for calculation of CD spectra. Ten scans per sample were integrated to obtain one spectrum and the baseline was corrected by subtracting buffer spectra.

RESULTS

Protein identification

A primary sequence of 18 N-terminus amino acids **(GFEETVCTLRLKHNIGRS)** was obtained through Edman degradation. The sequence BLAST search was done via UniProtKB online server which yielded a high sequence identity (83%) with already reported 11S globulin of *Cucurbita maxima* as shown in Fig. 1. Further sequence comparison suggested a high homology to already known seed storage cruciferins of different plant species. It also showed high homologies with already reported cruciferins from *Pisum sativum*, *Vicia faba* and *Glycine max*.

Purification of *Cs*Cr

*Cs*Cr was precipitated from the crude extract of defatted powder by ammonium sulfate (40% saturation) precipitation. Excessive salt was removed by extensive dialysis and partially pure protein was subjected to gel filtration chromatography with 25 mM phosphate buffer (Fig. 2A) in order to remove further impurities. Ultimately, an optimized combination of ammonium sulfate precipitation along with chromatographic steps provided more than 95% pure *Cs*Cr protein (Fig. 2B) from seeds of *C. sativus*. One dimensional SDS-PAGE was run under both reduced and non-reduced conditions for further characterization of the protein. Under non-reduced condition, it showed one band corresponding to high molecular weight protein of approximately 50 kDa as shown in lane 1 of Fig. 2B. The corresponding α and β-polypeptides were further confirmed by running the *Cs*Cr under reduced conditions. This separated the monomer (50 kDa) in two smaller subunits of approximately 28 (α-polypeptide) and 19 (β-polypeptide) kDa bands as shown in lane 2 of Fig. 2B. This confirms the presence of an inter-chain disulfide bond between α and β-polypeptides which is very much typical for 11S globulins.

MALDI-Q-TOF MS/MS analysis

The *Cs*Cr from *C. sativus* seeds was purified by gel filtration chromatography and subjected to MALDI-Q-TOF MS/MS analysis in order to get more amino acid sequence. Twelve random fragments were generated which yielded a total number of 185 amino acids as shown in Table 1. These random fragmented amino acids sequences were aligned correctly considering the already deposited sequence of *C. sativus* cruciferin (UniProtKB - A0A0A0K9P5) in UniProtKB database as shown in Fig. 3. The residual sequence shaded black is representing the same twelve fragments of *Cs*Cr that were obtained through mass spectrometry. Additionally, the multiple sequence alignment was prepared considering the cruciferin sequences of other plant species e.g., *Cucurbita maxima*, *Morus notabilis* and *Ficus pumila*.

Dynamic Light Scattering analysis

The monodispersity of purified globulins were checked by dynamic light scattering (DLS) in 25 mM phosphate buffer (pH 7.0). The purified globulin solution was concentrated to 10 mg/ml in 25mM phosphate buffer (pH 7.0). The solution was centrifuged, filtered using micro-spin filter tubes and subjected again to DLS measurements. A single prominent signal showed a monodisperse protein (10 mg/ml) with R_H of 5.72 nm confirming the trimeric nature of the native globulin as shown in Fig. 4 A and B.

Secondary structure determination of globulins

Circular Dichroism (CD) data suggested the presence of both α-helical and β-sheeted secondary structure of the 180 kDa native globulin, showing a broad negative band which is typical for α and β type protein (Fig 5). The secondary structure content was calculated to approx. 33% helical structure, 13% β-strands and 25% turns.

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Table 1: MALDI-Q-TOF MS/MS generated twelve random fragments of varying lengths along their corresponding amino acid sequences of *Cs*Cr protein.

Sr. No.	Peptide sequences
1	GLLLPGFTNAPK [12]
2	GDLLVVPAGVSHW [13]
3	NVANQIDPFAR [11]
4	GFEETVCTLRLKHNIGRSEHADVFNPR [27]
5	LSTANFNNLPFLR [13]
6	LSAERGVLYSNAISAPHYTVNAHTVAYATR [30]
7	VQVVDNYGQAVFDGEVR [17]
8	EGQVLVIPQNFVVMTRASER [20]
9	GFEWIAFK [8]
10	TNDNAITNLLAGR [13]
11	LLPLGVLSNMYR [12]
12	LKYGQPEMR [9]

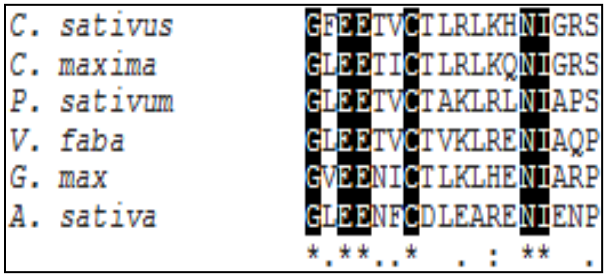

Fig. 1: The NH_2-terminal amino acid sequences of the 11S Globulin basic subunits (β-polypeptides) from *Cucumis sativus, Cucurbita maxima, Pisum sativum, Vicia faba, Glycine max* and *Avena sativa.* Regions of identity are represented by (*), similarity by (:) and dissimilarity by (.). Sequence of *C. sativus* cruciferin showed more than 80% sequence homology with other cruciferins.

