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Research Article

Methods for Extraction of Muscle Proteins from Meat and Fish Using Denaturing and Nondenaturing Solutions

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The objective of the present study was to test two extraction methods including solutions with different ionic strengths on the extraction and separation of the myofibrillar proteins from meat and fish muscles of different species. Meat samples from *longissimus thoracis* muscle of beef and lamb, *pectoralis major* muscle of chicken, and dorsal white muscle of fish from sole (*Solea solea*), European hake (*Merluccius merluccius*), and sea bass (*Dicentrarchus labrax*) were analyzed. The extraction method using nondenaturing solution led to a major extraction of high molecular-weight proteins as myosin heavy chain, α-actinin, and desmin; on the contrary, the denaturing method provided a good protein extractability of proteins and fragments with low molecular-weight as actin, troponin-T, tropomyosin, and myosin light chain 1 and 2 proteins for the most meat and fish samples. The nondenaturing extraction method showed several advantages resulting in time and labour saving and in minimizing the use of toxic and polluting agents.

1. Introduction

Muscle proteins are grouped into three categories based on location in the skeletal muscle and solubility as sarcoplasmic, stromal, and myofibrillar proteins. Myofibrillar proteins are the main component of the skeletal muscle accounting for about 50% of total proteins and are mainly constituted by myosin and actin, involved in muscle contraction. Due to their structure and localization [1], myofibrillar proteins require denaturing conditions, e.g., high ionic strength solution to be solubilized and extracted [2]. Sarcoplasmic proteins, localized in the sarcoplasm of the muscle fibers, are recognized to be soluble in water or in solutions of low ionic strength, whereas stromal proteins, such as collagen and elastin, are reported to remain insoluble in high-salt solutions [3].

Proteomics techniques have been extensively applied to separate, characterize, and identify proteins in animal food products [4, 5]. Sample preparation and extraction are the most crucial steps in the electrophoretic analysis for obtaining reliable results [6]. The choice of the extraction method of muscle proteins is essential for obtaining samples with high protein concentration and free of salt and other disturbing factors, such as lipids, that could interfere with the electrophoretic analysis. The most commonly used procedures for myofibrillar protein extraction involves denaturing solutions containing urea, thiourea, reducing agents (DTT, beta-mercaptoethanol), detergents (SDS, sodium dodecyl sulfate), and salts [7, 8]. However, it has to be considered that the use of these reagents is regarded as toxic and highly polluting and require proper disposer procedures. Chen et al. [9] reported the use of water or low ionic strength media for the extraction and solubilization of myofibrillar proteins from skeletal muscle. To the best of our knowledge, no research is available on the comparison of the extraction capacity of denaturing and nondenaturing solutions. In the light of this consideration, the objective of the present study was to provide a comparison between two methods for the extraction and separation of myofibrillar proteins including solutions with different ionic strength in meat and fish muscles.

2. Materials and Methods

2.1. Chemicals and Reagents. All reagents used in the experiment were of analytical grade. Potassium chloride, disodium phosphate, monopotassium phosphate, urea, thiourea, dithiothreitol, cholamidopropyl dimethyl hydroxy propanesulfonate (CHAPS), IGEPAL® CA-630 NP 40, glycerol, and tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acrylamide, bis-acrylamide, ammonium persulfate (APS), N,N,N,N-Tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), tris(hydroxymethyl)-aminomethane, glycine, bromophenol blue, β-mercaptoethanol, and Coomassie Brilliant Blue G-250 were purchased from Bio-Rad Laboratories (Hercules, CA).

Phosphate buffer (pH 7, 0.003 M), KCl phosphate buffer (pH 7.5), and Tris-HCl (pH 8, 20 mM) were freshly prepared. Ultrapure water was obtained in the laboratory using a Water Purification System Barnstead™ Pacific TII (ThermoFisher Scientific, USA).

2.2. Samples Collection and Preparation. Meat samples from *longissimus thoracis* muscle of beef and lamb; *pectoralis major* muscle of chicken; and dorsal white muscle of fish from sole (*Solea solea*), European hake (*Merluccius merluccius*), and sea bass (*Dicentrarchus labrax*) were purchased from a local market and immediately transferred under refrigeration to the laboratory. For each species, a total of fifteen animals were included in the experiment. Adipose and connective tissues were removed from meat samples, while bones, scales, and fat were discarded from fish samples. All fresh samples were finely minced prior to protein extractions.

2.3. Protein Extraction Methods. The flowchart of the extraction of muscle protein fractions from different species analyzed is shown in Figure 1. Meat and fish proteins were fractionated based on different solubility. Samples were homogenized with 0.03 M phosphate buffer (pH 7) containing a protease inhibitors cocktail (P2714, Sigma-Aldrich, St. Louis, MO) on ice for 2 min using an Ultra-Turrax T18 basic (IKA, Wilmington, Germany). The homogenate was centrifuged at 8,000 $\times g$ (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) for 20 min at 4°C. After centrifugation, the supernatant (sarcoplasmic proteins) was discarded, and the extraction of myofibrillar proteins were obtained as follows.

