Proteomic analysis of processing by-products from canned and fresh tuna: identification of potentially functional food proteins

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ABSTRACT

Proteomic approaches have been used to identify the main proteins present in processing by-products generated by the canning tuna-industry, as well as in by-products derived from filleting of skeletal red muscle of fresh tuna. Following fractionation by using an ammonium sulphate precipitation method, three proteins (tropomyosin, hemoglobin and the stress-shock protein ubiquitin) were identified in the highly heterogeneous and heat-treated material discarded by the canning-industry. Additionally, this fractionation method was successful to obtain tropomyosin of high purity from the heterogeneous starting material. By-products from skeletal red muscle of fresh tuna were efficiently fractionated to sarcoplasmic and myofibrillar fractions, prior to the identification based mainly on the combined searching of the peptide mass fingerprint (MALDI-TOF) and peptide fragment fingerprinting (MALDI-LIFT TOF/TOF) spectra of fifteen bands separated by 1D SDS-PAGE. Thus, the sarcoplasmic fraction contained myoglobin and several enzymes that are essential for efficient energy production, whereas the myofibrillar fraction had important contractile proteins, such as actin, tropomyosin, myosin or an isoform of the enzyme creatine kinase. Application of proteomic technologies has revealed new knowledge on the composition of important by-products from tuna species, enabling a better evaluation of their potential applications.

Keywords: *Thunnus alalunga*, proteomics, sarcoplasmic, myofibrillar, by-product, food protein.

1. INTRODUCTION

Fish by-products may contain valuable protein and lipid fractions as well as vitamins and minerals highlighting the nutritional and health benefits attributed to fish consumption. Currently, more than 33 million metric tones (23%) of world fish production are destined for non-food uses (FAO, 2008). In addition, the percentages of processing by-products generated from commercial filleting of fish species such as cod, tuna, and trout are even higher, ranging between 60 to 70% (Chen & Jaczynski, 2007). It is anticipated that these high levels of fish by-products are going to be increased during next years as consequence of a raise in fish production in response to expected demand growth for fisheries products (Delgado, Wada, Rosegrant, Meijer & Ahmed, 2003). In this context, there are major ongoing research addressed to improve the management of these by-products in terms of searching new bioactive compounds and developing new technologies that allow a more profitable utilization of this material (Rustad, 2003).

Particularly, given that recent studies have identified a number of bioactive compounds from remaining fish muscle proteins, collagen and gelatine (Kim & Mendis, 2006), the recovery of proteins from fish by-products for their utilization as potential food ingredients is becoming of increasing interest in the food industry (Sanmartin, Arboleya, Villamiel & Moreno, 2009). Therefore, to assess the potential applications for proteins recovered from fish by-products, it is of paramount importance the characterization of the protein fraction of the discarded material. To the best of our knowledge, despite their global economic importance and intensive international trade for canning, protein fractions contained in by-products generated from processing tuna

63 species have not been characterized to date. Likewise, considering that fresh tuna species
64 have high economic value and its protein-rich muscle tissue is a valuable food source, by-
65 products generated from commercial filleting could also be an important and alternative
66 source for functional proteins or peptides.

67 Proteomic techniques have strong potential to accurately characterize the major
68 proteins found in complex food matrices. However, up to now, the use of proteomics in
69 fish biology and aquaculture has been limited (Forné, Abián & Cerdà, 2010).
70 Furthermore, fish protein identification is a challenging task due to the relatively low
71 number of fish protein sequences and expressed sequence tags (ESTs) present in the
72 public databases (Pineiro, Barros-Velazquez, Vazquez, Figueras & Gallardo, 2003;
73 Kjaersgard, Nørrelykke & Jessen, 2006). Therefore, it is essential to obtain protein
74 sequences by either Edman degradation or tandem mass spectrometry which enables
75 identification by matching with homologue proteins from different species as it has been
76 previously reported for hake (Pineiro, Vazquez, Marina, Barros-Velazquez & Gallardo,
77 2001) or cod proteins (Kjaersgard et al., 2006). In this sense, Matrix Assisted Laser
78 Desorption Ionization-Time Of Flight mass spectrometry (MALDI-TOF) provides high
79 sensitivity for peptide mass fingerprinting (PMF). Besides, latest developments in this
80 type of mass spectrometers allow performing tandem mass spectrometry analysis by
81 MALDI-LIFT TOF/TOF for peptide fragment fingerprinting (PFF) (Suckau, Resemann,
82 Schuerenberg, Hufnagel, Franzen, & Holle, 2003).

83 The aim of this study was the fractionation and identification of the main proteins
84 present in processing by-products generated from both commercial canning and filleting

of tunas species using proteomic approaches by combining peptide mass fingerprinting and peptide fragment fingerprinting (MS/MS) analysis.

2. MATERIALS AND METHODS

2.1. By-products homogenization

Canned tuna solid by-products were obtained from a local canned fish processing plant. Skin, viscera and red muscle were the main compounds of the raw material, and were collected after an industrial heat treatment at 95ºC for 1h. After deboning and mixing steps, fish wastes were homogenized, freeze–dried and defatted by the Soxhlet method using petroleum ether (Panreac, Barcelona, Spain) at 60ºC during 12 h. The protein, ash, lipid, and moisture contents of the canned by-product were 60.1%, 19.7%, 1.0% and 4.4% (w:w), respectively, as determined according to the methods of AOAC (2000).

Fresh tuna (*Thunnus alalunga*) red muscle was obtained from filleting wastes at a local fish store situated in the coast (Bermeo, Spain) immediately after the reception of tuna from a close landing centre in order to minimize changes in the quality of the product. Immediately after collection, fish wastes were homogenized using a blender and kept frozen at -20ºC.

