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Characterisation of Soluble Nitrogen and Muscle Proteins in Wastewater Throughout the Salting Process of Codfish (*Gadus morhua*)

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In Portugal, Atlantic codfish (*Gadus morhua*) is usually consumed after dry salting; this process is carried out by mixing deboned codfish with food–grade marine salt followed by stacking in a tank for 6 days. Along the salting process, codfish incorporates salt as well as it is partially dried by the release of water - up to 22 % (w/w). Currently, this wastewater is treated as a residue not being further valorized. However, the presence of a significant amount of valuable compounds in this rest, such as amino acids and proteins, may allow for valorization opportunities not yet explored. The present work focuses on the identification of the nitrogen-containing compounds present in such wastewater. Total nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCASN) and phosphotungstic acid-soluble nitrogen (PTASN) were evaluated by the micro-Kjeldahl method; Biuret method was used for total protein determinations and SDS-Page was performed for protein molecular weight screening. The results revealed an increase of WSN, TCASN and PTASN with time, with corresponding values of 3.17 g/L (WSN), 1.62 g/L (TCASN) and 1.16 g/L (PTASN) by the end of the process; the evolution of WSN versus the released water was approximately constant during the salting process, as well as the ratios of TCASN/WSN and PTASN/WSN with values of 51.25 and 36.55 % (w/w) at equilibrium, respectively.

1. Introduction

Salt-cured codfish continues to be widely produced due to low operating costs, simplicity of processing and highly appreciated sensory characteristics (Martínez-Alvarez and Gómez-Guillén, 2006). During the salting process codfish takes salt up to ca. 20 %(w/w) and drains concomitantly ca. 22 %(w/w) of physiological water — inducing important changes in the composition and structure of the muscle tissue (Andrés et al., 2005). Several major effects are imparted by salt such as modifications in muscle protein conformation, changes in water–holding capacity (with subsequent protein denaturation), and losses of nutrients (e.g. peptides and amino acids). The water

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released by codfish throughout the heavy salting is currently treated as a waste due to the high content of salt and organic load; however, it carries important compounds, which, although not regarded as essential nutrients, can be considered beneficial under certain circumstances; free amino acids and muscle proteins – myosin and actin – are examples of such active compounds (Larsen et al., 2007; Sikorski et al., 1990).
Free amino acids released by codfish muscle and skin can be used in food supplements or sport drinks (Sullivan et al., 2000) as well as in parental solution (Chiarla and Giovannini, 2004; Lourenço and Camilo, 2002).
Myosin and actin are two proteins currently applied for food rheology improvement, due to their emulsifying properties and to their ability to develop structuring gels (Venugopal, 2009). The aim of this work was thus to characterise the water drained away during the codfish salting process, in terms of muscle proteins and total nitrogen (WSN) along with its relative fractions TCASN – small peptides and free amino acids, and PTASN – free amino acids, respectively.

2. Materials and Methods

2.1 Wastewater sampling

Wastewater samples were supplied by *Pascoal & Filhos, S.A.* (Aveiro, Portugal). Atlantic codfish (*Gadus morhua*) was caught in the Northern Sea, at a depth of 20 m and 200 m away from the Southern coast of Norway; it was immediately gutted and beheaded, and kept cold on board (at −20 ºC), until inshore uploading. Upon arrival, codfish was thawed, deboned and washed; then it is "butterfly-split" and salted by dry (or "kench") salting, i.e. mixed with alternating layers of dry and alkaline food–grade marine salt (pH 8.5), and stacked in a tank for six days at ca. 17 ºC. Throughout salting treatment, each wastewater sample was collected every day at the same time, in duplicate and from the same tank, and frozen at −20 ºC until analysis. The volume of wastewater drained away from the codfish to the tank was also measured every day at the same time.

2.2 Assessment of nitrogen fractions

The nitrogen content was quantified using a Kjeltec system 1002 distilling unit (Tecator; Höganäs, Sweden). Total nitrogen was quantified by micro-Kjeldahl (ISO R-973:1978) and expressed as water-soluble nitrogen (WSN). Trichloroacetic acid-soluble nitrogen (TCASN), accounted for small peptides (2-20 residues) and free amino acids, and phosphotungstic acid-soluble nitrogen (PTASN), accounted for free amino acids, were determined as well. The TCASN fraction was quantified by adding 5 mL of an aqueous solution of 48 %(w/v) TCA (Merck) to 15 mL of the water-soluble extract; the mixture was allowed to stand for 30 min at room temperature, and then filtered through a Whatman No. 42 filter paper (Millipore-Interface). The PTASN fraction was determined by adding 7 mL of 3.95 M sulfuric acid (Pronalab; Lisbon; Portugal) and 3 mL of 33.3 %(w/v) PTA (Merck) to 10 mL of water-soluble extract. The mixture was then allowed to stand overnight at 4 °C, and subsequently filtered through Whatman filter paper.

