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Proteomic Analysis in Response to Germination in *Ceiba pentandra* (Kapok) Seeds

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Abstract

Background: 1-D, 2-D gel electrophoresis, mass spectrometry and bioinformatics analysis were established and utilized to examine the changes in protein expressions associated with germination of *Ceiba pentandra* seeds.

Materials and Methods: The change in protein pattern was analysed by 1-D and 2-D gel electrophoresis for better separation and to ascertain the pattern of protein mobilization during germination.

Results: Digital image analysis of a silver stained 2-D gel revealed that the total protein profile of *Ceiba pentandra* seeds was dramatically changed by four days germination. The protein band 48.45 kDa sizes, which is prominent both in raw and germinating seed extracts was cut into 10 slices and subjected to in-gel trypsin digestion, followed by mass spectrometry analysis and tandem peptide mass fingerprinting.

Conclusion: It was identified as vicilin resembling storage protein with nutrient reservoir activity and its structure is alike to that of "Crystal structures of recombinant and native Soybean β-Conglycinin β Homotrimers".

Keywords: 2D gel; Electroelusion; MALDI-TOF; Peptide fingerprinting; Proteomics; Seed germination

Introduction

Two-dimensional (2-D) gel electrophoresis in combination with advanced mass spectrometry has revolutionized the large-scale profiling of proteins, also referred to as proteomics [1]. Protein spots isolated from 2-D polyacrylamide gels and digested with proteases generate reproducible peptide fragments whose molecular mass can be accurately and rapidly determined using Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. These experimentally determined peptide masses can be utilized to search "in silico" digested protein or nucleic acid databases for statistical matches [1,2] This systematic approach for the identification of unknown proteins based upon peptide masses is referred to as peptide mass fingerprinting (PMF) [2]. Although PMF is a facile approach well-suited for automation it is strongly dependent upon high quality

protein databases for making accurate assignments [1,2] . Thus the key objective of this cram was to learn the impact of germination on protein profile and isolation and characterization of prominent protein present in four days germinating *Ceiba pentandra* seeds belonging to the order *Malvalea* and the family *Malvaceae* by proteomic approach.

Materials and methods

Chemicals

Chemicals and reagents used for protein profiling and characterization were purchased from Merck. All additional chemicals used were analytical grade. Altogether the experiments were performed at room temperature unless otherwise stated.

Collection of seeds

Mature dried fruits of *Ceiba pentandra* were obtained from in and around Andhra University area, Visakhapatnam, India. Healthy seeds were selected and washed thoroughly with running tap water and with 5% (w/v) Teepol for 10 minutes followed by treatment with bavistin, a commercial fungicide for 5 minutes. Then the seeds were subsequently surface sterilized with 0.1% (w/v) $HgCl_2$ for 5 minutes and then washed with sterile distilled water. The seeds were kept for germination in sterile Petri dishes with double

layered moistened filter paper, after awash for 24 hours. The germination was carried out at 30 ^{0}C, 16 hours light and 8 hours dark [3] . Radicle emergence of 1cm was used as a reference to consider seed germination. Due to high-flying disparities 4 days germinated and raw seeds were used for protein profiling and characterization.

Estimation of proteins

One gram of non-germinating (soaked overnight in 0.2M Tris HCl Buffer, PH 7.2) and germinating *Ceiba pentandra* seeds were homogenized separately with 20 ml of pre-chilled 0.2M Tris HCl Buffer, PH 7.2, containing 0.1mM EDTA in chilled pestle and mortar. The homogenates were squeezed through double layered cheese cloth and centrifuged (Sorvall Instrument RC5C, Rotor SS-34) at 16,000 rpm for 15 minute at 4 ^{0}C. One ml of above extract was taken and 1 ml of ice cold 20% TCA was added [4] . The pellet was washed twice with acetone and again centrifuged at 8000 rpm. Supernatant was discarded and pellet was dissolved in 5 mL of 0.1 N NaOH. This was used for protein estimation. Total proteins were estimated by the method of [5] using BSA as standard.