2.2. Protein extraction and fractionation

Canned industry fish by-products were processed following the method described by Maitena, Katayama, Sato & Saeki (2004) with some modifications. Firstly, material was resuspended (1:100 w/v) with 0.16 M KCl (Merck KGaA, Darmstadt, Germany) containing 20 mM Tris-HCl (Sigma-Aldrich, St. Louis, MO) (pH 7.5). After 30 min, the solution was centrifuged at 30,000 x *g* for 10 min and the supernatant was recovered as ***Sample 1***. This step was repeated up to three times, in order to remove sarcoplasmic proteins. The final pellet was then dissolved in 0.5 M KCl (Merck) containing 20 mM Tris-HCl (pH 7.5) for 1 h. After addition of 40% saturation of ammonium sulphate (Panreac), the protein solution was centrifuged at 30,000 x *g* for 1 h obtaining a precipitate termed ***Sample 2***. Afterwards, the ammonium sulphate saturation in the supernatant was raised up to 55%, and again was subjected to centrifugation at 100,000 x *g* for 1h. Proteins in the precipitated were collected, dissolved in 0.5 M NaCl (Panreac) containing 20 mM Tris-HCI (pH 7.5), and dialyzed against the same buffer to remove ammonium sulfate (***Sample 3***)**.**

Regarding the protein extraction from fresh tuna by-products, a method described by Mohan, Ramachandran, Sankar & Anandan (2007) with some modifications was followed. Thus, fresh tuna (*Thunnus alalunga*) red muscle was mixed (1:5 w/v) with sodium phosphate buffer (Merck) 0.1M at pH 7.0 and homogenized using an ultraturrax (Miccra RT-D9®). The homogenate was centrifuged at 4 ºC, 12,000 x *g* for 15 min and the supernatant was collected as ***Sarcoplasmic*** proteins. Final precipitate was washed thoroughly using the same buffer in order to remove the soluble proteins. Myofibrillar fraction was recovered from the pellet by solubilization (1:5 w/v) with sodium phosphate buffer 0.1M pH 7.0 containing 0.5M NaCl. After homogenization, the solution was

centrifuged at 4ºC, 12,000 x *g* for 15 min and the supernatant was collected as a ***Myofibrillar*** proteins.

All the steps in both protein fractionation methods were carried out below 6ºC. Furthermore, the extraction procedures were repeated at least three times in order to evaluate difference between batches. According to the electrophoresis gels, no remarkable differences were observed regarding the number of visualized bands.

The protein concentration was determined by the bicinchoninic acid (BCA) method (Smith et al., 1985) using bovine serum albumin (Sigma-Aldrich) as a standard. Thus, total protein content corresponding to the myofibrillar and sarcoplasmic fractions isolated from the homogenized by-products of skeletal red muscle of fresh tuna was 5.5% (w:w).

2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure

For SDS-PAGE analysis, 32.5 μL of the fractionated samples was added to 12.5 μL of 4×NuPAGE LDS sample buffer (Invitrogen, CA, USA) and 5 μL of 0.5 M dithiothreitol (DTT, Sigma-Aldrich) and the mixture heated at 70 °C for 10 min. The samples were loaded (20 μL) onto a 4-12% polyacrylamide NuPAGE Novex® Bis-Tris precast gel (Invitrogen), and a continuous MES-SDS running buffer (Invitrogen) was used for proteins extracted from the canned tuna solid by-products and a MOPS-SDS running buffer (Invitrogen) from the proteins derived from the fresh tuna by-products. The gels were run for 35 min (MES-SDS running buffer) and 50 min (MOPS-SDS

running buffer) at 120 mA/gel and 200 V and stained using the Colloidal blue staining kit (Invitrogen). Marker proteins used were: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), insulin chain B (3.5 kDa) and insulin chain A (2.5 kDa) (Invitrogen).

2.4 In gel-tryptic digestion and peptide extraction

Bands were excised manually from 1-D stained electrophoresis gels and subjected to in-gel tryptic digestion according to Shevchenko, Wilm, Vorm & Mann (1996) with minor modifications. The gel pieces were reduced with 30 μL of 10 mM DTT at 56°C for 20 min, followed by an alkylation in 30 μL of 50 mM iodoacetamide for 20 min at room temperature in darkness. Then, bands were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/μL of trypsin (Roche Diagnostics, recombinant, proteomics grade trypsin, Penzberg, Germany) in an ice bath. After 30 min the supernatant was removed and discarded, 20 μL of 50 mM NH_4HCO_3 were added to the gel piece and the digestion allowed to proceed at 37 C overnight. After trypsinization, the supernatant was transferred to an empty eppendorf tube and acidified with 0.1 % TFA.

2.5 Sample preparation for MALDI MS analysis

Recovered peptides were desalted prior to MALDI analysis by home-made nano-columns consisting of 200 nL of POROS R2 + R3 material (PerSeptive Biosystems, Framingham, MA) as described by Gobom, Nordhoff, Mirgorodskaya, Ekman, & Roepstorff (1999) with some modifications. Columns were equilibrated with 0.1 % TFA and the bound peptides subsequently eluted directly onto the MALDI target with 0.5 µL CHCA solution (20 µg/µL in ACN, 0.1 % TFA, 70:30, vol/vol).

2.6 Mass Spectrometric analysis

Peptide mass fingerprinting and MS/MS analysis were performed on a Bruker Autoflex III TOF/TOF smartbeam mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with an ion LIFT selector and reflector. Positively charged ions were analyzed in reflector mode, using delayed extraction. The ionization is performed by solid-state laser with a 360 nm wavelength in pulses of 200 Hz. Resolution remains always above 7500, throughout the mass window. Routinely, 1400 scans were collected for MALDI-TOF PMF analysis. When feasible, best candidates were chosen in manual fashion for the corresponding LIFT MS/MS PFF analysis (400 MS scans for selection of parental ion, 1600 MS/MS scans for fragments). Spectra were first externally calibrated resulting in a mass accuracy of <50 ppm. Afterwards, internal calibration was performed, reaching an accuracy of <20 ppm. Protein identification was performed by searching NCBInr database (version 09/2009; 10490613 sequences; 3896452119 residues) and using Mascot 2.1 search engine (Matrix Science, Boston, MA). The following parameters were used for database searches: up to two missed cleavages; allowed modifications:

199 carbamidomethylation of cysteine (fixed) and oxidation of methionine (variable). For
200 peptide mass fingerprinting, 0.7 Da of tolerance was allowed for fragment-ion masses
201 obtained by tandem mass spectrometry. In all searches performed, protein scores greater
202 than 63 are considered statistically significant ($p<0.05$).

