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Food Chemistry 93 (2005) 385–393
Food Chemistry
www.elsevier.com/locate/foodchem

Effects of the hydration process on water-soluble proteins of preserved cod products

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Received 17 May 2004; received in revised form 26 September 2004; accepted 28 September 2004

Abstract

Electrophoresis and chromatography were used to analyse the water-soluble proteins of fresh, salted and dried cod (*Gadus morhua* L.) and of re-hydrated products. Sodium dodecyl sulphate pore gradient gel electrophoresis showed that there was a great loss of water-soluble proteins during stockfish re-hydration. Furthermore, comparison of the electrophoresis patterns of cod products, under reducing and non-reducing conditions, showed the presence of cross-linked protein aggregates, related to the oxidation of SH groups and oxidative stress. Similar results were obtained by high performance liquid chromatography, which confirmed a great loss of water-soluble proteins and a higher level of SH oxidation in hydrated stockfish samples. Finally, capillary electrophoresis allowed definition of the unfolded state of denatured proteins in salted cod. This technique also proved a fast and useful way to differentiate the two products by analysis of water-soluble proteins.

Keywords: Atlantic cod; Sarcoplasmic proteins; Dried cod products; Cross-linked aggregates

1. Introduction

The Atlantic cod (*Gadus morhua* L.) is a lean fish, which lives in the North Atlantic, and its main fishing area lies along the coast of Newfoundland-Labrador, Greenland, Iceland and Lofoten Island (Norway). Fishing only takes place during a short period of the year (from December to February), and traditional systems dating back to the 12th century are used to ensure availability of this fish all the year round.

Cod made the fortunes of Nordic peoples when modern storage techniques were still unknown. Fished with a simple but efficient system, cod was sun-dried on wood wattles to obtain stockfish and finally beaten to make it edible. While Norwegians monopolised stockfish, Basque fishermen, supported by Vikings, were engaged in whaling, salting the whale-meat before storage. When uncontrolled, whaling caused whales to emigrate to the North Atlantic; the Basque fishermen followed them and found cod. They began to fish for cod, which, unlike the Norwegians, they salted before storage to obtain the product called salted cod (Kurlansky, 1997).

Today Denmark and Norway are the major producers and exporters of dried preserved cod. It is prepared from fresh fish, which are decapitated, gutted and left to dry for about three months during winter or spring. Afterwards, the product is stored under cool, dry conditions.

The preparation of traditional salted cod is a different process because, after gutting the fish is cut along the

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doi:10.1016/j.foodchem.2004.09.034

ventral median line and opened to remove the spinal column for about 3/4 of its length. The fish is then arranged in piles with the skin-side down, in consecutive layers alternating with fine-grained salt. After one week, another salting is carried out, this time using coarse-grained salt. The maturation process of this product requires at least one month. Finally, the salted cod storage period is increased by dehydrating the fish in a hot-air tunnel or using atmospheric agents. Salted cod is mainly produced in Spain and Portugal and consumed in the Mediterranean area (Beraquet, Okada, Ferriera, & Menezes, 1975).

Both products are subjected to a reduction of their water content: from 65–95% to 10–15% by natural drying processes, or to 30–35% by artificial drying procedures (Cappelli & Vannucchi, 1998); the dried products (salted or dried) are each then subjected to different re-hydration processes which allow marketing and retailing. The dried products are also suitable for storage and transport, since they are light and do not occupy much space; this has favoured their spread in Mediterranean countries and Latin America.

Italians first came into contact with stockfish when a ship belonging to the Venetian trader, Piero Quercini, was wrecked near Lofoten Island, north of Norway. On his way back, in 1432, he described cod fishing, stockfish preparation, and cooking of cod (Birri & Coco, 1999; Kurlansky, 1997).

Preserved cod soon became one of the most popular traditional Italian dishes, and Italy became a major importer of these products (Bernardi, Rorato, & Zorzi, 2001).

Recent works have reported analyses of technological processes of stockfish (Santoro, Sarli, Murru, Nappo, & Cortesi, 2001) and salted cod (Barat, Rodríguez-Barona, Andrés, & Fito, 2003) as well as biochemical aspects of salting cod (Martinez, Solberg, Lauritzen, & Ofstad, 1992; Thorarinsdottir, Arason, Geirsdottir, Bogason, & Kristbergsson, 2002). However, to our knowledge, there is no previous study regarding the protein fraction modifications of stockfish and hydrating processes.

