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ELECTROPHORETIC ANALYSIS FOR THE SEPARATION OF MUSCLE PROTEIN OF FIDDLER CRABS OF PAKISTAN

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ABSTRACT

Fiddler crabs muscle tissue was used to estimate molecular weight of protein through SDS-PAGE electrophoresis. A detailed study was carried out from Sandspit, Karachi area to observe the relative mobility and molecular weight of proteins by using standard marker BSA. Results showed that all four species of fiddler crabs vary apart from each other by their molecular weight and their relative mobility. *Austruca analyses* revealed a maximum number of bands (total seven). While, *A. annulipes* showed 4. Out of 7 bands found heavier in size (>66 kDa) while three bands from each species shown as smaller in size (<66 kDa) from standard marker BSA. During the current study unveiled that the BSA can effectively be used for calculating the molecular weight of protein in family Ocypodidae.

KEYWORDS: Fiddler crabs, SDS-PAGE, BSA, Sandspit, Karachi.

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is widely used technique in biochemistry, molecular biology and biotechnology to separate biological macromolecules such as proteins or nucleic acids according to their electrophoretic mobility. To separate the protein sodium dodecyl sulfate (SDS) is applied to sample for protein transformation into a linear form and convey negative charge to linearize the protein, therefore, this procedure is well known as SDS-PAGE (Roy and Kumar, 2014). SDS is technique used to separate proteins based on their ability to move within an electrical current which is use of polypeptide length and their molecular weight (Khan *et al.*, 2012; Kresge *et al.*, 2006; Nowakowski *et al.*, 2014).

Protein electrophoresis received a prime improvement, when Davis (1964) described the polyacrylamide gel procedure for the separation of serum proteins. In this procedure, the muscle proteins were not separating out because of myofibril proteins, which were insoluble under those conditions. Therefore, Kresge *et al.* (2006) introduced a new approach by including the sodium dodecyl sulphate (SDS) in the sample and gel solutions. This detergent was found very much effective and binds to protein in a relatively constant weight ratio (Greaser *et al*., 1983). SDS-PAGE is simple technique through which proteins can be identified easily by their molecular weight. The SDS-PAGE is routinely employed to estimate the molecular weight of proteins due to its convenience, speed and cost effectiveness. In general electrophoresis move the proteins with relation to its weight, shape and charge but SDS has quality to minimize the shape and charge elements by binding to proteins. SDS denatures the protein and binds with each fragment, by binding each fragment of protein with SDS neutralizes the charge of bound molecule. In addition, with SDS 2-mercapto-ethanol is also used which further reduces the disulphide bonds in the protein. Therefore, it is assumed that proteins in SDS-PAGE are fully denatured and fully masked with SDS which subsequently causes the migration of proteins fully based on weight. Such assumptions may not be true always.

SDS-PAGE is widely followed by the Laemmli system in which tris-glycine gels comprised of a stacking gel and resolving gel. This system consists of discontinuous buffer system where the pH and ionic strength of the buffer used for running the gel (Tris, pH 8.3) is different from the buffers used in the stacking gel (Tris, pH 6.8) and resolving gel (Tris, pH 8.8).

Fiddler crabs are commonly found in coastal habitat of Pakistan. They are small creatures approximately 20 mm in size, construct burrows near the coastal backwaters mostly near mangroves. They play an important role in nutrient recycling and are important components of food web of coastal waters (Saher, 2008). The objective of this study was to observe the apparent molecular weight in 4 species of family Ocypodidae. The perceptible molecular weight can be determined by comparing it with known molecular weight marker BSA.

Materials and Methods

The four species (*Austruca annulipes, A. iranica, A. sindensis* and *Tubuca urvillei*) of family Ocypodidae were collected through random method from the Sandspit (24.84º E, 66.91º N) backwaters mangrove area from the year 2013-2014 during the low tide. All the specimens were brought to laboratory alive and kept in the freezer prior to start biochemical work followed by identification through available taxonomic key (Crane, 1975; Saher, 2008; Shih *et al*., 2016).

Tissue extraction

Total 0.1 g of muscle tissue was extracted from each specimen's cheliped and placed into handheld Homogenizer for mincing then the extraction buffer (Tris–citrate, 0.687M Tris and 0.157M Citrate pH 8.0) was added and homogenized efficiently until it become a thick solution then transferred this into well labelled 1.5 mL Eppendorf tubes.