203 PMFs and the corresponding tables describing the characterized peptides of all
204 proteins identified in the analyzed by-products are shown in the Supporting Information.

3. RESULTS

3.1 Proteomic identification of by-products from canned tuna

Following the ammonium sulphate precipitation procedure described in Materials and Methods section, different electrophoretic patterns (**Figure 1**, lanes 2 and 3), as well as a strong and highly resolved band of M_r ~36 kDa (**Figure 1**, lane 4) were obtained. Despite the high heterogeneity of the starting material which includes tuna bones, skin, muscle or internal organs, five major electrophoretic bands were visualized after Coomassie staining. These five bands were observed at 1D SDS-PAGE within a molecular weight of 6 and 200 kDa and were cut off for further identification. Three proteins were successfully identified by combination of PMF and PFF analyses (**Table 1** and Supporting Information).

Band no. 3 was identified as ubiquitin, a small protein of 74 amino acids abundantly present in all eukaryotes. It is highly conserved among eukaryotic species and this protein differs only in three residues in yeast and animals (Sharp & Li, 1987). Habu, Ohishi, Mihara & Yanaihara (1994) indicated that the amino acid sequence of tuna ubiquitin is identical to that of mammalian counterparts. The most prominent function of ubiquitin is labeling proteins for proteasomal degradation. Many kinds of stress appear to stimulate ubiquitin expression (Okubo et al., 2002), including heat shock in hamster (Fornace, Alamo, Hollander & Lamoreaux, 1989), chicken (Bond & Schlesinger, 1985) and yeast (Cheng, Watt & Piper, 1994), as well as starvation and respiration stress also reported in yeast (Cheng et al., 1994). Thus, the identification of ubiquitin in by-products

from canning tuna is not surprising considering the large number of factors can influence the up-regulation of ubiquitin genes.

Analysis of band no. 4, a predominant band present in the solution derived from the second step of the fractionation method (**Figure 1**, lane 3), revealed the unambiguous presence of the β-subunit of hemoglobin (**Table 1**). Furthermore, an additional fractionation step led to the apparent purification of a single and well-resolved band of M_r ~36 kDa (band no. 5, **Figure 1**, lane 4). Proteomic analysis (PMF + PFF) confirmed the presence of only one protein identified as tropomyosin of bluefin tuna from fast skeletal muscle (**Table 1** and Supporting Information). Huang, Ochiai & Watabe (2004) reported that tropomyosin is a mixture of nearly equimolar amounts of two isoforms (designated α and β) in the fast skeletal muscle of bluefin tuna, although only the sequence of the α-type is known so far. These results indicate that the precipitation method reported in this work is useful for obtaining tropomyosin with a high degree of purity from by-products of tuna processed for canning.

Regarding bands no. 1 and 2 with M_r ranging between 100 and 200 kDa (**Figure 1**, lane 2), no positive identification was accomplished by combination of PMF and LIFT analyses.

3.2 Proteomic identification of by-products from skeletal red muscle of fresh tuna

After fractionation steps, two well-differentiated 1D SDS-PAGE patterns, presumably corresponding to the sarcoplasmic and myofibrillar fractions were obtained (**Figure 2**). Up to 12 bands with M_r ranging between 14 and 116 kDa from the

sarcoplasmic fraction and 8 bands with M_r between 14 and 200 kDa from the myofibrillar fraction were excised, tryptic digested and analyzed by MALDI-TOF/TOF (Supporting Information). These bands were selected according to their abundance in both fractions.

3.2.1 Myofibrillar fraction

The most common myofibrillar proteins in the muscle of aquatic animals are myosin, actin, tropomyosin, and troponins C, I and T representing around 40-60%, 15-30%, 4-6% and 4-6% of the total myofibrillar protein of muscle, respectively, depending upon species (Mackie, 1997). Seven proteins corresponding to the myofibrillar fraction could be identified (**Table 2**). Five bands matched proteins involved in muscle contraction such as myosin (heavy and light chains), actin or tropomyosin. As an example, **Figure 3** shows the sequence coverage map of the combined PMF and five MALDI LIFT-TOF/TOF spectra corresponding to the identification of tropomyosin (**Figure 2**, lane 3, band 5). The peptides released at higher intensities from this band were identified as fragments (by decreasing order of abundance): ^{168}K-R^{178} (*m/z* 1284.8), ^{92}I-R^{101} (*m/z* 1243.7), ^{169}L-R^{178} (*m/z* 1156.7), ^{91}R-R^{101} (*m/z* 1399.8), ^{161}K-R^{167} (*m/z* 894.5), ^{252}T-K^{264} (*m/z* 1552.8) and ^{92}I-R^{105} (*m/z* 1727.9) (**Figure 3A** and Supporting Information). These peptides were obtained as a result of tryptic cleavages occurring at the carboxyl side of basic residues (R and K). Thus, three peptides spanning the region ^{161}K-R^{178} were found as consequence of the tryptic cleavages at bonds ^{160}R-K^{161}, ^{167}R-K^{168} and ^{168}K-L^{169}. Another set of three main peptides was derived from tryptic cleavages at bonds ^{90}R-R^{91}, ^{101}R-A^{102}, ^{91}R-I^{92} and ^{105}R-L^{106}. Finally, peptide ^{252}T-K^{264} was obtained

as a result of tryptic cleavages at bonds ^{251}K-T^{252} and ^{264}K-L^{265} (**Figure 3A**). Five of these peptides, i.e. ^{91}R-R^{101}, ^{92}I-R^{101}, ^{161}K-R^{167}, ^{168}K-R^{178} and ^{169}L-R^{178}, were analyzed by MALDI-TOF/TOF. The information of the peptide mass fingerprinting (PMF), together with that of the peptide fragment fingerprinting (PFF) obtained from the five selected peptides, allowed the unambiguous protein identification. The information derived from the tandem mass spectrometry analysis of the highest scored peptide (i.e., ^{92}I-R^{101}) for this protein is shown in **Figure 3B**. The detected y_n-type ions owing to the free carboxyl-terminus, as well as to the b_n-type ions owing to the free amino terminus, were detected and they are marked in red (**Figure 3B**).

