Low-Field Nuclear Magnetic Resonance Relaxation Study of Stored or Processed Cod

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Proton transverse relaxation has been measured in heated, pressure-treated, and frozen stored cod by pulse low-resolution NMR spectroscopy. These treatments resulted in the appearance of an additional component in the proton transverse relaxation which can be explained in terms of water exudation. The transverse relaxation time associated with this supplemental component increased with the temperature of the thermal treatment or with the temperature and duration of the frozen storage. For cod, these variations in NMR relaxation parameters can be used to detect nonreversible deterioration due to frozen storage or high hydrostatic pressure treatment.

Keywords: *NMR; protein; denaturation; cod; DSC; pressure*

INTRODUCTION

The texture of fish flesh is altered during processing and storage. This texture modification is mainly a result of damage caused to the protein structure (Suzuki, 1981). Indeed, heating (Wright, 1982; Davies et al., 1988; Howell et al., 1991; Chan et al., 1992), frozen storage (Shenouda, 1980; Curtil and Masson, 1993), and high hydrostatic pressure treatment (Ohshima et al., 1993; Balny and Masson, 1993) are processes known to denature proteins. This unfolding denaturation leads to a decrease in the solubility of the proteins and a loss of their functional properties, for instance, gel-forming ability and water-holding capacity.

A number of methods for studying protein unfolding denaturation have been described in the literature. Low-field pulsed nuclear magnetic resonance (NMR) spectroscopy has been introduced as an alternative to current methods [differential scanning calorimetry (DSC), circular dichroism] for studying thermal denaturation of proteins in aqueous solution. Since water protons interact with biopolymer protons by chemical exchange and/or spin exchange, structural modifications of the biopolymer can be recorded by the measurement of water proton relaxation times (Pumpernik et al., 1975; Oakes, 1976; Rydzy and Skrzynsky, 1980; Goldsmith and Toledo, 1985; Lambelet et al., 1989, 1992; Lee et al., 1992). The T_2 relaxation of water protons in systems containing myofibrillar proteins is a complex phenomenon. In these systems water proton relaxation has a multiple phase behavior due to the existence of a number of water proton populations which do not interact with one another or which interact slowly on the NMR time scale (Nakano and Yasui, 1979; Lee et al., 1992). For example, water proton T_2 relaxation in heart muscle is a three-exponential process (Lee et al., 1992). The NMR method can, nonetheless, be applied to such complex systems: heating heart muscle leads to the observation of a major change in value of the longest T_2 relaxation time (Lee et al., 1992).

The present study was undertaken to determine whether the water proton T_2 relaxation time could be a probe for investigating heat-, cold-, and pressure-induced denaturation of cod proteins. Cod was selected among edible fishes for its very low fat content. Fat protons could relax in a way similar to water protons and consequently complicate the interpretation of NMR data. The transverse relaxation parameter was chosen as it is more sensitive to protein unfolding denaturation than the longitudinal relaxation parameter (Oakes, 1976; Lambelet et al., 1989). Therefore, the transverse water proton relaxation was measured in cod after heating and frozen storage, as well as after hydrostatic pressure treatment. Modifications of this relaxation were interpreted in terms of water exudation associated with protein denaturation, as well as with physical damage and enzymatic reaction in the case of frozen storage.

MATERIALS AND METHODS

Sample Preparation. Fresh cod fillets were purchased locally. For thermal treatments and frozen storage, a piece of fresh cod fillet was introduced into an NMR sample tube (7.5 mm o.d.) and the tube sealed. High hydrostatic pressure treatments were applied to cod fillets contained in a waterproof plastic bag.

Thermal treatments were achieved by heating the samples for 30 min successively at increasing temperatures between 20 and 90 °C (10 °C interval). The samples were cooled by immersion into a water bath set at 20 °C. NMR measurements were performed following each thermal treatment.

For frozen storage a series of samples were kept at −40, −20, and −10 °C for a number of weeks. At various intervals they were removed from the storage room, thawed in a water bath at 20 °C, and analyzed.