Sodium Dodecyl sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to study the protein profiles of *Ceiba Pentandra* raw and germinating seeds according to the method of Laemmli (1970) on 10% (W/V) polyacrylamide gel in tris-Hcl buffer, pH 8.3 containing 0.1% SDS. Sample incubation buffer also contain 5% (V/V) β-mercaptoethanol. About 10µl of test sample containing protein (27.3±0.26 and 11.7±0.12 mg/g of tissue for non germinating and germinating seeds respectively) was loaded in each well and electrophoresis was carried out for about 3h.The gel was stained with 0.2% (W/V) coomassie brilliant blue R-250 and destained in 5% methanol, 7% acetic acid solution. Different marker proteins along with their molecular weights, they were Lysozyme (14.4kDa), Trypsin (23.3 kDa), pepsin (34.6kDa) and BSA (66.5 kDa) were used for calibration. The protein bands on the destined gel were quantitated using gel doc. Gels with similar samples were replicated thrice to ensure repeatability in results.

Two-dimensional gel electrophoresis (2-DE)

Protein profiles of raw and germinated seeds were compared by two-dimensional gel electrophoresis according to the protocol of O`Farrell (1975). 50µl sample solutions (27.3±0.26 and 11.7±0.12 mg/g of tissue for non germinating and germinating seeds respectively) were loaded on to the acidic side of the IEF gels (24-cm-long IPG strip) for the first dimensional, and anodic and cathodic electrode solutions were filled in the upper and lower electrode chambers respectively. SDS-PAGE in the second dimension was performed with 10% separation and 5% stacking gels. Protein spots in 2-DE gels were visualized by silver staining [6]. Each sample was run three times and the best visualized gels were selected.

Removal of CBB-R250 and electrophoretic elution of proteins from gel

In order to extract the protein from polyacrylamide gel, the method of electrophoretic elution was applied using dialysis membrane for protein retention [7,8] . Protein band with 48.45 kDa size was excised and cut into small fragments. Removing of CBB R250 from the gel fragments was performed according to the described method [9] . Briefly, destaining solution containing 50% isopropanol and 3.0% SDS was added to gel pieces in 12×75 mm glass test tubes and the tubes were capped with parafilm. Tubes were placed in a 37 °C water bath set for overnight without agitation. After cooling to room temperature, the liquid was removed and the gel fragments containing the appropriate protein were used for electrophoretic elution. For electophoretic elution, gel fragments were equilibrated twice in 0.125

M Tris-HCl buffer (pH 6.8) and 2.0% solution of 2-mercaptoethanol for 15 min. A final equilibration of the gel fragments in 0.125 M Tris-HCl buffer (pH 6.8) containing 1.0% (w/v) SDS was performed. The equilibrated gel fragments were then placed in a dialysis tube with a minimum amount of tris-glycine buffer containing SDS (25 mM Tris, 192 mM glycine, and 0.1% SDS). The dialysis tubes were taking care of and electroelution was carried out as described by previously [10]. Briefly, electroelution was performed at 50 V for 12 h at 4 °C in Tris-glycine buffer containing 0.1% SDS (pH 8.3). At the end of electrophoretic elution, the polarity of the electrodes was changed for 60 S in order to avoid the absorption of protein on the dialysis tubes. SDS-PAGE was performed again using electrophoretically eluted protein.

Protein in-gel digestion, MALDI-TOF-MS and Peptide mass fingerprinting

Protein spots of interest were excised from the stained gels and in-gel digestion was performed as follows. Gel pieces were washed three times with Milli-Q water and 50% (v/v) acetonitrile (Sigma) containing 100 mmol/L ammonium bicarbonate (Genebase) was used to remove the dye. Proteins in the gels were reduced in 10 mmol/L Dithiothreitol (DTT; Bio-Rad) dissolved in 50 mmol/L NH_4HCO_3 (Genebase) solution for at least 1 h at 56 °C and then incubated with 50 mmol/L iodoacetamide in 50 mmol/L NH_4HCO_3 at room temperature for 40 min. Gels were then dried by vacuum centrifugation and incubated for 14 h at 37 °C with 10 µl of 12.5 µg/ml trypsin (modified porcine trypsin, sequencing grade, Promega) in 50 mmol/L NH4HCO3. The resulting tryptic fragments were eluted by diffusion into 50% (v/v) acetonitrile and 0.5% (v/v) trichloroacetic acid (TCA), and dried in a speed vacuum. The dried samples were resuspended in 2 µl 0.5% (v/v) trifluoroacetic acid (Genebase). Each sample was mixed with the supernatant of 60% (v/v) acetonitrile saturated with α-cyano-4-hydroxycinnamic acid (Genebase) (1:1, v/v), and then air-dried on the flat surface of a sample plate. The samples were then analyzed with MALDI-TOF-MS (Reflex III, Bruker, Germany) in positive ion reflector mode at an accelerating voltage of 20 kV. Spectra were calibrated using trypsin autolysis products (m/z 842.51 and 2211.10) as

internal standards, and a mixture of standard peptides as external standards [11]. TOF/TOF MS of selected polypeptides was performed when PMFs obtained from a particular spot did not provide sufficient information for protein identification. TOF/TOF MS analyses were repeated using preparations from the same protein spot for accuracy [12].

