TEMPERATURE AND pH EFFECTS ON TOTAL WATER SOLUBLE PROTEIN FROM CORMS OF *FREESIA REFRACTA* AND *SPARAXIS TRICOLOR* PLANT SPECIES

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Abstract

This study is primarily focused to see the effects of different temperature and pH values on total protein extractions from corms of *Freesia refracta* and *Sparaxis tricolor* species belonging to medicinally important Iridaceous family. Comparative analyses of the two plants were made at two different temperature (4 & 25 ºC) and four pH (3.0, 5.0, 7.5 & 10) values. The results showed clearly that alkaline conditions were more suitable for obtaining better total protein quantities. In this study results shown that proteins of both plants have inter-chain disulfide linkages, the SDS-PAGE was performed in reduced and non-reduced conditions. *F. refracta* showed thicker bands on pH 5.0, 7.5 and 10.0. It showed maximum protein concentration of 120 mg/g of corm tissue at pH value 7.5 and 4 °C temperature. *Sparaxis tricolor* also showed maximum protein (112 mg/g of corm tissue) at pH 7.5 of the temperature 4 °C. However *S. tricolor* showd better protein concentration and banding patterns on SDS-PAGE as compared to *F. refracta.* The results of the present study showed that bands of 30 kDa in both species indicate the presence of cysteine protease at both temperatures under alkaline pH conditions. Cysteine proteases can be used to degrade the other proteins and can be used in the food industries.

Key words: Water soluble protein, Temperature, pH, Seed, SDS- PAGE

Introduction

Plants are being used in the synthesis of medicines since past. The modern pharmacy has developed from medicinal plants and most medicines are synthesized from medicinally important plants, and these plants are being cultured for isolation of bioactive compounds (Schmidt *et al.,* 2008). Plants produce a broad spectrum of proteins i.e. 2S albumins storage protein, chitin binding proteins, defensin-like proteins, ribonucleases, peroxidases protease inhibitors, cyclophilin-like proteins, thaumatin-like proteins, glucanases, embryo-abundant protein-like proteins, chitinases, ribosome-inactivating proteins, lipid transfer proteins, lectins deoxyribonucleases and many more (Wong *et al.,* 2010). These proteins show the effect against the heart diseases epidemic, autoimmune diseases and tumors (Blohm *et al.,* 1988). There are 50 % prescribed products are synthesized from the natural compounds in which plants and their derivate are included (Cordell, 2002; Newman *et al.,* 2003). On Earth, there are 250000m to 500000 plants present on which only 1 to 10 % plants is being studied in chemical synthesis and medicines development (Verpoorte, 2000). The proteins and peptides are utilized as active beneficial for the last two decades due to developments in modern recombinant DNA technology and biotechnology. Plant originated cysteine proteases play a role in immune modulation, digestion as mammalian serine proteinases and wound healing (Salas *et al.,* 2008).

These enzymes are also used in many industries. Bromelain, papain and ficin are the most frequently used plant cysteine proteases with their applications in brewing, milk-clotting, meat softening, cancer treatment and viral disorders (Reddy *et al*., 2002; Behnke *et al*., 2008; Baker and Numata, 2012; Hu *et al*., 2012; Tuck *et al*., 2012).

The species of Iridaceae have a long history of medicinal uses in different parts of the world with rich sources of secondary metabolites (Wang *et al*., 2010). *Freesia refracta* and *Sparaxis tricolor* belong to the family Iridaceae. *Freesia* species are very important in the perfume and cosmetic industry due to their flower volatile compounds (FVCs) (Ao *et al*., 2013) and have found their applications in aromatherapy as well. Plants are extremely useful in practicing production technologies for inducing expression of recombinant proteins on larger scale, but such a practice is still dealing with issues of how to improve product quality and quantity (Khanzada *et al.,* 2008).

In this context, it is of immense importance to optimize the plant proteins extraction conditions for the maximum quantity and as well as quality. Therefore, a temperature and pH screening was performed in order to have good protein profiles from the plants *F. refracta* and *S. tricolor*.

Materials and Methods

Collection of sample: The research work was conducted in the Proteomics Lab, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan. The non-germinated corms of the *F. refracta* and *S. tricolor* plants were obtained and identified from taxonomist.

Temperature and pH values: The protein was extracted at two different temperatures i.e. 4 and 25 °C and at four different pH values i.e. 3.0, 5.0, 7.5 and 10.0 as shown in table.1. Extraction at 4°C was performed in the ice boxes due to avoid the effect of heat shock. The experimental lay out is shown in Fig. 1.

