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Purification of Cytosolic Ribosome in *Arabidopsis thaliana* using different proteases

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ABSTRACT

The ribosome is a macromolecular system that has a significant role in synthesizing protein within all living cells. Ribosome in eucaryotic cell consists of two subunits; including small (40S) and large (60S) ribosomal subunits. Moreover, each subunit contains one or more molecules of ribosomal RNA (rRNA) and several ribosomal proteins (r-protein). The purification and isolation of ribosomal proteins from the other cellular organelles are a highly complicated process that needs to do several purification steps. In this study, the cytosolic ribosomal proteins in the *Arabidopsis thaliana* cell culture have been purified from other organelles. Also, several types of proteases have been studied to find the best one that hydrolyzes peptide bonds to be analyzed by mass spectrometry protein identification applications. The results have shown that by using double addition of sucrose cushion into the purified sample and using trypsin enzyme, we could purify large number of ribosomal proteins in the sample.

Keywords: Ribosomal proteins, ribosomal AGIs, Trypsin, Lys-C protease, Glu-C protease, Asp-N proteases.

1. Introduction

The hole living cells with biological protein production have ribosomes. Ribosomes are considered a complicated molecular machine. Amino acid can be joined together by a peptide bond to create polypeptide chains[1]. Ribosomes are existed as free ribosomes which is floating in the cytoplasm and also present as bound ribosomes that linked to the endoplasmic reticulum. Indeed, the major role of ribosomes is converting the genetic code into an amino acid chain and generating polymers of protein[2]. The ribosome that synthesizes protein by translating mRNA is a large complex of RNA protein made up of two subunits in eukaryotes, a 40S small subunit (SSU) and a 60S large subunit (LSU), Thus, for eukaryotes, 12 ribosomal proteins (6 in each subunit) of yeast are unique. The 80S ribosome in higher eukaryotes, such as humans contains an extra ribosomal protein, L28e, and thus have a total of 13 eukaryotic-specific r-proteins and in total 80 r-proteins including 33 in small subunit and 47 in large subunit[3].

The smaller subunit of both bacteria (30S) and eukaryotes (40S) contains a single ribosomal RNA (rRNA) chain including 16S rRNA in bacteria and 18S rRNA in eukaryotes, whereas the large ribosomal subunit in 50S bacteria contains two rRNAs include and three rRNAs in 60S eukaryotes include (23S and 5S rRNAs) and (28S, 5.8S, and 5S RRNAs) respectively[4]. Bacterial small subunit contains around 22 r-proteins and about 32 r-proteins in the eukaryotic small subunit, which 15 of these r-proteins are common to both. The large bacterial subunit usually contains about 32 proteins, while in eukaryotic, the large subunit contains approximately 45 ribosomal proteins, 18 of which are common to both[5 ,6]. Initially, the system of ribosomal protein nomenclature evolved organically, leading to some ambiguity and possible misunderstanding[7]. For each of the subunits, the majority of proteins are numbered sequentially, with the prefix S denoting as a small ribosomal subunit protein and L denoting a large subunit protein[8].

The large subunit (LSU) is about two times bigger in size than the small subunit (SSU). The SSU works to put both messenger RNAs (mRNAs) and aminoacyl-transfer RNAs (tRNAs) together as the decoding hub to decipher the genetic code [9]. In plants like *Arabidopsis thaliana*, cytosolic ribosome has shown that contains 81 different ribosomal proteins. . These ribosomal proteins are encoded by more than 240 genes (AGIs), among them, 102 genes encode the 33 r-proteins of the small subunit, and 146 genes encode the 48 r-proteins of the large subunit; this means that some r-proteins are encoded by more than one gene[7].

