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Cod (*Gadus morhua*) Muscle Proteome Cataloging Using 1D-PAGE Protein Separation, Nano-Liquid Chromatography Peptide Fractionation, and Linear Trap Quadrupole (LTQ) Mass Spectrometry

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Because Atlantic cod (*Gadus morhua*) has high economic value and its protein-rich muscle tissue is a food source, an increased understanding of the effects and consequences of environmental, nutritional, biological, and industrial factors on meat quality is necessary. To gain insight into cod muscle tissue protein composition, a large-scale proteomics approach has been used. One-dimensional polyacrylamide gel electrophoresis, nanoflow liquid chromatography peptide separation, and linear trap quadrupole mass spectrometry were used to identify 4804 peptides, which retrieved 9113 cod expressed sequence tags (ESTs), which in turn were mapped to 446 unique proteins. The same data set identified 3924 proteins from the zebrafish protein database, which highlights the complementary value of the two approaches. The generated data sets will act as a foundation for studies related to physiological status assessment of cod under different environmental conditions, screening for diseases, and biomarker identification for assessment of fish quality during industrial processing and preservation.

KEYWORDS: Atlantic cod; muscle; proteome analysis; SDS-PAGE; LC-MSMS

INTRODUCTION

Atlantic cod (*Gadus morhua*) is an economically important fish of the Gadidae family of demersal fish species that inhabits the North Atlantic from the coastal shorelines to continental shelves of the sea (*1*). Overexploitation, climate, and local environmental changes have led to a sharp decline in the global Atlantic cod catch from ~3.5 million tonnes in the mid-1970s to < 1 million tonnes in recent years (*2*). This in turn has stimulated increasing interest in cod farming due to increasing market demands (*3*). Currently, Norway has the largest industry of farmed cod among the North Atlantic countries (*1*, *4*).

The rapidly growing cod aquaculture industry requires a well-developed and carefully selected Atlantic cod broodstock, and because of this, extensive breeding programs have been established, which include the development of genetic markers for analyzing quantitative trait loci (QTL), expressed sequence tag (ESTs) libraries, identification of single-nucleotide polymorphisms (SNPs), and in-depth Atlantic cod genome and transcriptome analyses. A Norwegian consortium of researchers has generated a first sequence draft of the Atlantic cod genome using a shotgun sequencing approach (*5*).

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Analysis of the Atlantic cod genome has revealed important features such as microsatellite repeats and SNPs as genetic markers (*6*−*8*). At the transcriptome level, cDNA libraries and ESTs analyses have led to the generation of a high number of ESTs and the identification of a list of unique Atlantic cod transcripts (*9*). From this 16000 probes have been generated for microarray analysis, which has become an important tool for comparing gene expression profiles at different developmental stages, different tissues, and different physiological states of farmed and marine species (*10*). Although genome and transcriptome analyses have increased our understanding of fish genetics and physiology, it has become clear that analysis of proteins and their functions in relation to genome and transcriptome data is required not only to appreciate fish biology in general but to identify, understand, and map important biological pathways and processes (*11*). Indeed, proteomics approaches (*12*) have recently been employed for species authentication (*13*−*16*), to differentiate between farmed and wild fish (*17*), and in assessing general fish quality and freshness, which might be affected by fish handling, processing, and storage (*18*−*22*).

A typical proteomics study involves polyacrylamide-based fractionation of proteins extracted from biological samples followed by mass spectrometric (MS) analyses of the peptides to acquire protein structural information for use in protein identification and characterization (*23*). The sensitivity of MS analyses can be greatly increased when a second round of fractionation is

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applied to the mixture of peptides using nano-flow liquid chromatography (LC) before introduction to the MS (*24*). De novo sequencing of peptides using tandem mass spectrometry (MS/MS) provides an invaluable tool for identifying proteins on the basis of amino acid sequence homology searches from available databases (*25*, *26*). Currently, only five ray-finned fish species have complete (or nearly complete) genome sequences deposited in databases, including zebrafish, fugu, medaka, tetradon, and three-spined stickleback, which can be used for identification of proteins from proteomics approaches (*27*, *28*).