Protein identification

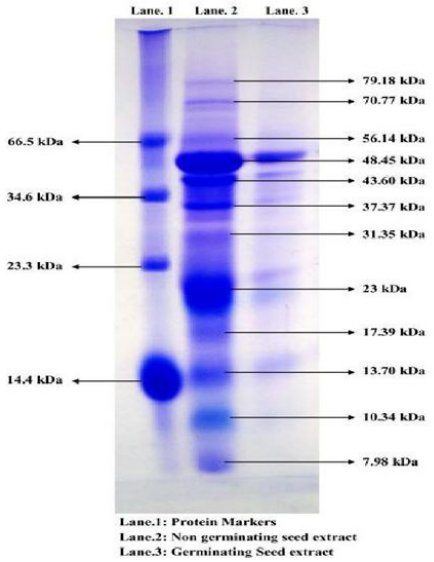
The proteins were identified by searching NCBI non-redundant database using the MASCOT program (http://www.matrixscience.com, Matrix scienc, UK). In support of Monoisotopic mass values and unrestricted protein mass, the search parameters allowed for fixed modifications of carbamidomethyl (C) and variable modifications by oxidation (M), by means of peptide mass tolerance ± 100 ppm and fragment mass tolerance ± 0.6 Da. For MS/MS searches, the fragmentation of a selected peptide molecular ion peak is used to identify with a probability of less than 5%. Thus, MS/MS spectra with a MASCOT score higher than the significant score ($p<0.05$) were assumed to be correct. When more than one peptide sequence was assigned to a spectrum with a significant score, the spectra were manually examined. Sequence length and gene name were identified by searching Swiss-Prot/ TrEMBL database using UniProtKB (http://www.uniprot.org/) [13].

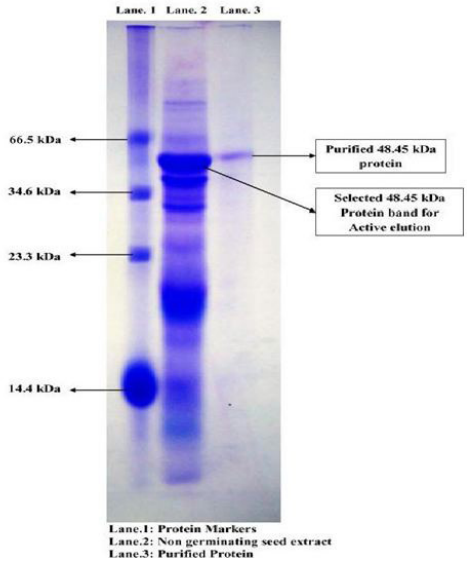
Results and Discussion

Protein identification

Germination improves the nutritive value of cereals and legumes by decrease the level of anti-nutrients and maximizes the level of utilizable nutrients [14]. It is an important technology for improving the nutritional quality of legumes and variably affects the proximate composition of seeds [15, 16]. During germination metabolic enzymes are activated and utilization or synthesis of wide range of chemical compounds occurs in seeds and results in the enhancement of nutritional quality [17]. Germinated seeds are rich in vitamins, minerals and are reported to contain important phytochemicals for disease prevention [18].

1-DGE analysis given the distinct morphological differences between the two seed extracts, we hypothesized that 1-DGE (1-Dimensional gel electrophoresis) would differentiate the seed extracts based on broad protein level differences. The profile of proteins extracted from the raw and germinated *Ceiba pentandra* seed samples were analysed using SDS- PAGE. As shown in (Figure 1), the proteins present in raw *Ceiba pentandra* seeds have a molecular weight ranging from 7 - 80 kDa (lane 2). The total protein profile of *Ceiba pentandra* seeds was dramatically changed by germination after day 4 as shown in lane 3 in (Figure 1). During the germination process, there were some marked changes in the protein composition. For example, proteins with molecular weights of around 7,10,13,17,23,31,37,43,56,70 and 79 kDa more or less fade away at day 4 of germination (Figure 1, lane 3). Whilst, a single band with molecular weight at 48 kDa remained until day 4 of germination (Figure 1, lane 3). SDS-PAGE was performed again using electrophoretically eluted protein and the follow-on is displayed in (Figure 2).