The aim of this work was to use electrophoresis and chromatography to investigate the effect on preserved cod products, of different drying and hydrating processes, using water-soluble proteins as molecular markers.

2. Materials and methods

2.1. Sample preparation

Three samples each of fresh cod, stockfish, dried salted cod, re-hydrated stockfish and re-hydrated salted cod were acquired in a local market.

The dried products were freed of skin and bones, and the fresh cod was cut along the ventral median line; 5 g of minced muscle from fresh, salted and sun-dried cod were homogenised for 3 min with 10 ml distilled water, and the homogenates were immediately centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant was filtered through a 0.45 μm filter membrane (Millipore, Bedford, MA, USA), recovered and subjected to protein assay by the Coomassie method (Bio-Rad, Hercules, CA).

2.2. Sodium dodecyl sulphate–discontinuous pore gradient gel electrophoresis (PGGE)

The samples, under reducing conditions, were prepared by mixing 50 μl of protein solution with an equal volume of sample buffer [0.125 M Tris pH 6.8, 4% SDS, 10% 2-ME, 60% sucrose and 0.005% bromophenol blue], after which they were kept for 2 min in boiling water; the samples under non-reducing conditions, were prepared without boiling in SDS and 2-ME. Finally, 7 μg of proteins was loaded onto the wells of stacking gels.

Sodium dodecyl sulphate–discontinuous pore gradient gel electrophoresis (SDS–PGGE) [45 T, 2.8% C stacking gel and 10–20% T, 2.8 % C polyacrylamide pore gradient gel 120 × 140 × 0.75 mm (Sheele, 1975)] was carried out using discontinuous buffer (Laemmli, 1970) at a constant current of 10 mA, 500 V_{max} and 14 °C until the tracer reached the running gel. Subsequently, the current was increased to 20 mA. The separated proteins were revealed by Coomassie Blue G-250 (Krause, Buchberger, Weib, Pfugler, & Klostermeyer, 1988) and subjected to densitometric analysis, using a laser densitometer (Ultrascan XL, Pharmacia-LKB, Uppsala, Sweden) equipped with a Gelscan 2.0 integration system.

2.3. Reversed phase high performance liquid chromatography (RP-HPLC)

A liquid-chromatograph (Pharmacia mod. 2551), equipped with a UV–Vis detector (Pharmacia-LKB-uvicord VW 2251) and a Shimadzu integration system, was used. Proteins were separated on an analytical Vydac large-pore (300 Å) C_8 column (150 × 4.6 mm). The chromatogram was developed with two linear gradients between buffer A (0.1% trifluoroacetic acid/Milli-Q water) and buffer B (0.1% trifluoroacetic acid/acetonitrile) at a flow rate of 1.5 ml/min and at 40 °C. The first gradient was 35–60% buffer B in 30 min, followed by 60–100% buffer B in 3 min and finally 100% buffer B for 5 min. The sample injection volume was 20 μl and protein elution was monitored at 280 nm.

2.4. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was carried out using a Beckman mod. P/ACE 2000 controlled by Gold

software. Proteins were separated in a fused-silica capillary column (57 cm × 0.075 mm I.D.), the injection time was 3 s at 25 °C and the first voltage gradient was 0–15 kV for 0.17 min, followed by 15–20 kV for 22 min, and 20–25 kV for 5 min.

3. Results and discussion

3.1. General

Preserved cod products are made edible by a process of hydration. The hydration processes differ in the soaking time: 48–60 h for salted cod and about 9 days for stockfish (Santoro et al., 2001), as shown in Fig. 1. Neapolitans living in the Vesuvius area prefer thicker and therefore more re-hydrated stockfish, and this involves a time increase in Stages III, V and VI, which prolongs the hydration process to 15 days (Santoro et al., 2001). In the hydration phase, exchanges occur between the components of preserved products and the soaking medium, and soluble components move into the water. Among these components, there are muscle proteins, mostly sarcoplasmic proteins, which we have considered as a possible molecular marker to evaluate the influence of hydration on the finished products.

3.2. Protein assay

The protein concentrations of water-soluble extracts and crude protein of preserved and re-hydrated cod products are shown in Table 1. Stockfish presented the highest protein content, which decreased greatly after re-hydration, whereas dry salted cod had a protein content which was similar to that of the re-hydrated product.