Two different extraction methods were carried out for myofibrillar proteins using denaturing and nondenaturing solutions. The extraction of myofibrillar proteins with nondenaturing solution is based on the method reported by Hashimoto et al. [10] with the modifications reported as follows: the pellet recovered was resuspended in 10 volumes of KCl phosphate buffer pH 7.5 (0.45 M KCl, 15.6 mM Na_2HPO_4, 3.5 mM KH_2PO_4) and vortexed for 2 min. The vortexing step was introduced to optimize the homogenization of the pellet and to prevent the formation of a mellow complex. The mixture was centrifuged twice at 5,000 $\times g$ (Eppendorf 5810R, Eppendorf AG, Germany) for 15 min at 4°C. After centrifugation, the supernatant containing the myofibrillar proteins was recovered, aliquoted, and frozen at −80°C.

For comparison, myofibrillar proteins were extracted using denaturing solution according to Marino et al. [11]. Briefly, the pellet was resuspended in a solution (8.3 M urea, 2 M thiourea, 64 mM dithiothreitol (DTT), CHAPS 2% (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), IGEPAL 2%, glycerol 10%, and 20 mM Tris-HCl, pH 8) and incubated overnight at 4°C in an orbital shaker. Subsequently, samples were centrifuged at 15,000 $\times g$ (Eppendorf 5810R, Eppendorf AG, Germany) for 20 min at 10°C. After centrifugation, the supernatant containing myofibrillar proteins was recovered, aliquoted, and frozen at −80°C until further protein analysis to avoid calpain protease activation.

For each species, all myofibrillar extracts obtained with the different methods were quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Absorbance was measured at 580 nm by the spectrophotometric assay (Power Wave XS, Biotek, UK), with a bovine serum albumin (BSA; >98% pure, Sigma-Aldrich) standard curve.

2.4. SDS-PAGE Analysis. The fifteen myofibrillar extracts of each species obtained by the denaturing or nondenaturing method were pooled and resolved by SDS-polyacrylamide gel electrophoresis in a gradient gel 8–18% [11]. Gels were loaded with 50 μg of proteins and run with a Protean II xi vertical slab gel unit (Bio-Rad Laboratories, Hercules, CA). Coomassie Brilliant Blue G-250 was used to visualize bands of interest. Gels were destained in an aqueous solution of acetic acid and methanol (10% v/v, and 7% v/v, respectively) and acquired by the ChemiDoc EQ system (Bio-Rad Laboratories, Hercules, CA). The relative quantity of each band was determined as percentage of the signal intensity of the defined band in a lane with the Quantity One software (Bio-Rad Laboratories, Hercules, CA). Identification of the protein molecular weight was done by comparison with the precision plus protein standard-broad range (Bio-Rad Laboratories, Hercules, CA).

2.5. Statistical Analysis. Protein concentration and electrophoretic data were analyzed using the GLM procedure of the SAS statistical software [12]. The tested effect was the extraction methods on the myofibrillar fraction of muscle proteins from beef, lamb, chicken, sole, hake, and sea bass. When significant differences were found (at $P < 0.05$), the Student t-test was used to locate significant differences among means.

3. Results and Discussion

3.1. Protein Extractability. Solubility is an indicator of protein extractability; indeed, a solubilized protein could be easily extracted into a solution from muscle fibers or myofibrils [9]. The amount of myofibrillar proteins extracted using denaturing and nondenaturing solutions from beef,

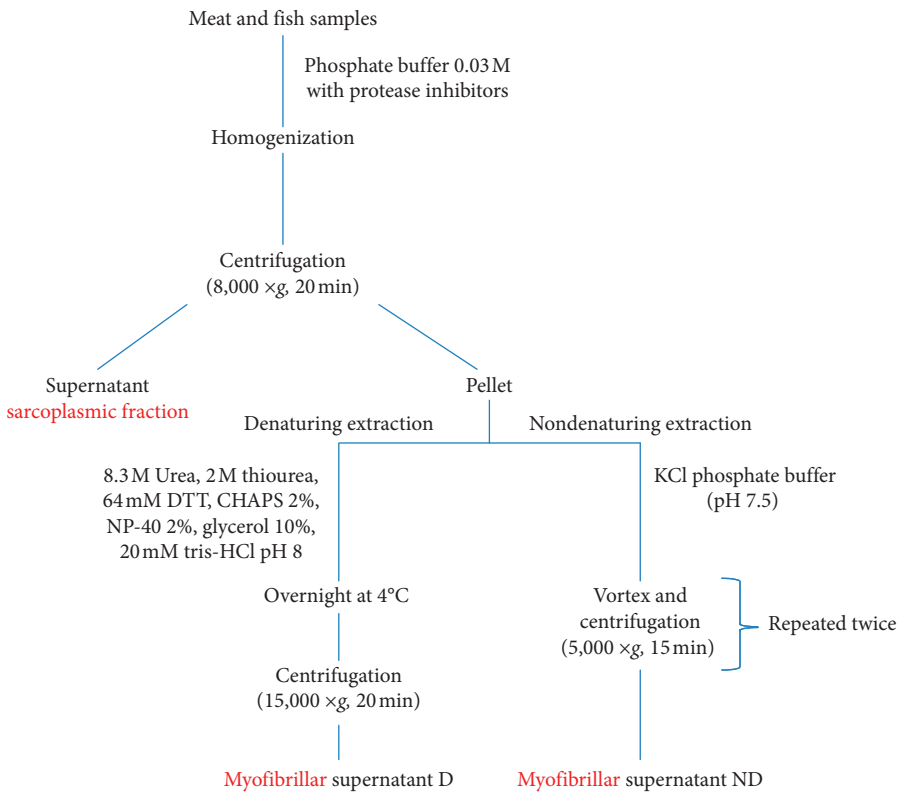

Figure 1: Flowchart of the extraction of sarcoplasmic and myofibrillar protein fractions from different species.