In this study, we have identified 446 skeletal muscle proteins by searching the Atlantic cod EST database and 3924 proteins based on the identification of at least one specific peptide mapped for each protein in the zebrafish protein database. To our knowledge the reported muscle proteome represents the most comprehensive data set for a fish muscle's proteins and for an economically important edible fish species, which will be of considerable interest and value to both academia and industry.

MATERIALS AND METHODS

Fish. Whole degutted cod fish were provided by Nofima (Nofima, Norway). Fish were caught off the northern Norwegian coast and kept in ice until they were used in experiments within no more than 3 days from the date of catching.

Fish Muscle Samples. White muscle tissue samples free of connective tissue were taken from the fish dorsal fin muscle using clean dissecting tools (scalpel, scissors, and tweezers). Samples were rapidly prepared, weighed, and transferred to Eppendorf tubes containing Laemmli buffer for direct solubilization of muscle tissue proteins.

Extraction and Solubilization of Cod Muscle Protein. Ten milligrams of connective tissue free cod muscle tissue (corresponding to at least 2 mg of proteins) was transferred into an Eppendorf tube containing 90 μL of 2× Laemmli buffer comprising 0.09 M Tris-Cl (pH 6.8), 20% glycerol, 2% SDS, 0.025% bromophenol blue, and 0.1 M DTT. Protein extraction and solubilization were enhanced by vortexing tubes three times for 30 s with 5 min intervals. Tubes were then heated at 95 °C for 5 min, vortexed briefly, placed on ice, and vortexed again before being centrifuged at 13000*g* for 5 min at room temperature to obtain a clear supernatant of cod muscle lysate.

Cod Muscle Protein Fractionation Using SDS-PAGE. Ten microliters of supernatant obtained from the centrifuged muscle lysate was applied onto a discontinuous 8 × 10 cm SDS polyacrylamide gel composed of a 1 cm long 4% stacking gel on top of a 12.5% separating gel. The applied lysate was subjected to 100 V for the first 15 min before the voltage was raised to 150 V. The run was terminated when the bromophenol dye front reached the baseline of the gel. A protein molecular weight standard was run in parallel to estimate the approximate molecular weights of the proteins separated. The gels was then washed and fixed in a solution of 50% ethanol and 7% acetic acid in distilled water three times for 15 min each before they were washed in distilled water for an additional 15 min. Gels were then immersed in colloidal Coomassie staining solution (*29*) overnight. Destaining was performed using distilled water prior to scanning.

Protein Band Excision and Tryptic Digestion. Protein bands were excised and subjected to tryptic digests using OMX tube devices (OMX, Germany) following the manufacturer's protocol. Gels were placed on a clean glass plate, and all protein bands were excised in descending order (from top to bottom) with the sampler of the OMX tube (1.5 mm diameter). The whole gel lane gave a total of 26 samples. The OMX tube sampler was used to pick at least two gel pieces from each band (three pieces for faint bands) by pressing the sampler vertically through the gel band against the glass plate. Tubes were then spun inverted in a benchtop centrifuge at 13000*g* for 2 min to bring the gel pieces to the reaction bulb. Twenty microliters of trypsin working solution was added to each tube, and the tubes were briefly centrifuged inverted to drain the trypsin solution into the reaction bulbs. The tubes were then incubated at 50 °C in a shaking incubator for 45 min before the tryptic digests were collected by centrifugation at 1000*g* for 2 min. The tryptic digests were stored at 4 °C prior to mass spectrometric analysis.

Peptide Separation and MS Analysis. LC-ESI MS/MS analysis was performed using a Dionex Ultimate 3000 HPLC with a 300 μm i.d. × 0.5 cm Acclaim PepMap100 C_{18} trap column (Dionex) and a 75 μm i.d. × 15 cm Acclaim PepMap 100 C_{18} analytical column (Dionex). The HPLC was coupled to a LTQ-Orbitrap (Thermo Scientific). The protein digests were loaded (5 μL) onto the trap column using 0.1% formic acid (VWR) in water (Milli-Q, Elga) at a flow rate 2 μL/min. The mobile phases for the analytical separation consisted of 0.1% formic acid in 2.5%/97.5% acetonitrile/water (A) and 0.1% formic acid in 80%/20% acetonitrile/water (B) and were pumped with a flow of 300 nL/min. The peptides were separated on the analytical column using a linear gradient from 5 to 60% B in 165 min after a 10 min delay postinjection. The gradient was then run to 100% B in 10 min and held there for 30 min to wash the columns. A total run time of 256 min was used, including the washing step and 30 min re-equilibration of the columns. A PicoTip emitter (SilicaTip, New Objective) with a 10 μm tip and without coating was used as an ESI interface. The electrospray voltage was set to 1 kV, and no sheath gas was used.