In band no. 4 (**Figure 2**, lane 3), two different proteins were identified by combining PMF and PFF, i.e. the enzyme muscle-type creatine kinase CKM1 together with skeletal α-actin. Creatine kinase is known to catalyze, through the consumption of adenosine triphosphate, the conversion of creatine to phosphocreatine, an organic compound capable of storing and providing energy for muscular contraction, whilst skeletal α-actin belongs to the actin family of proteins which are highly conserved proteins involved in various types of cell motility and ubiquitously expressed in all eukaryotic cells. Five MS/MS spectra were acquired from peptides generated from band no. 4. Three of them matched to creatine kinase CKM1: *m/z* 1125.6 (GFTLPPHNSR), *m/z* 1657.9 (TFLVWVNEEDHLR) and *m/z* 1995.0 (GTGGVDTASVGGVFDISNADR), and two matched to α-actin *m/z* 1198.7 (AVFPSIVGRPR) and *m/z* 1790.9 (SYELPDGQVITIGNER).

3.2.2 Sarcoplasmic fraction

297

298 Eight proteins corresponding to 9 bands were identified in the sarcoplasmic
299 fraction (**Figure 2,** lane 2 and **Table 2**). Seven of these bands matched proteins known to
300 be involved in energy metabolism, mainly enzymes of carbohydrate metabolism.
301 Glycolytic enzymes are responsible for quality deterioration of fish after death and before
302 bacterial spoilage (Hui et al., 2006), and they have been proposed as markers for post-
303 mortem changes during storage of fishes such as cod (Gebriel, Uleberg, Larssen,
304 Bjornstad, Sivertsvik & Moller, 2010). Post-mortem biochemical and structural changes
305 in proteins are directly related to sensorial properties and water-holding capacity (Huff-
306 Lonergan & Lonergan, 2005).

307 Glycogen phosphorylase A (band no. 9) participates in the breakdown of
308 glycogen, catalyzing the phosphorolytic cleavage of the α(1-4) glycosidic linkages,
309 releasing glucose-1-phosphate as the reaction product. This enzyme has been reported to
310 be predominant in skeletal muscle of mammalians, among other tissues, in response to its
311 greater demand for specific glycogenolytic control. Furthermore, four enzymes directly
312 involved in glycolysis, and thus serving to breakdown glucose for energy and carbon
313 molecules, were also identified: triose-phosphate isomerase (band no. 19),
314 glyceraldehyde 3-phosphate dehydrogenase (band no. 17), β-enolase (band no. 13) and
315 pyruvate kinase (bands no. 10 and 11). Interestingly, in addition to a long established
316 metabolic function, glyceraldehyde 3-phosphate dehydrogenase enzyme has also been
317 implicated in several non-metabolic processes, such as binding to actin and tropomyosin,
318 and thus, it might have a role in cytoskeleton assembly (Dugaiczyk, Haron, Stone,
319 Dennison, Rothblum & Schwartz, 1983). Thus, interaction of glycolytic enzymes with

actin has been suggested to be a mechanism for compartmentation of the glycolytic pathway (Waingeh, Gustafson, Kozliak, Lowe, Knull & Thomasson, 2006). Additionally, enolase has also been reported as an early marker of human myogenesis (Fougerousse et al., 2001), as well as it has been identified as an early oxidative protein in bonito muscle (Kinoshita, Sato, Naitou, Ohashi, & Kumazawa, 2007). The identified β-isoform is the predominant (~90%) form of enolase in skeletal muscle (Foucault, Vacer, Merkulova, Keller & Abrio-Dupont, 1999). Lastly, the identification of pyruvate kinase in two different bands (no. 10 and 11) of apparently similar electrophoretical mobility might be indicative of the presence of at least two post-translationally modified forms, and/or as consequence of a partial degradation.

Isocitrate dehydrogenase, an enzyme that participates in the Krebs cycle catalyzing the oxidative decarboxylation of isocitrate to produce α-ketoglutarate and CO_2 while converting NAD^+ to NADH, was detected in the band no. 12. Finally, the enzyme muscle-type creatine kinase, previously matched within the myofibrillar fraction (band no. 4 in **Figure 2**, lane 3), was also identified in the sarcoplamisc fraction as the isomer CKM2 (band no. 14 in **Figure 2**, lane 2). The fact that the tuna red muscle could have at least two isoforms of creatine kinase is consistent with the reported presence of multiple forms of this enzyme in muscle tissue of other fish species, such as carp (Sun, Hui & Wu, 1998), antarctic icefish (Winnard, Cashon, Sidell & Vayda, 2003) or zebrafish (Bosworth, Chou, Cole & Rees, 2005).

Finally, the well-defined and separated band no. 20 was identified by PMF and PFF analyses as myoglobin, a protein abundantly found in the skeletal red muscle, from different tuna species including *Thunnus alalunga* (Albacore), *T. albacares* (Yellowfin),

T. thynnus orientalis (North Pacific bluefin), *T. thynnus* (Bluefin) and *T. obesus* (Bigeye) (**Figure 4**). However, the highest similarity score "Mowse score" corresponded to myoglobin from *T. alalunga* with a high sequence coverage of 71% that includes the distinctive peptide EHPDTQK derived from the hydrolytic action of trypsin on bonds ^{31}K-E^{32} and ^{38}K-L^{39} (*m/z* 854.3, **Figure 4A**). Likewise, additional peptides released at high intensities were identified as fragments ^{115}A-R^{126} (*m/z* 1129.6), ^{10}C-R^{28} (*m/z* 2108.1), ^{43}F-K^{66} (*m/z* 2267.3), ^{127}N-K^{141} (*m/z* 1663.9) and ^{93}H-K^{101} (*m/z* 1110.6) (Supporting Information). All these abundant peptides were also obtained as a result of tryptic cleavages occurring at the carboxyl side of R and K residues without missed cleavages, with the exception of peptide ^{93}H-K^{101} which contained one missed cleavage (Figure 4A). Furthermore, the C residue at peptide ^{10}C-R^{28} was carbamidomethylated following the treatment with iodoacetamide. Lastly, MS^2 spectra from peptides ^{115}A-R^{126} and ^{10}C-R^{28} (Figure 4B) were dominated by the sequence informative y-ions resulting from cleavages on the carboxy-terminal side of peptide.