lamb, chicken, sole, European hake, and sea bass is reported in Figure 2. No differences were found in the protein extractability of beef, European hake, and sea bass when the different extraction methods were tested, evidencing that nondenaturing extraction method led to successful protein extraction as denaturing extraction method. The physical force applied by repeated centrifugations damaged the structures of myofibrillar proteins partly allowing the dissolution of myofibrillar proteins in water.

On the contrary, the extraction capacity of the denaturing solution seemed to be more efficient in lamb, chicken, and sole with an amount of myofibrillar proteins extracted of about 30% in lamb and of about 10% in chicken and sole higher than nondenaturing solution. It is known that the extraction of protein from skeletal muscles is a complex phenomenon that is influenced by the parameters of the extraction, by the tissue structure, and by the *post-mortem* changes that occur during the transformation of muscle [13]. The greater extractability of myofibrillar proteins by the denaturing solution in lamb, chicken, and sole samples could be due to the type and structure of muscle [14], suggesting that the power of solubilization could be species-specific.

3.2. Myofibrillar Fraction. The densitometric profile and SDS-PAGE of myofibrillar fraction extracted with denaturing and nondenaturing solutions from meat and fish species are showed in Figures 3 and 4, respectively. Both extraction methods provided an adequate separation of

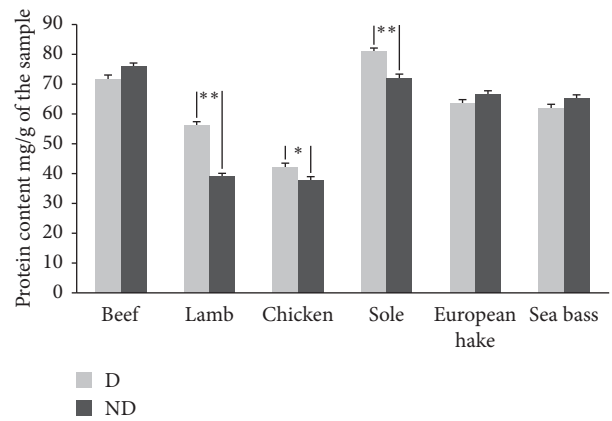

Figure 2: Protein solubility of myofibrillar proteins from the muscle of beef, lamb, chicken, sole, European hake, and sea bass using denaturing and nondenaturing solution (D = denaturing; ND = nondenaturing; $^{*}P < 0.05$; $^{**}P < 0.01$) (means ± SEM).

myofibrillar proteins and derived fragments as showed by the electrophoretic profile with well-defined bands and the absence of any contaminants (e.g., lipids). In meat samples, the main protein bands identified in the range of molecular weights from 250 to 10 kDa were myosin heavy chain (MHC), α-actinin (α-act), desmin, actin (ACT), troponin T (TnT), tropomyosin (TPM), myosin light chains 1 (MLC1), troponin C (TnC), and myosin light chains 2 (MLC2). On the contrary, the electrophoretic profile of fish samples revealed the absence of troponin complex. However, all

