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Myocardial and pericardial 2-DE protein profiles of African catfish, Clarias gariepinus

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Summary

The present study was carried out to introduce a proteomic workflow based on two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) as a tool for creating protein profiles of the myocardium and pericardium of African catfish (Clarias gariepinus). The experiment was carried out on five healthy 8-month-old males. Myocardial and pericardial proteins within the isoelectric point ranging from 3.0 to 10.0 were separated by high resolution 2-DE. Subsequently, 335 reproducible protein spots (125 from myocardial and 210 from pericardial tissue) were excised from the gels and submitted for identification by PMF (peptide mass fingerprinting). As a result, 38 protein spots were successfully identified and corresponded to 32 distinct myocardial and pericardial gene products. These proteins included binding, enzymatic, structural and regulatory proteins. Two-dimensional (2-D) maps presented in the current study provide a basic overview of myocardial and pericardial proteins of African catfish. These maps might be useful for designing further proteomic studies aimed at elucidating the patterns of cardiac adjustments to various physiological and/or pathophysiological factors, including environmental stressors.

Keywords: Clarias gariepinus, myocardium, pericardium, 2-DE, MALDI-TOF MS

Two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) have recently emerged as powerful tools for the study of highly complex biological systems (8). These techniques make it possible to visualise hundreds of proteins present in a given biological material, including myocardial and pericardial tissues (4, 13). Over the past few years, many studies have been undertaken to create protein expression patterns of various fish tissues, including blood plasma (2), brain (1, 2, 15), liver (1, 2), skeletal muscle (1, 2, 11), intestine (2), sperm (9) and seminal plasma (14). Proteomic tools have also been successfully employed to establish protein profiles of the heart tissue of zebrafish (*Danio rerio*) (2) and Gulf killifish (*Fundulus grandis*) (1), and to assess the influence of an excessive concentration of fluoride in water on cardiac proteome changes in Japanese pufferfish (*Takifugu rubripes*) (10). Lu et al. (10), using 2-DE, MALDI-TOF MS and MALDI-TOF/TOF MS, showed that a 3 h exposure to sodium fluoride (35 mg/l) triggered changes in the expression of 21 myocardial proteins. These proteins are involved mainly in the process of apoptosis and other functions associated with fluorosis (e.g. telomerase reverse transcriptase, 4S Nc-tudor domain protein, protein disulfide isomerase ER-60, tuba1 protein, mitogen-activated protein kinase 10, SMC4 protein) (10).

However, not many proteomic studies aimed at analysing cardiac proteins have been carried out in fish that are commercially important in aquaculture. Recently, Klaiman et al. (8) investigated changes in the abundance of cardiac protein in Rainbow trout (*Oncorhynchus mykiss*) in response to acclimation to both low (4°C) and high temperatures (17°C). Using two-dimensional difference gel electrophoresis (2-D DIGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), these authors found that cold acclimation triggered changes in the phosphorylation state of the slow skeletal isoform of troponin T and myosin binding protein C. Similar studies have been performed on African catfish species. For example, Hanson et al. (6) conducted an experiment aimed at assessing the influence of temperature acclimation (15, 22, 32°C) on myocardial β-adrenoceptor density and binding affinity in *Clarias gariepinus*.

African catfish (*Clarias gariepinus*) has recently gained considerable attention as an excellent species for artificial breeding in Europe. It is regarded as a good candidate for aquaculture because of its fast growth rate, ability to withstand variable environmental conditions, including very low oxygen content, and resistance to diseases (12).

This study attempted to determine 2-D maps reflecting characteristic patterns of myocardial and pericardial proteins in healthy adult African catfish (*Clarias gariepinus*). These maps might be useful for designing further proteomic studies aimed at elucidating the patterns of cardiac adjustments to various physiological and/or pathophysiological factors, including environmental stressors.

Material and methods

Fish. A total of five healthy 8-month-old male African catfish (*Clarias gariepinus*) were used. Fish were maintained in an artificial breeding pool in rain water at 24-27°C, without access to light throughout the whole experiment. Water chemistry: dissolved oxygen averaged 4 mg/l, pH ranged from 7.5 to 8.0, and total ammonia nitrogen was approximately 1.0 mg/l.