When the dried cod is soaking, the water exercises an osmotic pressure, which swells the muscle tissue and

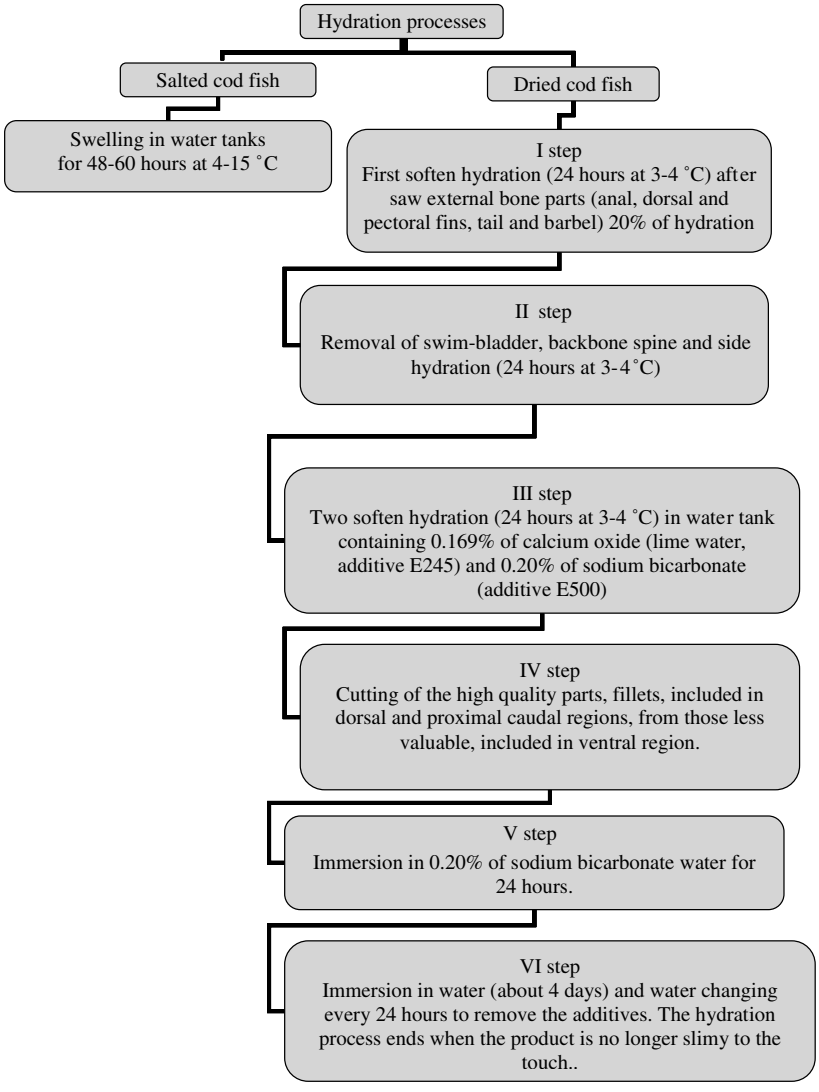

Fig. 1. Flow chart of hydration process of salted and dried cod.

Table 1
Concentrations of water-soluble (g/l) and crude (g/100 g of edible part) protein of preserved and hydrated cod fish

Samples	Dried		Hydrated	
	Soluble proteins	Crude protein	Soluble proteins	Crude protein
Stockfish	2.10 ± 0.13	79.8 ± 2.87	0.38 ± 0.02	19.5 ± 1.17
Salted cod	1.44 ± 0.11	29.2 ± 1.64	1.36 ± 0.08	22.6 ± 1.04

causes the flow of soluble protein into the soaking water (Yao & Le Maguer, 1996). Moreover, the weakening of bonding myosin heads to actin and the weakening of transversal structural elements caused by endogenous enzymes facilitate the extension of myofibrils of the thick filaments.

The biggest loss of water-soluble proteins from stockfish, after re-hydration, can be ascribed to protein solubilisation and to resulting movement into the soaking medium. The addition of calcium hydroxide and sodium bicarbonate to the hydration solution causes an alkaline pH, from 10 to 12 (Santoro et al., 2001); this favours a loss of muscle protein into the soaking medium due to solubilisation (Undeland, Kelleher, & Hultin, 2002, 2003). In addition, the alkaline environment acts as a bactericidal agent, thus permitting a long re-hydration process to be used (Santoro et al., 2001). Finally, calcium hydroxide bleaches the yellow dried products, which would not be appreciated by consumers.