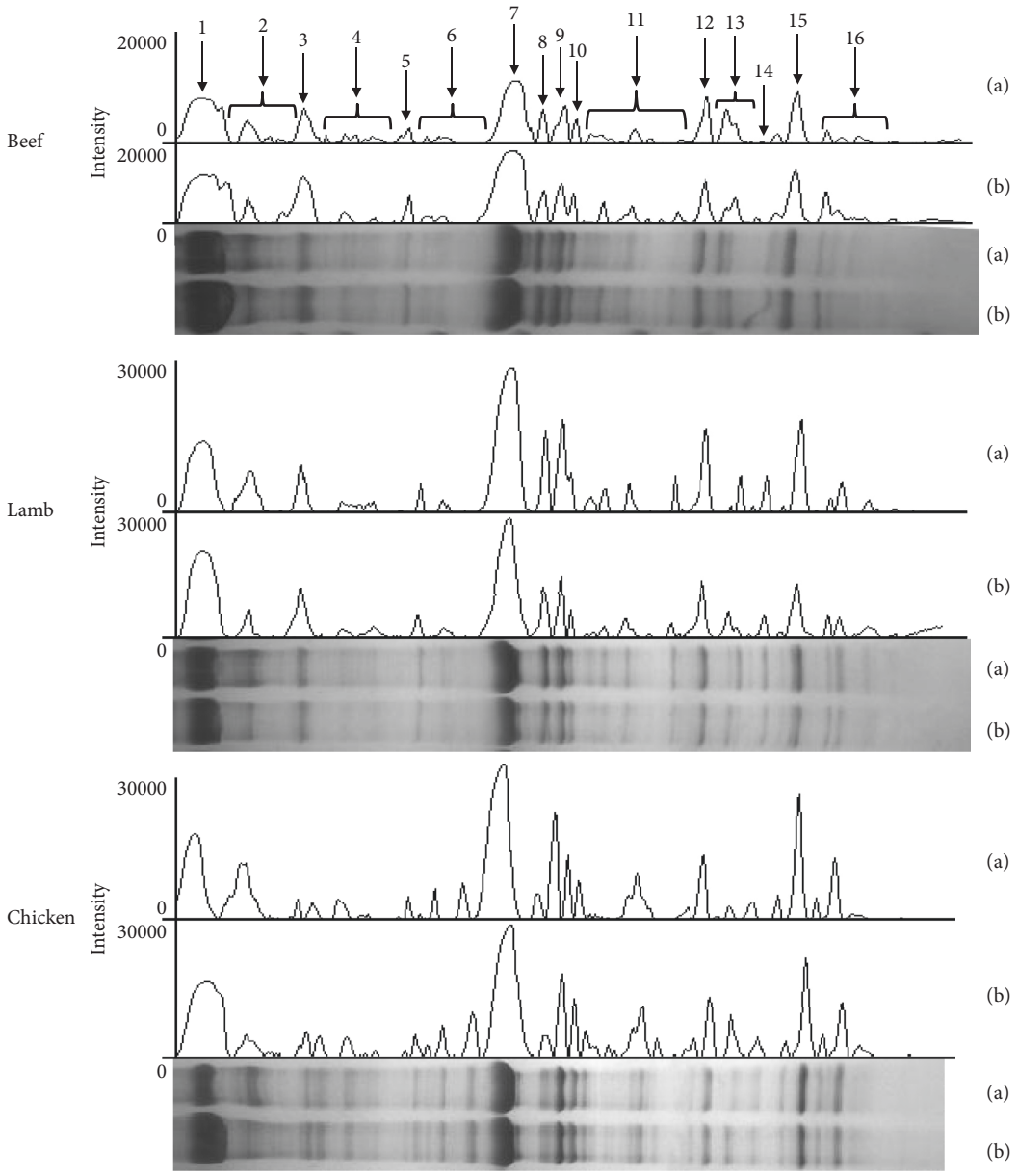

Figure 3: Densitometric profile and SDS-PAGE of a pool of 15 myofibrillar extracts obtained from beef, lamb, and chicken samples extracted with denaturing (a) and nondenaturing (b) solutions (1 = myosin heavy chain; 2 = 180–110 kDa; 3 = α-actinin; 4 = 95–55 kDa; 5 = desmin; 6 = 51–47 kDa; 7 = actin; 8 = 39 kDa; 9 = troponin T; 10 = tropomyosin; 11 = 33–23 kDa; 12 = myosin light chain 1; 13 = 21–18 kDa; 14 = troponin C; 15 = myosin light chain 2; 16 = 14–10 kDa).

species analyzed showed protein fragments in the molecular weight ranging from 180 to 110 kDa, from 95 to 55 kDa, from 51 to 47 kDa, from 40 to 38 kDa, from 33 to 23 kDa, from 21 to 18 kDa, and from 14 to 10 kDa and bands at 39 and 16 kDa.

The densitometric profile of SDS-PAGE revealed that the use of the denaturing solution led to a more complex profile in terms of number of bands and fragments extracted (30, 32, and 32 vs 26, 27, and 28 bands in the nondenaturing profile of meat samples and 35, 29, and 30 vs 31, 26, and 28 bands in the nondenaturing profile of fish samples) while the use of the nondenaturing solution revealed a major intensity for most of the myofibrillar protein analyzed.

The percentage of the main myofibrillar proteins extracted using nondenaturing and denaturing solutions from meat and fish species are reported in Figures 5 and 6, respectively. All samples extracted with the nondenaturing solution showed the highest values of MHC ($P < 0.001$ in beef, lamb, chicken, sole, and European hake; $P < 0.01$ in sea bass), α-actinin ($P < 0.001$ in lamb; $P < 0.01$ in beef, chicken, and sole; $P < 0.05$ in European hake), and desmin ($P < 0.001$ in beef; $P < 0.01$ in sea bass; $P < 0.05$ in lamb, not detected in European hake).