High-pressure treatments (hydrostatic pressures of 1000, 2000, 3000, and 3800 bar corresponding to 100, 200, 300, and 380 MPa, respectively) were applied to cod fillet for 20 min using a cold isostatic press (National Forge Europe/4 kbar/8.6 l). Cold (~12 °C) water was introduced into the press at the beginning of the treatment so that the sample temperature did not exceed 20 °C during the whole process. After pressure treatment, pieces of cod fillet were introduced into 7.5 mm NMR tubes and analyzed.

NMR measurements were performed on samples equilibrated for 30 min at 20 °C, using a Minispec PC 20 (Bruker Physik, Karlsruhe) at a resonance frequency of 20 MHz. The samples were kept at 20 °C during the measurements by using a variable temperature probe head. T_2 relaxation times were determined from the spin echo decay curve stemming from a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence. Each decay curve (390 experimental points) resulted from a combination of three decays obtained by sampling the signals every 2, 6, and 10 echoes. Thus, the range of measurements extended from 2τ to $1690 \times 2\tau$, where τ is the interpulse spacing (Le Botlan and Hélie, 1994). An additional decay curve (160 experimental points) measured with an interpulse

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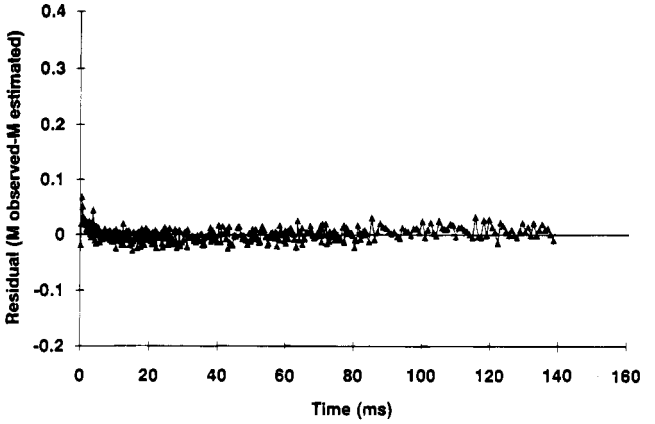

Figure 1. Residuals plot of water proton T_2 magnetization decay measured in fresh cod fillet with an interpulse spacing of 41 μs (one-component relaxation model).

spacing of 12 μs was recorded for each sample to detect the shortest transverse relaxation time.

Relaxation Component Analysis. T_2 relaxation times were calculated on a Hewlett-Packard 950 computer by performing nonlinear least-squares fitting of exponentials to the corresponding decay curves.

$$M(t) = \sum[M_{0i} \exp(-t/T_{2i}] + C$$

The constant C was determined from the decay curve obtained with an empty tube. Curve fitting was achieved with the program STEPT written in 1973 by J. P. Chandler, Department of Computing and Information Sciences, Oklahoma University (QCPE Program 307). The STEPIT algorithm was used for fitting. The multidimensional optimization consisted of finding local minima of a smooth function of the parameters M_{0i} and T_{2i}. Each experiment was repeated three times, and mean values of the T_2 relaxation time and percentage of population were calculated.

Calorimetric measurements were performed with a Micro-DSC (Setaram, Lyon, France) used in scanning mode between 20 and 97 °C. Heating rate was 1 °C/min. Sample weights were around 600 mg. Reference material was water.

RESULTS

Fresh Cod. Figure 1 shows the residuals between the T_2 water proton relaxation data measured in fresh cod fillet with an interpulse spacing of 41 μs and values calculated with a one-exponential model. As can be seen in this figure, there is no systematic discrepancy between experimental data and calculated values. A T_2 relaxation time of 65 ms was calculated from this relaxation decay. This value showed dispersion as the interpulse spacing increased (Figure 2). If the relaxation data were collected using the shortest interpulse spacing available, 12 μs, there were small but systematic discrepancies between experimental data and values calculated with a one-exponential model (Figure 3). This is indicative of a two-exponential phenomenon with a small component characterized by a short relaxation time. The latter could not be precisely determined due to experimental limitations, but it was estimated to be around 1 ms. Accordingly, the T_2 water proton relaxation decay measured in fresh cod fillet was interpreted in terms of two exponentials characterized by a short relaxation time $T'_2 \approx 1$ ms and a long relaxation time $T''_2 = 65$ ms.