4. DISCUSSION

MALDI has historically been considered a "soft" ionization technique that produces almost exclusively intact protonated pseudomolecular ion species. Later studies have shown that a significant degree of metastable ion decay occurs after ion acceleration and prior to detection. The ion fragments produced from the metastable ion decay of peptides and proteins typically include both neutral molecule losses (such as water, ammonia and portions of the amino acid side chains) and random cleavage at peptide

bonds. The observance of these metastable ion decay products in MALDI mass spectra is dependent on the TOF instrumental configuration. As it is indicated in Materials and Methods section, our MALDI TOF spectrometer is equipped with a LIFT pusher/filter that enhance post source decay (PSD) observance, simulating a real TOF/TOF device (Suckau et al., 2003). This is particularly important for protein identification in *Thunnus alalunga* (Albacore) whose genome is not sequenced and, consequently, it is essential to obtain protein sequences by tandem mass spectrometry allowing their identification by matching with homologue proteins from different related fish species.

Knowledge about quality and composition of fish by-products is essential to obtain a profitable utilization. Results presented in this work show the usefulness of proteomic techniques like peptide mass fingerprinting and peptide fragment fingerprinting performed by MALDI-TOF/TOF to identify proteins extracted from different tuna by-products, although *T. alalunga* is an organism without a sequenced genome. Such information is important for future studies investigating the evaluation of their potential applications.

If the recovered protein material is intended to be used for human food products, the knowledge of the nutritional value of these proteins will be essential. It is well-known that tuna species such as *T. alalunga* has proteins of high nutritional value (Castrillón, Navarro & García-Arias, 1996). Regarding essential aminoacids, previous works reported that the composition of proteins from tuna source was of very high quality (Pigott & Tucker, 1990).

From a technological point of view, fish myofibrillar proteins have shown excellent functional properties as food ingredients, such as gel-forming ability,

389 emulsifying properties, and water-holding ability, which are related to their solubilization
390 in salt solutions (Saeki & Inoue, 1997). Surimi is a good example of a product created
391 with myofibrillar proteins isolated from previously undervalued fish parts. Likewise, a
392 gel-enhancing effect as well as good water holding and oil binding capacities have been
393 recently reported for sarcoplasmic fish proteins indicating their potential use as a
394 promising food ingredient with good functional properties (Jafarpour & Gorczyca, 2009;
395 Yongsawatdigul & Hemung, 2010).

396 The analyzed by-products could also be suitable sources of bioactive peptides
397 according to the identified proteins. Recently, Je, Qian, Lee, Byun & Kim (2008) and Hsu
398 (2010) reported that protein hydrolysates of tuna dark muscle by-product derived from
399 canned processing possessed strong *in vitro* antioxidative activity. Likewise, peptides
400 possessing ACE (Angiotensin Converting Enzyme) inhibitory properties and, hence, with
401 potential beneficial effects in the treatment of hypertension, derived from muscle proteins
402 of fish species such as bonito or tuna have been previously identified. Thus,
403 glyceraldehyde 3-phosphate dehydrogenase from tuna muscle, protein identified in the
404 band no. 17 of the sarcoplasmic fraction isolated from by-products of red muscle of fresh
405 tuna (**Figure 2**, lane 2, **Table 2**), has an octapeptide (PTHIKWGD) with a potent ACE
406 inhibitory activity (Kohama, Matsumoto, Oka, Teramoto, Okabe & Mimura, 1988). This
407 specific peptide was one of the first identified ACE inhibitory peptides in marine
408 products.

409 Later on, protease digests of bonito muscle had ACE-inhibitory activity in
410 addition to a good taste (Yokoyama, Chiba & Yoshikawa, 1992). These authors
411 demonstrated that the most potent ACE-inhibitory peptides were derived from actin,

412 which has been identified in the band no. 4 of the myofibrillar fraction isolated from by-
413 products of red muscle of fresh tuna (**Figure 2**, lane 3, **Table 2**). In fact, this mixture of
414 peptides derived from bonito actin has been proved to exert not only *in vitro* ACE
415 inhibitory activity but also *in vivo* antihypertensive activity after oral administration in
416 spontaneously hypertensive rats (Fujita, Yokoyama, Yasumoto & Yoshikawa, 1995) and
417 borderline hypertensive humans (Fujita, Yasumoto, Hasegawa & Ohshima, 1997).
418 Consequently, it is commercially available following official approval as a "Food for
419 Specified Health Use" in Japan (Fujita, Yokoyama & Yoshikawa, 2000).

420 Likewise, one peptide (DMIPAQK) derived from muscle-type creatine kinase
421 (Yokoyama et al., 1992), protein identified in this work in both myofibrillar and
422 sarcoplasmic fractions from red muscle, and another heptapeptide (SVAKLEK) derived
423 from tropomyosin (Yamamoto, Ejiri & Mizuno, 2003), which was also identified in by-
424 products from canned tuna in addition to by-products from red muscle, also showed to
425 posses ACE-inhibitory activity.

426 On the other hand, the genus *Thunnus* comprises many species that differ in
427 commercial value depending on their organoleptic features and, consequently, these
428 species are often subjected to fraudulent substitution (Pepe, Ceruso, Carpentieri,
429 Ventrone, Amoresano & Anastaio, 2010). The proteomic identification of myoglobin in
430 the sarcoplasmic fraction revealed the presence of an *alalunga*-specific peptide
431 (EHPDTQK) that might be used as a specific marker. This fact supports the potential use
432 of proteomic tools for species identification of fish.