Preparation of extracts: Corms of the two plants were peeled to remove the outer papery layer and then washed thoroughly with distilled water. Plant material was ground in liquid nitrogen using mortar and pestle to a very fine powder to disrupt the cell structure maximally for the smooth release of water soluble proteins. The fine powders of each of the two plants were divided into two sets of four equal parts (i.e., 5 grams each for each of the above mentioned four pH values). One set was kept for room temperature (25 ºC) extraction while the other was used for low temperature (4 ºC) extraction. Furthermore, experiment was performed in six replicates for every temperature and pH values. To see the pH effects on the extraction of crude proteins, four buffers (Citrate: 3.0, Acetate: 5.0, Phosphate: 7.5 and Carbonate: 10.0) with different pH values were prepared according to the standard procedures. The pH of every buffer solution was checked and confirmed on pH meter. The molar strength of each respective buffer was kept to 100 mM with 0.1 M NaCl, 0.001M PMSF and 5 % glycerol. Along with PMSF, protease inhibitor cocktail tablets (S8820, Sigma) were also used to stop the attack of protein catalytic enzymes. Each 5 g powdered sample was taken into sterilized mortar with little volumes of the respective buffer and once more was given harsh treatment of pestle in order to have maximum cell rupturing. The slurry was shifted in 0.25 L flask with magnetic bars of 1 cm length and the flasks were filled up til 0.05 L of the extraction buffer. Stirring was done overnight. The samples were centrifuged (Ogawa 6470, Japan) for 30 minutes at 10,000 rpm. Pellets were throw away and the supernatants were filtered through Whatman filter paper no. 1 to remove any particulate matter from the liquid crude extract. 0.01 % Sodium azide was added in these samples to stop the growth of microbes. All the above steps were kept constant for all the extractions except the usage of ice and cold buffers kept at 4 ºC for the cold temperature extractions.

Protein quantification and gel analysis: Total protein concentration was quantified by Bradford assay (Bradford, 1976). Bovin Serum Albumin (BSA) was used as a standard curve and 5 mg/mL concentration of BSA used as stock solution to quantify the protein. Color change was observed by spectrophotometer at 595 nm. Protein prolife of two extracts was studied at 25 ºC and 4 ºC temperatures and at four pH values by performing the Sodium Dodecyle Sulphate Polyacrylamide Gel Electrophoresis. The standard procedure for SDS-PAGE was used (Laemmli, 1970) and to visualize the protein profile of both plant extracts. Plant extracts were made reduced by adding β-mercaptoethanol in the sample buffer and then the samples were heated in a water bath for 5 minutes at 95 ºC to denature the samples and gel was stained according to strandard protocol. (Sambrook *et al.,* 1989) to see the protein bands in the plant extracts.

Results

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The objective of this study was to optimize the temperature and pH values regarding the maximum total protein extractions from two plants of family Iridaceae. It was noted that corm protein concentration significantly affected by different temperature and pH values.

Total protein quantifications of *F. refracta* and *S. tricolor* at two temperatures and four pH values: Results have shown that protein extractions at 4 °C are much better as compared to extractions made at room temperature (25 °C). Similarly, as compared to acidic pH, alkaline pH is showing more promising results in terms of total protein quantities. Comparison of total protein contents of the two species quantified through Bradford reagent are shown in Fig. 2. Results shown protein concentration pattern is different at both temperatures and at four different pH values. Maximum protein was obtained at basic extractions in both plant extracts. In *F. refracta*, in pH 7.5 maximum protein (120 mg/g of corm tissue) was obtained at temperature 4 °C as compare to pH 3.0 minimum protein (7.0 mg/g of corm tissue) was extracted at the temperature 25 °C. However, after this second high protein concentration was obtained at pH 7.5 of the two temperatures. However again, at temperature at 4 °C higher protein concentration was obtained as compared to room temperature. Extreme acidic condition (pH 3.0) altogether has been very deleterious for the protein extractions at all temperature values.

In *S. tricolor*, in pH 7.5 at temperature 4 °C maximum protein 112 mg/g of corm tissue was obtained as compared in pH 3.0 at temperature 25 °C. However, second higher protein concentration can be seen at pH 7.5 of the temperature 4 °C.

