EFFICACY OF VARIOUS METHODS OF PROTEIN EXTRACTION FROM PURSLANE SUITABLE FOR PROTEOME ANALYSIS

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ABSTRACT *The purslane leaf is characterized by high content of protein, vitamins and minerals. However, its use is limited due to the high fiber content and anti-nutritional substances. In this perspective, the objective of this research was to optimize the protein extraction processes, aiming the use of purslane leaves as an alternative protein. The important characteristics of the protocol include, 1) coagulation of proteins with mild acid precipitation, 2) extraction of protein by three-phase partitioning, 3) extraction of protein by TCA/Acetone method. The protein concentrations among three methods were 802±0.2 µg/g in mild acid precipitation, 625±0.3 µg/g in TCA Acetone and 81.6±0.2 µg/g in TPP, respectively. The solubilization of proteins (method 1) showed a higher extraction yield. Based on the literature survey, the comparison of protocol for protein extraction from purslane leaf was demonstrated first time in this work and showed reproducible results.*
Subtitle: *Protein extraction from purslane leaves for proteome analysis*

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I. INTRODUCTION

In the underdeveloped countries, the ordinary people are following a vegetarian diet. The meat is too expensive that many families cannot afford more than once a week. A few fractions of the world edible plants are being utilized for human food. Furthermore, only 20 crops are being utilized to produce about 90% of the world plant food. In fact, in a number of countries only 6 crops are exported/imported (Parrish et al, 1974). Those food plants which gives about 12% or more than that of their calorific value of protein considered as a good source of protein by Food and Nutrition Board (2001).

The leaves are often considered for the production of high-value recombinant proteins since they have a large quantity of the vegetative substance (Shanker and Debnath, 2016). Type of sample is the main factor to choose which technique or process used for extraction, considering the limitations of the sample and the literature survey. Our interest of molecule can be found in several sources. The source of the sample also plays a significant role in deciding which method to choose. The sample can be obtained from plant cells, animal cells, human cells, microorganisms etc. The plant tissues have a relatively low protein concentration compared to bacterial or animal tissue that makes the study of plant proteome more challenging. Moreover, the cell wall and the vacuole are connected with numerous substances, such as polysaccharides, lipids, proteolytic and oxidative enzymes and polyphenols that interfere with protein analysis (Ashoub et al., 2011).

Separation and isolation of proteins from a complex biological sample is cumbersome. The main challenge in proteomics is to find the most efficient protocols for different sample types. The three-phase partitioning (TPP) is used to extract and purify biomolecules (Duman and Kaya 2013; Narayan et al., 2008). Since purslane is a lesser-known plant and studies on its characteristics are not well established, this plant was chosen for the purpose. This work focuses on various leaf proteins present in purslane and its amino acid profiling. Proteins, long chains of amino acids, vary according to the amino acid sequence. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was generally performed to find out the estimated molecular weight of the protein of interest. Considering leaves, it is said that purslanes are the significantly largest source of protein. Dry matter of leaves contains approximately 10-20% protein. There are soluble as well as insoluble proteins present in leaves. The (SDS-PAGE) is one of the most widely used laboratory methods to separate biological macromolecules such as proteins and nucleic acids. High-quality proteomic profiles are preliminary required to study the proteomic profile of plants (Syed et al., 2016). The objective of the study is to optimize the protein extraction processes from purslane, aiming the use of purslane leaves as an alternative protein and for the application of the proteomic analysis.

II. MATERIALS AND METHODS

2.1 Processing of plant material

Purslane leaves were procured from local shops. Leaves were washed with de-ionized water, blot-dried with filter paper, frozen in liquid nitrogen and stored at -20 ℃ until extraction.

2.1.1Extraction of protein using mild acid precipitation (A)

The protein precipitation test was carried out to validate the effect of pH on the precipitation of proteins in the juice of purslane leaves. The experiment was done according to the protocol followed by (Glória and Regitano-d'arce 2000). Leaf juice was prepared with ~50 grams of leaves and 500 ml of distilled water, ratio 1:10 (w/v). The leaves were homogenized in a grinder (Kenstar, Karishma classic model). The fibrous part was removed by filtering with a muslin cloth. The pH range considered to confirm the precipitation of proteins was between 2 to 12, varying in every unit. The pH was determined using Eutech instrument, Singapore. For each pH value, two beakers were taken with 50 ml of leaf juice each. The first pH of the juice was 5.5. The NaOH (0.1 N) and HCl (0.1N) were used for adjustment of pH. The pH was set at the start of the test, after 10 minutes, and every 1 hr for a period of 4 hr. Finally, for the pH adjustment, the solution was maintained at rest for 1 hr so the precipitate could completely form sediment. Then, every sample was centrifuged using a cooling centrifuge at 20 °C (REMI, CL 30), for 5 min at 7000 rpm. Aliquots of each phase (precipitate and supernatant) were removed for the determination of crude protein and moisture.