Unlike stockfish, the salting of cod causes protein denaturation, leading to a small loss of protein during the re-hydration process, due to protein aggregation and precipitation and the short soaking time (Ito, Kitada, Yamada, Seki, & Arai, 1990; Tambo, Yamada, & Kitada, 1992; Thorarinsdottir et al., 2002). At a high salt concentration, protein solubility decreases because of the solvation competition between salt ions and proteins. The higher solvation capacity of ions reduces the hydrodynamic radius of proteins, and protein–protein interactions become stronger than protein–water interaction. In this way, polar and hydrophobic interaction of proteins increases, favouring their hydrophobicity, aggregation and precipitation (Caflisch & Karplus, 1994; Tanford, 1970).

We believe that the high salt concentration initially causes protein loss by the osmotic effect of the salt (Ooizumi, Kawase, & Akahane, 2003; Yao & Le Maguer, 1996). Concomitantly, aggregation and precipitation are determined by protein denaturation of the more external part of the muscle tissue and create a barrier against more internal proteins being drawn into the soaking medium while internal salt diffusion occurs. This hypothesis seems to be supported by the similar amounts of protein in salted and re-hydrated cod muscle tissues, determined both by crude protein and by protein assay of the water extract. It receives support also from the results of Garcia, Diez, and Zumalacarregui (1997) who observed a reduction in the extractability of sarcoplasmic and myofibrilar proteins of about 80% during the processing of Spanish ''cecina'' dried beef. This was thought to be due to denaturation of proteins and/or proteolysis. Moreover, the salting process also involves the diffusion of salt into fish muscle tissue (Ooizumi et al., 2003), causing denaturation of sarcoplasmic and myofibrillar proteins as well as a possible solubilisation of myofibrillar protein and heavy myosin chain fragmentation (Thorarinsdottir et al., 2002).

These results clearly show how protein composition of cod products can be influenced by preservation and hydration methods.

3.3. Sodium dodecyl sulphate–discontinuous pore gradient gel electrophoresis

Fig. 2 shows the SDS–PGGE patterns of water-soluble proteins from fresh muscle, preserved and re-hydrated cod products. The pattern of fresh cod (Lane 1) was quite similar to that of preserved products and stockfish (Lane 2). Significant differences can be noted in Lane 3, where the pattern of salted cod is shown, in particular the appearance of a band at about 94.1 kDa and disappearance of the bands with estimated molecular weights of 74.0 and 45.0 kDa. It is notable that this latter band was also absent in re-hydrated products, whereas the lower-intensity band in the stockfish profile (Lane 2) could originate from the reducing condition, it being a product of protein aggregation caused by thiol oxidation.

As regards the re-hydrated products, stockfish surprisingly presents only three main bands at 42.0, 24.3 and 14.4 kDa (Lane 4), whereas re-hydrated salted cod (Lane 5) was similar to dried salted cod except for the absence of bands with an estimated MW of 94.1 kDa. These results reflected those listed in Table 1, where a greater loss of water-soluble proteins was found after the hydration process of stockfish.

The profiles from 6 to 9 correspond to dried and re-hydrated preserved cod products, running under non-reducing conditions (see Section 2). It is worth noting that dried stockfish (Lane 6) exhibits a band at about 166 kDa while, in re-hydrated stockfish (Lane 8), there appear two protein bands with estimated molecular weights of 112.3 and 108.0 kDa; the former does not appear in the reducing pattern (Lanes 2 and 4); the latter, instead, appears as a faint band only in Lane 2. The absence of these three bands in reducing patterns suggested

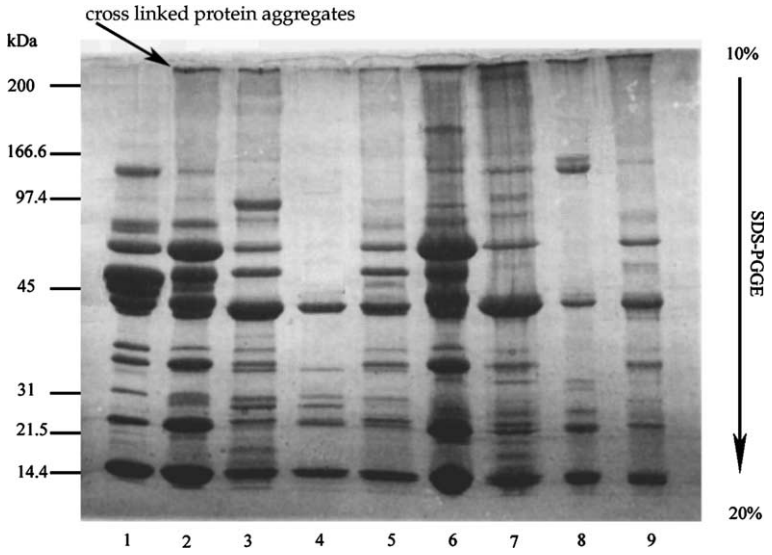

Fig. 2. SDS–PGGE of sarcoplasmic proteins from fresh cod and preserved products. Lanes 1–5, samples under reducing conditions: fresh cod (1); dried and hydrated stockfish (2 and 4); dried and hydrated salted cod (3 and 5). Lanes 6–9, samples under non-reducing conditions: dried and hydrated stockfish (6 and 8); dried and hydrated salted cod (7 and 9).