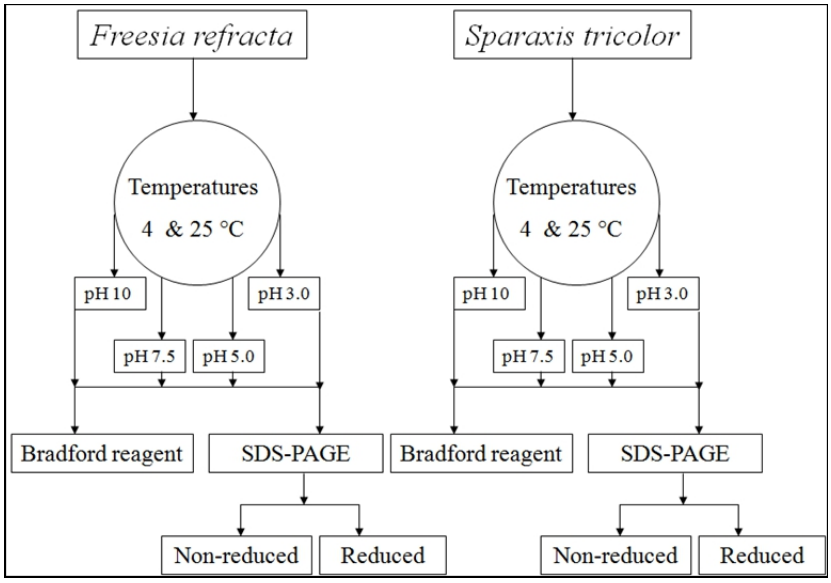

Fig. 1. Illustration of the experimental lay out.

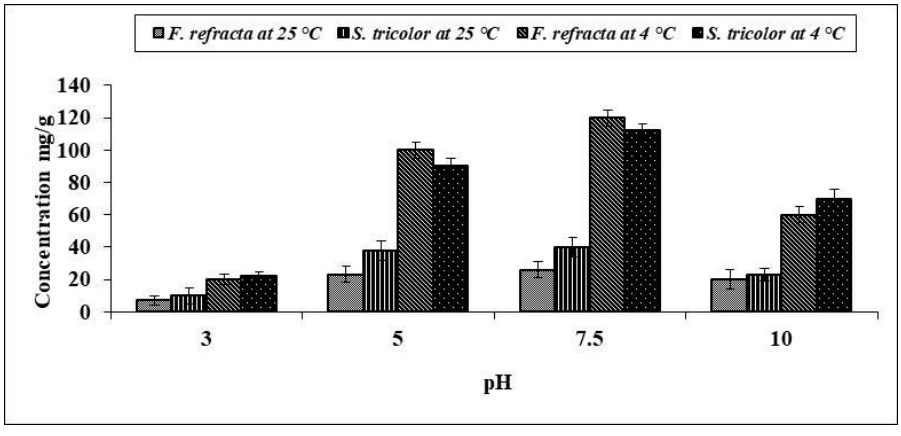

Fig. 2. Graph depicting the total protein comparison of *F. refracta* and *S. tricolor* at different pH with varying concentrations.

It is very much evident that in two temperatures, the lower temperature (4 °C) extractions for both the plants are better than the extractions done at the room temperature (25 °C). Similarly, comparison between the different pH has shown the best extractions around physiological pH values than extreme acidic or basic conditions. However, the basic pH value (10.0) is still much better than the acidic pH value (3.0) which proved to be highly unfavorable for the extractions of this biomolecule.

Gel analyzed protein profile of *F. refracta* at two temperatures: For a single temperature value, a single gel of *F. refracta* contains both reduced and non-reduced protein profiles together to make a better comparison between them.

At 4 °C, it was very much clear that almost all the protein bands more than 50 kDa were totally disappeared under reduced condition. At pH 3.0, three bands were visible under non-reduced condition which was again almost disappeared under reduced condition. At pH 5.0, three major bands ranging between 15 to 30 kDa were visible and such a banding pattern was also very much similar to the one present in lane of pH 7.5. However, lane of pH 10.0 exhibited a major band of 30 kDa under both reduced and non-reduced conditions. Overall this 30 kDa protein band was constant in almost all lanes with drastic reduction in its concentration under pH 3.0.

At 25 °C, a similar type of banding pattern can be observed like the one existing in 4 °C. However, a much decreased concentration of the bands was present. It was very much evident that in comparison between the two temperatures, the low temperature treatment was much better for the total protein extraction from corms of *F. refracta*.

Gel analyzed protein profile of *S. tricolor* at two temperatures: For a single temperature value, a gel of *S. tricolor* contains both reduced and non-reduced protein profiles together to make a better comparison between them. Surprisingly a very similar attitude like *F. refracta* has been experienced in protein banding pattern of *S. tricolor* as well. The banding pattern developed at low temperature treatment has clearly showed the disappearance of higher molecular weight proteins (> 50 kDa) under reduced conditions of the SDS-PAGE. This band is easily visible in first three crude samples of pH 3.0, 5.0, 7.5, but at pH 10.0, third band is very light and not easily visible. Two prominent bands are present throughout all the four pH extractions with molecular weights approximately around 15 and 30 kDa respectively.