The mass spectrometer was used in positive mode. Full scans were performed in the Orbitrap in the m/z range from 200 to 2000, and data-dependent MS/MS scans performed in the linear ion-trap for the five most abundant masses with $z \geq 2$ and intensity ≥10000 counts. Dynamic exclusion was used with 3 min of exclusion after fragmentation of a given m/z value four times. Collision-induced dissociation (CID) was used with a collision energy of 35% and with activation Q setting of 0.400 and activation time of 30 ms for MS^2. The mass spectrometer was tuned daily and calibrated weekly using the calibration solution recommended by Thermo Scientific.

Each raw file was analyzed using the Proteome Discoverer 1.0 (Thermo Scientific) with the following workflow: Rawfile selector, Spectrum selector, and Sequest search. Files were searched against the zebrafish (*Danio rerio*) (Tax.id 7955) database at NCBI (downloaded on July 10, 2010, with > 76000 protein entries) with trypsin as digestion enzyme, allowing for two missed cleavages. Precursor ion and fragment ion mass tolerances were set to 50 ppm and 0.8 Da, respectively. Oxidation (M) was set as dynamic modification, and carbamidomethyl (C) was set as static modification. Results were filtered for high-confidence X-correlation as follows:

$z = 1$	high-confidence XCorr	**1.2**
$z = 2$	high-confidence XCorr	**1.9**
$z = 3$	high-confidence XCorr	**2.3**
$z > 4$	high-confidence XCorr	**2.6**

Database Searches and Identifications of Peptides and Proteins. Peptide sequences generated from searching the cod EST database at NCBI using the mass spectra were used to map EST entries using the NCBI's TBLASTN algorithm. EST hits retrieved were then clustered using the NCBI UniGene, and only protein hits from zebrafish were used to map the list of peptides. Peptides generated from searching the zebrafish protein databases were

searched against the zebrafish protein databases to identify candidate proteins to which they belong. Only high score matches of proteins were accepted. In a parallel approach, the *G. morhua* (tax ID 8049) EST database was downloaded from the NCBI Website, on October 24, 2010 (containing 229,090 entries). Poly-A tails were removed using *trimest* (EMBOSS), and entries containing nine or fewer nucleotides were removed. Translation of the three forward reading frames was conducted using *transeq* (EMBOSS), resulting in a translated EST database of 686,595 entries. Peptides searched against translated cod ESTs retrieved the same number and identity of proteins.

RESULTS AND DISCUSSION

In an attempt to complement the recently sequenced Atlantic cod genome with a parallel annotation of tissue-specific protein profiling, we have performed a large-scale and high-throughput proteome analysis of Atlantic cod muscle proteins. We have used a combination of 1D-PAGE for protein fractionating followed by an additional fractionation step at the peptide level using reversed phase (RP) LC, which increases the efficiency of the subsequent peptide electrospray ionization (ESI) and the sensitivity of the mass spectrometric analysis as well as a combination of searching both the cod ESTs database and the zebrafish protein database. The high sensitivity and resolution of the LTQ mass spectrometer instrument used ensured a low degree of false positives and a high confidence value of the peptide sequences generated and the proteins identified. Using this setup we identified 446 unique proteins from the cod EST database (Supporting Information Table 1) and 3924 proteins from the zebrafish protein database (Supporting Information Table 2), where each protein identified had a minimum of one reliably identified peptide match of a minimum of 8−10 amino acid residues.

Solubilization and SDS-PAGE Separation of Cod Muscle Proteins. Extraction and solubilization of proteins from cod muscle tissue using Laemmli buffer and their subsequent separation on a 12% SDS polyacrylamide gel resulted in a clear separation of cod muscle lysate into distinct protein bands spanning the molecular mass range between >200 and <20 kDa (**Figure 1**). Most bands appeared as clear distinct entities, well separated from adjacent protein bands, whereas a few bands appeared stacked with adjacent bands of close molecular weights. The well-defined, smear-free, and sharp protein banding pattern indicates a high degree of solubilization efficiency of the buffer composition and concentration used with respect to the amount of tissue used for protein extraction. Indeed, subsequent MS/MS analysis of the 1D SDS-PAGE separated cod muscle proteins revealed efficient extraction and solubilizing of membrane proteins (including the ryanodine receptor and ion voltage channel proteins), myofibrillar proteins, and sarcoplasmic proteins (Supporting Information Table 2).