Heat-Treated Cod. Results similar to those recorded with fresh cod were observed in cod heated at temperatures below 40 °C. Thus, the magnetization decay in these samples could also be interpreted in terms of a two-component model with $T'_2 \approx 1$ ms and $T''_2 = 65$ ms.

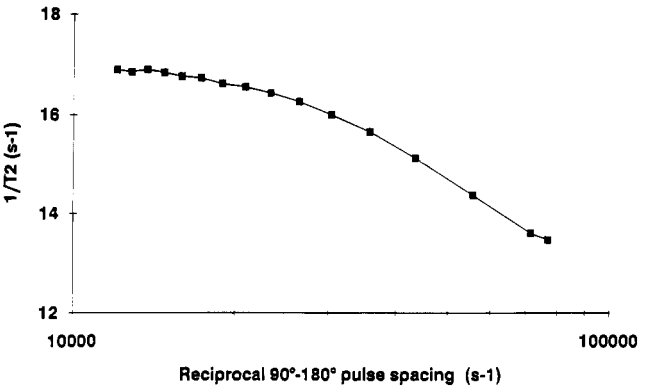

Figure 2. T_2 water proton relaxation rate vs reciprocal interpulse spacing in fresh cod.

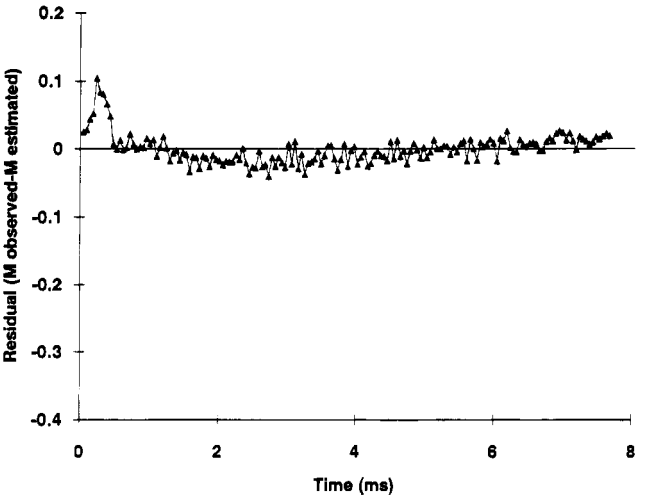

Figure 3. Residuals plot of water proton T_2 magnetization decay measured in fresh cod fillet with an interpulse spacing of 12 μs (one-component relaxation model).

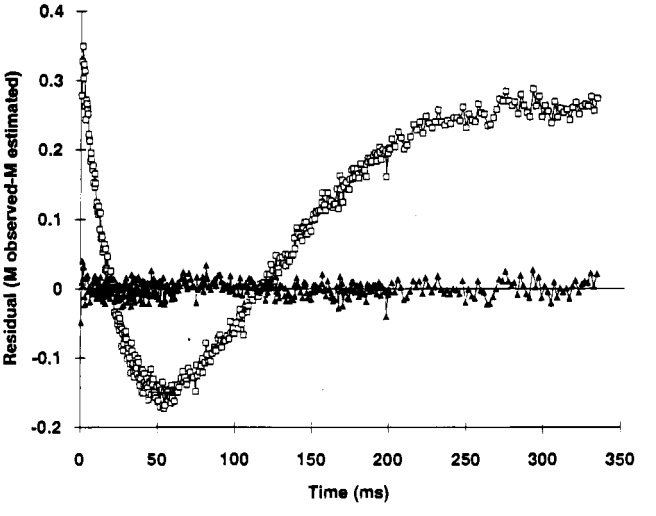

Figure 4. Residuals plot of water proton T_2 magnetization decay in cod fillet heated for 30 min successively at 30 and 40 °C, measured with an interpulse spacing of 99 μs (□, one-component, and, ▲, two-component relaxation models).