433

434 **5. CONCLUSION**

Procedures described in this work allowed the isolation and characterization of proteins from processing tuna by-products that could be potentially used in a wide number of human food applications. Although further studies are needed to evaluate specific bioactivities, these findings could contribute to expand the use of tuna processing by-products to the development of new commercial applications for the human health promotion.

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601

FIGURE CAPTIONS.

Figure 1. SDS-PAGE analyses under reducing conditions showing fractionation of proteins from canned tuna by-products. Lane 1: Marker proteins. Lane 2: sample 1- soluble proteins at 0.16 M KCl 20 mM Tris-HCl (pH 7.5). Lane 3: sample 2 - soluble proteins at 0.5 M KCl, pellet after 40% ammonium sulphate saturation. Lane: 4: sample 3 - soluble proteins at 0.5 M KCl, pellet after 55% ammonium sulphate saturation. Labelled bands are described in the text and Table 1.

Figure 2. SDS-PAGE analyses under reducing conditions showing fractionation of proteins from fresh red muscle tuna by-products. Lane 1: Marker proteins. Lane 2: Soluble proteins at 0.1 M sodium phosphate buffer (pH 7) corresponding to the sarcoplasmic fraction. Lane 3: Soluble proteins at 0.1 M sodium phosphate buffer, 0.5 M NaCl (pH 7) corresponding to the myofibrillar fraction. Labelled bands are described in the text and Table 2.

Figure 3. A) MASCOT results obtained for band no. 5 (identified as α-tropomyosin) corresponding to the myofibrillar fraction extracted from fresh tuna by-products. Above is represented the sequence coverage map obtained by PMF. Matched peptides are represented in the table below including: start and end positions of the peptide sequence starting from the amino acid terminal of the whole protein, the observed *m/z*, transformed to its experimental mass (Mr(expt)), the calculated mass (Mr(calc)) from the matched peptide sequence, as well as their mass difference (in ppm), the number of missed

cleavage sites for trypsin (Miss), and the peptide sequence. **B)** List of fragmented ions obtained (in red) vs. total predicted ions for MS/MS fragmentation of peptide at 2107.0408 Th, which achieve extensive statistical significance (MASCOT score of 137).

Figure 4. A) MASCOT results obtained for band no. 20 (identified as myoglobin) corresponding to the sarcoplasmic fraction extracted from fresh tuna by-products. Above is represented the sequence coverage map obtained by PMF. Matched peptides are represented in the table below including: start and end positions of the peptide sequence starting from the amino acid terminal of the whole protein, the observed *m/z*, transformed to its experimental mass (Mr(expt)), the calculated mass (Mr(calc)) from the matched peptide sequence, as well as their mass difference (in ppm), the number of missed cleavage sites for trypsin (Miss), and the peptide sequence. **B)** List of fragmented ions obtained (in red) vs. total predicted ions for MS/MS fragmentation of peptide at 1242.6456 Th.which achieve extensive statistical significance (MASCOT score of 56).

Table 1. Identified proteins of by-products from canned tuna after in-gel trypsin digestion under reducing conditions and combined PMF and PFF analyses.

SDS-PAGE band[a]	Identified protein (NCBI database entry)	Theoretical mass (kDa)	Number of matched peptides / total observed peptides	% Covered sequence	Mowse score	Sequenced peptides by MS/MS (ion score)	Actinopterygii Genera (common name)
3	Ubiquitin (GI:223646272)	8.6	6 / 11	52.7	177	IQDKEGIPPDQQR (68)	*Salmo salar* (Atlantic salmon)
4	β-Hemoglobin (GI:122701)	16.2	3 / 12	16.4	132	CLIVYPWTQR (88)	*Thunnus thynnus* (Bluefin tuna)
5	Tropomyosin (GI:38175083)	32.7	16 / 23	40.0	304	IQLVEEELDRAQER (105)	*Thunnus thynnus* (Bluefin tuna)

[a] Bands corresponding to Figure 1.

Table 2. Identified proteins corresponding to the myofibrillar and sarcoplasmic fractions isolated from by-products of skeletal red muscle of fresh tuna after in-gel trypsin digestion under reducing conditions and combined PMF and PFF analyses.

Protein Fraction	SDS-PAGE band[a]	Identified protein (NCBI database entry)	Theoretical mass (kDa)	Number of matched peptides / total observed peptides	% Covered sequence	Mowse score	Sequenced peptides by MS/MS (ion score)	Actinopterygii Genera (common name)
Myofibrillar	1	Ventricular myosin heavy chain (GI:82175142)	223.3	20 / 50	12	139	ILNPAAIPEGQFIDSR (46) STHPHFVR (25)	*Danio rerio* (Zebra fish)
	2	Myosin, heavy polypeptide 1.1, skeletal muscle (GI:288856329)	222.5	13 / 34	8	78		*Danio rerio* (Zebra fish)
	3	LOC554876 protein (GI:113674097)	24.8	7 / 47	23	76		*Danio rerio* (Zebra fish)
	4	Muscle-type	43.3	12/47	30	236	GTGGVDTASVGGVFDISNADR	*Oreochromis*

		creatine kinase CKM1 (GI:21694041)					(70) GFTLPPHNSR (40) TFLVWVNEEDHLR (29)	*mossambicus* (Tilapia mossambica)
	4	skeletal α-actin (GI:6653228)	42.2	12/47	40	231	SYELPDGQVITIGNER (98) AVFPSIVGRPR (30)	*Sparus aurata* (Sea bream)
	5	Tropomyosin (GI:38175083)	32.8	14 / 51	34	270	IQLVEEELDR (56) KLVIIEGDLER (46) KYEEVAR (20) RIQLVEEELDR (20) LVIIEGDLER (18)	*Thunnus thynnus* (Bluefin tuna)
	7	Fast skeletal myosin Light chain 3 (GI:68132180)	16.2	5 / 18	26	65		*Misgurnus fossilis* (Weatherfish)
Sarcoplasmic	9	Phosphorylase, glycogen (muscle) A (GI:66472494)	97.4	15 / 51	21	126		*Danio rerio* (Zebra fish)