During the first two weeks of life, alevins were fed crustacean larvae (*Artemia salina*) every two hours at 10% of the body weight. From the third week, fish were fed CatCo CRUMBLE Excellent (Coppens International) every two hours, with the addition of β-glucans. Subsequently, from the second month until the end of the experimental period, they were fed CatCo SELECT-13 EF twice a day at 1% of the body weight. The use and handling of animals for this experiment was approved by the Local Ethical Committee (no. 11/2012 of 23.05.2012).

Sample collection. Fish were anaesthetized with an overdose of benzocaine (Sigma) prior to dissection. Dissected tissue samples (heart and its surrounding sack – pericardium) were washed twice with 0.65% NaCl (0°C) and thereafter twice with 20 mM Krebs-HEPES buffer (0°C, pH 7.4) containing (in mM): 99 NaCl, 4.69 KCl, 2.50 $CaCl_2 \times 2 H_2O$, 1.20 $MgSo_4 \times 7 H_2O$, 25 $NaHCO_3$, 1.03 KH_2PO_4, 5.6 D-glucose and 20 Na-HEPES. Subsequently, all tissue fragments were pulverized under liquid nitrogen and resuspended in lysis buffer containing (5 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM (w/v) Tris, 0.2% (v/v) Bio-Lyte 3-10 ampholyte, 2 mM TBP, pH 8.5). The samples, prepared in this manner, were then stored at –80°C until further analysis.

2-DE. Proteins were separated by a modified 2-DE method as previously described by Görg et al. (5). Each tissue sample was processed in duplicate to ensure reproducibility. Total protein concentration was estimated by the modified Bradford assay (Bio-Rad Protein Assay, Bio-Rad). Total myocardial and pericardial proteins (1 mg) were mixed with the rehydration buffer (9 M urea, 4% (w/v) CHAPS, 100 mM DTT, 0.2% (v/v) Bio-Lyte 3-10 ampholyte) to a total volume of 350 µl and applied to 3-10, 17 cm L (linear) ReadyStrip™ IPG Strips (Bio-Rad). Strips were first rehydrated with rehydratation buffer and samples passively (6 h, 0V, 20°C) and then actively (12 h, 50V, 20°C). Isoelectrofocusing (IEF) was run (Protean® IEF Cell, Bio-Rad) for a total of 75 000 Vh. After IEF, IPG strips were reduced in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% v/v glycerol) containing 1% (w/v) DTT for 15 minutes and then alkylated with equilibration buffer containing iodoacetamide (2.5% w/v) for 20 minutes. The second dimension was performed (Protean Plus™ Dodeca Cell™ electrophoretic chamber, Bio-Rad) on 12% SDS polyacrylamide gels (20 × 25 cm) at 40 V for 1 hour and subsequently at 120 V for 16 hours at 15°C. After 2-DE separation, gels were stained with colloidal Coomassie Brilliant Blue G-250 according to Westmeier (17).

Image acquisition and analysis. All gels were scanned with the aid of a GS-800™ calibrated densitometer (Bio-Rad). The 2-D image computer analysis was performed by PDQuest Analysis software version 8.0. Advanced (Bio-Rad). Gel images were cropped prior to analysis according to the same pattern. The following analyses were performed: spot background substraction, spot detection and matching. The parameters used for between-gel comparison were the size of the faintest spot, the smallest spot and the size of the largest spot. Each individual spot was normalised by the local regression model (LOESS). The gels resolved, on average, 280 spots in myocardial tissue and 350 spots in pericardial tissue. To present the variation of protein expression, 280 spots of myocardial and 350 of pericardial tissue were quantified on each replicate group. Only the spots whose locations and stain intensities on 2-D gels were similar between the replicate gels were included in further analysis. On this basis, we selected 125 spots from myocardial and 210 spots from pericardial tissue that were reproducible on each analysed 2-D gel. These selected spots constituted 45% (myocardium) and 60% (pericardium) of all detected protein features. Additionally, intersample variability was calculated. The analysis revealed an average coefficient of variation (CV) of 36.75% for the five analysed samples (performed in duplicate) from myocardial tissue, and of 43.28% for the five analysed samples (performed in duplicate) from pericardial tissue. On the basis of a molecular range standard (Plus Protein™ Kaleidoscope™ Standards for SDS-PAGE by Bio-Rad), observed molecular mass (kDa) was computed for each identified protein spot.