the presence of proteins aggregated by S–S bridges as a consequence of cysteine-SH residue oxidation.

In the salted cod profiles (Lane 7) the presence of bands can be seen with estimated molecular weights of 205.0 and 108.0 kDa, which were absent in Lanes 3 and 5 under reducing conditions. The disappearance of these bands in Lanes 3 and 5 also demonstrates that these proteins are aggregated by S–S bridges due to cysteine residue oxidation, whereas the absence of the 108.0 kDa band in the non-reducing re-hydrated salted cod (Lane 9) led to the supposition that this band was drawn into the soaking medium.

Finally, the presence of newly-formed bands between 21.5 and 14.4 kDa in salted cod suggested a proteolytic activity during salting, even if some of these bands disappeared with re-hydration. Although the fragmentation of denatured protein by proteolytic systems is due to free radicals or pro-oxidants such as salts (Davies, 1987; Pacifici & Davies, 1990; Sirivasan & Hulting, 1995; Tambo et al., 1992), in the case of muscle proteins endogenous enzyme action also should be considered. In fact, Martinez et al. (1992), studying the effect of Ca^{2+} and Mg^{2+} salts on water-soluble fractions of cod and surimi whole muscle proteins by two-dimensional gel electrophoresis, revealed new additional protein fragments. They suggested that these fragments originated by increased proteolytic activity of Ca^{2+}-actived proteases.

Large cross-linked protein aggregates (CLPA) were present above the separating gel in every sample except for fresh cod (Lane 1). The main variations in the different patterns were quantified by densitometry and compared with average optical density values of absolute areas, as shown in Table 2. Under non-reducing conditions, small differences in band intensity after re-hydration of two preserved products, were observed. The variations of band intensity observed under reducing conditions can be explained by considering the effect of soaking which causes transfer of large CLPA and consequently a reduction of intensity in re-hydrated products. Stockfish presented a lower intensity than salted products, demonstrating that more SH oxidation occurred. However, the incomplete disappearance of large CLPA in reducing profiles suggested the probable occurrence of bonds between oxidised amino-acid residues generated by reactive oxygen species (ROS), produced as a consequence of lipid peroxidation (Davies & Delsignore, 1987). Sodium chloride is known as a pro-oxidant (Astwan, Wahyuni, Tadokoro, & Maekawa, 1995; Gorelik & Kanner, 2001; Lubis & Buckle, 1990) and the dry salting process involved exposure to air, allowing accelerated lipid oxidation (Karaçam, Kutlu, & Köse, 2002).

External stockfish lipids, mainly unsaturated lipids, are oxidised by the combination of air oxygen, cellular transition metal, xenobiotics and light (Emerit, Beaumont, & Trivin, 2001; Mortensen, Sørensen, & Stapelfeldt, 2002) producing ROS during the long drying period. Transition metals catalyse the formation of hydrogen peroxide via the Fenton reaction, which is capable of abstracting a hydrogen atom from polyunsaturated fatty acids to initiate lipid peroxidation (Stohs & Bagghi, 1995). Lipid-free radicals and lipid oxidation products could be initiators of protein denaturation (Srinivasan & Hultin, 1995). A number of amino-acid residues (methionine, cysteine, and aromatic amino-acids) are highly susceptible to ROS-mediated oxidations, which might lead to protein oxidation and

Table 2
Mean absolute areas (OD/mm) of cross-linked protein aggregates (CLPA) obtained by image analysis of SDS–PGGE shown in Fig. 2

	Lanes								
	Reducing conditions					Non-reducing conditions			
	1	2	3	4	5	6	7	8	9
MW (kDa)	Fresh cod fish	Dried stockfish	Salted cod fish	Hydrated stockfish	Hydrated salted cod fish	Dried stockfish	Salted cod fish	Hydrated stockfish	Hydrated salted cod fish
CLPA		98.0	111	54.9	86.1	140	117	105	107

carbonyl derivatives (Gutteridge, Rowley, & Halliwell, 1982; Stadtman & Berlett, 1997). However, no hydrolysis products were detected in the electropherogram, as in the case of salted cod; this agrees with the results of Undeland et al. (2002) who found negligible hydrolysis in homogenates at alkaline and neutral pH.