Nano-LC-MS/MS Analysis of Peptides and Identification of Proteins. The high degree of proteome complexity makes comprehensive proteome analysis challenging and highlights the need for strategies to reduce complexity prior to MS/MS analysis. In our study, two levels of sample fractionation were performed to reduce complexity. Initially, we employed 1D SDS-PAGE to separate proteins, on the basis of their relative molecular mass, from the cod muscle lysate. Following this, we employed RP chromatography to fractionate peptides on the basis of their relative hydrophobicities. In addition, we used a nanoflow rate of eluting peptides by applying a very slow but increasing elution solution (acetonitrile/alcohol) concentration, which increases the overall sensitivity of MS in terms of the number of peptides it receives and analyzes per unit time. This in turn reduced the complexity of peptides generated from the tryptic digestion of proteins found in each excised gel slice before being introduced to MS/MS. Ultimately, this strategy increased the overall proteome coverage in terms of the total number of proteins identified.

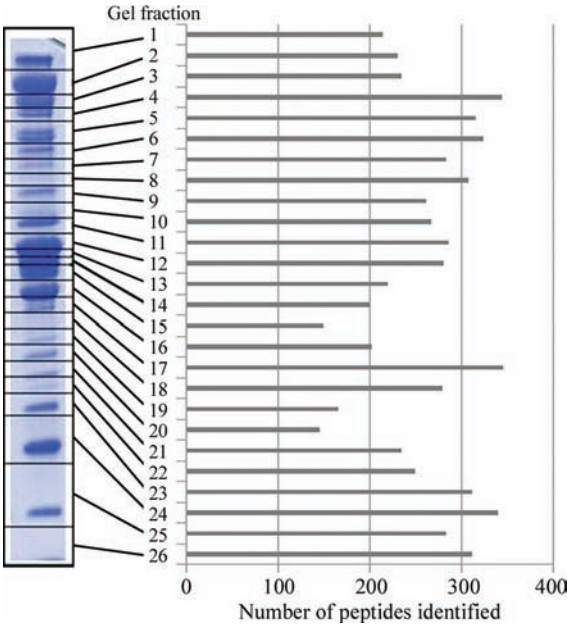

Figure 1. 1D SDS-PAGE and LC-MSMS analyses of Atlantic cod muscle proteins. Laemmli buffer-solubilized muscle proteins were electrophoretically separated on a 12% SDS-PAGE gel. The gel lane was divided into 26 protein-containing gel slices, which were tryptically digested into mixtures of peptides. Horizontal black gray bars show the number of proteins identified in each gel slice using LC-MSMS analysis.

We fractionated the entire SDS gel lane into 26 individual samples as shown in **Figure 1**. The number of peptides generated from tryptic in gel digestion of proteins found in each sample, as detected by MS/MS, ranged from 1 peptide to >500 peptides depending on the number and amount of proteins found in each gel sample, type, and size of each protein in terms of number of lysine and arginine residues (Supporting Information Table 2; **Figure 1**). Some proteins were found in more than one sample (gel slice), which can be attributed to a partial proteolysis and/or the existence of multiple isoforms of these proteins.

Protein identifications were conducted by searching both the cod EST databases and the zebrafish protein database. Using the 4804 MS/MS-generated peptides sequences (Supporting Information Table 3) we identified 9113 EST entries (Supporting Information Table 4) from the cod EST database, which were mapped to 446 unique proteins (Supporting Information Table 1). In contrast, a total of 3924 muscle-specific proteins were identified upon direct searching of the zebrafish database (Supporting Information Table 2). Because of the higher number of proteins identified when using the zebrafish database, we have based our further analysis on this data set. Proteins identified comprise all major classes of structural and functional proteins including extracellular matrix proteins, membrane-integral and associated proteins, sarcoplasmic proteins, myofibrillar proteins, and organellar proteins.