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The process of ribosome biogenesis consists of numerous steps including biosynthesis of ribosomal proteins in the cellular cytoplasm and importing them to the nucleus, which ribosomal DNA (rDNA) transcription, and followed by creating the 47S (pre-RNA) precursor for the synthesis of each 28S, 5.8S, and 18S ribosomal RNAs (rRNAs). Then followed by the processing of 47S pre-RNA to ribosomal RNA in the nucleolus. Finally, the assembly of both rRNA and r-proteins occurs to make the ribosome complex[1]. 47S ribosomal genes are transcribed to 47S (pre-RNA) by RNA polymerase I (Pol I), and it undergoes synthesis and processing to make three of the four rRNA (28S, 5.8S, and 18S rRNAs). The fourth rRNA (5S rRNA) which occurs in the nucleoplasm is the only one that is transcribed by RNA polymerase III (Pol III). RNA polymerase II (Pol II), on the other hand, transcribes all the mRNA for r-proteins which are later translated in the cytoplasm [1, 10]. It was estimated that about 50% of RNA polymerase II is devoted to transcription of ribosomal mRNAs[11].

Ribosomal isolation and purification in different organisms, including plants is a highly complicated process that needs several purification steps to get the unique ribosomal protein. In *Arabidopsis thaliana* there are numerous studies have shown the purification of ribosomal protein for different purposes[6, 7, 12]. In this study, the ribosomal proteins in *Arabidopsis thaliana* cell culture have been isolated by using different steps of purification process. Furthermore, the effect of different proteases has been investigated to find the best one that can hydrolyze peptide bonds into short peptide chains.

2. Methods and Materials

2.1 Arabidopsis thaliana cell culture

Every week 20 ml of a seven days old of the *Arabidopsis thaliana* (cv. Landsberg erecta) cell culture was kept in 100 ml of new autoclaved medium that consisted of 19 Murashige and Skoog Modified Basal Salt Mixture (phytotechnology M524) with addition of each of supplements includes 0.05 mg/L kinetin, 3% (w/v) sucrose, and 0.5 mg/L naphthalene acetic acid. Cell culture was kept under constant light (100µmol $m^{-2}sec^{-1}$) with orbital shaking at 100-120 rpm at 22°C, as described previously[13].

2.2 Ribosome purification process

From *A. thaliana* cell culture, 10 g of the 7-day-old were frozen in liquid nitrogen and grinded. The powder was extracted in a solution containing 100 mM KCl (Sigma Aldrich P9333), 0.45 M mannitol (Ajaxs chemical A310), 0.5% (w/v) polyvinyl pyrrolidone-40 (Sigma Aldrich 9003-39-8), 30 mM HEPES (Sigma Aldrich H3375), 20 mM $MgCl_2.6H_2O$ (Ajax Finechem A296), and 0.5% (w/v) bovine serum albumin (Bovogen biologicals BSAS1.0) with addition of 20 mM cysteine-L (Sigma Aldrich A-9165).The solution was filtered through two layers of Mira cloth (Merk Millipore, Darmstadt, Germany and transferred into a pre-cooled of 25 ml of Beckman Coulter centrifuge tubes and centrifuged for 20 minutes at 4°C at 30000 *xg*. The supernatant was transferred into 1.5 M sucrose dissolved in 2x mixture of a buffer solution containing 30 mM HEPES, 100 mM KCl and 20 mM $MgCl_2.6H_2O$, pH adjusted to 7.5 and ultra-centrifuged for 90 minutes at 60000 *x g*. After the first centrifugation, the pellet was resuspended in 2 ml of a solution of 5 mM DTT and 1.5 M sucrose. The resuspended sample was mixed with 2x ribosome extraction buffer followed by second centrifugation for 150 minutes at 60000 *xg*. Then, the ribosome pellet was resuspended in ribosome resuspension (2 ml of a solution of 5 mM DTT and 1.5 M sucrose) buffer and stored in -80 °C.

