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RESEARCH PAPER

Characterization of Proteins from Popovo Minnow (*Delminichthys ghetaldii* Steindachner, 1882) Muscle

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Abstract

In this research protein profiles of Popovo minnow muscle were presented and partially characterized for the first time. *Delminichthys ghetaldii* (Steindachner, 1882) is an endemic fish species which can be found in springs and streams in fields Ljubomir, Dabar and Fatnica in Bosnia and Herzegovina, and Bacina lakes in Croatia. Most of the year minnows dwell in underground water habitats of karst region in eastern Herzegovina. During the autumn and spring flooding of the surrounding fields, Popovo minnows emerge from the underground waters in estavele and spawn in newly formed temporary lakes. In order to obtain protein profiles from fish muscle, we used several extraction protocols and buffers. Sarcoplasmic proteins were extracted from muscle tissue using Tris/EDTA and Tris/$CaCl_2$ buffers. The results showed that the most abundant sarcoplasmatic proteins had molecular weight of 40 and 34 kDa while high MW band appeared only in samples extracted by Tris/EDTA. Re-extraction with 1% SDS resolved the presence of myofibrillar proteins and showed to be qualitatively and quantitatively more efficient than the extraction by nonionic detergent Tween. Our results also indicate that protein extraction with commercial SDS buffer from a biopsy muscle tissue gives good screening results and is applicable for field work. The results are discussed in terms of the efficiency of different buffers in protein extraction from Popovo minnow muscle tissue.

Keywords: *Delminichthys ghetaldii*, *muscle*, *Popovo minnow*, *protein profile*.

Introduction

Protein profile in general is defined as a method for identifying proteins expressed in a specific tissue, under determined conditions and at a particular time (Bradley *et al*., 2009). This information is useful for understanding the response mechanisms to the changes in the environment at the protein level. Fish muscle protein profiles can likewise give us important information about taxonomy, phylogeny and ecology of the species (Ladewig and Shwantes, 1984; Bezrukov, 1987; Rashed *et al*., 1998; 2009; Saad *et al*., 2002). Three major groups of muscle proteins which differ in functions and solubility properties are sarcoplasmic, myofibrillar and stromal proteins. Sarcoplasmic group of proteins includes enzymes involved in energy metabolism of muscle e.g. enolase, creatin kinase, aldolase, gliceraldehyde phosphate dehydrogenase (Nakagawa *et al*., 1988). Myofibrillar proteins are the major proteins of fish muscle and include myosine, actine and regulatory proteins tropomyosine, troponine and actinine (Hassan and Javed, 2000) while stromal proteins (collagen, elastin) are main components of lamina. There are many papers with different approaches for the solubilisation and extraction of fish muscle proteins. Various pH values, ionic strengths, detergents and incubation temperatures were used for the improvement of the protein extraction (Kim *et al*., 2005; Balan *et al*., 2004; Meric and Demir, 2012; Rehbein and Karl, 1985; Ladrat *et al*., 2003; Mitsuhashi *et al*., 2002; Helander, 1957). Our research focuses on Popovo minnow (*Delminichthys ghetaldii* Steindachner, 1882), an endemic fish species which can be found in springs and streams in Ljubomir, Dabar and Fatnica fields in Bosnia and Herzegovina, and Bacina lakes and some springs in Croatia (Kottelat and Freyhof, 2007). Earlier, three species, *Paraphoxinus metohiensis*, *Paraphoxinus pstrossi* and *Paraphoxinus ghetaldii* were classified in genus *Phoxinellus*. Current classification suggests two genera, *Telestes* (with species *Telestes metohiensis*) and *Delminichthys* (with species *Delminichthys ghetaldii*) (Freyhof *et al*., 2006; Kottelat and Freyhof, 2007). Popovo minnow is characterized by a specific way of life. Most of the year it inhabits underground waters of karst region in specific parts of eastern Herzegovina. During autumn and spring flooding of the surrounding fields, Popovo

minnows emerge from the underground waters in estavele. There they spawn and spend several months. When the water withdraws to the underground basins, they redraw with it. In the literature there are several reports about distribution (Čučković, 1983; Dekić *et al*., 2011; 2013), phylogeny (Freyhof *et al*., 2006; Bogutskaya *et al*., 2012), and morphology (Aganovic and Kapetanovic, 1971) of *Delminichthys ghetaldii*, however little information is available about biochemical characteristics of this species. Accordingly, no reported studies were found about the minnow muscle protein extraction and characterization.

The aim of our work was to give an insight in partial characterisation of Popovo minnow muscle protein profile using different solubilisation protocols.