It is known that myosin mainly contributes to the tensile strength of the muscle, while α-actinin and desmin are cytoskeletal proteins responsible for the maintenance of

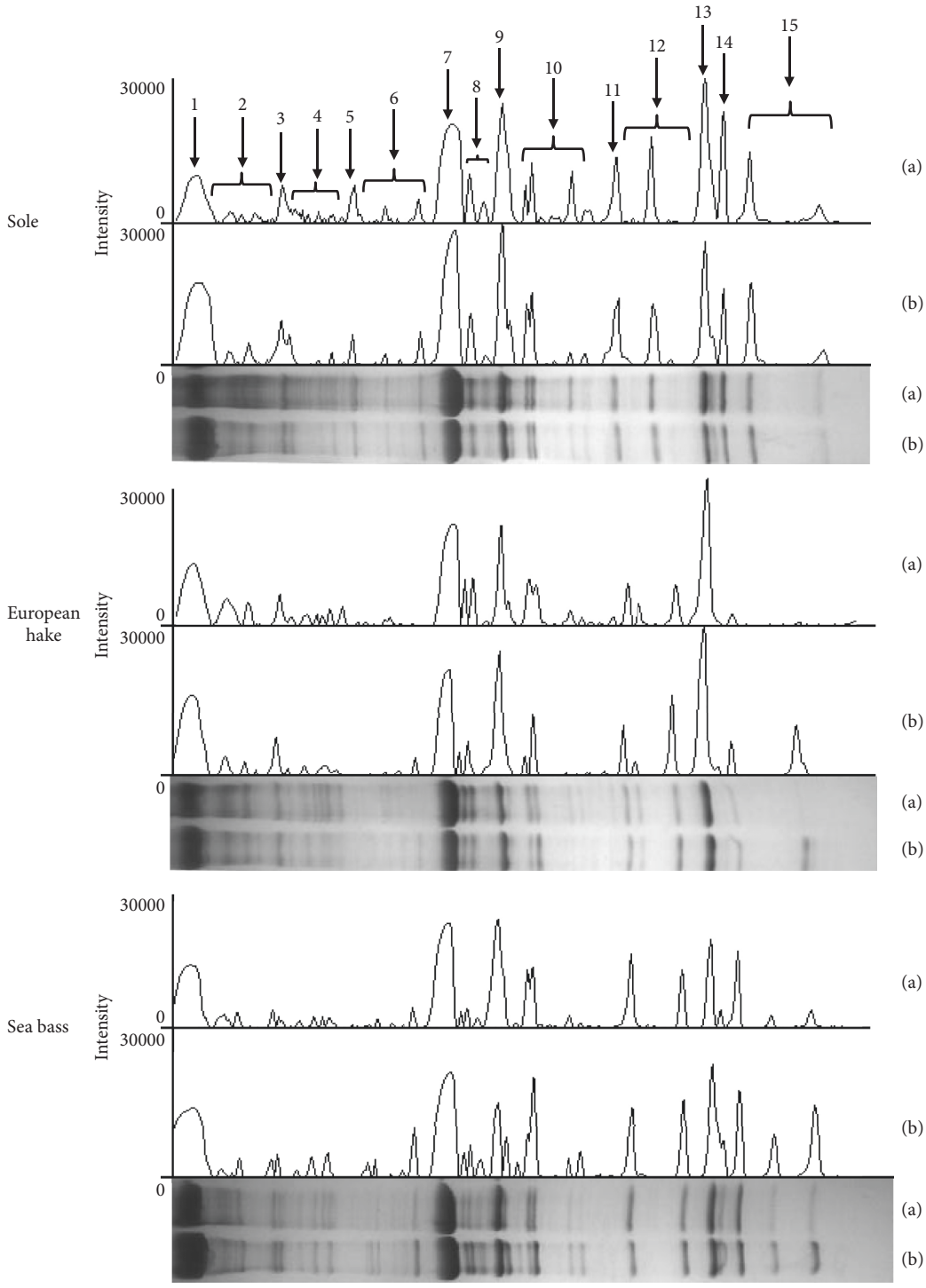

Figure 4: Densitometric profile and SDS-PAGE of a pool of 15 myofibrillar extracts obtained from sole, European hake, and sea bass samples extracted with denaturing (a) and nondenaturing (b) solutions (1 = myosin heavy chain; 2 = 180–110 kDa; 3 = α-actinin; 4 = 95–55 kDa; 5 = desmin; 6 = 51–47 kDa; 7 = actin; 8 = 40–38 kDa; 9 = tropomyosin; 10 = 33–23 kDa; 11 = myosin light chain 1; 12 = 21–18 kDa; 13 = 16 kDa; 14 = myosin light chain 2; 15 = 14–10 kDa).

structural and mechanical integrity of actin filaments in the Z-disk [15]. In any case, whether the reduced relative quantity of all these proteins is due to proteolysis, denaturation, or a combination of both, desmin has also been considered a marker of freshness in some fish species [16]. In the present study, the use of a solution with low ionic strength, in the nondenaturing extraction method, led to a major extraction of these proteins with high molecular weight.