2.3 Assessment of proteins

The concentration of water–soluble proteins was determined by the Biuret method (Copeland, 1994; Chance and Redfearn, 1961), using an UV mini 1240 spectrophotometer (Shimatzu; Carnaxide, Portugal). The Biuret color yield was standardised against an aqueous solution of analytical bovine serum albumin (Sigma-Aldrich) covering the range 1-10 mg/mL. A volume of sample of 0.5 mL was used for the aliquots. All determinations were performed in triplicate.

2.4 Electrophoretic analysis

Prior to electrophoresis, a sample of wastewater collected at the end of the salting process was heated at 80 ºC for 15 min, and then mixed with an equal volume of sample buffer. This buffer was prepared by adding 6 g of glycerol (Merck) and 1.6 g of sodium dodecyl sulphate (Sigma-Aldrich) to 4 mL of Tris-SDS solution (Sigma-Aldrih). SDS-PAGE was then performed in a Protean II, XL Cell (Bio-Rad), using 4% stacking gel, 10% spacer gel and 16.5% running gel - as described in details by Schägger and von Jagow (1987). The protein bands were stained with 0.25% Coomassie brilliant blue solution (Merck) for 1 h and then washed with a destaining solution of 2.5:1 (v/v) acetic acid and methanol (Frilabo), until protein bands became clearly visible in a colourless gel matrix. Band analysis was performed using a GS-700 Densitometer equipped with Molecular Analyst v.1.0 Software (Bio-Rad).

2.5 Statistical analysis

The analysis of variance (one-way ANOVA), was performed with the software STATISTICA v.9.0, to access the variation of nitrogen and proteins content with time.

3. Results and Discussion

By 6 days of salting of 800 kg of codfish, 155 l of water were drained away. According to Xiong (1997) the water release occurs due to myofibrillar protein modifications arising from changes in ionic strength, polarity and capillary forces associated with the high salt supplied. The composition of the wastewater generated along the codfish salting process is displayed in Table 1, in terms of amount of water released by codfish, total nitrogen (WSN), non-protein nitrogen (TCASN and PTASN), and ratios TCASN/WSN and PTASN/WSN, whereas the ratios water released/WSN, water released/TCASN and water released/PTASN are also plotted in Figure 1. By the end of the process, the WSN concentration was 3.1 g/L, which is equivalent to 18.1 g/L of total protein, assuming a nitrogen-to-protein Kjeldahl conversion factor of 5.82 (Sosulski and Imafidon, 1990). As expected, the non-protein nitrogen TCASN concentration was higher than its PTASN counterpart, and both fractions were below WSN; recall that TCASN is accounted for mainly small peptides (2–20 residues) and free amino acids, whereas PTASN corresponds only to free amino acids (Kuchroo and Fox, 1982). Analysis of the ratios PTASN/WSN and TCASN/WSN (see Table 1) indicated that about 54.62 % (with a range 49.30–67.21 %) of total nitrogen corresponded to small peptides and free amino acids, so the remaining nitrogen, 45.38 % (with a range 32.79–50.70 %), represented soluble proteins.

Table 1. Wastewater composition and concentration of total muscle proteins (mean ± standard deviation) throughout the codfish salting process

Entity concentration	Processing time (h)					
	9	33	57	81	105	129
Water released (l)	75	102	123	135	147	155
WSN (g/l)	1.19±0.13	1.79±0.12	2.40±0.23	2.79±0.21	2.92±0.12	3.17±0.13
TCASN (g/l)	0.79±0.19	0.88±0.06	1.35±0.17	1.44±0.23	1.50±0.20	1.62±0.20
PTASN (g/l)	0.45±0.08	0.46±0.11	0.63±0.10	0.79±0.91	1.05±0.11	1.16±0.21
TCASN/WSN (%)	67.21±15.69	49.30±4.05	56.51±8.23	51.91±8.04	51.52±6.16	51.25±5.79
PTASN/WSN (%)	37.93±0.06	25.54±0.03	26.36±0.04	28.39±0.06	36.14±0.04	36.55±0.06
Water released/WSN (l^2/g)	63.80±7.38	57.10±4.08	51.56±5.08	48.64±3.72	50.38±2.09	48.96±2.00
Water released/TCASN (l^2/g)	98.85±24.54	116.13±7.36	92.16±11.12	95.36±15.36	98.94±13.26	96.59±12.63
Water released/PTASN (l^2/g)	171.55±29.02	226.37±31.94	199.21±33.73	172.86±19.85	140.44±14.51	137.21±26.60
Total muscle proteins (g/l)	3.015±0.253	3.104±0.292	3.235±0.344	3.344±0.272	3.517±0.186	3.668±0.201

