JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Modifications of Trout (*Oncorhynchus mykiss*) Muscle Proteins by Preslaughter Activity

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The effect of two different preslaughter procedures (limited or 15-min intense muscular activity) on muscle trout proteins was investigated. Muscle was sampled 45 min and 24 h post-mortem, proteins were separated using two-dimensional electrophoresis, and spots of interest were tentatively identified by MALDI-TOF spectrometry. Twenty-nine and 4 spots were differentially represented between the two groups of fish at 45 min and 24 h post-mortem, respectively. Spots that could be identified corresponded mainly to proteins involved in energy-producing pathways (triosephosphate isomerase, enolase, pyruvate dehydrogenase) or to structural proteins (desmin, cap-Z, myosin heavy chain fragment). Persistent under-representation of desmin, a key cytoskeletal protein, in fish submitted to intense muscular activity suggests that such a preslaughter treatment can have an effect on post-mortem muscle integrity.

KEYWORDS: Proteome; muscle; trout; preslaughter procedure

INTRODUCTION

Slaughter of farmed fish involves crowding and water removal by netting, and optional steps such as transport or individual handling/stunning. All preslaughter steps generate stress and/or muscle activity, both having an influence on muscle biochemistry and product quality. Thus, muscle of salmonids subjected to preslaughter activity is characterized immediately after death by lower levels of the high-energy reserves phosphocreatine and ATP (*1*, *2*), by a lower pH, and by higher lactate concentrations (*3*, *4*). In later stages of storage, several detrimental effects of high preslaughter activity on product quality have been reported in many fish species: earlier onset of rigor mortis (*2*, *5*), greater susceptibility to gaping (*4*), enhanced lipid oxidation (*6*), and softer texture (*2*).

Studies on acute stress or exercise and their biochemical impacts on fish muscle generally focus on metabolites and ions (*7*, *8*), on specific proteins such as enzymes of energetic pathways (*9*), or on heat shock protein (HSP) transcripts (*10*). Proteomics, which allow one to observe concomitant changes in hundreds of proteins, is expected to be a powerful means of bringing into evidence regulations in one or more biochemical pathways and also in structural proteins. For instance, effects of preslaughter conditions on post-mortem muscle proteome have been described in pig (*11*). Recently, proteomics has been applied to zebrafish normoxic and hypoxic muscle (*12*). The objective of the present study was therefore to use proteomics, combining two-dimensional electrophoresis and mass spectrometry, to characterize the modifications of proteins in rainbow trout white muscle induced by preslaughter activity.

MATERIALS AND METHODS

Animals and Sampling. Rainbow trout (*Oncorhynchus mykiss*) used in this study were hatched and reared in Station Expérimentale Mixte IFREMER INRA (SEMII) facilities, Sizun, France. At the time of the experiment, stocking density in the ponds was 14 kg/m^3. Feeding ceased 2 days before the experimentation. To produce "rested" fish, 10 trout were quickly netted and placed in a small vat containing water to which the anesthetic eugenol had been added at the concentration of 50 ppm. After 10 min, fish were bled by section of the gill arches. For the "exercised" group, the water level of the rearing tank was lowered to 20 cm for 15 min, resulting in pronounced crowding (density > 50 kg/m^3). Ten fishes were subsequently sacrificed following the same sedation and killing protocol as previously described.

Muscle samples were taken ~45 min after bleeding (t_i). Samples of 1 cm^3 were taken from the deep dorsal white muscle, in the area below the dorsal fin toward the head. Fish were subsequently stored in ice. A second muscle sample was taken 24 h post-mortem (t_{24}) from the same location in the opposite fillet. All muscle samples were frozen in liquid nitrogen and stored at −80 °C until experimental use. Five fishes for each preslaughter procedure were selected on the basis of comparable morphometric parameters (average live weight, 250 g; average condition factor, 1.32), the condition factor being calculated as live weight (g)/total length (mm) × 100000.