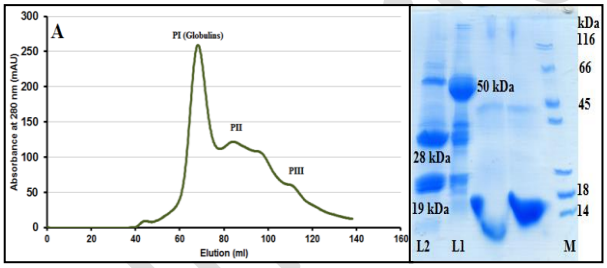

Fig. 2: Purification profile of *C. sativus* cruciferin (*Cs*Cr). (A) A highly purified globulin was obtained after gel filtration (Hi-Load 16/60 Superdex 200 column) chromatography and concentrated in PI peak of the eluting sample. (B) SDS-PAGE showing the corresponding purification quality of *Cs*Cr on gel. Lane M is the standard protein ledder. Lane 1 is showing the overloaded 50 kDa band of *Cs*Cr ran under non-reduced condition while Lane 2 is showing the separation of α (19 kDa) and β-polypeptides (28 kDa) due to the oxidation of disulfide linkage between two subunits in the presence of β-mercaptoethanol.

Fig. 3: Multiple sequence alignment of globulin sequences from *Cucumis sativus, Cucurbita maxima, Morus notabilis* and *Ficus pumila*. Identically conserved sequences are represented by Asterisks (*) under sequences while conserved substitutions and semi-conserved substitutions are represented by double dots (:) and single dots (.) respectively. The *Cs*Cr fragmented amino acid sequences obtained through MALDI-Q-TOF MS/MS are highlighted by white type on black background.

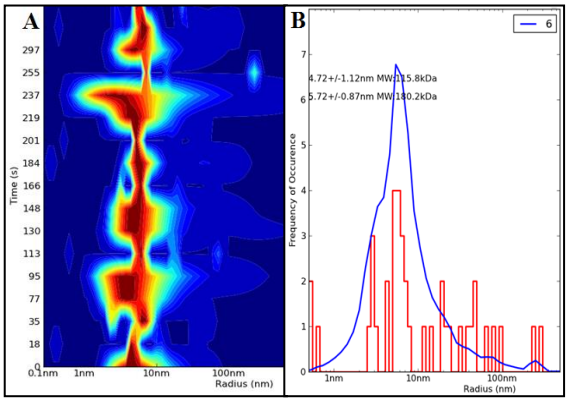

Fig. 4: Dynamic light scattering (DLS) measurements of the purified globulin. (A) Purified *Cs*Cr dissolved in 25 mM phosphate buffer (pH 7.0) showed a monodispersity of oligomeric form of a 50 kDa monomer. (B) A monodisperse stable protein solution with a hydrodynamic radius (R_H) of approximately 5.72 nm indicating the presence of trimeric (~180 kDa) nature.

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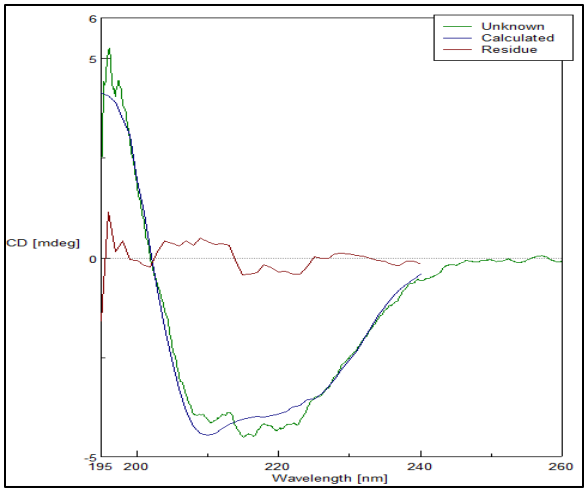

Fig. 5: Secondary structure analysis of purified *Cs*Cr with Circular Dichroism (CD) spectroscopy. Far-UV CD spectra ranging 195 to 260 nm is showing α-helical and β-sheeted secondary conformation of the protein.