Among the total proteins identified, we have confirmed the presence of 1459 predicted proteins in addition to 687 novel proteins that have not been described to be present in vertebrate muscle tissue before, in addition to 12 hypothetical proteins.

Most Abundant Proteins and Protein Isoforms. As observed from the SDS-PAGE protein banding pattern, we found that nano-LC-MS/MS analyses of the different gel samples revealed

Table 1. Relative Abundance of Myofibrillar Sacromeric Proteins Identified

GI no.	protein	peptides identified
gi48734671	myosin, heavy polypeptide 2, fast muscle specific	522
gi3421386	myosin light chain 2	494
gi37903435	actin	485
gi269148252	actinin 3a	397
gi41351010	actinin α 3b	388
gi238776848	myosin, heavy polypeptide 1.2, skeletal muscle	358
gi269148248	actinin1 isoform c	275
gi32766291	actinin, α 4	270
gi7649818	fast skeletal myosin light chain 3	245
gi82414763	actinin, α 2	108
gi63021930	slow myosin heavy chain 1	98
gi110005908	titin b	91
gi8698673	skeletal α1 actin	71
gi136083	tropomyosin α1 chain	44
gi8698679	fast skeletal muscle troponin C	21
gi8698677	fast skeletal muscle troponin T	15

proteins of different abundance, which matched the SDS-PAGE gel pattern. The most abundant proteins, defined as the first 250 proteins with the highest number of peptides identified for each of them (down to 20 peptides), were found to comprise both structural and functional proteins. These included myofibrillar structural proteins, sarcoplasmic proteins, and receptor proteins. The most abundant myofibrillar proteins identified were myosin protein isoforms, nebulin, titin, actin, actinin, tropomyosin, and troponins I, C, and T, which make up the muscle sarcomere structure (**Table 1**). The fact that all of the main protein components of the sacromere structure were identified demonstrates the comprehensive nature of the reported approach. Other less abundant structural proteins found included α- and *β*-catenin, laminin, talin, and integrins (Supporting Information Table 2).

Abundant sarcoplasmic proteins including enzymes involved in different metabolic and signaling pathways, such as those involved in glycolysis, gluconeogenesis, and the TCA cycle, were also identified. These include creatine kinase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, sarcoplasmic/endoplasmic reticulum calcium ATPase, enolase, glycogen phosphorylase, phosphoglucomutase, phosphoglucose isomerase, enolase, glutamate dehydrogenase, phosphoglycerate kinase, l-lactate dehydrogenase, phosphoglycerate mutase, and triose phosphate isomerase (Supporting Information Table 2). Interestingly, glycolytic enzymes have been shown to interact with skeletal muscle F-actin postulated to act as part of a compartmentation strategy (*30*). In terms of post-mortem changes glycolytic enzymes are also of great interest as glycolysis is temperature dependent and chilling leads to increased water-holding capacity. In addition, receptors and channel proteins (especially for calcium ions) were identified as being highly abundant proteins (Supporting Information Table 2). Specifically, the ryanodine receptors, which form a class of intracellular calcium channels in muscle tissue (*31*), and other anion- and cation-voltage gated channel proteins, such as chloride channel protein 1, voltage-dependent anion channel protein 2 (VDAC2), anion exchanger protein, and sodium, potassium, and calcium channel proteins, were identified, reflecting the need for muscle cells in maintaining appropriate ion homeostasis for general muscle physiology. Extracellular matrix (ECM) proteins were also found to be abundant including collagens (*32*). A number of proteins were repeatedly found in more than one gel sample, which suggests either partial hydrolysis and/or the presence of different protein isoforms due to multiple gene loci, alternative splicing, and/or different types of post-translational modifications. Of the identified proteins with possible multiple isoforms, myosin and keratins represented the most abundant ones (Supporting Information Table 2).