Two-Dimensional (2D) Electrophoresis. Total protein extraction, protein content measurement, sample application, and 2D electrophoresis were performed according to the methods of Morzel et al. (*11*). Proteins were separated in the first dimension on 3−10 NL Bio-Rad ReadyStrip, 17 cm, followed by 11% polyacrylamide gels. The protein

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10.1021/jf0528759 CCC: $33.50
Published on Web 03/28/2006

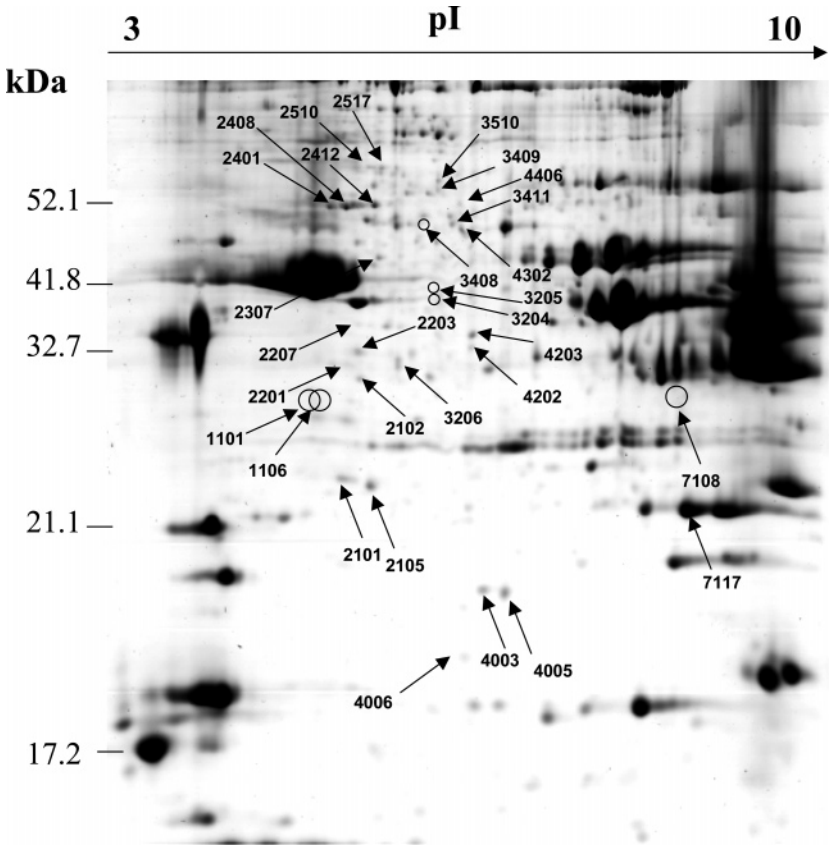

Figure 1. Two-dimensional electrophoretic separation of proteins of rainbow trout white muscle. Proteins of interest (see text) are indicated by arrows and a unique number.

load was adjusted to 800 μg per gel. Gels were stained in 0.02% colloidal Coomassie Blue after fixing in 0.2% (v/v) phosphoric acid−30% (v:v) ethanol, washing in 2% (v/v) phosphoric acid, and equilibrating in 2%(v/v) phosphoric acid−18% (v/v) ethanol and 15% (w/v) ammonium sulfate. When satisfactory spot intensity was reached, gels were destained in water.

Image Analysis and Statistical Treatment. Gel images were acquired through a GS-800 densitometer and analyzed using PDQuest software (Bio-Rad). Detected and matched spots were normalized by expressing the relative quantity of each spot as the ratio of individual spot quantity on the total quantity of valid spots. Relative quantities were expressed in parts per million. To ensure a normal distribution of values, quantities in parts per million were transformed into $\log(x + 1)$ before statistical treatment. The statistical effect of preslaughter activity on spot quantities was tested by ANOVA (5% level) and by a nonparametric Wilcoxon test (10% level), using SAS v8.10 (SAS Institute Inc., Cary, NC). Significant spots were those reaching the significance level in both tests.

Identification of Proteins by Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF). Spots excised from the gels were placed into 96-well plates, destained, digested, and desalted using the Montage In-Gel $Digest_{96\ ZP}$ Kit (Millipore, Bedford, MA). Resulting peptide mixtures were loaded directly onto the MALDI target. The matrix solution (5 mg mL^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid v/v) was added immediately and allowed to dry at room temperature. A Voyager DE-Pro model MALDI-TOF mass spectrometer (Perseptive BioSystems, Farmingham, MA) was used in positive-ion reflector mode for peptide mass fingerprinting. External calibration was performed with a standard peptide solution (Proteomix, LaserBio Labs, Sophia-Antipolis, France). Internal calibration was performed using peptides resulting from autodigestion of porcine trypsin. Monoisotopic peptide masses were assigned and used for NCBI database searches with Mascot or Profound software (http://www.matrixscience.com and http://prowl.rockfeller-.edu). Search was performed at a 40 ppm mass tolerance and within the "ray-finned fishes" or "chordata" taxonomy group, respectively. When a protein was declared unnamed or hypothetical, a similarity search of the putative amino acid sequence was performed using the BLAST algorithm (*13*).