The polyacrylamide gel electrophoresis - SDS (PAGE-SDS)

The complete protocol for PAGE-SDS preparation was followed by Laemmile (1970). The 10% resolving gel was prepared for the separation of the proteins. The stain Coomassie brilliant blue R-250 was used for staining. Prior to gel preparation all the stock solutions were prepared (Table 1). Followed the series of steps such as gel assembly, casting a gel, staining and de-staining process, relative frequency calculations and creating graphs and tables for approximate band size estimation by using molecular marker Bovine Serum Albumin (BSA).

Sodium dodecyl sulfate (SDS) is an ionic detergent which denatures the proteins and 2-mercapto ethanol have ability to cleave the disulphide bonds. Subsequently, the charges on proteins become insignificant because of SDS. SDS combines with proteins to neutralize them by providing opposite charge. Consequently, polypeptides have almost same charge so the movement is according to their molecular mass. The molecular mass of protein/peptide can be determined using a molecular mass standard.

Table 1. The buffer system and other solutions used in preparation of gel and their stock solution.

Discontinuous buffer system:	**Stock solution**
Tissue homogenizing buffer Tris-Citrate (pH 8.0)	200 mL
Stacking gel buffer (pH 6.8)	100 mL
Resolving gel buffer (pH 8.8)	100 mL
Reservoir buffer (pH 8.3)	1000 mL
Sample dilutes buffer (pH 6.8)	100 mL
Tracking dye	50 µL
Solution A Acrylamide: bis-acrylamide (30:0.8)	100 mL
Solution B 3.0 *M* Tris – HCl (pH 8.8)	100 mL
Solution C 0.5 *M* Tris – HCl (pH 6.8)	100 mL
Solution D 1% SDS	100 mL
Solution E Ammonium Per Sulphate	1mL
Staining solution	500 mL
Destaining Solution	2000 mL

Gel preparation and gel casting

Vertical slab gel electrophoresis (PLAS- EV243) were selected to identify banding patterns. Gel Assembly and solutions were prepared prior to cast the gel. The gel was prepared by using the discontinuous buffer system in two parts, one is staking gel (5%) and other resolving gel (10%). Staking gel function is to hold the proteins and dissociate them into small fragments to analyze each band separately. After gel preparation, 40-µl sample was poured into each well and run on 130 volts. BSA was used as molecular markers.

Staining and De-staining

Coomassie Brilliant Blue was used for staining the gel. The gel was poured into the solution for 30 minutes and then replaced with de-staining solution for 30 minutes again. Process for replacing the de-staining solution was repeated if bands were not clearly visible.

Banding pattern analysis

After de-staining, the bands observed manually by gel photographed and their migration rate recorded in a notebook for relative mobility calculation. The relative mobility (*Rf*) can be calculated by using the following formula:

$$\text{Relative frequency} = \frac{\text{Distance travelled}}{\text{Dye front}}$$

MW estimation by SDS PAGE

The 10% percent acrylamide gel applied for the estimation of molecular weight of protein. The percentage of acrylamide gel regulates the molecular weight of protein. Sodium Dodecyle Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was used to find out the molecular weight of active bands of the sample. SDS-PAGE was performed in separating gels, according to the method described by Laemmli (1970). Different samples were used to determine the molecular weight of proteins in the muscle tissue. The muscle tissue containing proteins was detected. Protein marker (BSA) with molecular weight (66 kDa) was used. The bands were detected in the gel that represents proteins. The BSA was used to analyze and observed the bands with a protein marker that which band is higher in molecular weight from the BSA.

RESULTS

The SDS-PAGE was shown to have protein patterns of muscle tissue samples from each species of fiddler crabs. SDS-PAGE easily distinguish the species of family Ocypodidae by using a single molecular marker. However, it would effectively if all the markers would be used to identify the appropriate molecular weight of species. It can be demonstrated through SDS-PAGE, that two species (*A. annulipes* and *T. urvillei*) have demonstrated the additional proteins to muscles. At least two proteins showed larger size than BSA (66 KDa) in all four species of Fiddler crab. *A. iranica* showed total 5 bands on SDS among which two bands showed up larger than BSA and two smaller in size while one was equal to BSA. *A. annulipes* showed total seven bands among which three bands appeared to larger in size from BSA while four bands showed smaller size as compared with BSA. In case of *A. sindensis, the* two protein bands appeared with a higher molecular weight than BSA (66.6 KDa) and two smaller than BSA in size, however one band was equal to the molecular weight of BSA. *T. urvillei* showed total 6 bands among which four bands appeared as larger than BSA while two showed up smaller than BSA none of them showed equal size with BSA (Table 2; Figs. 1 and 2).