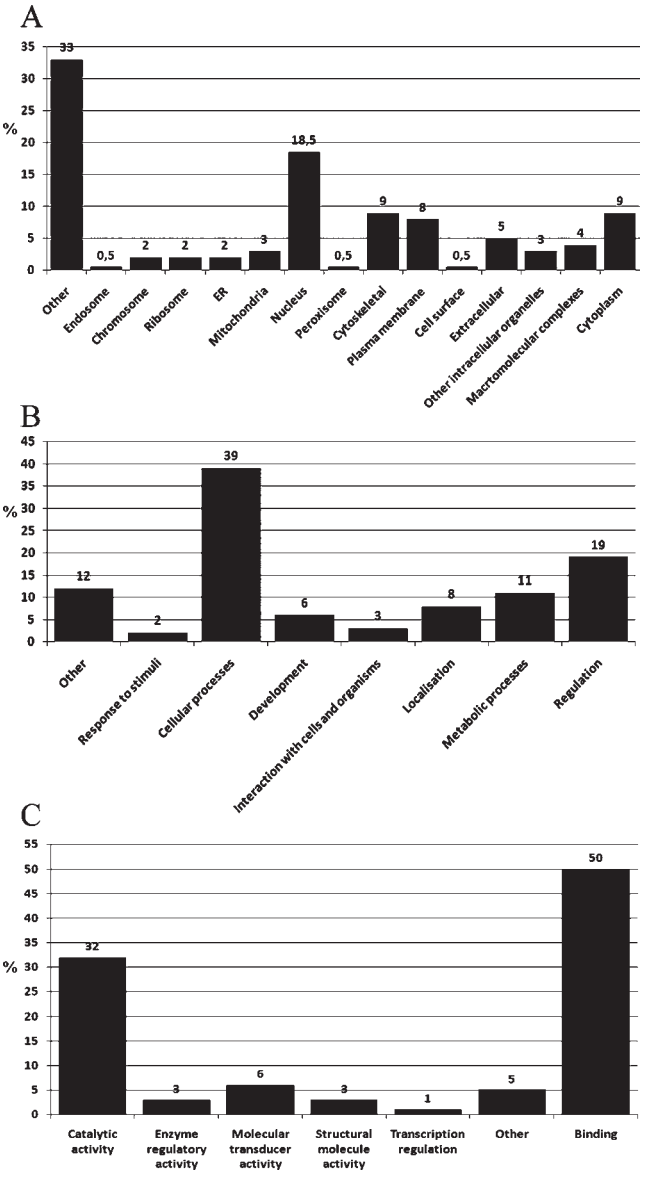

Figure 2. GO-based analysis of all proteins identified showing (**A**) cellular component analysis, (**B**) biological process analysis, and (**C**) molecular function analysis. Different classifications are shown on the *x* axis, whereas the percentage of identified proteins in each category is shown on the *y* axis. Percentages are also shown above each category.

Functional and Cellular Distribution Analysis of Proteins Identified. The first sensory changes in fish during storage relate to appearance and texture, and it is therefore important to understand the protein-induced post-mortem changes that occur in cod. The effect of temperature on rigor is particularly high in cod, where high temperatures result in rapid and strong rigor mortis. In most teleost fish, glycolysis supplies ATP; however, under post-mortem anaerobic conditions the muscle cannot maintain normal ATP levels, which leads to a decrease in pH due to accumulation of lactic acid. As the pH decreases, the net surface charge on muscle proteins is reduced, causing partial denaturation and loss of water-holding capacity. Indeed, many glycolytic enzymes were identified in this study, which may act as good markers for post-mortem changes during storage of cod.