RESULTS AND DISCUSSION

Fish Activity. Fish quickly netted and placed in the anesthesia vat showed an initial swimming response that lasted for ∼1 min before reaching immobility. However, after gill arch sectioning, most fish responded by occasional bouts of intense muscle activity over a period of 5−10 min. In the second batch, lowering the water level of the rearing tank to 20 cm resulted in intense and prolonged struggling throughout the 15 min. Such an activity has been reported to mobilize the whole musculature, including deep white muscle (*14*). It can therefore be expected that constitutive proteins will be affected by the treatment. Fish responded in a manner similar to the rested group during netting, sedation, and gill arch cutting, although bouts of physical reactions were less frequent and intense, probably because the muscular reserves of those fishes were partly depleted. Eugenol has been reported to be an effective anesthetic for rainbow trout. However, the reported time to reach so-called deep anesthesia varied from 2 min at 150 ppm (*15*) to 30 min at 17 ppm (*4*). The conditions used in the present study resulted in light anesthesia. Although such a treatment did not suppress all muscular activity in the "rested" group, there was a clear difference of muscular activity between the two groups.

Protein Patterns and Protein Identification. After automated detection and matching and manual correction, 190 spots were successfully matched across the whole set of images. A gel, representative of t_i, is shown in **Figure 1**. Twenty-nine spots were differentially expressed at t_i. Four spots, three being already significant at t_i, were differentially represented at t_{24}. All of these

Table 1. Proteins Differentially Represented in White Muscle of "Rested" or "Exercised" Fish

time	spot	identification by peptide mass fingerprinting	score		identification by Blastp	quantity (ppm)	
			Mascot	Profound		in rested fish	in exercised fish
t_i	1101	ni				174	67
	1106	ni				138	65
	2101	ni				1924	954
	2102	pyruvate dehydrogenase (*D. rerio*)	48	1.35		430	194
	2105	cytoplasmic carbonic anhydrase (*O. mykiss*)	127	2.28		1335	542
	2201	ni				382	180
	2203	capping protein–muscle Z-line (*D. rerio*)	68	2.21		1026	488
	2207	ni				139	58
	2401	desmin (*O. mykiss*)	92	1.56		2593	1708
	2408	desmin (*O. mykiss*)	118	2.37		1718	940
	2412	desmin (*O. mykiss*	181	2.28		1128	657
	2510	ni				538	248
	2517	myosin heavy chain/fragment (*O. mykiss*)	56	2.19		546	220
	3204	ni				193	93
	3205	ni				290	152
	3206	ni				444	101
	3408	ni				354	189
	3409	ni				292	133
	3411	gi|50417157	83	2.24	sarcalumenin-like (*E* value: 0.0)	371	199
	3510	ni				220	108
	4003	ni				1081	420
	4005	gi|53734023 (*D. rerio*)	56		DJ 1-like (*E* value: 1 e^{-24})	1679	985
	4006	gi|37589643 (*D. rerio*)	55	1.46	no detected homology	123	61
	4202	ni				354	126
	4203	α enolase 1 (*S. trutta*)	67	2.37		850	409
	4302	α enolase 1 (*D. rerio*)	144	2.35		886	500
	4406	ni				134	62
	7108	ni				7	91
	7117	triosephosphate isomerase (*O. mykiss*)	179	2.36		20862	29221
t_{24}	2105	cytoplasmic carbonic anhydrase (*O. mykiss*)	127	2.28		1079	642
	2412	desmin (*O. mykiss*)	181	2.28		2154	1004
	2307	ni				456	210
	4202	ni				862	407

[a] Samples were taken 45 min (t_i) or 24 h (t_{24}) after death.

spots of interest are indicated by arrows in **Figure 1**. Surprisingly, only two spots were over-represented in the exercised group at t_i and none at t_{24}. The discrepancy in numbers of significant spots at t_i and t_{24} indicates that intense exercise induces a rapid disturbance in protein pattern, in accordance, for example, with studies describing the up-regulation of HSPs immediately after stress or exercise (*16*). In later post-mortem stages, events such as denaturation, oxidation, or proteolysis probably override those early modifications.

Table 1 provides identification and relative quantity of the spots of interest. Using strict significance scores (>61 for Mascot and >1.75 for Profound), 10 spots of 29 were identified. However, additional protein identifications are proposed under two conditions: the obtained score was near the significance level (>48 for Mascot and >1.35 for Profound), and the position of the spot was consistent with the theoretical molecular weight and isoelectric point of the proposed protein. Using those rules, another three proteins were identified. Although these results are far from optimal, they compare well with other published works on fish muscle proteomics (*12*, *17*, *18*) where few differentially expressed proteins were identified. We here demonstrate the validity of using peptide mass fingerprinting for the identification of proteins by cross-species matches, even in a rather poorly documented species.