The change in protein pattern was further analysed by 2-DGE for better separation and to ascertain the pattern of protein mobilization during germination. Proteins were separated in a pH range of 3–10 and a MW range varying from 10 kDa to 100 kDa. Digital image analysis of a silver stained 2-DE gel revealed 59 protein spots in non germinating seeds and 26 down regulated protein spots were observed in four days germinating seeds and obvious proteomic difference between raw seeds and germinating seeds was observed. Outcomes were put on view in (Figure 3)

Hsu and co-workers (1982) observed a progressive decrease in large protein subunits and formation of small subunits as germination progressed. They found more changes in proteins for lentils than pea and faba bean. The significant changes in protein content of

seeds were observed in the final part of germination could be related to protein hydrolysis, synthesis and rearrangement. This was explained mainly through the initial alteration of the seeds component proportion (the rundown of the seeds reserve), then through the de novo synthesis. The results of this study were supported by other reports showing selective protein band disappearance and appearance [19,20]. suggested that protein disappearance represents degradation of reserve proteins, while new proteins appearing at specific times during germination and seedling development have stage specific developmental functions. The decrease in protein content in the cotyledons during germination also occurs in horse gram (*Macrotyloma uniflorum*) [21]. lupine (*Lupinus albus* L.) [22] and Lathirus sativus [23]. Storage globulin mobilization was observed in pea (*Pisum sativum* L.), soyabean (*Glycine max* L.), vetch (*Vicia sativa* L.) and garden pea (*Phaseolus vulgaris* L.) [24,25]. Suda and Giorgini (2000) reported that proteins, including albumins (49%), globulins (51 %) and prolamins (0.3%) in Euphorbia heterophylla comprise about a quarter of seed dry mass. These protein fractions exhibit different degradation patterns, globulins being degraded in the early stages after the start of imbibition and albumins between 60 and 84 hours. Globulin depletion is accompanied by an increase in free amino acids in the endosperm, whereas intense albumin depletion is not, suggesting that during albumin depletion there is a rapid transfer of amino acids to the growing embryo. In soybean dry seeds, the ROS (mainly superoxide anion) were accumulated on the testa. These superoxide anions might be produced during storage and could be quickly converted into H_2O_2 within 12 hours upon imbibition. Interestingly, the H_2O_2 on the testa could be hardly detected 24 hours after imbibition. The H_2O_2 on testa might have either diffused into the water or been detoxified by the antioxidant proteins such as catalase and peroxidase. The accumulation of H_2O_2 was latter than superoxide anions, which is similar to that happened in rice seed germination [27], total, 36 redox regulatory proteins were identified in the germinating soybean seeds. However, these proteins just accounted less than 0.5% of the total proteins in terms of abundance.