There are different types of acrylamide gel depending on how much percentage is being used for. There is also another use of acrylamide by using it as gradient gel from 4 to 20% and there are a few advantages with gradient gels. For example, few people use a 4 to 20% gradient of acrylamide concentration as this will allow the variable sizes of (large and small) proteins to separate more widely in their molecular weight of 5,000 KDa to 30,000 KDa. But the major problem with gradient gels is that, it does not produce the same gradients and gel patterns in every used.

Table 2. The percentage of acrylamide gel that regulate molecular weight of protein (Modified from Dube and Flynn 1998).

Percent acrylamide	Molecular weight (KDa)
6%	65-220
10%	20-80
16%	10-50
4-20%	5-300

DISCUSSION

The scientists continued to follow (Laemmli, 1970) approximately 40 years since the methodology of using the SDS-PAGE has been introduced. This methodology gives higher resolution of protein separation which increase the intrest to follow the same methodology since it is introduced (Nowakowski *et al.,* 2014). This method is generally used to examine the presence, relative concentration, purity of protein and their approximate molecular mass (Gallagher and Wiley, 2008; Walker, 2002). Dubey and Flynn (1998) suggested that protein resolution is controlled by percentage of acrylamide as used in any study. They also suggested that if the acrylamide concentration is uniform then linear relationship between logarithm molecular weight and relative mobility is limited. For example, with the help Laemmli protocol system, the linear ranges varied such as if gel is 10% then, logarithmic molecular weight range from 20 KDa to 80 KDa.

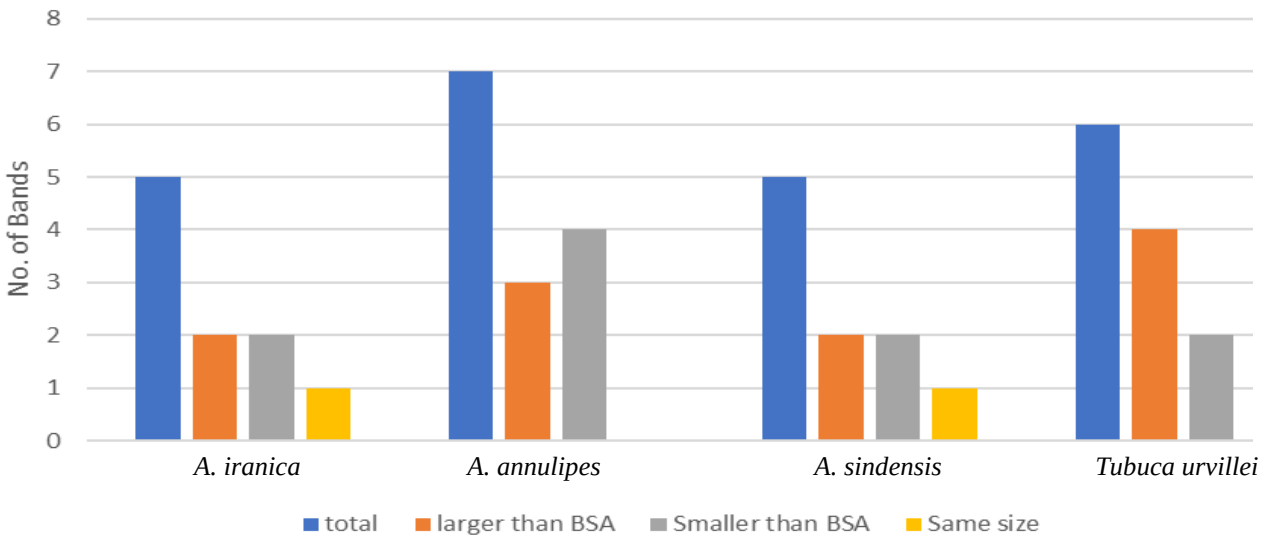

Fig. 1. The total banding position representing in each species, in which *A. annulipes* shows a higher number of bands (4) larger in size than the BSA.