Finally, the effect of decreased solubility of water-soluble proteins because of increased hydrophobicity, due to their denaturation (Ito et al., 1990; Thorarinsdottir et al., 2002), may have caused a smear during the sample migration in Lanes 6 and 7, particularly evident in Lane 6.

Therefore, protein denaturation, due to accumulation of lipid free radicals and free fatty acids during storage, could have caused formation of S–S bridges and/or hydrophobic interactions as well as amino-acid modifications leading to cross-linking of monomers and aggregates to make them larger and more stable. Numerous works demonstrate that free radicals or oxidants are responsible for both the formation of CLPA and the fragmentation of denatured protein by proteolytic systems (Davies, 1987; Davies, Del Signore, & Lin, 1987; Davies, Lin, & Pacifici, 1987; Pacifici & Davies, 1990). This hypothesis is supported by the findings of Martinez et al. (1992), whose analysis of the water-soluble fraction of cod and surimi whole muscle proteins by two-dimensional gel electrophoresis revealed new additional

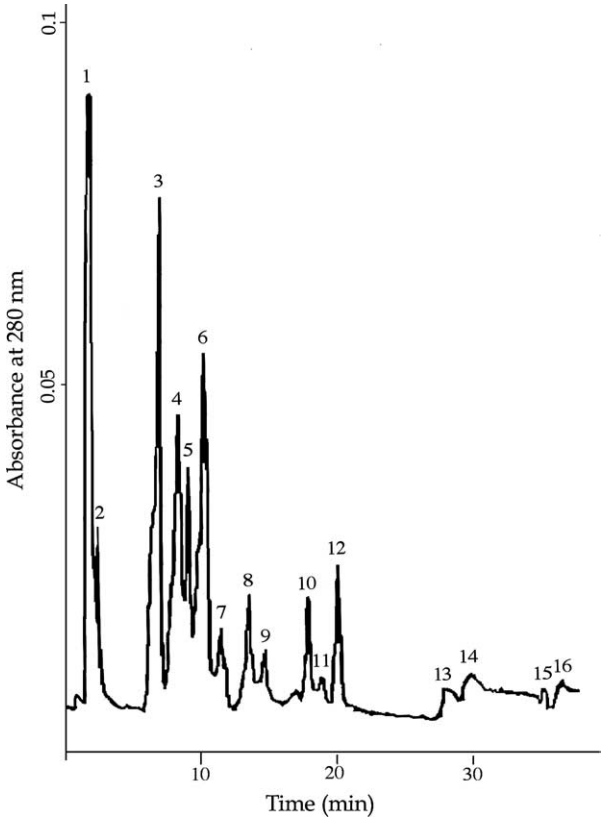

Fig. 3. RP-HPLC chromatogram of fresh cod.

protein fragments. They suggested that these fragments were due to increased proteolytic activity during surimi production because of the addition of Ca^{2+} and Mg^{2+}.

3.4. Reversed phase high performance liquid chromatography

Fig. 3 shows the RP-HPLC chromatogram of extracted water-soluble proteins of fresh cod; Figs. 4(a) and (c) show preserved dried products; and Figs. 4(b) and (d) show the re-hydrated products. Polypeptide components were eluted in 20 min, the rest were the most restrained components (eluted with 100% of buffer B), which give a wider and poorly defined peak. This strongly increases in salted cod and is the main component of re-hydrated stockfish.

The occurrence of hydrophobic polypeptides is a consequence of protein denaturation (Caflisch & Karplus, 1994; Tanford, 1970), and RP-HPLC was useful for showing monomer aggregates caused by hydrophobic interaction, occurring in preservation and/or re-hydration processes. Ito et al. (1990), studying cross-linking reactions of the myosin heavy chain in cured Walleye Pollack and Alaska Pollack fish, and Tambo et al. (1992) studying the effect of soaking in NaCl solution on fish myofibrillar proteins, both reported that