In the purification process, we aimed to identify the proteins associated with the core ribosomal particle. The ribosomal proteins were extracted from *Arabidopsis thaliana* cell culture through three centrifugation steps, and they were analyzed in triplicate. The homogenized cell culture was powdered, filtered, and centrifuged at 30000 *xg* for ½ hour. This process aimed to reduce organelle contaminants and cell wall residue while retaining soluble protein and was termed as 'Without sucrose cushion' solution. Part of the supernatant (described in the section of the method) was kept for protein quantification and LC-MS/MS analysis, and the pellet was discarded. The rest of the remaining supernatant was used for another centrifugation process by addition of 1.5M of sucrose cushion which uses as a discontinuous density gradient which enables the fractionation of macromolecules, and this step was termed as "1st cushion addition". In this step the insoluble materials were removed, and again part of the supernatant was kept for later analysis, and the rest of them were used for another centrifugation step which is known as "2nd cushion addition". In this step, another 1.5M of sucrose was added to the supernatant and centrifuged for 150-180 minutes (Figure 1). Again, the insoluble materials were removed, and part of the supernatant was kept for analysis. The three samples were collected in triplicate and analyzed by reversed phase LC on an Orbitrap-Fusion mass spectrometer.

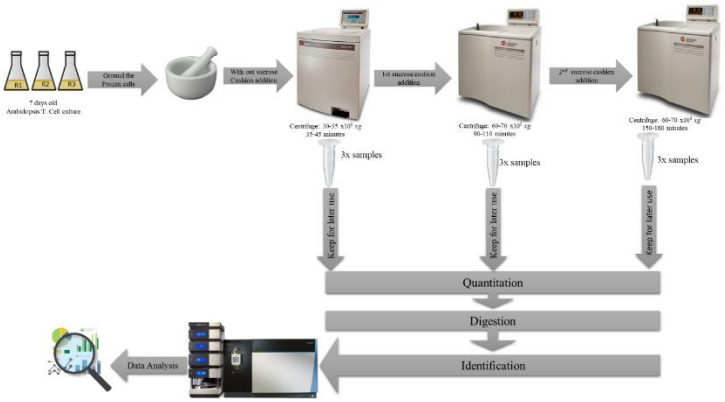

Figure 1: The diagram represents the step-by-step purification of 80S cytosolic ribosomes from Arabidopsis thaliana cell culture.

2.3 Quantification of ribosomal proteins

A standard solution of bovine serum albumin (BSA) from 2 mg/ml of stock and protein sample was prepared. The Amido black method was used to quantify protein content in the purified ribosome sample by dissolving 26 mg of amido black in 100 ml of (1:10) acetic acid (Ajaxs Finechem 2789): methanol (Ajaxs chemical A318) solution. To begin with the quantification, 300 µl of amido black solution was added to each protein sample and centrifuged for 4 minutes at 20800 x g at 20°C. Pellet was

resuspended in 1ml of acetic acid: methanol (1:10) and centrifuged as before, and the supernatant removed with a vacuum line and the pellet dissolved in 1 ml of 0.1 N NaOH (Ajaxs chemical A482). Finally, to measure the protein content in the sample, a UV mini-1240 UV-VIS Spectrophotometer (Shimadzu Scientific Instruments) was used, and the absorbance of each sample read at 615 nm.

2.4 Precipitation, denaturation, digestion, and cleanup

50 µg /100µl of purified ribosome was precipitated based on the quantification result, by using 1ml cold acetone (Sigma Aldrich 666-52-4) and incubated for 1 hour in -80 °C. Following 20 minutes of centrifugation for at 20800 x g at 4°C. later, the supernatant was gently removed, and the pellet denatured, reduced, and alkylated by using 1.5 M urea (Ajaxs chemical A817), 10 mM DTT (Sigma Aldrich D0632) and 25 mM of iodoacetamide. Then, the denaturated protein was digested by using trypsin and incubated overnight at 37°C and the reaction terminated by acidification of the mixture through the addition of 1% formic acid (BDH 101154E). By using silica C18 (micro spin column) in a reverse phase column (50-450 µl loading, 30-300 µl capacity) the digested protein was purified and concentrated. Before adding the protein, 500 µl of 100% of methanol (Ajaxs chemical A318) was added to the column to rinse the column wall and wet resin as an activation solution, then centrifuged at 500 xg for 2 minutes. Then the column was equilibrated with 500 µl of a solution consisted of 5% ACN (RCI Labscan C2502) and 0.1% formic acid (FA) and then centrifuged for 2 minutes at 500 xg. Next, the protein sample was mixed with 500 µl of a mixture (5% ACN, 0.1% FA) and loaded into the wetted column, centrifuged at 500 xg for 2 minutes. Then, 500 µl of (70% ACN, 0.1% FA) was added to the column, centrifuged at 500 xg for 2 minutes, and the extracted peptide was dried by vacuum centrifugation at 30°C overnight. Finally, 1µg of the protein sample in the dried sample was resuspended in 1 µl of a mixture of (5% ACN and 0.1% FA).