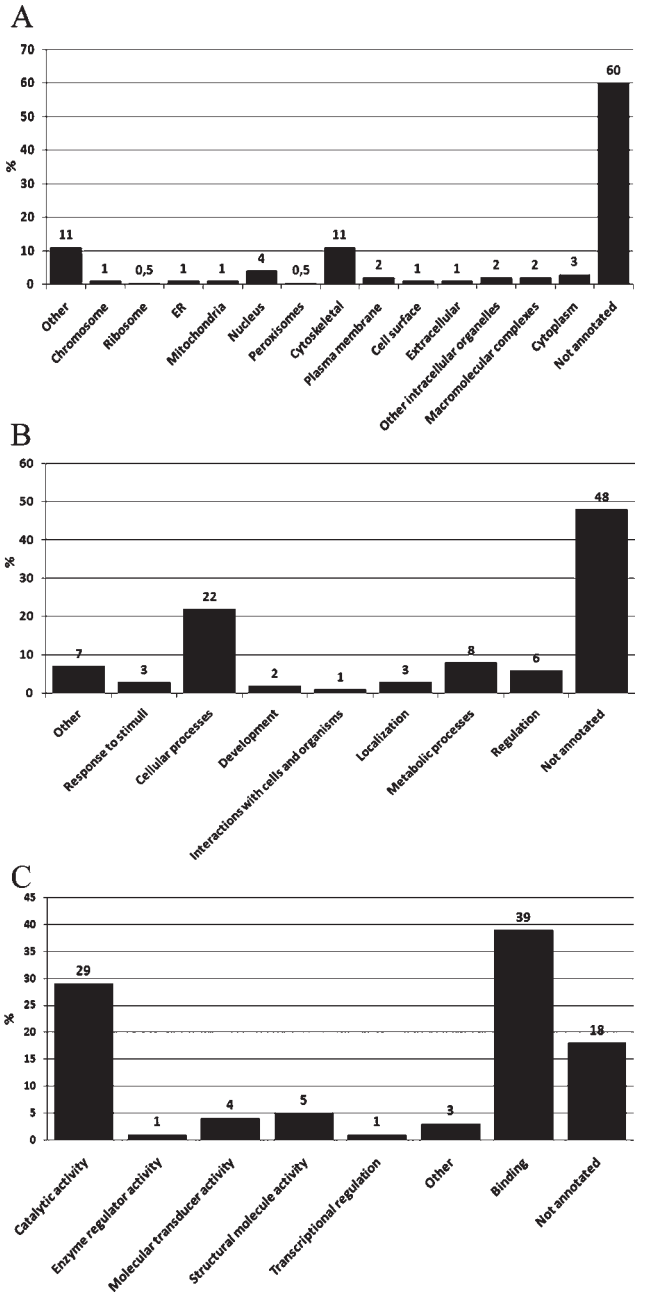

Figure 3. GO-based analysis of the most abundant proteins (229) identified showing (**A**) cellular component analysis, (**B**) biological process analysis, and (**C**) molecular function analysis. Different classifications are shown on the *x* axis, whereas the percentage of identified proteins in each category is shown on the *y* axis. Percentages are also shown above each category.

Softening of fish muscle has also been linked to proteolytic processes and although many proteases have been associated with muscle texture, cathepsins are the most studied. Cathepsins are acid proteases, and cathepsin D, which is among the proteins identified in cod muscle tissue, is thought to be the major autolytic protease involved in textural changes in trout (*33*, *34*); furthermore, cathepsin D is more active at low ATP levels, suggesting high activity in post-mortem fish. The abundance of this entry was, however, low, which may relate to the fact that this study used fresh cod samples and that the relative abundance becomes higher after a longer post-mortem period. With respect to this, it appears that cathepsin D could be a valuable post-mortem marker in Atlantic cod. The calcium-activated calpain proteases are also involved in fish muscle autolysis. In fish, calpains digest myosin, and it has been shown that calpain activity is higher at low temperatures, suggesting that fish species such as cod that live in cold waters are more susceptible to calpain autolysis (*35*). As part of our study we identified calpain 1-, calpain 3-, and calpain 5-like proteins.

Functional and Cellular Distribution Analysis of Proteins Identified. To gain insight into their subcellular location and their functional annotation, all proteins identified from cod muscle were searched against the UniProt database (**Figure 2**). On the basis of these analyses, 9% of the proteins identified were found to be localized to the cytosol, 9% were assigned to membranes, and 18.5% appeared to have a nuclear origin, whereas 9% were found to be associated with cytoskeleton (**Figure 2A**), which indicates a degree of high-order protein complexes in muscle tissue. Indeed, 50% of the proteins identified are assigned general binding functions, indicating their involvement in transient and/or permanent protein complex formation (**Figure 2C**). At a functional level 32% of the proteins identified were found to be enzymes catalyzing different biological processes (**Figure 2C**), which reflects the 39% of all identified proteins being involved in cellular processes (**Figure 2B**).

Identical analysis performed on the 229 most abundant proteins found in the cod muscle tissue showed similar trends in terms of cellular component, biological process, and molecular function analysis (**Figure 3**). However, within the most abundant proteins there was a higher proportion of proteins assigned to the cytoskeleton (11%) than to the nucleus (4%), which is reasonable considering the overall biological function of muscle cells.

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Supporting Information Available: Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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