At 25 °C, a much fuzzy and disturbed banding pattern was obtained this is due to less protein quantity was extracted at this temperature as compared to 4 °C. It is very much evident that in comparison between the two temperatures, the low temperature treatment is much better for the total protein extraction from corms of *S. tricolor*.

Discussion

The activity of any biological macromolecule depends on its structural stability which is depending on the biophysical nature of surrounding microenvironment. Every metabolic

pathway in living organism is strictly depending upon pH and temperature factors. The main goal of the present study was to find out the optimum temperature and pH values for the protein stability during their extractions. The importance of proteins of underground storage plant organs like tubers, bulbs or corms have already been reported by Shewry, 2003. In addition to their storage function, these proteins play many other important roles for plants such as enzymatic (Tonón *et al*., 2001), antioxidant (Hou *et al*., 2001), antibacterial and as well as antifungal (Flores *et al*., 2003). The present study was aimed to prove that family Iridaceae is not only an ornamental family but also contains a very rich protein banding pattern present in its corms. Already pharmaceutically important proteins has been discovered from its genera like *Crocus* (Akrem *et al*., 2011), *Gladiolus* (Yamagami *et al*., 1998) and *Iris* (Hao *et al*., 2001). Nevertheless, still a great number has to be investigated in search of better protein profiles. Since purification and characterization of the proteins is strictly dependent upon their better quantitative as well as qualitative extractions. The two plants were analyzed for their protein profiles under different temperature and pH values. The pH normally affects the protein in two ways, either influencing its solubility or changes the charge of its amino acids.

Weight by volume (w/v) ratios (1g of corm tissue/10ml of the buffer) were selected very carefully after repeated checks avoiding the super saturation condition in order to protect the unnecessary protein precipitation. However, the unnecessary dilution was also avoided keeping in view the proper thickness and visibility of the protein bands on SDS-PAGE. It is obvious from the data analysis that *Freesia refracta* extractions made at 4 °C showed significantly different results from that of 25 °C. There was a high protein content quantified as 120 mg/g of the corm at pH 7.5 of the low temperature treatment while for 25 °C, maximum protein quantity of only 26 mg/g was observed at pH 7.5. Very low protein content (7 mg/g) was experienced at pH 3.0 of the 25 °C. Similarly, Protein quantification of *Sparaxis tricolor* samples indicated a maximum of 112mg/g at pH 7.5 of 4 °C as compare to 40mg/g at pH 7.5

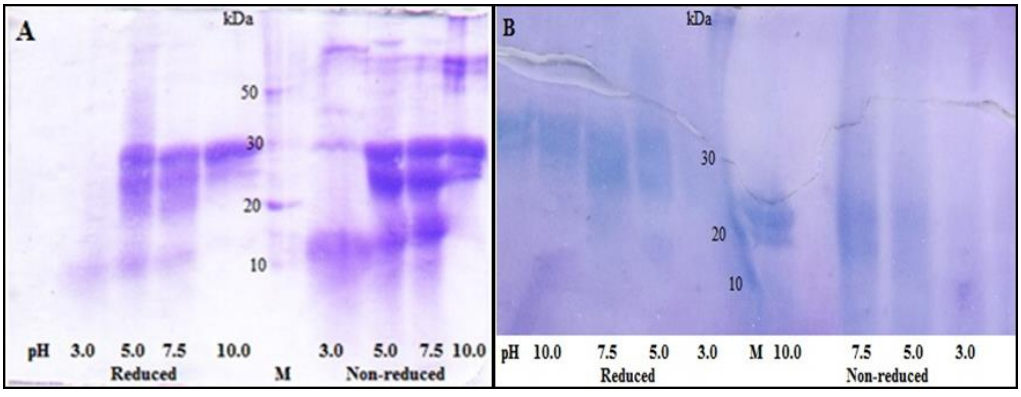

Fig. 3. pH based protein profiles of *F. refracta* corms at 4 °C (A) and 25°C (B).