2.5 LC-MS/MS and Data analysis

Mass spectrometry analysis was performed with Orbitrap Fusion mass spectrometer[14]. The raw data files (. raw) files were converted to .mzML files by using open-source MS convert software. Then the .mzML files were converted to .mgf files via the Convert mZ[X]ML tool. The Matrix Science was used to search tandem mass spectra against proteins from the TAIR10 release of *Arabidopsis thaliana* (https://www.arabidopsis.org/) database to convert the. mgf files to both .dat and .csv files, as was discussed before[14].

3. Results and Discussion

3.1 Protein digestion

Proteins are a large molecular complex that are formed from the polymerization of amino acids through the transcription process. In order to study proteomics, the protein molecule must be hydrolyzed into short peptides by using proteolytic enzymes (proteases) to be analyzed via liquid chromatography–mass spectrometry (LC-MS). The two major groups of proteases are the exopeptidases, which target the C-terminal and N-terminal of proteins also called carboxy peptidases and amino peptidases, and the other one is endopeptidases, which target amino acid residue within proteins such as trypsin. In the isolation and purification of the cytosolic ribosome in *A. thaliana* cell culture, four different proteases were tested including trypsin, Lysin-C, Asp-N, and Glu-C during digestion of the isolated protein to maximize the proportion at which ribosomal proteins were identified in samples.

Among 248 ribosomal AGIs that were identified previously[6, 7, 14], we found different numbers of AGIs by using different types of proteases. For example, by using Glu-C protease we identified a few ribosomal AGIs which were only 51 AGIs in comparison with trypsin enzyme, which we found more than 165 ribosomal AGIs, as shown in Figure 2 and supplemental Table 1.

Among all the identified ribosomal proteins that were isolated from other cellular components by using different enzymes, 32 AGIs were found by using all proteolytic enzymes as shown in Table 1A and Figure 2. Whereas by using each of trypsin and Lys-C proteases, we could identify 68 shared AGIs, at which 41 of them were located on the large ribosomal subunit, and the rest of them were found in the small ribosomal subunit (Table 1B). Here, this finding indicates that trypsin is the best type of protease that can be used for isolation and purification of ribosome from other cytosolic organelles in comparison with using other proteases.

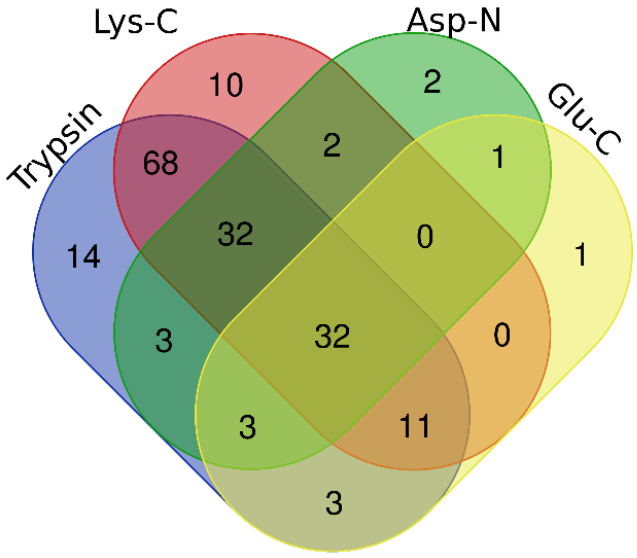

Figure 2: The Venn diagram represents the total and common ribosomal AGIs that found in Arabidopsis thaliana cell culture by using different proteases including Trypsin, Asp-N, Glu-C and Lys-C (n=3).