After heating at temperatures equal to or greater than 40 °C, however, the proton magnetization decay measured with a long pulse spacing could no longer be analyzed with a one-exponential model. It required the use of two exponentials to be correctly fitted (Figure 4). Hence, the proton magnetization relaxation in cod fillets heated at 40 °C was interpreted in terms of a three-

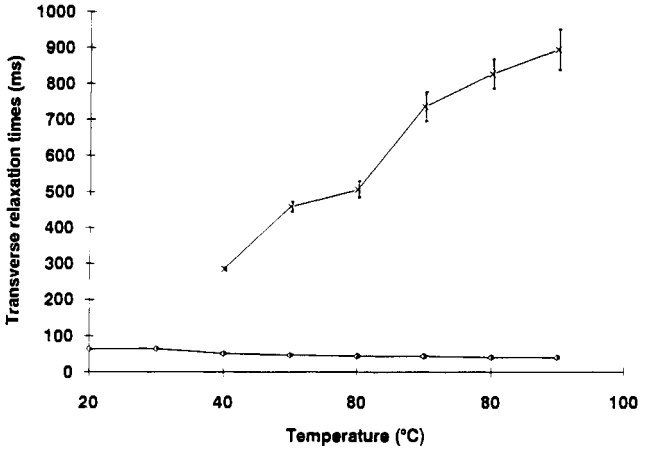

Figure 5. Water proton transverse relaxation times measured at 20 °C in cod fillet heated for 30 min successively at increasing temperatures between 20 and 90 °C (10 °C interval): ◇, intermediate relaxation time (T''_2); ×, longest relaxation time (T'''_2).

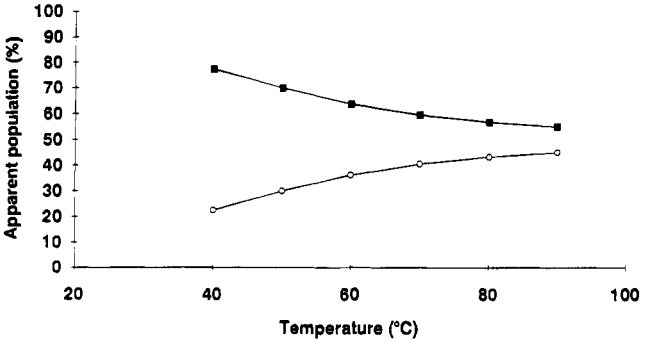

Figure 6. Apparent population associated with the intermediate relaxation time (■) and the longest relaxation time (○) measured at 20 °C in cod fillet heated for 30 min successively at increasing temperatures between 20 and 90 °C (10 °C interval).

component model with $T'_2 \approx 1$ ms, $T''_2 = 50$ ms, and $T'''_2 = 290$ ms. The intermediate relaxation time (T''_2) did not change significantly during the heating process. Thus, the tiny, regular decrease of its value from 65 to 45 ms recorded during the heating of cod between 20 and 90 °C was probably due to the progressive variation in the 90−180° interpulse spacing used for these measurements (41 μs for fresh cod to 322 μs for cod heated at 90 °C). The longest relaxation time (T'''_2), however, varied markedly during heating, increasing from 290 ms after heating at 40 °C to 890 ms after the treatment at 90 °C (Figure 5). The apparent population associated with this longest relaxation component increased regularly as a function of temperature. Without taking into account the rapidly relaxing protons, it went from 23% after heating at 40 °C up to 45% after thermal treatment at 90 °C (Figure 6).

A typical DSC curve of fresh cod fillet is depicted in Figure 7. The endothermic transitions observed around 40 °C are associated with changes in the structure of collagen, myosin (major transition), and sarcoplasmic proteins. The endothermic transitions observed around 70 °C are mainly due to actin with a small contribution from the sarcoplasmic proteins. The small transitions between 40 and 70 °C are associated primarily with sarcoplasmic proteins and to a smaller extent with myosin (Hastings et al., 1985; Poulter et al., 1985).