MALDI-TOF MS. Protein spots were manually excised from the gels and decolorized by washing with buffer containing 25 mM NH_4HCO_3 in 5% v/v acetonitrile (ACN) followed by two washes with solution (25 mM NH_4HCO_3 in 50% v/v ACN). The excised gel pieces were subsequently dehydrated with 100% ACN and vacuum dried (Concentrator 5301, Eppendorf). Samples were then incubated with trypsin (20 µl/spot of 12.5 µg/ml in 25 mM NH_4HCO_3; Sigma-Aldrich, St. Louis, MO) for 16 h at 37°C. After incubation, the resultant peptides were extracted with 100% ACN, combined with an equal volume of matrix solution (5 mg/ml CHCA, 0,1% v/v TFA, 100% v/v ACN) and loaded onto a MALDI-MSP AnchorChip™ 600/96 plate (Bruker Daltonics, Germany). For calibrating the mass scale, Peptide Mass Standard II (Bruker Daltonics, Germany within mass range 700-3200 Da) was used. Mass spectra were acquired

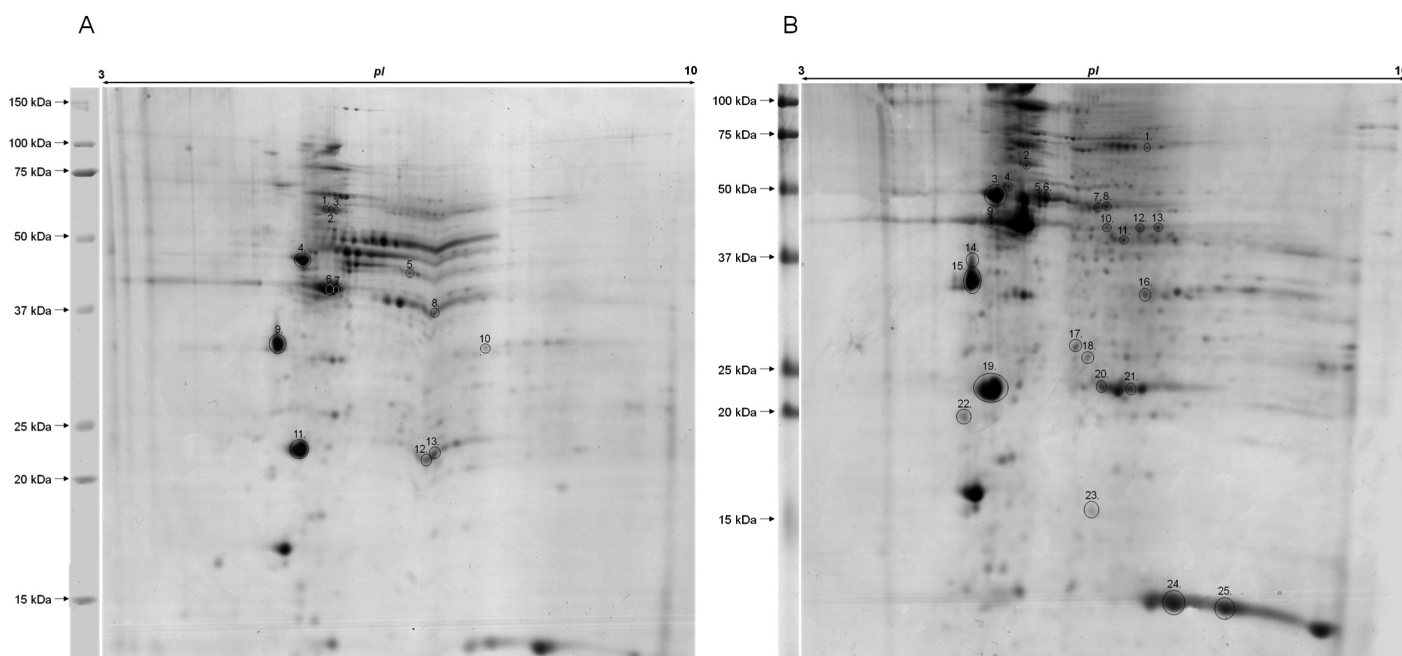

Fig. 1. A 2D protein map of myocardial (A) and pericardial (B) proteins of an eight-month-old male *Clarias gariepinus.* For the first dimension, 1.0 mg of proteins was applied on a IPG strip (17 cm, pH 3-10). The second dimension was performed on 12% SDS-PAGE gels, and the gels were stained with Coomassie brilliant blue G-250. Spot numbers correspond to those in Table 1 (myocardium) and Table 2 (pericardium)