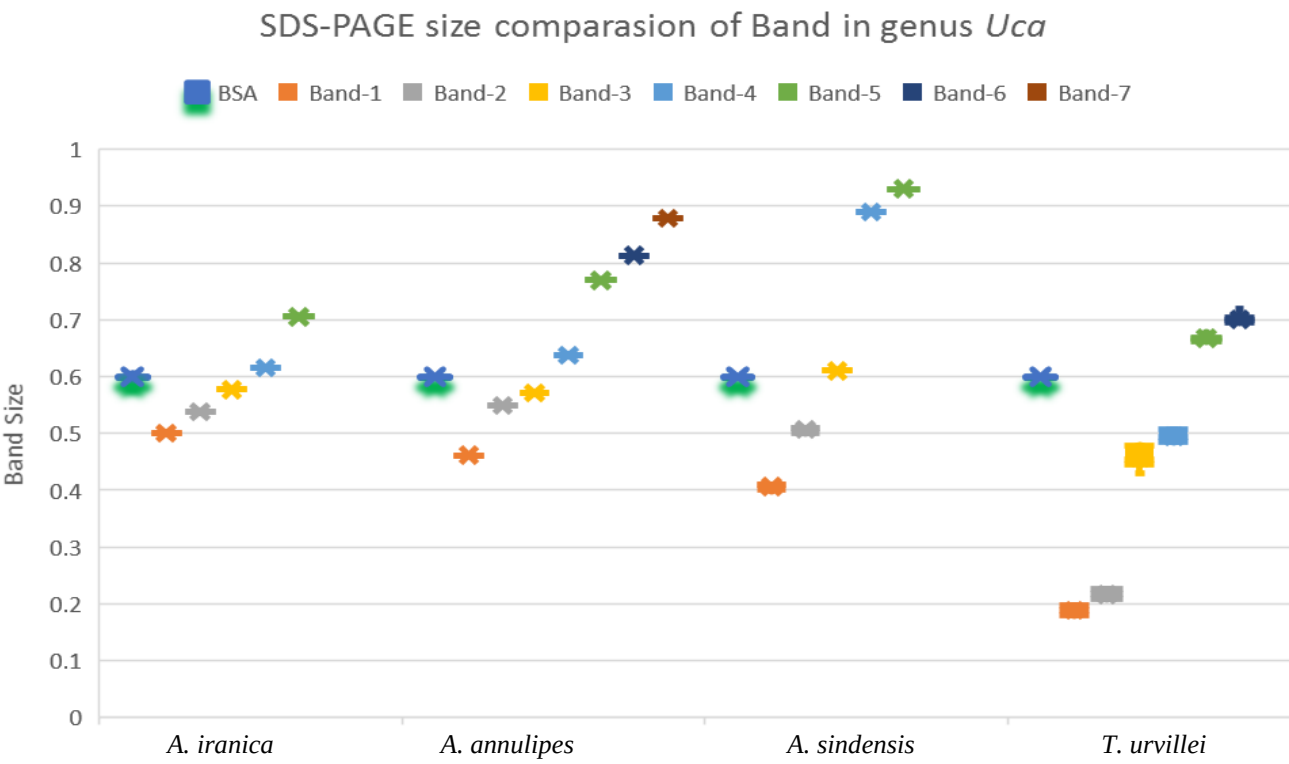

Fig. 2. The position of bands with relation to the molecular marker (BSA) as resolved in the muscle tissues of four species of Fiddler crabs.

Basically, SDS is a soap (detergent) that have ability to liquify the hydrophobic molecules of a protein and also possess a negative charge which help the protein molecule to move towards the positive charge. Therefore, when SDS is applied to a protein the protein losses its 3D structure and coated with SDS and the proteins are converted into two important features that all protein converted into primary structure and all proteins possess a negative charge as migrate towards the positive pole when electric current is applied. Nowakowski *et al.* (2014) described that the SDS-PAGE deliberately denatures the proteins prior to electrophoresis limits the analysis of proteins such as enzymatic activity, protein cofactors detection and binding interaction of proteins. Therefore, for such results PAGE-Native is employed to investigate the structure-function relationship of proteins (Walker, 2002; Witting *et al*., 2005).

Shapiro *et al.* (1967) described that proteins could be separated by using the SDS-PAGE depending upon the molecular weight of Proteins. This is also confirmed by Weber and Osborn (1969) suggesting the SDS-PAGE technique can be used with commercially available proteins as a standard to determine the molecular weights of polypeptide

chains. For this purpose, they designed proteins marker with recognized molecular weight of 40 different proteins and assessed the utility of electrophoresis which gave the molecular weights of unknown proteins.

During the current study, only one known protein standard BSA was used. That gave the sufficient results to analyze the molecular weight of proteins. But, this is further noted that molecular weight reported with the help of SDS-PAGE using different protein markers not always agree with the mass spectrometry or protein sequencing. So, the bands observed during the study can be assumed that these bands range from 20 KDa to 80 KDa. As we have one known molecular marker with 66 KDa so we can say that bands larger from BSA were between 66 KDa to 80 KDa while the bands lower from the BSA can be assumed between 66 KDa to 20 KDa depending on their size shows the clear distinction between bands and their relative molecular weight BSA.

So, we can conclude that all the species analyzed here are separate species and results showed that these can easily be identified by using the SDS-PAGE and molecular marker. There is a need of complete usage of molecular markers to identify the exact molecular weight of proteins obtained from the muscle tissues of these crabs.

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