Frozen Stored Cod. Observations similar to those recorded during heating of cod were made during its frozen storage. Thus, the short transverse relaxation time (T'_2) observed in fresh cod could also be observed

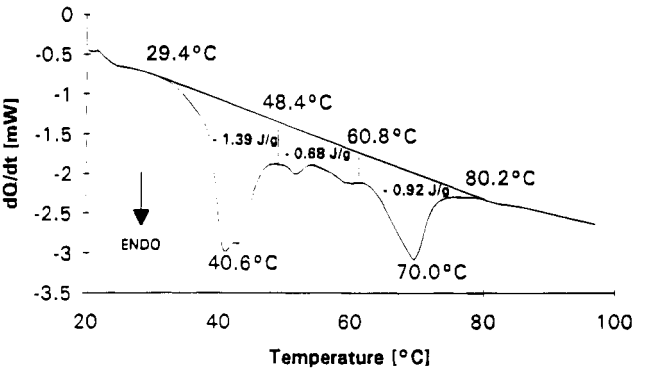

Figure 7. Typ al DSC curve of fresh cod fillet.

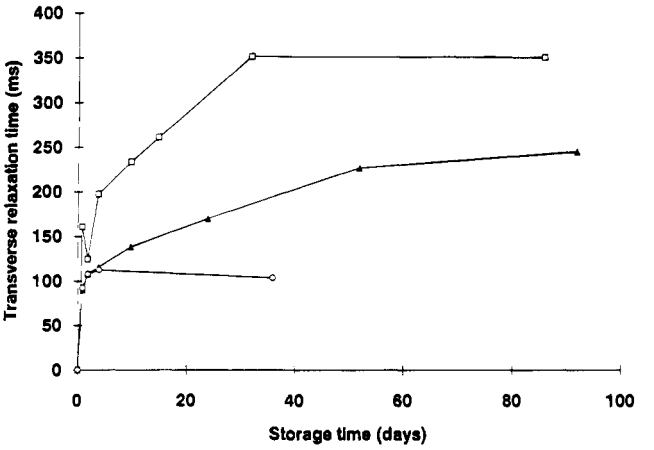

Figure 8. Longest water proton transverse relaxation time observed during storage of cod fillet at −10 °C (□), −20 °C (▲), and −40 °C (○).

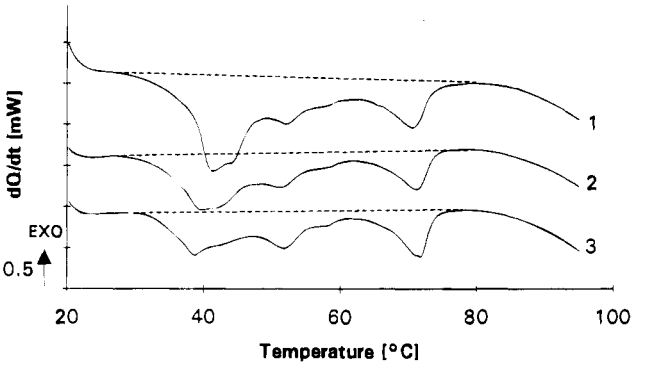

Figure 9. DSC curves of cod fillet: (1) untreated sample; (2) sample stored at −10 °C for 16 days; (3) sample stored at −10 °C for 49 days.

in frozen stored cod. In the same way, the intermediate transverse relaxation time (T''_2) decreased from about 60 ms to about 40 ms during frozen storage, presumably due to the increase in the interpulse spacings used for the measurements. Moreover, a supplemental exponential component, characterized by a long relaxation time (T'''_2), was observed during frozen storage. For storage at −40, −20, or −10 °C this additional component always appeared within the first 24 h. The value of the relaxation time associated with the additional component, however, varied according to the storage temperature; at a given storage time, the higher the temperature, the longer the relaxation time (Figure 8).