in the positive-ion reflector mode with a Microflex™ MALDI TOF mass spectrometer (Bruker Daltonics, Germany). The PMF (peptide mass fingerprinting) data were compared with the available databases (SWISS-PROT; http://us.expasy.org/uniprot/ and NCBI; http://www.ncbi.nlm.nih.gov/) by means of the MASCOT search engine (http://www.matrix-science.com/). Search parameters were as follows: trypsin as an enzyme, carbamidomethylation of cystein as fixed modification, methionine oxidation as variable modification, mass tolerance to 150 ppm, and a maximum of one missed cleavage site. On this basis, the results were further validated by the MASCOT score, sequence coverage and the coincidence of expected and measured molecular mass values.

To define the subcellular location of the identified proteins, a bioinformatic tool was employed: CELLO v.2.5 (http://cello.life.nctu.edu.tw/).

Results and discussion

The present study was carried out to introduce a proteomic workflow based on 2-DE and MALDI-TOF MS as a tool for creating protein profiles of the myocardium and pericardium of African catfish (*Clarias gariepinus*). Figure 1 presents patterns of myocardial and pericardial proteins resolved in the pH range of 3-10 and the molecular masses of 15-150 kDa (Fig. 1A) and 15-100 kDa (Fig. 1B).

A total of 335 reproducible protein spots (125 from myocardial and 210 from pericardial tissue) were excised from the gels and submitted for identification by PMF (peptide mass fingerprinting). As a result, only 13 spots from myocardial tissue (identification rate of 10.40%) and 25 spots from pericardial tissue

Tab. 1. Summary of the myocardial proteins identified by MALDI-TOF MS

Spot	Protein name	Accession No UniProt	Score values/ Sequence coverage	Theoretical pI/M_r	Experimental M_r	Taxonomy
1 2	Alanyl-tRNA synthetase domain-containing protein 1-A	Q08B09	75/23% 76/23%	6.48/46.14	61.40 61.10	*Xenopus laevis*
3	Tyrosyl-tRNA synthetase, cytoplasmic	Q6DIJ1	51/17%	6.33/59.32	60.80	*Xenopus tropicalis*
4	ATP synthase subunit beta, mitochondrial precursor	Q9PTY0	204/55%	5.05/55.33	46.00	*Cyprinus carpio*
5	40S ribosomal protein S6	Q9YGF2	51/23%	10.90/28.83	40.10	*Oncorhynchus mykiss*
6 7	Actin, alpha cardiac	P53480	103/33% 141/57%	5.22/42.29	40.50 40.50	*Fugu rubripes*
8	Hemoglobin subunit beta	P04245	75/59%	6.64/16.10	34.10	*Tragelaphus strepsiceros*
9	Tropomyosin-1 alpha chain	P13104	84/44%	4.70/32.76	34.00	*Danio rerio*
10	L-lactate dehydrogenase B chain	Q9YGL2	57/27%	7.68/36.68	29.40	*Anguilla rostrata*
11	Prefoldin subunit 1	Q5D016	60/60%	8.83/13.98	23.20	*Danio rerio*
12 13	Triosephosphate isomerase	P00940	53/24% 63/24%	6.71/26.83	24.70 24.80	*Gallus gallus*