Table 1 A: Common AGIs found by using all proteases.

Ribosomal AGIs	Ribosomal Proteins	Ribosomal AGIs	Ribosomal Proteins
At3g05560.1	RPL22B	At3g09630.1	RPL4A
At3g49010.1	RPL13B	At2g41840.1	RPS2C
At4g27090.1	RPL14B	At2g32060.1	RPS12C
At3g09200.1	RPP0B	At1g48630.1	RACK1B
At1g07770.1	RPS15aA	At1g33120.1	RPL9B
At2g37270.1	RPS5A	At5g16130.1	RPS7C
At3g57490.1	RPS2D	At2g44120.1	RPL7C
At3g11250.1	RPP0C	At1g48830.1	RPS7A
At1g15930.1	RPS12A	At3g25520.1	RPL5A
At5g35530.1	RPS3C	At3g14600.1	RPL18aC
At1g04270.1	RPS15A	At3g28500.1	RPP2C
At3g07110.1	RPL13aA	At5g02870.1	RPL4D
At1g43170.1	RPL3A	At1g58380.1	RPS2A
At2g40010.1	RPP0A	At2g31610.1	RPS3A
At2g40590.1	RPS26B	At3g18130.1	RACK1C
At1g04480.1	RPL23A	At2g43460.1	RPL38A

Table 2 B: The common AGIs that were found by using Trypsin and Lys-C proteases in both small (40S) and large (60S) ribosomal subunit.

Ribosomal AGIs	Ribosomal Proteins	Ribosomal AGIs	Ribosomal Proteins
60S Large Subunit		**40S Small subunit**	
At1g14320.1	RPL10A	At1g25260.1	RPP0D
At1g26910.1	RPL10B	At5g41520.1	RPS10B
At2g37190.1	RPL12A	At3g48930.1	RPS11A
At5g60670.1	RPL12C	At4g30800.1	RPS11B
At3g48960.1	RPL13C	At5g23740.1	RPS11C
At5g23900.1	RPL13D	At3g60770.1	RPS13A
At4g16720.1	RPL15A	At2g36160.1	RPS14A
At1g27400.1	RPL17A	At3g52580.1	RPS14C
At1g67430.1	RPL17B	At3g46040.1	RPS15aD
At2g34480.1	RPL18aB	At3g04230.1	RPS16B
At1g29965.1	RPL18aD	At5g18380.1	RPS16C
At1g02780.1	RPL19A	At2g04390.1	RPS17A
At3g16780.1	RPL19B	At5g04800.1	RPS17D
At1g57660.1	RPL21E	At3g45030.1	RPS20A
At2g36620.1	RPL24A	At3g47370.1	RPS20B
At3g53020.1	RPL24B	At3g04920.1	RPS24A
At3g49910.1	RPL26A	At5g28060.1	RPS24B
At5g67510.1	RPL26B	At2g16360.1	RPS25A
At2g32220.1	RPL27A	At4g39200.1	RPS25E
At4g15000.1	RPL27C	At1g23410.1	RPS27aA
At2g19730.1	RPL28A	At3g61111.1	RPS27C

At4g29410.1	RPL28C	At5g47930.1	RPS27D
At1g36240.1	RPL30A	At3g02560.1	RPS7B
At1g77940.1	RPL30B	At5g59240.1	RPS8B
At4g18100.1	RPL32A	At5g15200.1	RPS9B
At1g26880.1	RPL34A	At5g39850.1	RPS9C
At1g41880.1	RPL35aB	At3g04770.1	RPSaB
At1g74270.1	RPL35aC		
At3g55750.1	RPL35aD		
At2g39390.1	RPL35B		
At3g55170.1	RPL35C		
At5g02610.1	RPL35D		
At2g37600.1	RPL36A		
At3g53740.1	RPL36B		
At5g02450.1	RPL36C		
At1g61580.1	RPL3B		
At5g39740.1	RPL5B		
At3g62870.1	RPL7aB		
At3g13580.1	RPL7D		
At4g36130.1	RPL8C		
At4g10450.1	RPL9D		