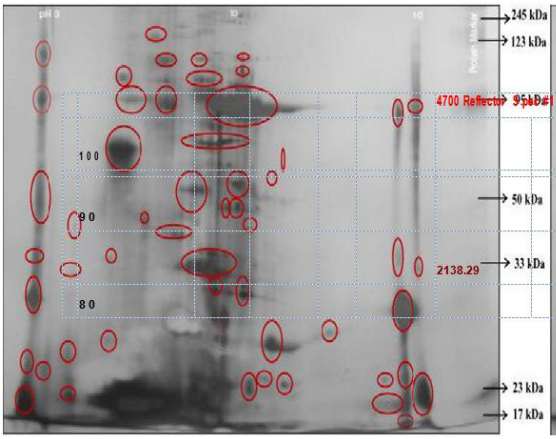


Figure 1: Protein profiling by SDS-PAGE

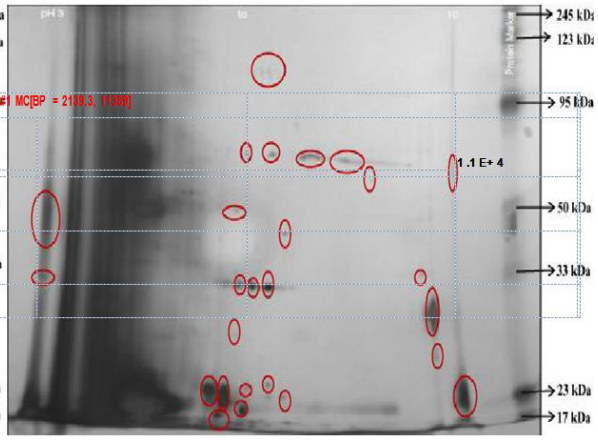


Figure 2: SDS-PAGE for electroeluted pure protein

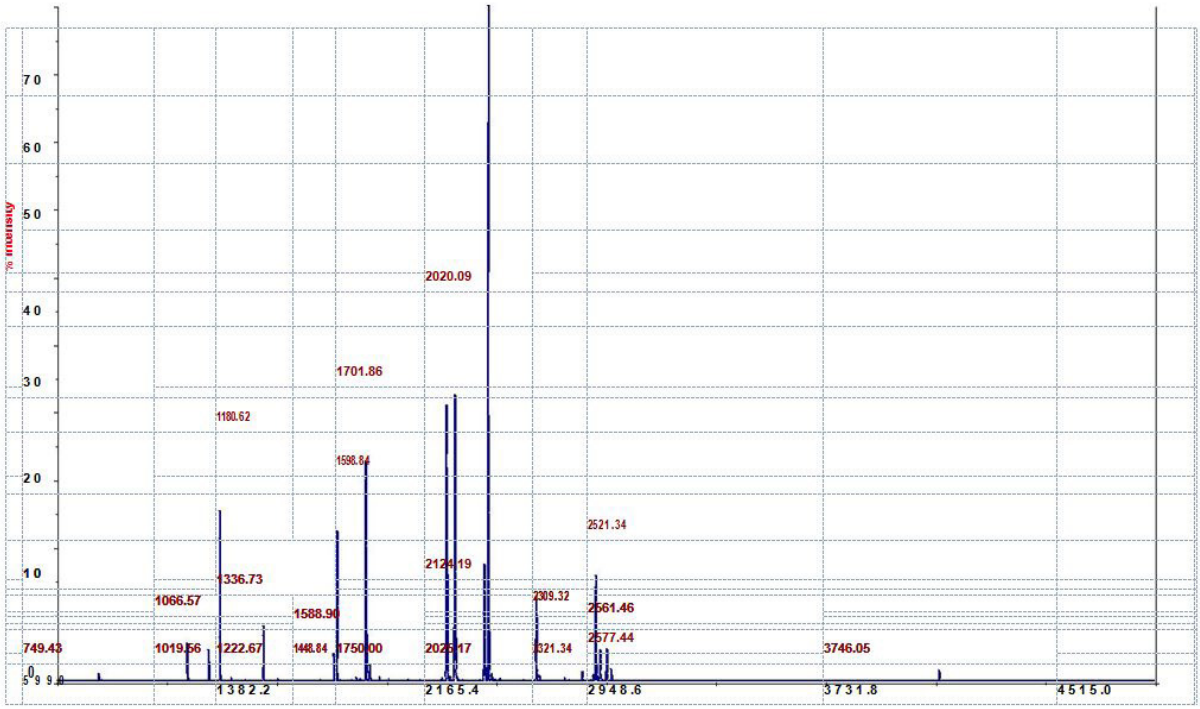


Figure 3: 2D protein patterns of Ceiba pentandra seeds (a) raw seeds and (b) germinating seeds. The proteins were separated using a linear pH 4–7 immobilized gradient and 10% SDS–PAGE The gels were silver stained

Protein identification

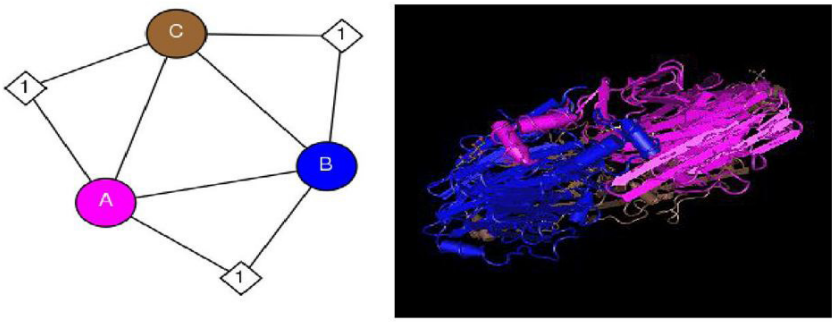
The protein band in the sample lane, 48.45 kDa size, which is prominent both in raw and germinating seed extracts was cut into 10 slices (Figure 2) and subjected to in-gel trypsin digestion, followed by mass spectrometry (LC-MS/MS) analysis and tandem peptide mass fingerprinting. Peptide mass fingerprinting (PMF) acquired by MALDI-TOF MS remains the most simple and powerful technique for high-throughout protein identification. This approach can be successfully applied and is more effective for plants, when their genomes have already been sequenced and fully annotated. For species without full genome sequence, when ESTs (Expressed Sequence Tags) are available, it is still possible to carry out identifications using this strategy. So, the species-specific EST databases have been used for protein identification as an alternative in species without full genome sequence information [28,29]. Although the EST raw sequences can be used to query by MS fingerprinting data, the accuracy and efficiency of protein identification would be suffered from the out-of-frame translation and short peptide translation. MALDI-TOF MS MS Spectrum for the trypsin digested gel fragment is kept on show in figure: 4 along with differentially prominent proteins identified by PMF