DISCUSSION

An 11S Globulin/Cruciferin has been identified and purified from the seeds of *C. sativus*. The N-terminal amino acids sequences of the globulin showed 83% sequence identity to the 11S globulin/cruciferin of *Cucurbita maxima* confirming the identity of cruciferin. The N terminal sequence of the basic polypeptide of 11S globulins from the various monocotyledonous and dicotyledonous plants were aligned with the sequences of the *C. sativus* basic polypeptide. This sequence homology is the fundamental similarity between the proteins described. The *C. sativus* globulin is composed of the two subunits under the reduced conditions having a monomer of 50 kDa. There is a large acidic and a small basic subunit. The similar reports have been presented from the other members of the *Cucurbitaceae* family. In *Cucurbita pepo*, a hexameric 11S globulin of 325 kDa with each monomer of 54 kDa was reported and each monomer in turn is composed of two subunits, a large acidic (33 kDa) and a small basic (22 kDa) part (Blagrove and Lilley, 1980). This confirms the presence of an inter-chain disulfide bond between α and β polypeptides which are very much typical for globulins (Robin et al., 1991). A very little variation in the molecular weights of 11S globulins and their subunit pairs are represented from the comparative studies on these cucurbits. In another study, *Cucurbita maxima* have been shown to have two subunit pairs of molecular weights of 62 and 57 kDa (Hara et al., 1978). The variations in the electrophoretic mobility of the seed proteins on SDS gel have been reported in the different lines of *Citrullus* (Navot and Zamir, 1987) and *Cucumis* (Sawant and Moore, 2002).

Larger random residual sequences of *Cs*Cr were obtained through mass spectrometry which were aligned correctly by the help of *C. sativus* cruciferin sequence already deposited in UniProtKB database and *Cs*Cr fragmented sequence is marked as black shades in Fig. 3. It is important to mention here that this above UniProtKB *C. sativus* cruciferin sequence is calculated from the gene (GeneID: 101215676) and is deposited as uncharacterized protein. The total 185 amino acids of *Cs*Cr showed 72% homology with the sequences of the *Cucurbita maxima* seed globulins. The alignment between the two globulins and with other monocotyledonous and dicotyledonous globulins is presented in Fig. 3. The amino acid sequences obtained from mass spectrometer may have differences from those sequences predicted from genes because during the translation of the proteins from mRNA, changes may be introduced due to codon degeneracy. This is observed when the amino acid sequences obtained from mass spectrometery were aligned with those of the predicted sequences from the gene (NCBI XP_004155945). The protein homology suggests that all 11S globulins have originated from the common ancestral gene. Since monocots and dicots bifurcated millions of years ago, there may have a strong selective pressure to maintain this sequence. The amino acids replacements in the sequences may have resulted from the single nucleotide change in the triplet codon (Walburg and Larkins, 1983). The amino terminal residues of their small basic chain are all glycine (Hara et al., 1976).

At a CsCr concentration of 10 mg/ml in phosphate buffer (25 mM, pH 7.0) showed monodisperse solution (Fig. 4A) in DLS measurement which further indicated the globular shape of the native *Cs*Cr molecules. Very interestingly DLS calculated a higher hydrodynamic radius (R_H 5.7 nm) which strongly confers the trimeric nature of the CsCr protein. Additionally, such value is an indicative that the native 50 kDa monomer (depicted by SDS-PAGE) is actually oligomerizing to a higher molecular weights of approximately 180 kDa as shown in histogram (Fig. 4B). These results are confirming the trimeric state of native *Cs*Cr protein. However, the oligomerization of such proteins is very much depending on many physical parameters especially of the ionic strength of extraction buffer. Similar results have been reported for rapeseed cruciferin which assembles in hexamers with ionic strength above 0.5 M and dissociated into trimeric forms with lower lower ionic strength (Schwenke et al., 1983).

A brief analysis of the structure has shown the dominance of helices over β-sheeted secondary structure which is just a minor part of the molecule as shown in Fig. 5. Similar properties have been observed in the CD spectra of *C. sativus* globulins as the characteristic band at 210 nm from α-helices was

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observed, in accordance with a lower content of β-sheeted structure of globulins (Fig. 5). Secondary structure of *Cucumis* globulins showed a well folded protein and CD spectrum results indicted 33% α-helix, 13% β-sheet, 25% turn,

In summary, a considerable electrophoretic and spectroscopic characterization has been presented for 11S globulins of *Cucumis sativus* plant. Such studies should be further strengthen by solving the molecular structure of native cruciferins in order to understand their mechanism of oligomerization targeting the interaction between specific amino acids and corresponding salts, as well as to better grip their pharmaceutical potential.

Authors' contributions

SB and BK together performed the lab work and collected the data, KHS helped in SDS-PAGE analysis, FB performed the N-terminal sequencing and mass spectrometry, AM, ZA and SM improved the draft language, CB provided the facilities for DLS and CD spectra while AA conceive the idea and design the whole project as group leader.

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