3.2 Number of peptides per each protein vary by using different proteases

Proteins are a long chain of molecules consisting of multiple polypeptide subunits, they can be digested and hydrolyzed by enzymes into short peptide fragments. Here, peptides are generally reflected to be short chains of two or more amino acids molecule. Here, in our experiment, for each hydrolyzed protein by using different proteases, we checked the total number of peptides that were present in each digested protein. Here, for the same amount of *Arabidopsis Thaliana* cell culture, the ribosomal proteins were isolated by using different enzymes included trypsin, Lysin-C, Asp-N, and Glu-C. It was found that the average number of unique ribosomal AGIs that were isolated by using trypsin enzyme in the sample is higher than the number that were found by using other proteases (Figure 3A). The small ribosomal protein RACK1 is one of the most important protein in *A. thaliana* that involves in several cellular processes, such as cell growth and division[15], apoptosis[16], stress response[17], translation and transcription[18], neuronal remodeling[19] and activity ,signaling pathway[20]. It consists of three isomers including RACK1A, RACK1B and RACK1C, and each is encoded by a specific gene which is AT1G18080.1, AT1G48630.1 and AT3G18130.1 respectively. We found that by using trypsin enzyme we can isolate a greater number of different peptides of RACK1 protein isomers than by using other proteases such as Glu-C, as shown in Figure 3B and Supplementary 1.

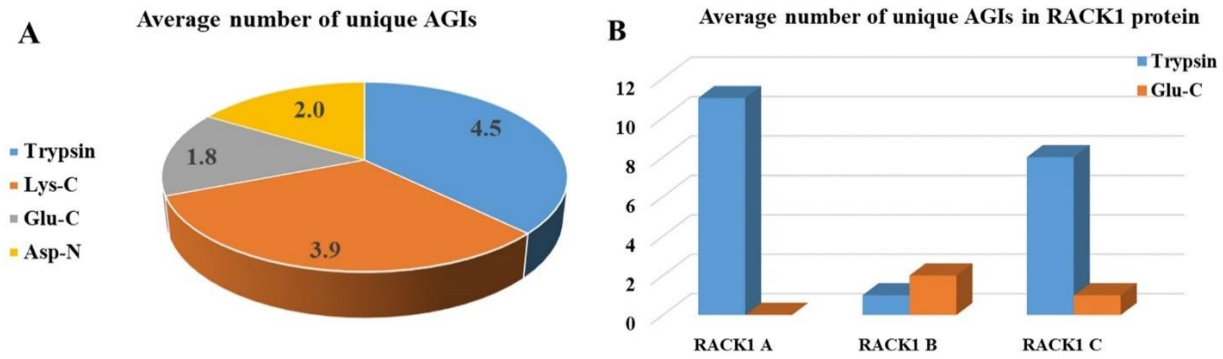

Figure 3: (A) Average number of unique ribosomal AGIs that were isolated by using different proteases. (B) Effect of using two proteases (Trypsin and Glu-C) on proteolysis of RACK1 protein isomers (n=3).

Furthermore, for each digested protein, we tested to check the number of unique peptides that were digested by using the above enzymes. The results have shown that the average number of peptides that were digested by using trypsin is higher than the average that were found by other enzymes, as shown in Figure 4. An example of the fragmented peptide of the RACK1 protein that was found by using trypsin is shown in Figure 5, as the digested peptides are highlighted in yellow color in RACK1A (At1g18080, NP_173248) sequence. From this result, we can propose that by using trypsin enzyme in the protein digestion, particularly the isolation of ribosome, we could get a higher number of ribosomal proteins with higher number of peptide proteins in comparison with using other types of proteases. This high number of small piece peptides has a significant role in LC-MS/MS analysis, which improves the quality of the analysis and can get a reliable result than those which are obtained from other digestive enzymes (Figure 5 and Supplemental table 1).