2.1.2 Extraction of protein by Three Phase Partitioning (TPP) followed by Dialysis (B) of protein

About 0.5 g of dried purslane leaves were weighed. To this sample, about 20 ml of distilled water was added. Ammonium sulphate salt (20 - 80%) was added to the sample and the later was incubated for overnight. After incubation, *tert*-butyl alcohol (1:1) was added. The final step was centrifugation (REMI, C30 BL) of the sample at 5000 rpm for 10 min. After separation, middle layer was collected and stored for further use. The *tert*-butyl alcohol was added to the mixture at the ratio of 1:0.5; 1:1; 1:2 and 1:4 (Jain and Singh, 2004).

Dialysis is a separation method that facilitates the deduction of small, useless compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane. After salt precipitation, it is necessary to purify the sample, so desalting step is carried out. Dialysis bag of the range of 6-8 kDa was used for this experiment. The phosphate buffer of pH 7.4 was used as dialysate which is usually 200-500 times the volume of the sample. The sample was then stored at -20 ºC for subsequent protein analysis.

2.1.3 Extraction of protein using TCA - acetone (C)

This method was modified little from a published TCA protocol (Pavoković et al, 2012). Tissue powder (1.5 - 5 g) was dissolved in 3 - 5 ml of ice-cold extraction buffer and extracted with acetone containing 10% TCA (w/v) and 1% DTT (w/v). The samples were kept at 20±1 °C for 2 h before centrifuging at 25,000 g for 20 min at 4 °C. The pellet was washed two times by suspending in acetone with 1% DTT, kept at -20 °C for 1 h and allowed to centrifuge. The pellets were then allowed for centrifugation at 15000 *g* at 4°C for 45 min and washed three times using an ice-cold solution of 2-mercaptoethanol in water/acetone. Supernatants were separated and discarded, and pellets were dried at room temperature.

2.2 Protein Quantification

2.2.1 Estimation of protein by Follin-Lowry method

Based on the protein concentration the whole protein sample exhibited by a color difference, are calculated using colorimetric techniques (SHIMADZU UV-1800 spectrophotometer). A blue-purple color complex is formed from the phenolic cluster of tyrosine and tryptophan residues (amino acid) in a protein which further showed absorption in the region of 660 nm wavelength with the Folin-Ciocalteau reagent containing sodium tungstate molybdate and phosphate. Thus, the amount of color depends on the amount of these amino acids that differ for different proteins. The incubation time is extremely critical for a reproducible assay. The Bovine Serum Albumin (BSA) is used universally as standard in most of the proteins evaluation, as of its low cost, high purity and ready accessibility. The method is sensitive down to about 10 µg/ml and is possibly the most widely used for protein assay despite its being only a relative process (Lowry et al, 1951).

2.2.2 Separation of proteins using Electrophoresis

The protein profile unidimensional SDS-PAGE (10% separating gel and 5% stacking gel) was carried out in a small vertical system to conduct the SDS-PAGE experiment. About 100 µg of protein test sample along with 10 µl of buffer containing bromophenol blue as tracking was loaded. A medium range of marker was loaded into the gel to find out the molecular weight of the bands. The gels were run at a continuous voltage of 100 V for 3 hr followed by staining in Coomassie brilliant blue (Hames and Rickwood, 1990) for

overnight. Relative mobility (Rm) of the protein bands was examined and Zymograms were constructed. The gel was photographed and stored in 3% acetic acid.

2.2.3 Estimation of amino acids

The samples were hydrolyzed using 6N HCl at 110 °C for 24 h. Derivatization has been conducted using ophthaldialdehyde prior to HPLC test. The total amino acids were tested by the Knauer (Germany) HPLC set by C18 column at the flow rate of 1 ml/min with a fluorescence detector (RF-530, Knauer, Germany).

2.2.4 Calculation of PER ratio

The protein efficiency ratio (PER) is an important score to examine the quality of protein. This is calculated by feeding a diet containing about 10% of the test protein to the rats and measuring their weight gain. This is a costly and time taking process. An alternative method was developed using experimental models by (Alsmeyer et al., 1974) to evaluate the protein efficiency ratio (PER).

2.3 Statistical analysis

Results were presented as mean ± S.D. ANOVA was used to evaluate the significant difference among the groups. All the experiments were repeated thrice.