	10	Pyruvate kinase (GI:197632483)	58.9	9 / 47	20	65		*Salmo salar* (Atlantic salmon)
	11	Pyruvate kinase (GI:197632483)	58.9	11 / 51	19	96		*Salmo salar* (Atlantic salmon)
	12	Isocitrate dehydrogenase 2 (NADP+), mitochondrial (GI:41054651)	50.9	9 / 49	21	72		*Danio rerio* (Zebra fish)
	13	Enolase 3 (β muscle) (GI:68086449)	47.8	9 / 51	27	79		*Danio rerio* (Zebra fish)
	14	Muscle-type creatine kinase CKM2 (GI:21694043)	43.0	15 / 49	32	156		*Oreochromis mossambicus* (Tilapia

								mossambica)
	17	Glyceraldehyde 3-phosphate dehydrogenase (GI:119943230)	36.0	6 / 51	24	63		*Misgurnus anguillicaudatus* (Weather Loach)
	19	Triose phosphate isomerase B (GI:126211567)	26.9	7 / 51	27	70		*Poecilia reticulata* (Guppy)
	20	Myoglobin (GI: 9930588)	15.7	10 / 51	71	301	CWGPVEADYTTIGGLVLTR (137) AGLDAGGQTALR (76)	*Thunnus alalunga* (Albacore tuna)

[a] Bands corresponding to Figure 2.

Figure 1. *Sanmartín et al.*

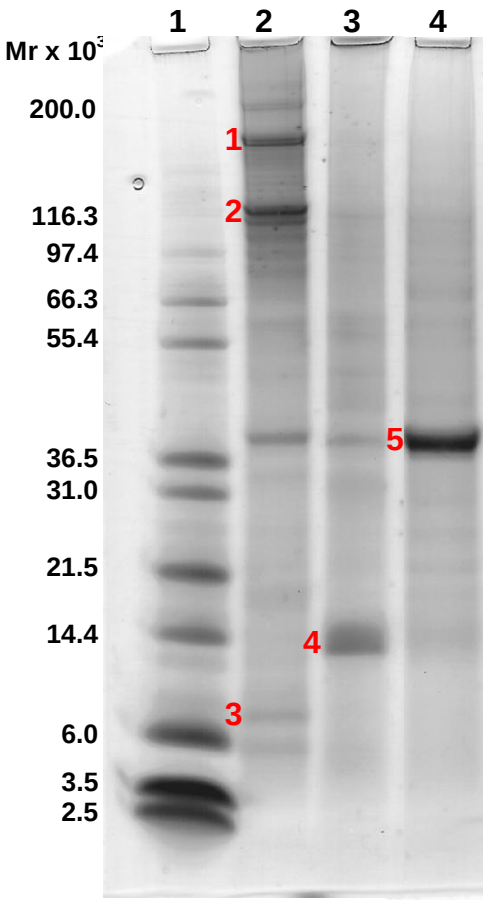

Figure 2. *Sanmartín et al.*

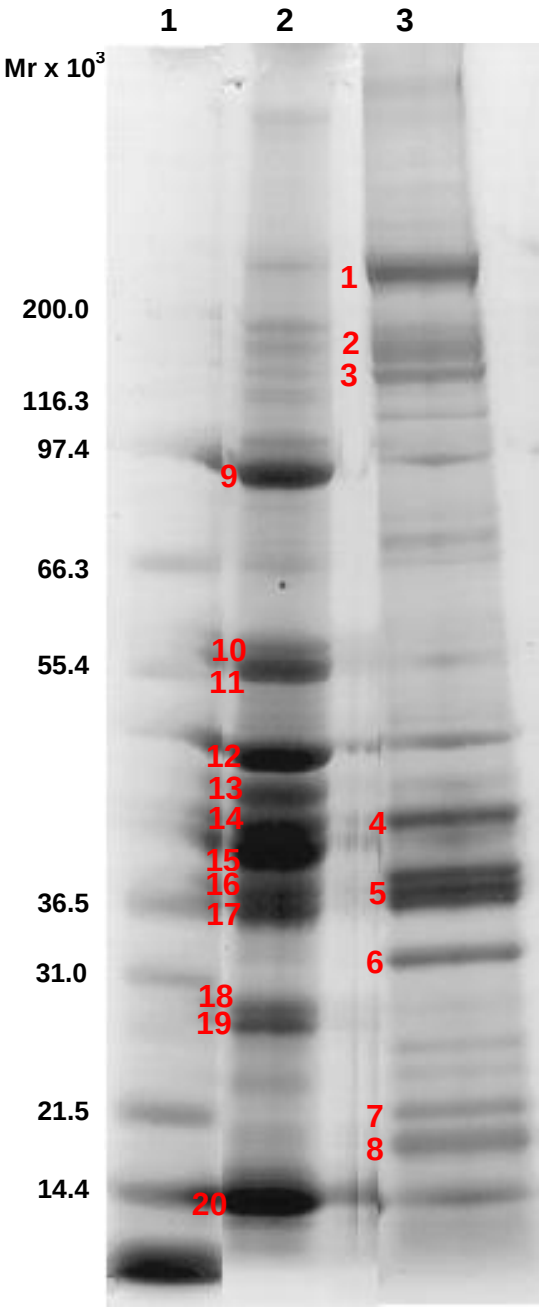

Figure 3. *Sanmartín et al.*

A)