Materials and Methods

Fish material

Fishes were collected in January during the flooding period in field of Fatnica using nets with 18 mm mesh size (Figure 1). Samples were transported in aerated containers filled with adequate volume of water. All experiments were performed after the acclimatisation period. The treatment of fish was conducted according to European directive 2010/63/EU and the "Animal welfare act" ("Official Gazette of Republic of Srpska", No 111/2008). We used ten female Popovo minnows weighing 16.5 ± 2.75 g, with average total length 119±8 cm and average standard length 102 ± 6 cm. Due to the spawning season fish population was mainly comprised of female individuals therefore only females were used in the research. White muscle tissue from the lateral muscle was removed and used for the protein profile characterization.

Protein Extraction

Protein extraction procedures were conducted using 5 different extraction buffers:

Buffer 1. 0.5 M Tris pH 8.3 with 1 mM EDTA,

Buffer 2. 0.05 M Tris pH 7.6 with 5mM $CaCl_2$,

Buffer 3. 0.1 M NaPi pH 6.4, 1% Sodium dodecyl sulfate, 8M urea, 0.01M dithiothreitol, 1mM EDTA and 1mM phenylmethanesulfonyl fluoride,

Buffer 4. 0.1 M Sodium-phosphate pH 7, 5 mM EDTA, 15% glycerol, 0.2% Tween 20 and 1 mM Phenylmethanesulfonyl fluoride,

Buffer 5: Commercial loading SDS buffer (Merck, Germany).

Aliquots (0.5 g) of white muscle tissue were pulverised in liquid nitrogen and homogenate thus obtained was used for the extraction procedure. Exception was made for the Buffer 5 protocol where extraction was done directly from the muscle tissue without using liquid nitrogen. In this protocol tissue was mixed with 300 µL of commercial loading SDS buffer and mixed thoroughly without heating.

For extraction procedures 1 and 2, tissue/buffer ratio was 1:3 (w/v), and in the case of extraction procedures 3 and 4 it was 1:6 (w/v). After the homogenization, samples were centrifuged at 10 000 rpm for 10 minutes at 4°C. Collected supernatants were heated at 60°C for 5 minutes and employed in electrophoretic analysis. Pellets remained after Buffer 1 and 2 extractions were washed 3 times with the corresponding buffer and re–extracted with Buffer 3. After the centrifugation supernatants were also collected for furthure analysis.

Electrophoresis and Staining

SDS PAGE was performed according to Laemmli (1970). Protein bands were separated on 1 mm thick slab gel comprised of 12% running gel and 5% stacking gel. Following the electrophoresis, gels were stained in Coomassie brilliant blue (0.1%), acetic acid (10%) and methanol (50%) and destained in mixture of 5% methanol and 7% acetic acid. Each analysis was performed in 5 replicates. Molecular weight (MW) of protein was determined using Prestained Rec Protein Ladder by Fisher Reagents. TotalLab software was used for the analysis of quantitative and qualitative distribution of protein bands.

Figure 1. Popovo minnow (*Delminichthys ghetaldii*).

Results

Different types of homogenisation and buffers were applied for protein extraction from Popovo minnow muscle. Due to their watersolubility, sarcoplasmic proteins are easily soluble in slightly alkaline buffer solution with low ionic strength (Balan *et al*., 2004; Meric and Demir, 2012). Presence of 12 sarcoplasmic protein bands was resolved using Buffer 1 ranging from 16 to 230 kDa (Figure 2a, Table 1). Protein extraction with Buffer 2 showed similar protein profile (11 protein bands) with exception of band with MW 230 kDa (Figure 2a). Eventhough the extraction with Buffer 1 resolved the presence of only one wide protein band with MW 40 kDa, results of extraction using Buffer 2 confirmed presence of two separate bands (39 and 42 kDa). Obtained protein pattern corresponds to sarcoplasmic fraction given by Nakagawa *et al*., (1988) and Ladrat *et al*., (2003).

Myofibrillar proteins can be solubilised using high ionic strength buffers and detergents (Rehbein and Karl, 1985; Kubota *et al*. 1994). With the purpose of solubilizing myofibrillar proteins, buffer containing 1% SDS and 8 M urea was used for re-extraction of the pellet remained after extraction with Buffer 1 and Buffer 2. After the electrophoresis and Coomassie blue staining 13 myofibrillar protein bands were detected (Figure 2b). We suggest that the most abundant protein band detected on the gel with molecular weight of approximately 230 kDa corresponds to Myosine heavy chain (MHC) according to myofibrillar patterns obtained by Ladrat *et al*., (2003). Re-extraction of the pellets showed new protein bands in the range between 40-170 kDa (Table 1, Figure 2b).