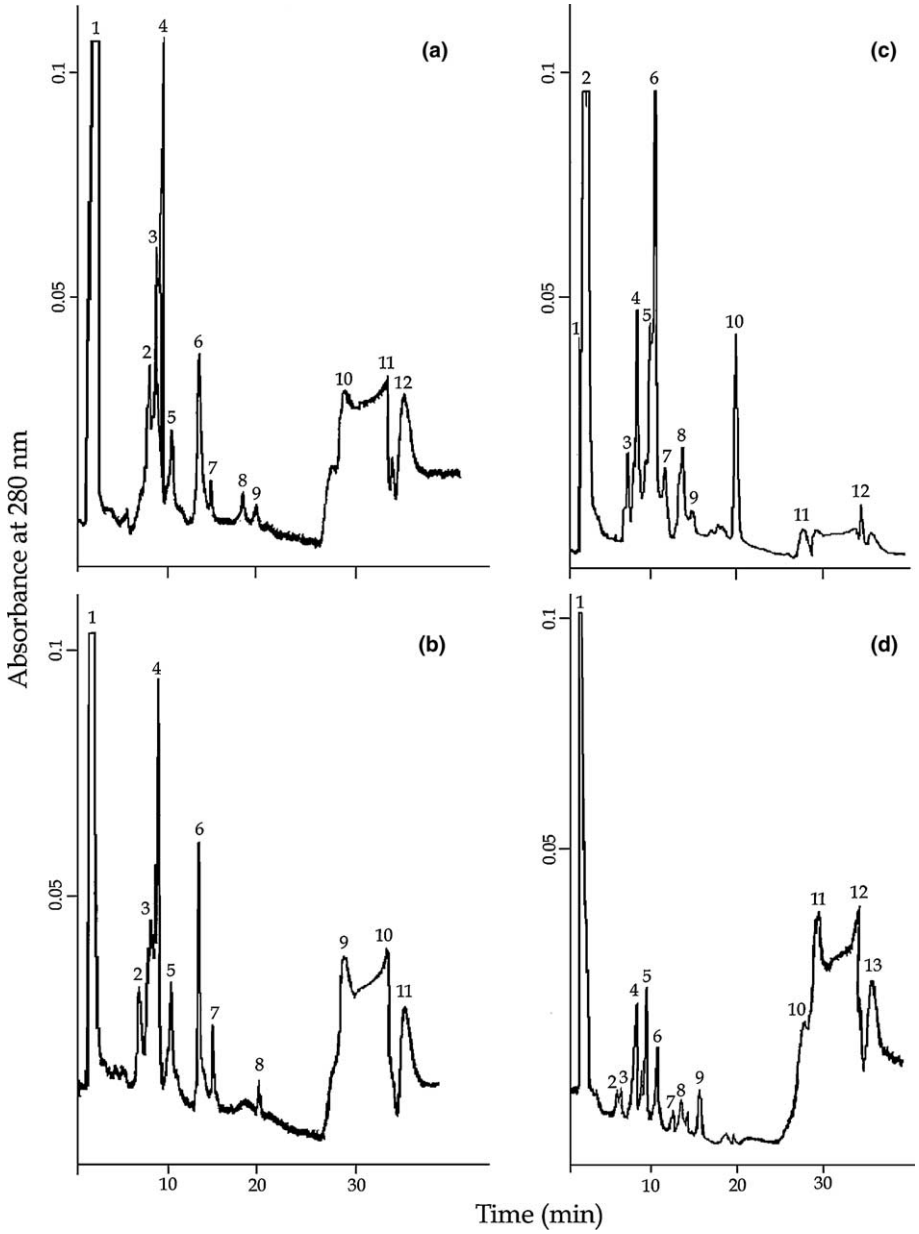

Fig. 4. RP-HPLC chromatograms of dried (a) and hydrated (b) salted cod and of dried (c) and hydrated (d) stockfish.