DSC curves of fresh cod fillet and cod fillets stored at −10 °C for 16 and 49 days are shown in Figure 9. Although the transitions associated with sarcoplasmin and actin were almost unaltered during frozen storage

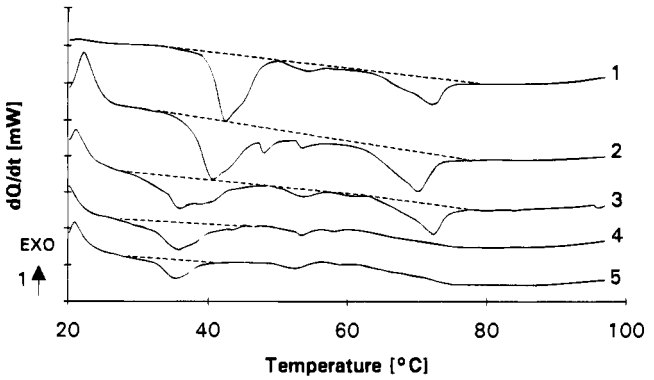

Figure 10. DSC curves of cod fillet: (1) untreated sample; (2) sample treated for 20 min at 1000 bar; (3) sample treated for 20 min at 2000 bar; (4) sample treated for 20 min at 3000 bar; (5) sample treated for 20 min at 3800 bar.

Table 1. Water Proton Transverse Relaxation in Cod Treated for 20 min at Various Pressures

pressure (bar)	T'_2 (ms)	SD	T''_2 (ms)	SD	T'''_2 (ms)	SD
	1.0	0.2	67	3		
1000	1.0	0.1	56	2.1		
2000	0.8	0.2	49	0.8	122	9
3000	0.8	0.1	49	6	119	9
3800	0.7	0.1	47	2	107	8

at −10 °C, the transitions related to myosin were significantly reduced during storage. This reduction increased with storage time (Figure 9).

Pressure-Treated Cod. The T_2 relaxation decays measured in fresh cod and in cod treated for 20 min at 1000 bar were found to be biexponential processes characterized by one relaxation time (T'_2) around 1 ms and the other (T''_2) in the range 55−70 ms (Table 1). However, an additional exponential component associated with a relaxation time (T'''_2) around 120 ms was found in cod treated for 20 min at 2000 bar or above (Table 1).

The corresponding DSC curves (Figure 10) showed a shift of the myosin transitions toward lower temperatures as pressure increased. Moreover, the intensity of this transition decreased drastically when a pressure greater than 1000 bar was applied to cod. The transition corresponding to actin was practically unaltered at pressures below 3000 bar, but could no longer be observed in samples treated with pressures equal or superior to 3000 bar.

DISCUSSION

As for myosin solutions (Nakano and Yasui, 1979; Yasui et al., 1979) and for fish tissue (Lillford et al., 1980) a multiexponential proton transverse relaxation has been found in fresh cod fillet. Although this multiexponential relaxation could *a priori* result from diffusive exchange processes (Belton, 1990), it probably reflects the presence in cod of two magnetically unequivalent states. Proton relaxation associated with the longer relaxation time (T''_2) is governed by chemical exchange. Indeed, the dispersion of the T''_2 relaxation time as a function of the interpulse spacing observed in fresh cod (Figure 2) is indicative of exchange between chemically shifted sites (Belton, 1990).

The appearance of an additional exponential component during heating reflects an increase in the heterogeneity of the sample. Indeed, to observe a supplemental relaxation component, spatial heterogeneity must exist on a large distance scale compared to the diffusion of water molecules. In this case the water diffusion is not sufficiently fast so that all water molecules experience all environments on a short time scale compared to the chemical exchange time scale (Hills et al., 1989).

The temperature at which this additional component appears (40 °C, Figure 5) corresponds to the temperature of transitions related to the denaturation of myosin (Figure 7). Accordingly, the third exponential component can be observed in heated cod fillet only after myosin has been denatured. This finding can be explained by the water-holding capacity of fish, which is reduced by myosin denaturation; in this case syneresis can take place, which will markedly increase the heterogeneity of the sample. Juice can actually be seen on the top of the sample after thermal treatment.