III. RESULTS AND DISCUSSION

3.1 Evaluation of protein obtained by mild acid precipitation

The extractable protein contents in the purslane leaves are shown in **Table 1**. The pH range considered to confirm the precipitation of proteins was between pH 2 to 12. The protein in the leaf cells was separated from the fiber by dissolving the protein in the cell sap and filtering off the sap. The protein suspension is strongly affected by the pH. At higher pH, the cell wall swells and releases pectase which initiate to weaken the wall. So the cell wall of cellulose and polysaccharides absorb more water and become soften. Other benefits of the alkaline form are (a) it counteracts the acidic state resulting from the rupture of acidic vacuoles through pulping which could denature the protein, making it unavailable for extraction (Kinsella, 1970) and (b) recoveries of the nutritious xanthophylls and carotene are better (Parrish et al, 1974).

Table 1. Protein concentration of purslane leaves samples extracted by different methods

Sample of extracted protein	Concentration (µg/g)
Fresh leaves	28.96±0.12
Mild acid participated 4.0 pH	697.36±0.6
Mild acid participated 4.5 pH	753.77±0.41
Mild acid participated 5.0 pH	788.12±0.65
Mild acid participated 5.5pH	802.03±0.23
TCA/Acetone	625.14±0.3
TPP 30% Salt conc.	81.60±0.23
TPP 40% Salt conc.	41.12±0.16

3.2 Evaluation of protein obtained by three-phase partitioning

The TPP was carried out varying the ammonium sulphate concentration (20 - 80%). This range was chosen as most proteins are precipitated within this range. It was reported that in 30% and 40% concentration, proteins are precipitated in the middle layer. The Follin-Lowry method was used for protein was analysis. The variation in the solvent was carried out. It was observed that 1:1 ratio had the three-phase separation, whereas the other ratios were not suitable for the extraction as no middle layer was observed (**Table 1**).

3.3 Evaluation of protein obtained by TCA/Acetone

The total amount of protein extracted from purslane tissues varied according to the protein extraction method used. A considerably higher amount of protein was found extracted with the direct IEF buffer extraction method, while precipitation was observed the significant removal of pigments (**Table 1**).

3.4 SDS PAGE of optimized proteins

Proteins were separated on 12% resolving gel, where lane-1 contains the molecular marker, lane-2 is loaded with TCA/Acetone precipitated and lane-3 is loaded with mild acid precipitated sample. The lane-4 is loaded with fresh leaves sample and lane-5 consists of sample extracted by three-phase partitioning. The bands of various protein are visible according to their molecular weight. An approximate range of the protein was deduced from this result. It is observed that most of the proteins separated are of high molecular range. The molecular weight of SDS protein bands is ranging from 14.3 kDa to 97.4 kDa. Relative

mobility of the bands varied from 0.41 to 0.78. Experimental data showed the presence of low, medium and high mobility bands in all the samples. The presence of one polypeptide band whose relative mobility is 0.41 representing MW 43 kDa was observed in all the methods. Healthy, 2nd, 3rd, 4th and 5th stages showed the highest quantity of bands i.e. all the five bands were noticeable. The low molecular weight polypeptide band of medium to light concentration by Rm 0.53 (MW 44 kDa) was excellent to every stage and healthy one. The 4th stage having a strong dark protein band of about 97 kDa which is varied by all other stages. The 2nd, 3rd and 4th stage could be distinguished from healthy, 5th stage by the lack of a protein band of 97 kDa. In the middle of the gel protein bands was well expressed. The lower side of the gel the bands were most common. The presence vs. absence type of polymorphism of SDS protein with varied intensities was observed through this study (**Fig. 1**). The electrophoresis of proteins has been used for the characterization of different taxonomic, evolutionary and genetic relationship studies (Virinhos and Murry, 1983).