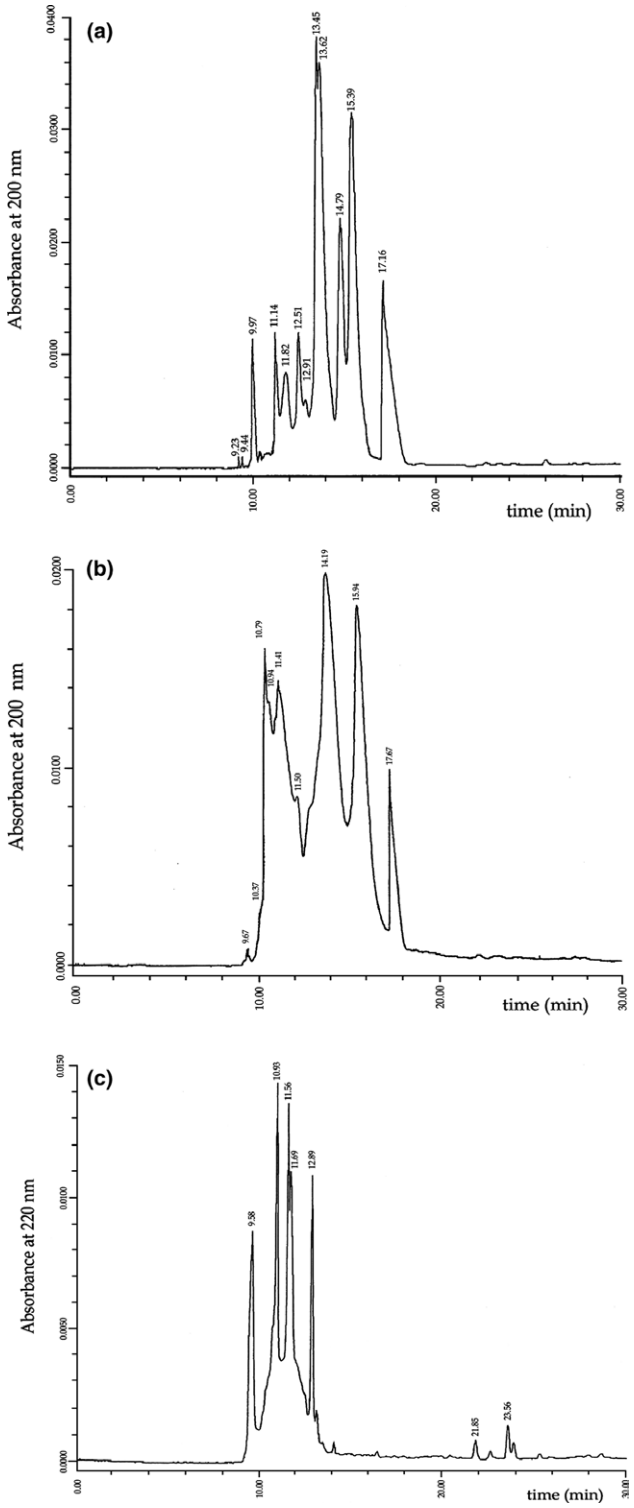

Fig. 5. Capillary zone electrophoresis of fresh cod (a) and of hydrated salted cod (b) and stockfish (c).

protein aggregation is dependent on the salt concentration in flesh, and the soaking time. In fact, increase of protein hydrophobicity was only found in salted product and re-hydrated stockfish, whereas the cod drying process did not produce hydrophobic polypeptide as observed in fresh cod.

3.5. Capillary zone electrophoresis

In free solution capillary electrophoresis, the electrophoretic mobility of a molecule is in general a function of the net charge, the hydrodynamic drag and the properties of the solution (viscosity, concentration and mobility of dissolved ions). Owing to these criteria, capillary electrophoresis has recently been used to monitor the folded or unfolded state of proteins (Carbeck & Negin, 2001; Verzola, Focolari, & Righetti, 2001). In the native or folded state, hydrophobic residues are shielded from the water environment, but denaturation and unfolding diminish this protection. Therefore, native proteins have a high narrow peak, but when unfolding (denaturated state) occurs, this peak becomes smaller and wider and its mobility decreases (Verzola et al., 2001).

In our case, the fresh cod electropherogram shows well-defined peaks and an intensity detection of 0.04 OD for the presence of protein in a native state (Fig. 5(a)); the profiles of re-hydrated preserved products show two different situations (Fig. 5(b) and (c)). Re-hydrated salted cod (Fig. 5(b)) shows large and poorly defined peaks with a lower intensity detection of 0.022 OD. This result can be explained by the increase of protein hydrophobicity and more protein–protein interactions, which can involve protein aggregation, causing an increase in size and a reduction in charge. Due to changes in size and charge of proteins, a stronger interaction with capillary walls occurs, which causes a resistance and slower movement in free solution.

In re-hydrated stockfish (Fig. 5(c)), five narrow peaks were observed, three of which were at the top of a low wide peak; in addition increased mobility and a lower peak intensity (0.015 OD) were observed. Lower peak intensity and better-defined peaks compared with those of re-hydrated salt cod, are consistent with the presence of folded proteins with a low molecular weight; this reduces the analysis time because there is less interaction between polypeptides and the capillary wall.

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