Therefore, the additional component (relaxation time superior to 250 ms) can be tentatively assigned to cooking exudate. This assignment is confirmed by the measurement of transverse relaxation in samples from which the cooking exudate has been eliminated. Indeed, removing exudate from a cod sample heated for 30 min at 90 °C with a Pasteur capillary pipet (16% of the total sample weight can be removed this way) leads to the observation of an additional component characterized by a much shorter transverse relaxation time (Table 2). Although no quantitative information can be gained from the apparent populations (Belton, 1990), the increase of the apparent population associated with the long relaxing component versus temperature (Figure 6) presumably reflects an increase in the amount of cooking exudate. T''_2 values recorded in cod heated successively at various temperatures between 20 and 90 °C (10 °C interval) are slightly higher than those observed in cod heated for 30 min at 90 °C (Figure 5 and Table 2). This is probably due to the longer period of time the sample was at elevated temperature in the former case and reflects presumably a greater extent of protein denaturation.

The same phenomenon, i.e. the appearance of a third exponential component in the decay curve, was observed in cod stored at frozen temperatures (−40, −20, and −10 °C). This additional component characterized by the longest relaxation time can reasonably be associated with thaw exudate. The appearance of a third component during frozen storage of cod could also be explained in terms of protein deterioration, as it is known that protein denaturation occurs during frozen storage of fish flesh (Connell, 1960; Shenouda, 1980; Hastings et al., 1985; Wagner and Añon, 1986; LeBlanc and LeBlanc, 1989). Some denaturation was observed during frozen storage of cod fillet at −10 °C (Figure 9). However, only myosin was partially denatured after 49 days. Therefore, other mechanisms must be also considered here. Among these are physical damage to the tissue caused by ice crystal formation during storage and cross-linking

Table 2. Influence of Removal of Cooking Exudate on Water Proton Transverse Relaxation in Cod Heated at 90 °C for 30 min

treatment	T''_2 (ms)	SD	apparent population (%)	SD	T'''_2 (ms)	SD	apparent population (%)	SD
	65	6						
30 min at 90 °C	49	1	71	3	743	102	29	3
30 min at 90 °C and removing 16% exudate	45	2	76	4	333	107	24	4

of proteins consecutive to action of the trimethylamine oxide demethylase. Both mechanisms will also result in production of thaw exudate.

In the same way, a third exponential component was observed in cod pressure-treated for 20 min at pressures higher than 1000 bar (Table 1). This can be explained in terms of pressure exudation due to protein denaturation. As a matter of fact, it is known that application of high pressure results in protein denaturation (Ohshima et al., 1993; Balny and Masson, 1993). In general, reversible effects are observed below 1000 or 2000 bar, whereas nonreversible protein denaturation is noticed above 2000 bar (Balny and Masson, 1993). DSC curves (Figure 10) show that protein denaturation is very weak in cod treated for 20 min at 1000 bar, while it is important when the pressure is equal or superior to 2000 bar. Accordingly, the third component associated with a long relaxation time found in cod treated for 20 min at pressures equal or superior to 2000 bar can be assigned to pressure exudate resulting from protein denaturation. Thus, although different from denaturation by heat (Ohshima et al., 1993), denaturation of fish muscle proteins by high hydrostatic pressure treatment can also be observed by T_2 relaxation measurements.

CONCLUSION

Proton transverse relaxation in cod fillets is modified by processing (heating, high hydrostatic pressure treatment) and by frozen storage. In all cases a supplemental component appears in the water proton relaxation which is presumably associated with juice exudation. The relaxation time associated with this additional component is sensitive to the temperature of the heat treatment or to the temperature and length of the frozen storage.

Accordingly, the NMR technique described here can be used to detect changes in the properties of fish muscle. NMR might be a useful tool for determining deterioration of the quality of fish muscle which takes place during frozen storage and, therefore, could be a potential method for evaluating the quality of frozen fish.

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