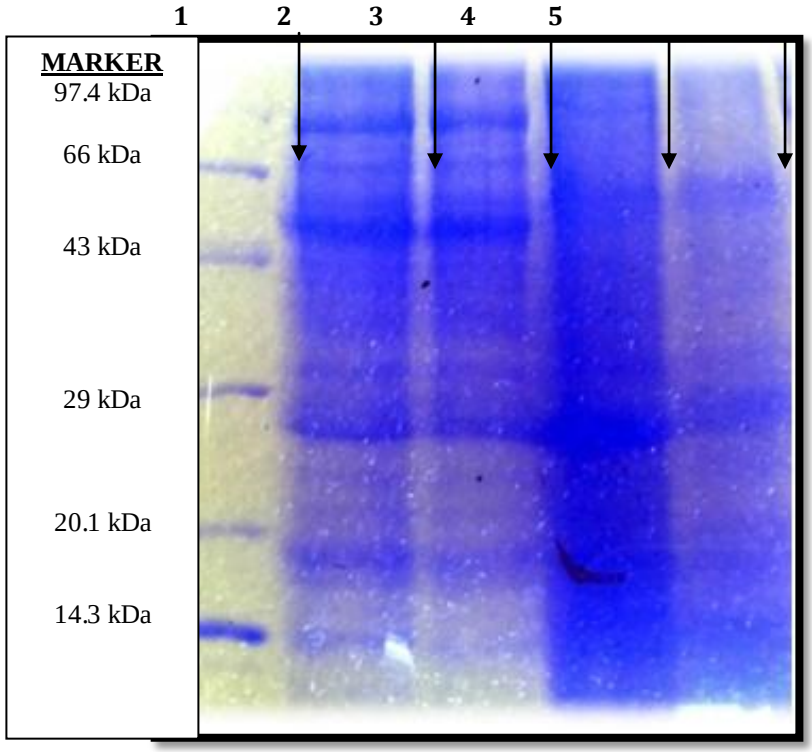

Fig. 1 Fig. 1 SDS-PAGE separation of purslane leaf proteins. Proteins extracted from different methods of purslane leaf are resolved using 12.5% polyacrylamide gel and visualized with CBB Coomassie Brilliant Blue. The arrow indicates the prominent polypeptide, characteristic of leaf tissue extracts. The protein standards sizes are indicated on the left. Lane 1- contains the molecular marker. Lane 2 TCA/Acetone sample Lane 3 - mild acid precipitation sample. Lane 4 fresh - leaves sample. Lane 5- TPP sample

3.5 Evaluation of amino acids of the optimized protein

The purslane leaves are a rich source of essential amino acids such as leucine, isoleucine, lysine, valine, threonine, and tryptophan as can be inferred by its amino acid profiling (**Table 2**).

Table 2. Amino acid profiling of purslane from mild acid precipitation (optimized method)

Amino acid	Purslane (g/100g)
Asx	7.47±0.08
Glx[b]	15.07±0.19
Ser[b]	4.30±0.21
Gly[b]	5.82±0.10
His[a]	2.18±0.16
Arg[a]	6.81±0.50
Thr[a]	4.00±0.22
Ala[b]	6.42±0.09
Pro	6.01±0.11
Tyr	2.81±0.37

Val[a]	6.35±0.26
Met[a]	2.34±0.01
Cys	2.36±0.23
Ile[a]	5.24±0.24
Leu[a]	9.61±0.28
Phe[a]	5.81±0.21
Lys[a]	5.44±0.05
∑AA	98.67
∑EAA	48.41
∑FAA	31.61
∑EAA/∑AA	0.49
∑FAA/∑AA	0.32
∑EAA/∑NEAA	2.59

Essential amino acids, [b] Flavor amino acids, AA: Amino acid, EAA: Essential amino acids, FAA: Flavor amino acid, NEAA: Non-Essential amino acid

It can be treated as all-rounder food as it is rich in nutrient content. The PER values lower than 1.5 and higher than 2 indicate low and high-quality proteins, respectively (Friedman, 1996). While high PER1, PER2 and PER3 values observed are due to high Leu content, high PER2 and PER3 due to low Tyr content (**Table 3**).

Table 3. Prediction equation for some of the nutritional indices of protein hydrolysate from purslane leaves (mild acid precipitation) using Alcalase and Protamex

PER Equation	
-0.468 + 0.454 [Leu] -0.104 [Tyr]	3.602
-1.816 + 0.435 [Met] + 0.780 [Leu] + 0.211 [His] -0.944 [Tyr]	6.2
0.08084 [X7] -0.1094	3.02
0.06320 [X10] -0.1539	3.08

[a]X_7 = Thr + Val + Met + Ile + Leu + Phe + Lys

X_{10} = X7 + His + Arg + Tyr

IV. CONCLUSION

Leaf protein concentrate is extracted from the leaves of plants. The present study demonstrated that different methods are efficient for downstream of proteome analysis. The present methods emphasize the importance of protein extraction method in achieving optimal separation and identification of proteins using SDS-PAGE and mass spectrometry. In terms of SDS- gel separation and identification of proteins by PMF (peptide mass fingerprinting), the mild acid precipitation method was found superior to other two tested methods (TPP and TCA/Acetone) for purslane leaves analysis. The present study also indicated that the purslane is susceptible to show the potential antioxidant activity and free radical scavenging properties owing to the presence of different of various functional compounds as revealed by FTIR spectral studies. The protocol is expected to be applicable and could be of interest to various food laboratories/industries involved in plant proteomics.

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