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  1 MDAIKKKMQM LKLDKENALD RAEQAESDKK ASEERSKQLE DDLVGLQKKL
 51 KGTEDELDKY SEALKDAQEK LELAEKKATD AEGEVASLNR RIQLVEEELD
101 RAQERLATAL TKLEEAEKAA DESERGMKVI ENRAMKDEEK MELQEIQLKE
151 AKHIAEEADR KYEEVARKLV IIEGDLERTE ERAELSEGKC SELEEELKTV
201 TNNLKSLEAQ AEKYSQKEDK YEEEIKVLTD KLKEAETRAE FAERSVAKLE
251 KTIDDLEDEL YAQKLKYKAI SEELDHALND MTSI
```

Matched peptides shown in **Bold Red**

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
13 - 21	1073.5678	1072.5606	1072.5513	9	1	K.LDKENALDR.A	
36 - 48	1472.7723	1471.7650	1471.7882	-16	1	R.SKQLEDDLVGLQK.K	
38 - 48	1257.6832	1256.6760	1256.6612	12	0	K.QLEDDLVGLQK.K	
77 - 90	1460.7561	1459.7489	1459.7267	15	1	K.KATDAEGEVASLNR.R	
78 - 90	1332.6736	1331.6663	1331.6317	26	0	K.ATDAEGEVASLNR.R	
91 - 101	1399.7773	1398.7700	1398.7467	17	1	R.RIQLVEEELDR.A	(Ions score 20)
92 - 101	1243.6707	1242.6635	1242.6456	14	0	R.IQLVEEELDR.A	(Ions score 56)
92 - 105	1727.9035	1726.8963	1726.8849	7	1	R.IQLVEEELDRAQER.L	
153 - 160	940.4485	939.4413	939.4410	0	0	K.HIAEEADR.K	
161 - 167	894.4632	893.4560	893.4606	-5	1	R.KYEEVAR.K	(Ions score 20)
168 - 178	1284.7732	1283.7659	1283.7449	16	1	R.KLVIIEGDLER.T	(Ions score 46)
169 - 178	1156.6689	1155.6616	1155.6499	10	0	K.LVIIEGDLER.T	(Ions score 18)
206 - 213	875.4231	874.4159	874.4396	-27	0	K.SLEAQAEK.Y	
252 - 264	1552.7724	1551.7652	1551.7304	22	0	K.TIDDLEDELYAQK.L	

B)

Monoisotopic mass of neutral peptide Mr(calc): 1242.6456

MS/MS Fragmentation of IQLVEEELDR
Found in gi|38175083, tropomyosin [Thunnus thynnus]

Ions Score: 56 Individual ions scores > 51 indicate identity or extensive homology (p<0.05).

#	Immon.	b	y	Seq.
1	86.0964	114.0913		I
2	101.0709	**242.1499**	1130.5688	Q
3	86.0964	**355.2340**	***1002.5102***	L
4	72.0808	**454.3024**	***889.4262***	V
5	102.0550	**583.3450**	***790.3577***	E
6	102.0550	712.3876	***661.3151***	E
7	102.0550	841.4302	***532.2726***	E
8	86.0964	954.5142	***403.2300***	L
9	88.0393	1069.5412	***290.1459***	D
10	129.1135		***175.1190***	R

Figure 4. *Sanmartín et al.*

A)

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  1 MADFDAVLKC WGPVEADYTT IGGLVLTRLF KEHPDTQKLF PKFAGIAQAD
 51 LAGNAAISAH GATVLKKLGE LLKAKGSHAS ILKPMANSHA TKHKIPINNF
101 KLISEVLVKV MQEKAGLDAG GQTALRNVMG IIIADLEANY KELGFTG
```

Matched peptides shown in **Bold Red**

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
10 - 28	2108.0934	2107.0861	2107.0408	21	0	K.CWGPVEADYTTIGGLVLTR.L	(Ions score 137)
32 - 38	854.3234	853.3161	853.3930	-90	0	K.EHPDTQK.L	
43 - 66	2267.2976	2266.2904	2266.2070	37	0	K.FAGIAQADLAGNAAISAHGATVLK.K	
43 - 67	2395.4254	2394.4182	2394.3019	49	1	K.FAGIAQADLAGNAAISAHGATVLKK.L	
76 - 92	1749.9171	1748.9098	1748.8992	6	0	K.GSHASILKPMANSHATK.H	
76 - 92	1765.8901	1764.8829	1764.8941	-6	0	K.GSHASILKPMANSHATK.H	Oxidation (M)
93 - 101	1110.6326	1109.6253	1109.6345	-8	1	K.HKIPINNFK.L	
115 - 126	1129.5968	1128.5895	1128.5887	1	0	K.AGLDAGGQTALR.N	(Ions score 76)
127 - 141	1663.8744	1662.8671	1662.8651	1	0	R.NVMGIIIADLEANYK.E	
127 - 141	1679.8795	1678.8723	1678.8600	7	0	R.NVMGIIIADLEANYK.E	Oxidation (M)

B)

Monoisotopic mass of neutral peptide Mr(calc): 2107.0408

MS/MS Fragmentation of CWGPVEADYTTIGGLVLTR
Found in gi|118595817, Myoglobin

Ions Score: 137 Individual ions scores > 51 indicate identity or extensive homology (p<0.05).

#	Immon.	b	y	Seq.
1	133.0430	161.0379		C
2	159.0917	347.1172	1948.0175	W

3	30.0338	**404.1387**	***1761.9381***	**G**
4	70.0651	501.1915	***1704.9167***	**P**
5	72.0808	**600.2599**	1607.8639	**V**
6	102.0550	729.3025	1508.7955	**E**
7	44.0495	800.3396	***1379.7529***	**A**
8	88.0393	915.3665	***1308.7158***	**D**
9	136.0757	1078.4299	***1193.6889***	**Y**
10	74.0600	**1179.4775**	***1030.6255***	**T**
11	74.0600	1280.5252	***929.5778***	**T**
12	86.0964	1393.6093	***828.5302***	**I**
13	30.0338	1450.6307	***715.4461***	**G**
14	30.0338	1507.6522	***658.4246***	**G**
15	86.0964	1620.7363	***601.4032***	**L**
16	72.0808	1719.8047	***488.3191***	**V**
17	86.0964	1832.8888	***389.2507***	**L**
18	74.0600	1933.9364	***276.1666***	**T**
19	129.1135		***175.1190***	**R**