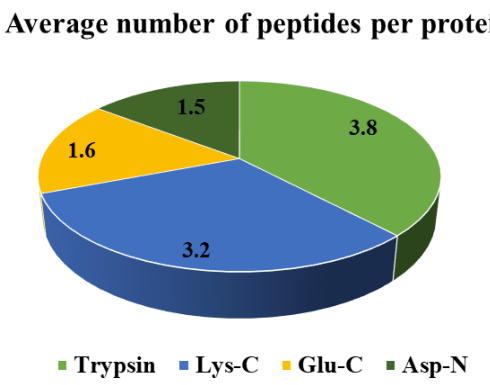

Figure 4: Effect of using different proteases on the average number of unique peptides that were used in isolation of ribosomal protein (n=3).

MAEGLVLKGTMRAHTDMVTAIATPIDNADIIVSASRDKSIILWKLTKDDKAYGVAQRRLTGHSHFVEDVV
LSSDGQFALSGSWDGELRLWDLAAGVSTRRFVGHTKDVLSVAFSLDNRQIVSASRDRTIKLWNTLGECKY
TISEGGEGHRDWVSCVRFSPNTLQPTIVSASWDKTVKVWNLSNCKLRSTLAGHTGYVSTVAVSPDGSLCA
SGGKDGVVLLWDLAEGKKLYSLEANSVIHALCFSPNRYWLCAATEHGIKIWDLESKSIVEDLKVDLKAEA
EKADNSGPAATKRKVIYCTSLNWSADGSTLFSGYTDGVIRVWGIGRY

Figure 5: RACK1A (At1g18080, NP_173248) amino acid sequence in *Arabidopsis thaliana*. The highlighted colour represents the peptides that were digested by using trypsin enzyme.

3.3 Identification of ribosomal and non-ribosomal proteins in *Arabidopsis thaliana* cell culture using differential separation method

At the beginning of analysis, we have checked the number of ribosomal proteins and non-ribosomal proteins that were present in the purified sample. As can be seen in Figure 6, in the first step of centrifugation, which the sugar has not been added to the sample, the number of isolated proteins is very much, this represented that the sample does not include pure ribosomal proteins only; however, there were other organellar proteins or proteins that bound to ribosome existing in the sample. Next, by addition of 1.5M sucrose to the previous supernatant and further centrifugation for more than one and a half hours, we found that the number of non-ribosomal proteins decreased sharply through a discontinuous density gradient technique, and this number decreased radially by further addition of 1.5M sucrose and then centrifuge of the sample for more than two hours (Figure 6) and supplemental table 2. Here, these results indicated that the numbers of identified proteins decreased as the level of enrichment increased.

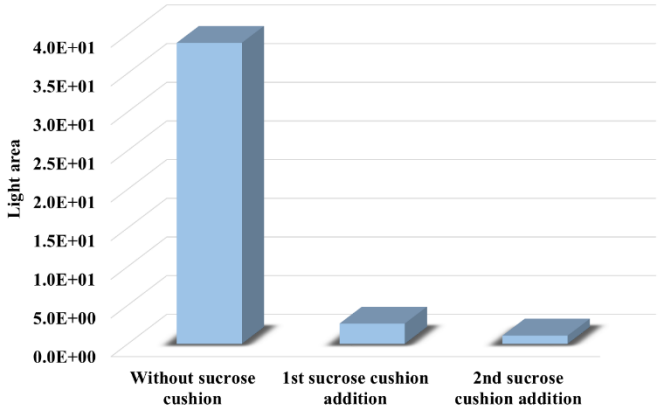

Figure 6: Purification of 80S cytosolic ribosome from other organelles proteins by using Sucrose cushion ultracentrifugation technique. X-axis: represents the numbers on the bar chart, represent the total number of proteins that are found in each step of the purification. Y-axis: the area of the pick that are found during the LC-MS/MS analysis **(**n=3).

Then, to see what these are proteins that were identified after the addition of 2X of (1.5M) sucrose cushion into the sample, we checked the functional category of each identified protein in the sample. As shown in figure 7, it showed that most of the isolated proteins which are more than 800 unique proteins belonged to the "protein synthase" group, which means that the higher number of purified proteins were related to ribosomal proteins. Once these isolated proteins are assembled, they create both small and large ribosome molecule, which later it uses as a protein synthesis machine through the translation process[1, 10].