Tab. 2. Summary of the pericardial proteins identified by MALDI-TOF MS

Spot	Protein name	Accession No UniProt	Score values/ Sequence coverage	Theoretical pI/M_r	Estimated M_r	Taxonomy
1	Pdcd8 protein	Q5XFY2	73/21%	8.58/67.42	66.20	*Danio rerio*
2	Chaperonin Cpn60	Q8JGM5	66/37%	9.13/33.42	54.80	*Danio rerio*
3	ATP synthase subunit beta, mitochondrial precursor	Q9PTY0	207/59%	5.05/55.33	46.50	*Cyprinus carpio*
4 9	Actin, alpha, cardiac muscle 1 like	Q6IQR3	178/70% 106/50%	5.22/42.29	48.10 41.80	*Danio rerio*
5 6	Desmin	Q90441	90/31% 67/28%	5.50/54.07	46.10 45.00	*Danio rerio*
7	Hox protein	Q58WY6	68/43%	10.88/18.89	44.00	*Oreochromis niloticus*
8	Rab-like protein 5	Q5M8K8	56/39%	5.08/20.97	44.60	*Xenopus tropicalis*
10	Actin, alpha skeletal muscle	Q98972	50/32%	5.23/42.27	41.80	*Oryzias latipes*
11	Creatine kinase	P24722	52/29%	6.20/43.26	39.60	*Oncorhynchus mykiss*
12	Mitochondrial isocitrate dehydrogenase 2-like	ABE98244	90/22%	7.55/50.89	42.00	*Oreochromis mossambicus*
13	Isocitrate dehydrogenase, mitochondrial precursor	Q4R502	76/29%	8.88/51.31	42.30	*Macaca fascicularis*
14 15	Tropomyosin-1 alpha chain	P13104	51/30% 66/32%	4.70/32.76	35.20 33.40	*Danio rerio*
16	L-lactate dehydrogenase B chain	Q9YGL2	50/22%	7.68/36.68	32.80	*Anguilla rostrata*
17	Enolase A	ABF60006	65/29%	5.98/47.47	27.50	*Acipenser baerii*
18	Fast myotomal muscle actin	NP_001117011	120/44%	5.22/42.25	26.20	*Salmo salar*
19	Prefoldin subunit 1	Q5D016	60/63%	8.83/13.98	21.60	*Danio rerio*
20	Glycosyltransferase-like protein LARGE2	Q66PG1	48/18%	6.44/87.73	22.90	*Danio rerio*
21	Triosephosphate isomerase B	AAK85204	85/39%	7.60/26.76	23.10	*Xiphophorus maculatus*
22	Coiled-coil domain-containing protein 65	Q6DHI2	48/17%	7.62/58.12	19.60	*Danio rerio*
23	Myoglobin	Q9DEN8	50/42%	6.15/15.73	15.60	*Notothenia coriiceps neglecta*
24	60S ribosomal protein L15	Q7T3P1	51/41%	11.51/24.13	13.30	*Anguilla japonica*
25	Unnamed protein product	CAG08583	68/39%	4.41/18.01	13.10	*Tetraodon nigroviridis*

(identification rate of 11.90%) were successfully identified. The results of the MALDI-TOF MS analyses are summarized in Table 1 and Table 2.

The subcellular location of the identified proteins was determined with a bioinformatic tool. The results of this analysis for proteins from myocardial and pericardial tissue are presented in diagrams (Fig. 2A, 2C). The proteins of the heart and pericardium showed similar patterns of diversification for molecular functions (Fig. 2B, 2D).

The identified 10 myocardial proteins were divided into four groups on the basis of their functions. The first group consist of structural proteins (4/13-30%), such as 40S ribosomal protein S6 (spot no. 5), actin alpha cardiac (spots no. 6, 7) and tropomyosin-1 alpha chain (spot no. 9). Proteins from the second group display enzymatic activity (7/13-54%). These include alanyl-tRNA synthetase domain-containing protein 1-A (spots no. 1, 2), tyrosyl-tRNA synthetase (spot no. 3), ATP synthase subunit beta (spot no. 4), L-lactate dehydrogenase B chain (spot no. 10) and triosephosphate isomerase (spots no. 12, 13). Hemoglobin subunit beta (spot no. 8) forms the third group: binding proteins (1/13-8%). The fourth group – regulatory proteins (1/13-8%) – is represented by prefoldin subunit 1 (spot no. 11).

The 22 identified pericardial proteins were sorted into five categories: binding proteins (5/25-20%) – group one, enzymatic proteins (9/25-36%) – group two, structural proteins (9/25-36%) – group three, a regulatory protein (1/25-8%) – group four, the remaining protein (1/25-8%), namely, coiled-coil domain-containing protein 65 (spot no. 22), without any functional information – group five. The first group was represented by the following proteins: chaperonin Cpn60 (spot no. 2), hox protein (spot no. 7), rab-like protein 5 (spot no. 8), myoglobin (spot no. 23) and unnamed protein product (spot no. 25). The second group consisted of pdcd8 protein (spot no. 1), ATP synthase subunit beta (spot no. 3), creatine kinase (spot no. 11), mitochondrial isocitrate dehydrogenase 2-like (spot no. 12), isocitrate dehydrogenase (spot no. 13), L-lactate dehydrogenase B chain (spot no. 16), enolase A (spot no. 17), glycosyltransferase-like protein LARGE2 (spot no. 20) and triosephosphate isomerase B (spot no. 21). The third group of myocardial proteins were structural proteins, which included actin, alpha, cardiac muscle 1 like (spots no. 4, 9), desmin (spots