The pH value of the solution in the salt-soluble method seemed to be favourable for protein extraction. Accordingly, Chen et al. [2] reported a greater solubilization of myosin

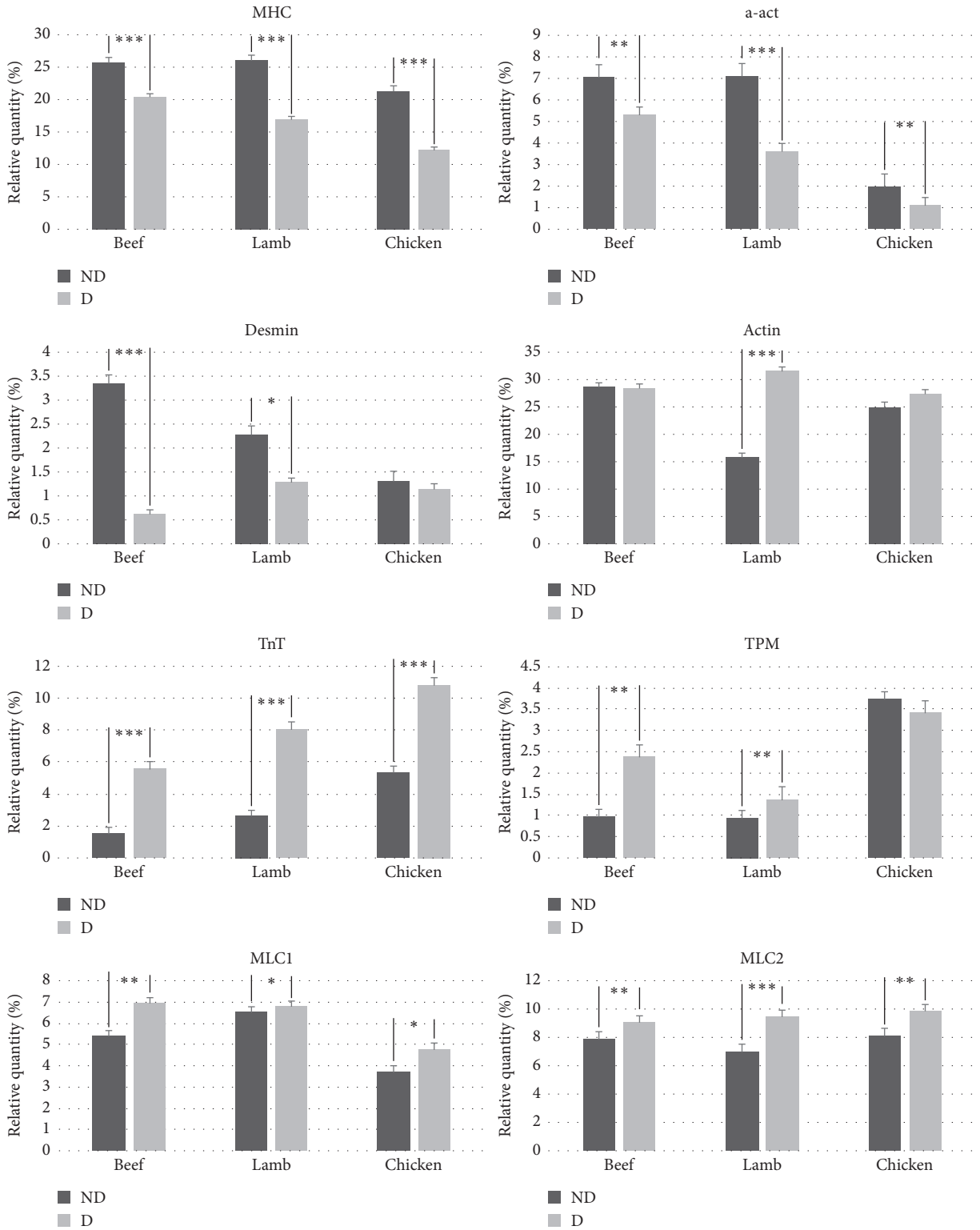

Figure 5: Percentage of the main myofibrillar proteins from beef, lamb, and chicken samples extracted with denaturing and nondenaturing solutions (D = denaturing; ND = nondenaturing; MHC = myosin heavy chain; a-act = α-actinin; TnT = troponin T; TPM = tropomyosin; MLC1 = myosin light chain 1; MLC2 = myosin light chain 2; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) (means ± SEM).

using a 0.6 M KCl solution pH 6.0 due to myosin filament dissociation induced by low ionic strength of the buffer solution. The reduction of salt content in the nondenaturing solution could have led to a modification of the physiological conditions of protein due to change of pH improving the solubility of proteins with high molecular weight.