Figure 4: MALDI-TOF MS spectra of excised gel piece. Proteins spot was subjected to in-gel digestion by trypsin and thereafter analyzed by MALDI-TOF MS in reflector mode over the mass range of 700–5000 Da
• E-value: 1.15e-149, bit-score: 444, aligned-length: 438, Identity to query: 54%

Protein name	Accession No.[a]	Mass (Daltons)	Score	Matches	Expect
Vicilin (Vicia faba)	VCL_VICF	52719	60	2	0.02
Protein ycf2	YCF2_CUSRE	235819	38	6	2.9
Limonene/alpha-pinene synthase,	TPSDB_ABIGR	73854	36	8	4.1
chloroplastic					
DNA-directed RNA polymerase	RPOB_SOLBU	121181	34	8	6.4
subunit beta (Solanum					
bulbocastanum)					
DNA-directed RNA polymerase	RPOB_SOLLC	121237	34	8	6.4
subunit beta(Solanum					
lycopersicum)					
DNA-directed RNA polymerase	RPOB_SOLTU	121237	34	8	6.4
subunit beta (Solanum tuberosum)					
CASP-like protein MTR_5g041900	CSPL2_MEDTR	20136	32	4	11
CASP-like protein MTR_4g081880	CSPL1_MEDTR	20694	32	4	11
Casparian strip membrane protein	CASP4_ARALL	21214	32	4	12
ARALYDRAFT_660474					
Histone H4 variant TH011	H41_WHEAT	11402	31	4	14

Table 1: Differentially prominent proteins identified by PMF query
a: Accession number in the NCBInr database

query in (Table 1). Currently, PMF data or peptide sequence tags of protein spots from *Ceiba pentandra* proteome profiles are usually used to query the Swiss-Prot, TrEMBL and NCBI protein databases to conduct cross-species protein identification [28, 29]. Homology or conservation in amino acid sequence is the basis for identifying proteins using the cross-species databases. [30,31] The results of peptide analyses from the three databases, SWISS-PORT, MASCOT AND NCBInr, were the same for prominent spot in the experiments. The sequences length and gene name were identified from Swiss-Prot/TrEMBL search. When protein was identified with likelihood score, mass accuracy of each peak was mostly above 50 ppm in mass range 600-3000 m/z. [32,33] This mass accuracy is consistent with the specification value of the MS instrument used in the stable condition. The prominent protein both in raw and 4 days germinating seed extracts was identified as vicilin (7S) resembling storage protein with nutrient reservoir activity, sequence similarity with *Vicia faba* (*Genbank common name: fava bean*; inherited blast name: eudicots) [34]. The structure of identified protein Vicilin is alike to that of "Crystal structures of recombinant and native Soybean β-Conglycinin β Homotrimers". It is composed of three kinds of subunits: A, B and C, their alignment and the correlated sequence [35] is displayed in (Figure 5).



A B C Three protein subunits are highlighted with three different colours

1 Chemical group N-acetyl-D-glucosamine

Figure 5: The matched sequence (marked bold red) and related 3D crystal structure (Crystal structures of recombinant and native Soybean β-Conglycinin β Homotrimers)

Conclusion

The present study nonetheless demonstrated that 1DGE, 2DGE and mass spectrometry could be successfully applied to the global study of protein expressions in *Ceiba pentandra* seeds. The established proteomes were well resolved for protein expression studies and identification of *Ceiba pentandra* seed proteins, even with limited amount of protein materials.Further examination of proteins present only in the germinating *Ceiba pentandra* may shed some light into their roles in the resumption of metabolic activity during germination.

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