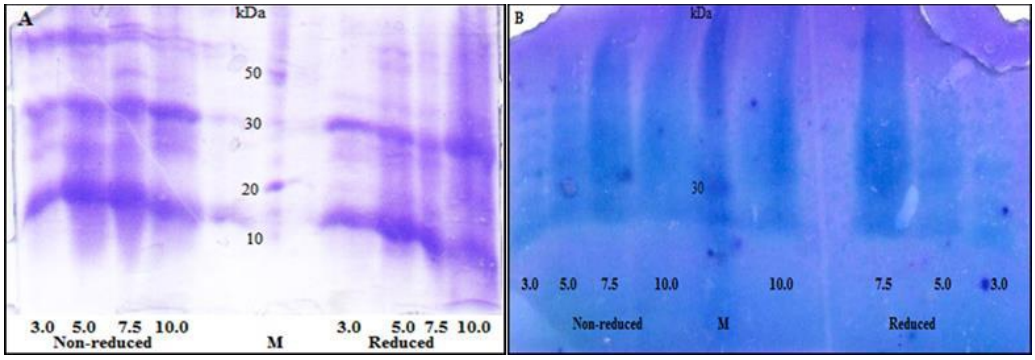

Fig. 4. pH based protein profiles of *S. tricolor* corms at 4 °C (A) and 25 °C (B).

of 25 °C. Very low protein content (10 mg/g) was experienced at pH 3.0 of the 25 °C. It has been very clearly observed that more acidic condition (pH 3.0) has more adverse effects on total protein content as compare to relatively high alkaline condition (pH 10.0). One possible reason for this could be that acidic condition is bringing most of the proteins near to their isoelectric pH or pI, as a result of which the proteins are getting insoluble and are flushed away during the centrifugation after overnight stirring; entangled in the hard debri pellet. Thermal denaturation of the proteins is relatively controversial phenomena. Some proteins are more prone to higher temperatures and some exhibited cold phenomenon of precipitation. Thermal denaturation normally disrupts either the hydrogen bonding or increases the hydrophobic contents. For the above two mentioned plants, the banding pattern is experienced at both the temperature values (4 and 25 °C), however much reduced concentration was observed at 25 °C which is an indication of the fact that for these two plants, the corm proteins are more sensitive to high temperatures. Almost similar quantities of the protein were experienced in both the plants with similar values of the physical factors. Additionally, both plants exhibited same reduction of protein bands under reduced conditions. The presence of β-mercaptoethanol in reduced samples has resulted in separating the proteins by reducing disulfide linkages and giving bands of lower weights.

In *Phaseolus vulgaris*, two cysteine proteinases, of 30 kDa (FLCP-1) and 25.1 kDa (FLCP-3) molecular weights were purified (Popovič *et al.,* 1998). From medicinal plants of family Cucurbitaceae, *Momordica charantia* and *Gelonium multiflorum* two anti HIV proteins with molecular weights of 30 kDa (MAP30) and 31 kDa (GAP31) respectively, were purified. These proteins are anti HIV, giving an indication of the presence of anti HIV properties in 30 and 31K Da proteases. Thus it can be predicted as cysteine proteases of these molecular weights have great medicinal value (Huang *et al.,* 1995). Another protein of molecular weight 36 kDa, purified from rice dark grown seed storage protein glutelin, was REP-1, with N-terminal amino acid sequence and experiments with its inhibitors indicated it to be a cysteine endopeptidase (Kato & Minamikawa, 1996). Similarly, a papain like cysteine protease of approximately 25 kDa has been reported from corms of *Crocus sativus* (Iqbal *et al*., 2011). Different cysteine proteases with different molecular weights as well as activities, between species of same genus have already been reported (Bah *et al.,* 2006). *Freesia refracta* contains protease (FP)-A in its regular corms (Kaneda *et al.,* 1997). *Freesia refracta* has been studied for analyzing the soluble proteins during somatic embryogenesis and different polypeptides with different molecular weights have been observed during development of embryogenic callus, globular embryoid, and embryoid with coleoptiles, except the embryoid with leaf (Abdullaev & Aguirre, 2004). When protein bands were observed in reduced samples of *Freesia refracta,* there was a protein band of 30 kDa present in all pH samples. 13mg of protein from 200mg of *Freesia refracta* corm was extracted at this temperature (Uchikoba, 2003). *Sparaxis tricolor* gel analysis via SDS-PAGE showed high protein visibility of thick protein bands. A similar protein band of approximately ~ 30 kDa can be seen in all pH samples and is stable also under reduced condition similar to the one present in *F. refracta*. Most probably it seems that it is also one of the cysteine protease present in the corms of *S. tricolor* as a part of the defensive mechanism of such underground storage organs.

Acknowledgement

This work was financially funded by "Directorate of Research and External Linkages, Bahauddin Zakariya University, Multan".

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