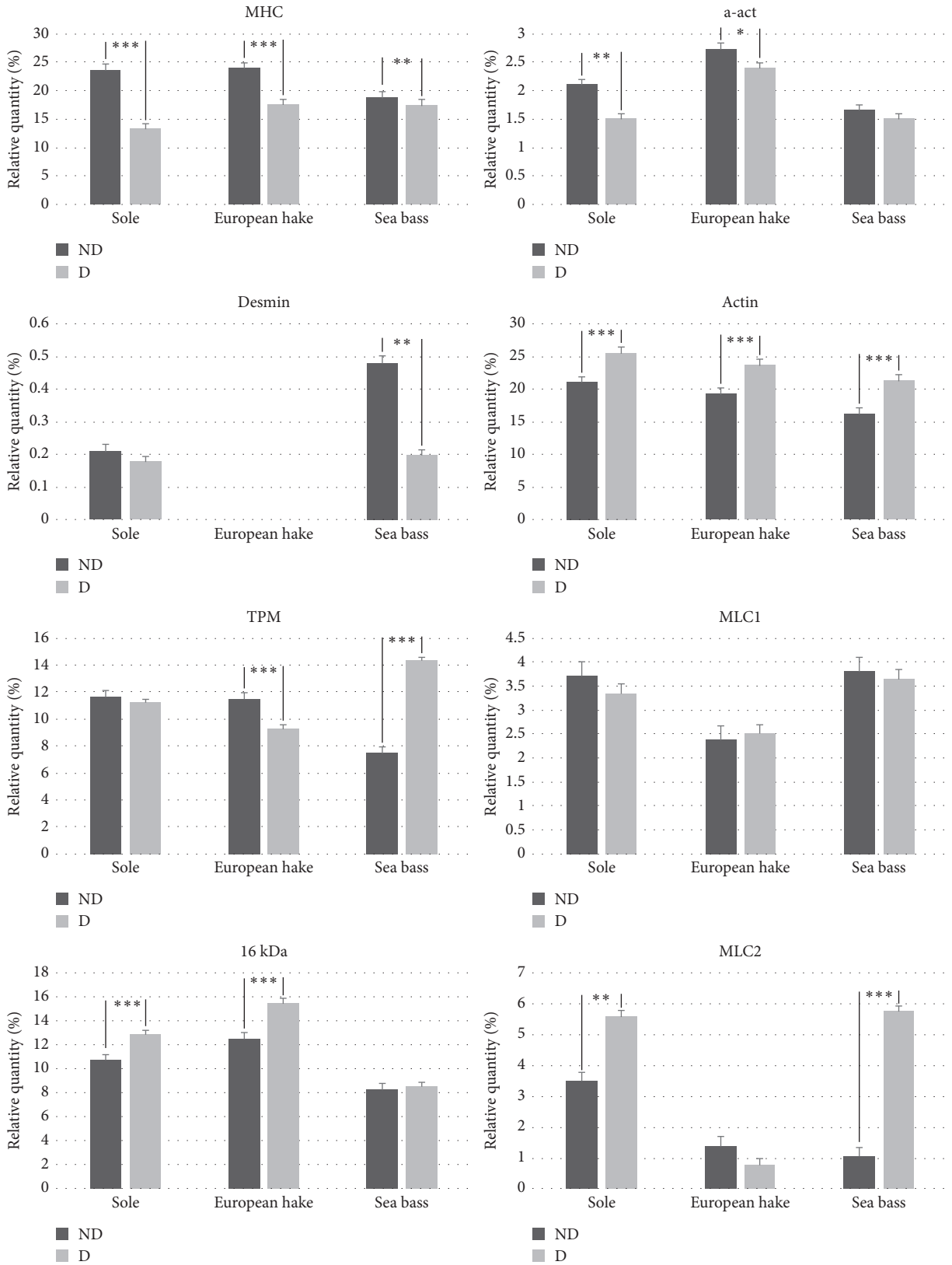

Figure 6: Percentage of the main myofibrillar proteins from sole, European hake, and sea bass samples extracted with denaturing and nondenaturing solutions (D = denaturing; ND = nondenaturing; MHC = myosin heavy chain; a-act = α-actinin; TPM = tropomyosin; MLC1 = myosin light chain 1; MLC2 = myosin light chain 2; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) (means ± SEM).

The use of denaturing solutions led to a major extractability of myofibrillar proteins with low molecular weight (under 45 kDa) as actin ($P < 0.001$ in lamb, European hake, and sea bass), troponin T ($P < 0.01$ in beef, lamb and chicken, European hake, and sea bass), tropomyosin ($P < 0.01$ in beef and lamb; $P < 0.001$ in European hake and sea bass), MLC1 ($P < 0.01$ in beef and $P < 0.05$ in lamb and chicken), and MLC2 ($P < 0.001$ in lamb and sea bass; $P < 0.01$ in beef, chicken, and sole) proteins. No significant differences were found between the two extraction methods in MLC1 of fish samples.

These results could be due to the compounds such as urea, thiourea, CHAPS, and DTT of the denaturing solution. It is known that urea is a chaotropic agent, efficient in the rupture of hydrogen bonds, denaturing proteins by breaking the noncovalent and ionic links between amino-acid residues [17]. Thiourea, indeed, breaks hydrophobic interactions leading to an increase in the solubilization of membrane proteins [18]. Previous studies [19, 20] reported that the combination of urea and thiourea exhibit a superior solubilizing power and increase dramatically the extraction of proteins. In addition, CHAPS and DTT affect protein solubilization because it prevents hydrophobic interaction and promote the reoxidation of disulphide bonds avoiding the lack of proteins by aggregation or precipitation [21]. The presence of denaturing compounds in the extraction solution led to an increase of extraction of myofibrillar proteins with low molecular weight probably due to differences in protein molecular size, conformation, and inter- and intramolecular bonds, resulting in more sensitivity to the strength of extraction of the denaturation method.

4. Conclusion

Nondenaturing and denaturing extraction methods were efficient to solubilize the main muscle proteins. Proteomic analysis revealed a good separation of proteins with well-defined bands without any contamination for all samples analyzed. The extraction method using nondenaturing solution lead to a major extraction of myofibrillar proteins with high molecular weight; on the contrary, the denaturing method provided good extractability of proteins and fragments with low molecular weight for the most meat and fish samples.