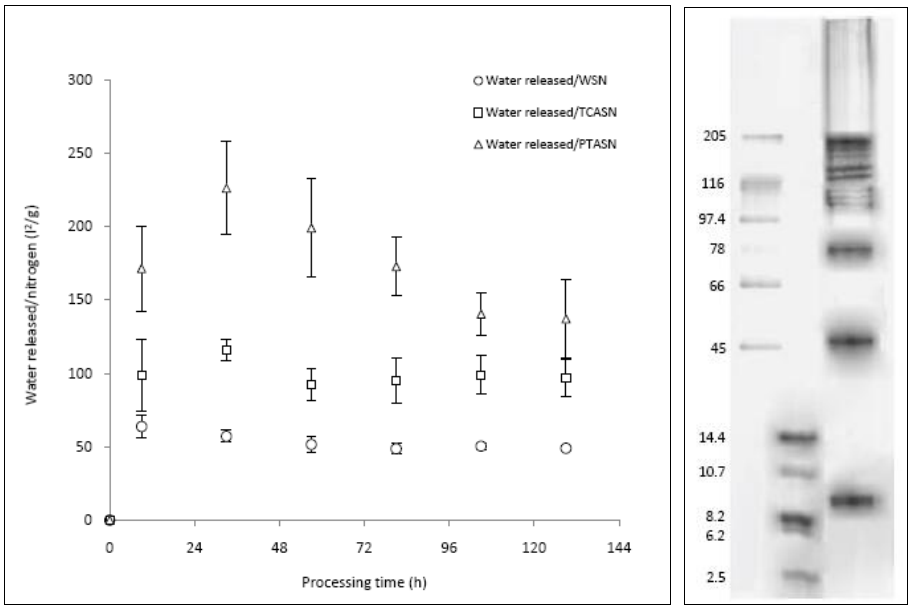

Figure 1 (left). Ratios of water release to nitrogen (mean ± standard deviation) throughout the codfish salting process (right). SDS-PAGE profile of wastewater of the codfish salting process. First lane: high molecular weight standard; second lane: low molecular weight standard; third lane: wastewater sample. Molecular weights are expressed in kDa.

The ANOVA results pertaining to the ratio TCASN/WSN revealed no significant changes throughout the salting treatment (at a significance level of 95 %), after the first 9 h. Conversely, the ratio PTASN/WNS showed significant changes ($p<0.05$) with time due to hydrolysis throughout the heavy salting (Martín et al., 1998). During the process the proportion of water release to WSN was approximately constant throughout

salting ($p>0.05$), with a mean value of 53.41 l^2/g and a range 63.80-48.96 l^2/g (see Table 1 and Figure 1). The proportion of water release to TCASN was also constant with time, with a mean value of 99.67 l^2/g and a range 92.16-116.13 l^2/g, as the changes were found not significant ($p>0.05$). Conversely, the proportion of water released to PTASN decreased from an initial 226.37 down to a final 137.21 l^2/g, after first 9 h, as proteins and peptides became hydrolysed (see also Table 1 and Figure 1). Hence, it can be hypothesized that proteins and peptides were released from codfish muscle promptly from the starting period of processing, whereas amino acids release took place gradually as a consequence of proteolysis.

Throughout the codfish salting process, the concentration of total muscle proteins increased with time ($p<0.05$) (Table 1). Myosin heavy chain was the most vulnerable to denaturation as induced by the heavy salting (Figure 1 right); its constitutive helix underwent denaturation to yield two light chains, represented by the 205 kDa band. Actin appeared to be more resistant and showed a clearly visible band at ca. 45 kDa. Bands between 205 and 97.4 kDa accounted for heavy meromyosin (and its subunits) and light meromyosin, all originated from myosin fragmentation. The band appearing at 78 kDa may be either tropomyosin or a remaining of light meromyosin, as reported elsewhere (Thorarinsdottir et al., 2002). The peptide of ca. 8.2 kDa, could also be a product of myosin degradation as also found by Ball et al. (1987).

4. Conclusion

The wastewater generated by the codfish salting process possesses a sufficiently high nutritional value to justify its upgrade: in 155 l of water released by the end of the codfish salting process were found ca. 1.62 g/L of phosphotungstic acid-soluble nitrogen (PTASN) accounted for free amino acids, and ca. 3.67g/L of muscle proteins that could be further valorized.

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