For the extraction of total muscle proteins we used buffers with detergents, Buffer 3 (containing 1% SDS) and Buffer 4 (containing 0.2% Tween 20). Electrophoretic patterns of the proteins extracted with these detergents differed in band number, mobility and protein yield (Figure 3a and 3b). Extraction with Buffer 3 resolved the presence of 15 protein bands while 11 bands were detected using Buffer 4. Five protein bands with MW 170, 130, 91, 70 and 30 kDa were not detected after the extraction with Buffer 4. Methodologically different extraction by Laemmli buffer (Buffer 5) resolved the presence of 10 protein bands (Table 1, Figure 3c), which in majority correspond to bands detected on the gel after the extraction of whole muscle proteins with Buffer 3. Differences were noticed in solubilization of MHC which was more efficient using Buffer 3.

Discussion

Besides their use in the identification of protein expression in muscle tissue, protein profiles may present useful tool for the understanding of response mechanisms triggered by changes in the environment. Popovo minnows are specific by their extraordinary

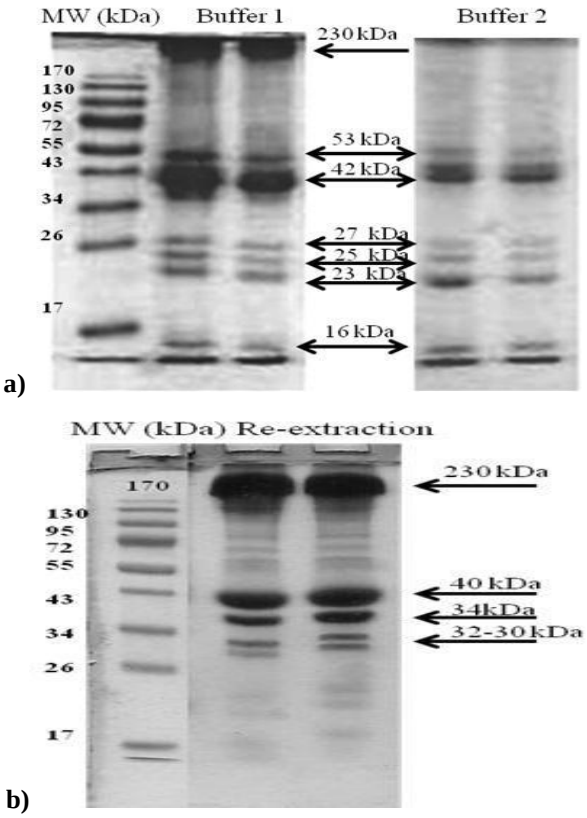

Figure 2. a) Sarcoplasmic and b) Myofibrillar proteins extracted from Popovo minnow muscle.

Table 1. Molecular weights of proteins extracted from the minnow muscle tissue using different buffers

Sarcoplasmatic proteins		Myofibrillar proteins	Total muscle proteins		
Tris EDTA	Tris CaCl2	Re-extraction (1% SDS)	1% SDS	0.2% Tween 20	Laemmli buffer
230	-	230	230	230	230
136	136	106	170	106	106
97	97	72	130	88	63
61	61	63	106	59	50
53	53	58	91	50	40
42	42	50	88	40	34
39	39	40	70	34	23
35	35	34	59	32	22
27	27	32	50	23	19
25	25	30	40	22	10
23	23	23	34	19	
16	16	22	32		
		19	30		
			23		
			19		

way of life. Therefore, it may be possible to observe adaptations at the protein level to the changes in the environment. For that reason, optimisation of the protocol for protein extraction from muscle is very important. Sarcoplasmic proteins were extracted using Tris EDTA (Buffer 1) and Tris $CaCl_2$ (Buffer 2) with slightely different pH value and ionic strength in order to distinguish possible qualitative differences in obtained protein profiles (Table 1, Figure 2a). Observed difference was the presence of two bands with MW 39 and 41 in samples extracted by Buffer 2, while only one wide band with corresponding MW was noticed in extractions with Buffer 1. We suggest that these two proteins bands were also present in the samples extracted by Buffer 1, but band with lower MW had a higher intensity and was overlapping upper protein band. Another difference recognized between these two protein profiles was high MW protein band (230 kDa) which was present only in extractions with Buffer 1. We assume this is related to different ionic strengths of buffers. Dagher *et al*., (2000) stated significant increasement in cod muscle proteins solubility in pH interval 8.9-9.2 at low ionic strength. Both buffers showed to be efficient for extracting main sarcoplasmic proteins - creatine kinase and aldolase (double band 42-39 kDa) and glyceraldehyde-3-phosphate dehydrogenase (35 kDa). Re-extraction enabled detection of myofibrillar proteins. The most abundant protein band with MW 230 kDa corresponds to MHC, and bands with MW 40 kDa and 34 kDa represent actine and tropomyosine (Ladrat *et al*., 2003). SDS as an ionic detergent has great abilities in solubilization of native and denaturated proteins (Waehnelt, 1975). Rehbein and Karl (1985) demonstrated almost complete solubilisation of proteins from fish fillets in SDS buffer. They have also showed that increase in SDS concentration did not effect solubilisation significantly. Kubota *et al*. (1994) used 1% SDS and 8 M urea for the solubilization of myofibrillar proteins in carp muscle tissue. SDS was also used by Mitsuhashi *et al*. (2002) for the solubilization of myofibrillar proteins.