The nondenaturing extraction method showed several advantages such as easy to carry out, less invasive, and minimal use of toxic and polluting agents.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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References

[1] Y. Au, "The muscle ultrastructure: a structural perspective of the sarcomere," *Cellular and Molecular Life Sciences*, vol. 61, no. 24, pp. 3016–3033, 2004.

[2] X. Chen, Y. F. Zou, M. Y. Han et al., "Solubilization of myosin in a solution of low ionic strength L-histidine: significance of the imidazole ring," *Food Chemistry*, vol. 196, pp. 42–49, 2016.

[3] A. Listrat, B. Lebret, I. Louveau et al., "How muscle structure and composition influence meat and flesh quality," *The Scientific World Journal*, vol. 2016, Article ID 3182746, 14 pages, 2016.

[4] J. Z. Han and Y. B. Wang, "Proteomics: present and future in food science and technology," *Trends in Food Science and Technology*, vol. 19, no. 1, pp. 26–30, 2008.

[5] B. Picard, L. Lefaucheur, C. Berri, and M. J. Duclos, "Muscle fibre ontogenesis in farm animal species," *Reproduction Nutrition Development*, vol. 42, no. 5, pp. 415–431, 2002.

[6] M. Fountoulakis, "Proteomics: current technologies and applications in neurological disorders and toxicology," *Amino Acids*, vol. 21, no. 4, pp. 363–381, 2001.

[7] M. J. Anderson, S. M. Lonergan, and E. Huff-Lonergan, "Myosin light chain 1 release from myofibrillar fraction during postmortem aging is a potential indicator of proteolysis and tenderness of beef," *Meat Science*, vol. 90, no. 2, pp. 345–351, 2011.

[8] B. C. Bowker, T. M. Fahrenholz, E. W. Parokzay, J. S. Eastridge, and M. B. Solomon, "Effect of hydrodynamic pressure processing and ageing on the tenderness and myofibrillar proteins of beef strip loins," *Journal of Muscle Foods*, vol. 19, no. 1, pp. 74–97, 2008.

[9] X. Chen, R. K Tume, X. Xu, and G. Zhou, "Solubilization of myofibrillar proteins in water or low ionic strength media: classic techniques, basic principles and novel functionalities," *Critical Reviews in Food Science and Nutrition*, vol. 15, no. 15, pp. 3260–3280, 2015.

[10] K. Hashimoto, S. Watabe, M. Kono, and K. Shiro, "Muscle protein composition of sardine and mackerel," *Bulletin of the Japanese Society of the Science of Fish*, vol. 45, no. 11, pp. 1435–1441, 1979.

[11] R. Marino, M. Albenzio, A. della Malva, A. Santillo, P. Loizzo, and A. Sevi, "Proteolytic pattern of myofibrillar protein and meat tenderness as affected by breed and aging time," *Meat Science*, vol. 95, no. 2, pp. 281–287, 2013.

[12] SAS Institute, *SAS/STAT User's Guide (Version 9.2)*, Statistical Analysis System Inst, Cary, NC, USA, 2011.

[13] M. Eady, D. Samuel, and B. Bowker, "Effect of pH and postmortem aging on protein extraction from broiler breast muscle," *Poultry Science*, vol. 93, no. 7, pp. 1–9, 2014.

[14] Y. M. Choi, S. H. Lee, J. H. Choe et al., "Protein solubility is related to myosin isoforms, muscle fiber types, meat quality traits, and postmortem protein changes in porcine longissimus dorsi muscle," *Livestock Science*, vol. 127, no. 2-3, pp. 183–191, 2010.

[15] A. Lana and L. Zolla, "Proteolysis in meat tenderization from the point of view of each single protein: a proteomic perspective," *Journal of Proteomics*, vol. 147, pp. 85–97, 2016.

[16] V. Verrez-Bagnis, J. Noel, C. Sautereau, and J. Fleurence, "Desmin degradation in postmortem fish muscle," *Journal of Food Science*, vol. 64, no. 2, pp. 240–242, 1999.

[17] M. M. Shaw and B. M. Riederer, "Sample preparation for two-dimensional gel electrophoresis," *Proteomics*, vol. 3, no. 8, pp. 1408–1417, 2003.

[18] T. Rabilloud, "Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis," *Electrophoresis*, vol. 19, no. 5, pp. 758–760, 1998.

[19] L. L. Chan, S. C. L. Lo, and I. J. Hodgkiss, "Proteomics study of a model causative agent of harmful red tide. Prorocentrum triestinum I: optimization of sample preparation methodologies for analyzing with two-dimensional electrophoresis," *Proteomics*, vol. 2, no. 9, pp. 1168–1186, 2002.

[20] M. P. Molloy, B. R. Herbert, B. J. Walsh et al., "Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis," *Electrophoresis*, vol. 19, no. 5, pp. 837–844, 1998.

[21] C. B. Malafaia, M. L. Guerra, T. D. Silva et al., "Selection of a protein solubilization method suitable for phytopathogenic bacteria: a proteomics approach," *Proteome Science*, vol. 13, no. 1, pp. 1–7, 2015.