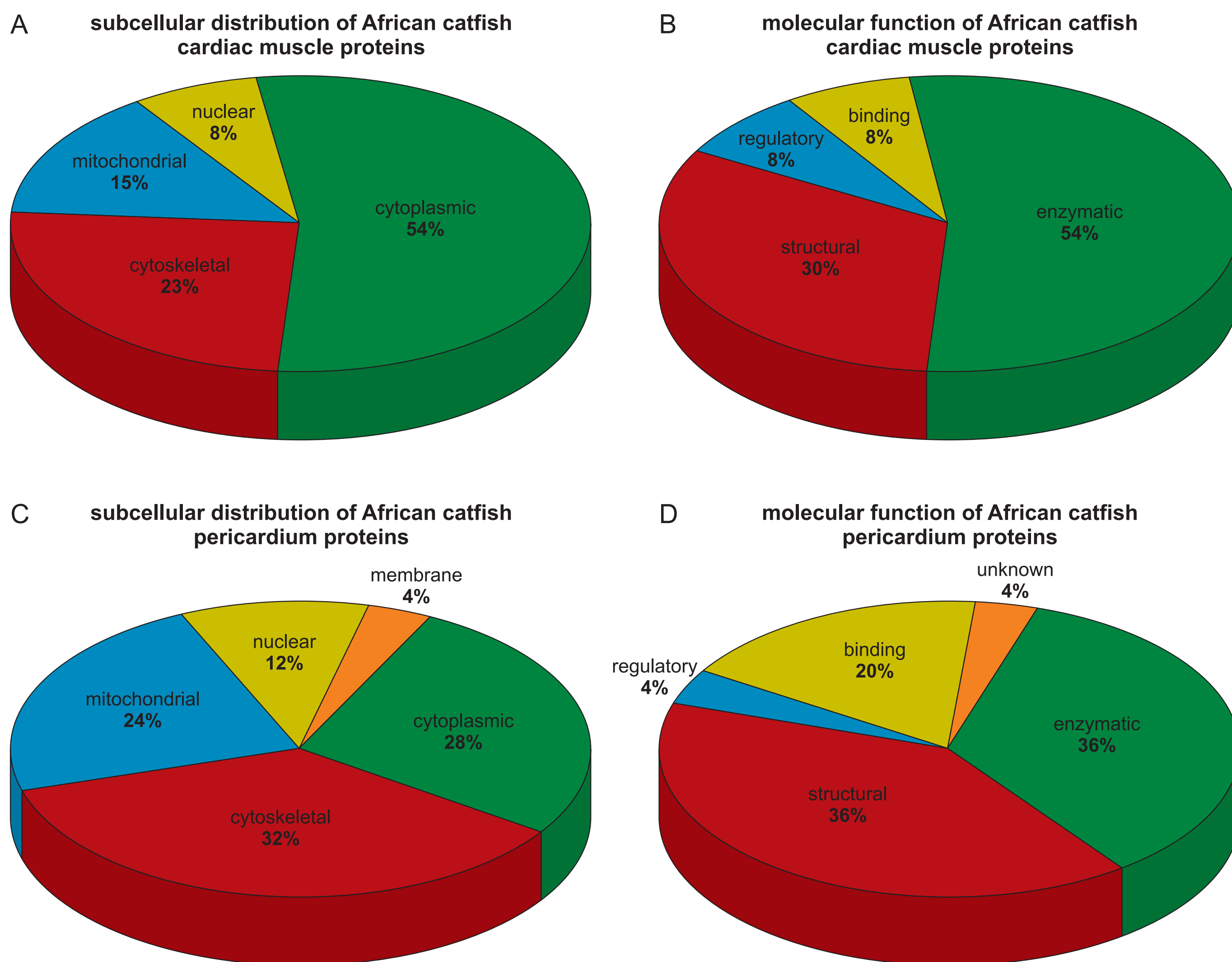

Fig. 2. The subcellular distribution of myocardial (A) and pericardial (C) proteins and their molecular functions (B – myocardium; D – pericardium) based on the subcellular location database (CELLO v.2.5) and on protein identities obtained from the MALDI-TOF MS analysis

no. 5, 6), actin, alpha skeletal muscle (spot no. 10), tropomyosin-1 alpha chain (spots no. 14, 15), fast myotomal muscle actin (spot no. 18), 60S ribosomal protein L15 (spot no. 24). Prefoldin subunit 1 (spot no. 19) formed the fourth group.