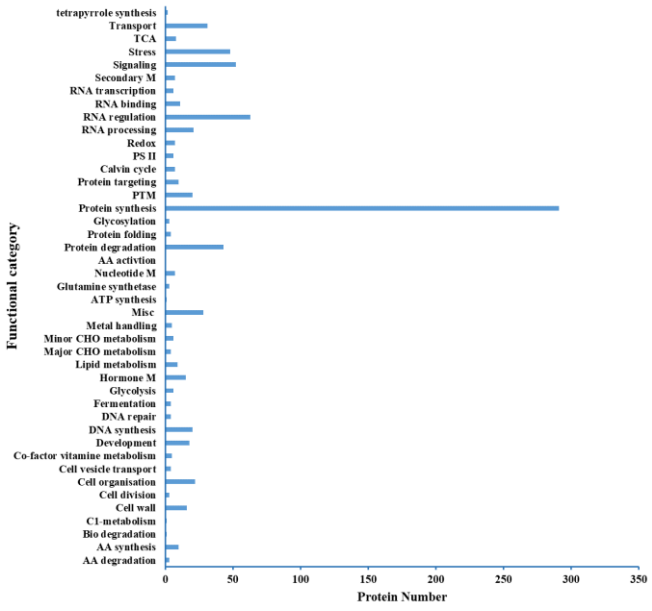

Figure 7: Identification of the proteins bused of their functional categories after two-times addition of 1.5M sucrose gradients (n=3).

Finally, we checked the number of ribosomal proteins that were found in each purification stage, and we found that in the last step of the purification process we could isolate more ribosomal proteins in comparison with the samples that did not contain 1.5M sucrose cushion (Figure 8) and Supplemental table 3. Overall, these results indicate that the relative abundance of known ribosome proteins increased relative to other proteins during the enrichment procedure and using 2X of 1.5M sucrose cushion and followed by ultracentrifugation process.

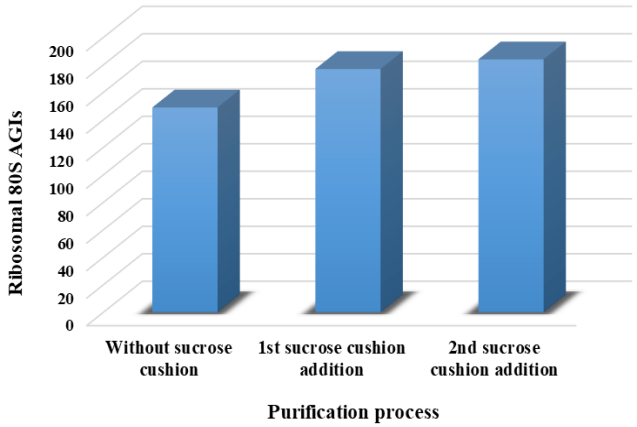

Figure 8: Differential purification process for isolation of cytosolic ribosome (80S) in *Arabidopsis thaliana* cell culture. X-axis: The three types of purification of ribosomal proteins. Y-axis: The number of 80S ribosomal AGIs that encode ribosomal proteins that increased after two times of addition of 1.5M sucrose gradients (n=3).

4. Conclusion

In the current study, it can be concluded that by using two times of 1.5M sucrose in the purification sample and followed by ultracentrifugation for about three hours, we could identify more proteins, and based on functional categories they belonged to the "protein synthesis" group. We further analyzed the number of the identified "protein synthesis" groups after 2X of 1.5M addition of sucrose into the solution, we found that most of the proteins of this group belongs to ribosomal protein. This result indicates that by using 2X of 1.5M sucrose into the sample followed by centrifugation, we can get a large number of isolated ribosomal proteins. Overall, this finding is important to help researchers to know which types of the proteases and/or which concentration of sucrose gradient are the best one in the process of protein digestion and purification, particularly in ribosomal protein purification process.

Conflict of interests

None.

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