For the extraction of total muscle proteins, we used two types of detergent, ionic SDS and non-ionic Tween. Ionic detergents are used for complete denaturation of proteins. On the other hand non-ionic detergents (non-denaturing detergents) are used for the solubilization of membrane proteins since they are able to break lipid-lipid and lipid-protein interactions, but have a limited ability to break protein-protein interactions and rarely interfere with enzymatic assays (Neugebauer, 1990). As we mentioned previously, additional protein bands appeared in the range between 40-170 kDa after the extraction with Buffer 3, which have not been detected during the pellet re-extraction. We suggest that these bands correspond to proteins from the sarcoplasmic fraction. Ability of sodium dodecyl suphate in solubilization of the bonds formed between myofibrillar proteins during frozen storage of cod muscle has been proved earlier (Tejada *et al*., 1996). Madovi (1980) showed high solubility od raw and cooked samples of meat from various animals (beef, cod) in 3% SDS.

Nevertheless, the extraction of total muscle proteins using Buffer 4 has showed medium efficiency. There are reports of using Tween 20 for the solubilization of membrane proteins from erythrocytes (Liljas *et al*., 1974). From our results, we can conclude that Tween 20 is not efficient for the solubilization of MHC.

In our experiment, we also tried to obtain a total muscle protein profile of Popovo minnow without homogenization of tissue in liquid nitrogen, by direct extraction of whole muscle proteins in commercial SDS buffer. Eventhough this type of extraction exhibited less efficiency than the extraction with Buffer 3, we consider this method of protein extraction appropriate for obtaining screening results in field work. Using this method, we could avoid

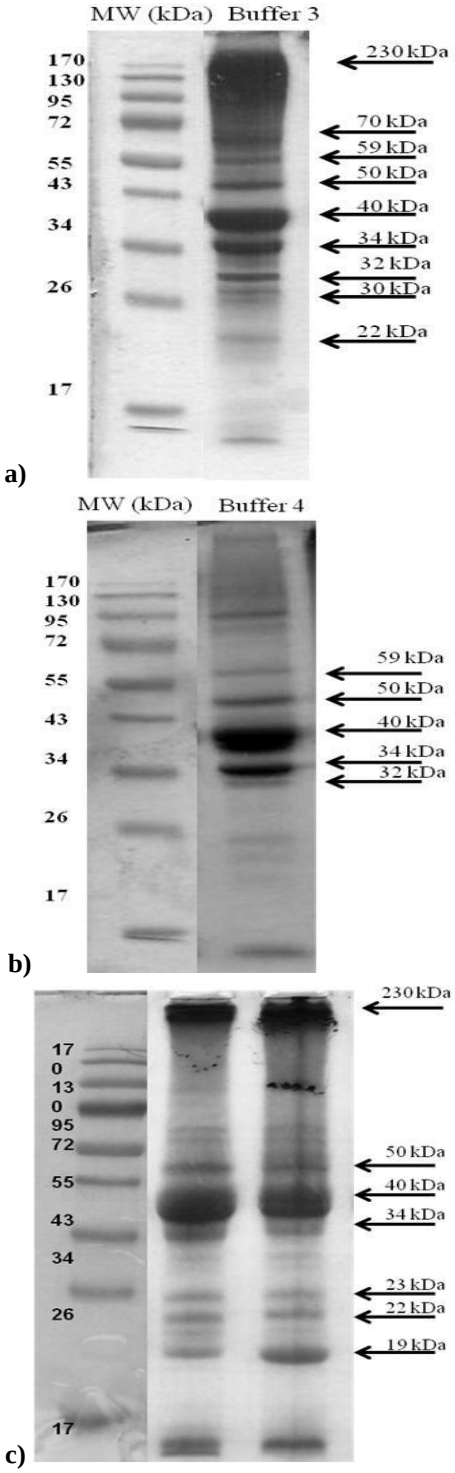

Figure 3. SDS PAGE profile of Popovo minnow muscle proteins solubilized with different extraction buffers. a) Extraction with Buffer 3 b) Extraction with Buffer 4 c)Extraction with Buffer 5.

proteolytic degradation (Ladrat *et al*., 2003), detect possible changes in the fish tissue during transport and additional stressing of the fish. Also, one of the advantages is a requirement for small amount of muscle tissue. To our knowledge, these are the first available data on Popovo minnow muscle protein profile. For the future work we will focuse on the utilization of gained results in relating protein profiles with changes in Popovo minnow life cycle and ecology.

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