In total, 38 protein spots corresponding to 32 distinct myocardial and pericardial gene products were successfully identified. Unfortunately, the majority of excised spots (in total 297 spots) remain unidentified despite repeated analysis. The failed identification can be attributed mainly to the low protein content, intrinsic limits of PMF, e.g. its inability to detect low molecular weight proteins, and most importantly to the limited number of entries in the available protein databases for fish species. Owing to the lack of information on the catfish genome sequence, the identification was based on homologies with sequences from other organisms (Tab. 1, Tab. 2). Undoubtedly, this leads to the identification of only conserved proteins.

The smallest molecular mass (M_r) of the identified myocardial proteins is assigned to prefoldin subunit 1 (23.20 kDa) and the largest to alanyl-tRNA synthetase domain containing protein 1-A (61.40 kda). Among the pericardial proteins, the lowest M_r is attributed to the unnamed protein product (13.10 kDa), whereas the highest to the Pdcd8 protein (66.20 kDa). For most of the named protein spots resolved in 2-D gels (Fig. 1), shifts between experimental and theoretical molecular masses were noted. Mass shifts between theoretical and measured M_r values were also observed by other scientists who published two-dimensional gel proteome reference map of the heart tissue of *Fundulus grandis* (1), the skeletal muscle of *Takifugu rubripes* (11) and early larvae of *Gadus morhua* (16). This phenomenon is due to post-translational modifications (PTMs), e.g. phosphorylation, glycosylation and proteolytic cleavage. PTMs results in a shift in the protein's electrophoretic mobility (3). Experimental mass values higher than theoretical ones, which have been observed for some proteins (e.g. chaperonin Cpn60, hox protein, 40S ribosomal protein S6, alanyl-tRNA synthetase domain-containing protein 1-A) probably result from glycosylation, whereas lower M_r values (e.g. ATP synthase subunit beta, isocitrate dehydrogenase, L-lactate

dehydrogenase B chain) are most likely due to proteolytic cleavage. Moreover, since the identification was based on homologies with sequences from other organisms, differences in aminoacid composition may occur between identified homolog proteins and those observed in our 2D maps.

Myocardial protein distribution on 2-D gel obtained in the current paper differs considerably from those previously determined for the heart of Gulf killifish (*Fundulus grandis*) (1) and Japanese pufferfish (*Takifugu rubripes*) (10). This may indicate interspecies differences in both the composition and physiochemical parameters of myocardial proteins. Abbaraju et al. (1) detected by 2-DE approximately 400 spots in *F. grandis* heart tissue and identified 84 out of 192 protein spots. They achieved a higher identification rate (43.80%) compared with the rate obtained in the present study (10.40%). This may have resulted from the fact that Abbaraju et al. determined the masses of tryptic digested peptides by MALDI-TOF/TOF mass spectrometry. Among 51 gene products identified by Abbaraju et al. (1) four were also present on our 2-D map. Nevertheless, we showed five additional cardiac proteins that were not identified by those authors. These proteins include alanyl-tRNA synthetase domain containing protein 1-A, tyrosyl-tRNA synthetase, 40S ribosomal protein S6, hemoglobin subunit beta and prefoldin subunit 1.

This is the first study attempting to map the myocardial and pericardial proteome of African catfish (*Clarias gariepinus*). Unfortunately, the majority of excised spots (in total 297 spots) remain unidentified despite repeated analysis. Thus, it seems that 2-DE and MALDI-TOF MS-based proteomic analysis of non-model fish species that are commercially important still poses a great challenge, mainly because of the limited number of entries in the available protein databases. Maps presented in the current study may be regarded as preliminary, and they await further refinements, such as the application of much more sophisticated proteomic tools. Nevertheless, these maps might be useful for designing further proteomic studies aimed at elucidating the patterns of cardiac adjustments to various physiological and/or pathophysiological factors, including environmental stressors.

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