Protein Pattern Modification: Enzymes and Regulatory Proteins. Triosephosphate isomerase is a key enzyme involved in the first phase of glycolysis, namely, the production of glyceraldehyde-3-phosphate (G3P). Its overexpression in the exercised group is consistent with a rapid increase in energy demand. On the basis of previous work imposing exercise to salmonids, in the form of nonsustainable swimming (*19*) or a combination of stress and exercise for 30 min (*8*), anaerobic metabolism is rapidly mobilized. In the present study, this is also indicated by the lower relative proportion of pyruvate dehydrogenase, necessary for the conversion of pyruvate to acetyl CoA before entering the aerobic Krebs cycle (aerobic metabolism). Another enzyme of glycolysis, α-enolase, was under-represented in exercised fish. However, because β-enolase is the largely predominant (~90%) form of enolase in skeletal muscle (*20*), it is difficult to infer the impact of the lower α-enolase relative quantity in the exercised group.

As to sarcalumenin, it possesses an activity of Ca^{2+} sequestration within the sarcoplasmic reticulum (*21*), which would need to be repressed during prolonged muscle contraction as induced in our study. More surprisingly, cytoplasmic carbonic anhydrase was also under-represented in exercised fish muscle, whereas its main function is to assist in the excretion of CO_2 and ammonia under conditions of maximal production, such as exhaustive stress (*22*). Finally, DJ-1 is a protein playing a pivotal role in several cellular functions: modulation of gene expression level, protection against protein misfolding after oxidative stress, apoptosis signaling (*23*). It has been studied almost exclusively in relationship with Parkinson's disease and in brain tissue. However, two recent studies (*24*, *25*) demonstrate its presence in skeletal muscle, more predominantly in mostly glycolytic muscles. Because of the multifunctionality of this protein, it

would be necessary to have access to related biochemical measurements, for example, protein oxidation level, to decipher its function in muscle.

Protein Pattern Modification: Structural Proteins. A fragment of myosin heavy chain (MHC) as well as two whole structural proteins (desmin and cap-Z) were under-represented in the exercised group at t_i. The presence of myosin fragments in very early post-mortem phases has been previously reported in bovine or porcine muscle (*11*, *26*), probably related to protein turn-over in live animals. However, because the half-life of MHC is ~5 days (*27*), it is unlikely that the treatment applied could induce observable changes in turn-over products. Oppositely, a 15 min of exercise exacerbated the degradation of MHC in muscle (*28*). However, the exercise was described as mild, far from the conditions used in the present study, and protein modifications are probably not comparable. Under-representation of the MHC fragment in our case could therefore be due to a mere reduced extractability of the fragment. Decreased solubility of MHC has been reported in defective pale soft exsudative (PSE) pork (*29*) and has been attributed to the structural changes of the molecule induced by a combination of low pH and high temperature (*30*). In fish, profound physicochemical disturbances of white muscle biochemistry are observed immediately after exhaustive exercise or a combination of stress and exercise: accumulation of lactate and metabolic H^+ and fluid volume modification (*31*), and significantly lower pH (*7*, *8*). Such an environment may therefore contribute to decrease the solubility and extractability of some structure proteins at t_i, either whole (desmin, cap-Z) or as fragments (MHC). However, regarding desmin and cap-Z, it is also possible that their reduced relative quantity is caused by early post-mortem proteolysis that would be more pronounced in exercised fish. The effect would be sustained over time in the case of desmin. Thus, Belcastro et al. (*32*) suggested that calpain activity is increased after eccentric exercise. Desmin has been described to be a potential substrate of proteolytic enzymes, including calpains, in meat and fish (*33*−35), as well as cap-Z (*36*). Desmin has also been considered to be a marker of freshness in some fish species (*37*). In any case, whether the reduced relative quantity of desmin is due to proteolysis, denaturation, or a combination of both, it most certainly can have an effect on muscle integrity because it is a main constituent of muscle cell cytoskeleton (*38*).

ACKNOWLEDGMENT

We sincerely thank the staff of SEMII facilities for fish rearing and for their technical assistance in measurements at the time of slaughter.

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Received for review November 18, 2005. Revised manuscript received February 3, 2006. Accepted March 7, 2006.

JF0528759