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Determination of Hydrophobicity and Reactive Groups in Proteins of Cod (*Gadus morhua*) Muscle During Frozen Storage

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This study, to elucidate the possible sources of protein denaturation and cross-linkage which cause textural deterioration, was a continuation of our work on the same lot of commercially harvested and processed fillets of Northern bank trawl cod (*Gadus morhua*), frozen stored for ca. 300 days at −30°C, −22°C, −15°C, −12°C and under conditions of fluctuating temperatures simulating industrial practice. Sarcoplasmic, myosin-rich and SDS-soluble protein fractions from each treatment were evaluated for surface hydrophobicity, total, available and unavailable SH—, SS—, aldehydes, free NH_2— and ester-link content. Changes in surface hydrophobicity in the three fractions may be caused by increases in SS— bonding, ester-links, aldehydes and free NH_2— groups. These results demonstrate that hydrophobicity is not only attributed to SS— formation but also to ester-links, aldehydes and free NH_2— groups.

INTRODUCTION

There are few reports on the biochemical changes resulting from frozen storage of fish muscle (Chu & Sterling, 1970; Mao & Sterling, 1970*a,b*; Fennema, 1981), particularly on the effect of temperature fluctuations along the distribution chain from processor to retailer. Since the early 1980s investigations have been carried out on the relationship between hydrophobicity and the physical and chemical changes in proteins (Kato & Nakai, 1980; Nakai, 1983; Nakai *et al.*, 1986; Niwa *et al.*, 1986*a,b*; Owusu-Ansah & Hultin, 1987; Ang & Hultin, 1989). Comparison of results is confounded by the variety of fluorescence probes employed (Shimizu *et al.*, 1986). Increased protein hydrophobicity is related to increased exposure of hydrophobic groups on the protein which, in turn, facilitates protein denaturation. New orientation of hydrophobic amino acid groups (either modified, unmodified or both) push non-polar groups to the surface and polar groups towards the interior of the molecule. Nakai (1983) and Nakai *et al.* (1986) have also reported that protein solubility is directly related to charge frequency and inversely related to hydrophobicity.

Limited work has been carried out on the relationship between hydrophobicity and fresh (Li-Chan *et al.*, 1985; 1987) or frozen (Ang & Hultin, 1989; Niwa *et al.*, 1986*a,b*; Owusu-Ansah & Hultin, 1987) seafood. Hence, the objectives of this study were to evaluate protein hydrophobicity and reactive groups as indicators of denaturation and cross-linkage using sarcoplasmic, myosin-rich and sodium dodecyl sulfate (SDS)-soluble extracts of fish fillets from the same lot of commercially harvested and processed Northern Bank trawl cod (*Gadus morhua*) subjected to the various frozen storage conditions previously reported (LeBlanc *et al.*, 1988). The data obtained were compared with those of unfrozen 48 h post-mortem age (PMA) cod.

MATERIALS AND METHODS

Experimental design

Fish samples were obtained from a single lot of commercially harvested and processed cod (*Gadus morhua*)

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produced by Fishery Products Ltd as premium grade individually polyethylene-wrapped fillets packaged in 2·2 kg waxed cardboard boxes overwrapped in cardboard cases. The fillets were taken from bled, gutted Northern Bank trawl cod which had been held in ice for 48 h after death to permit the resolution of rigor. At this time the fish were filleted, packaged as stated and frozen in a plate freezer at −40°C for 3–4 h with subsequent storage at −30°C prior to air shipment on dry ice from Newfoundland to Halifax, Nova Scotia. Same day shipments were transported from the airport to the University and randomly aliquoted for storage in microprocessor computer controlled temperature compartments at −30°C, −22°C, −15°C, −12°C and a fluctuating temperature program as follows: −30°C (14 days), −15°C (4 days), −22°C (21 days), −12°C (21 days) and a constant −30°C for the remaining storage period. Details of the storage experiment have been previously described (LeBlanc, 1988; LeBlanc & LeBlanc, 1989; LeBlanc *et al.*, 1988). After holding for *ca.* 1 year (346 days), fillets from each storage cabinet were thawed overnight in a cold room at 2°C. Fresh control cod fish were obtained from the live storage tanks of the Fisheries and Oceans Laboratory, Lower Water Street, Halifax, Nova Scotia. The fish were held on ice following decapitation and the control fillets were obtained at 48 h PMA.

Fractionation

Duplicate 900 g samples of the replicate fresh control and frozen fish fillets were cut into 1 cm^3 pieces, mixed and 50 g samples were homogenized in a Lourdes mixer, Model MM1-B. The samples were blended at high speed for four 15-s intervals with a 30-s cooling interval between each 15-s run. The homogenization container was continually cooled in an ice bath. The homogenates were kept cool on ice and weighed immediately into pre-chilled ultracentrifuge tubes held on ice. Sarcoplasmic proteins (SAR) were removed by ultracentrifugation at 45 000 × *g* for 30 min at −5°C in a Beckman Model L-2 preparative ultracentrifuge with a Type 30 rotor. The respective SAR aliquots from the various storage treatments were placed into vials for subsequent same day analysis.

The remaining pellet was gently divided and stirred for 1 h at 2°C with the extraction buffer (1:3 w/w except 1:4 w/w for the fresh tissue control) for myosin used by Connell (1960*b*). The solution was separated from the residue by centrifugation for 10 min at 10000 × *g*. Dilution of the supernate with nine volumes cold double-distilled deionized water brought about the precipitation of myosin-rich protein which was collected by centrifugation for 10 min at 10000 × *g*. The precipitate was dissolved in a 0·6M KCl–40 mM Tris buffer, pH 7·0 (Quass & Briskey, 1968). The resulting solution was then diluted with water to twice its volume (i.e. 0·3M KCl–20 mM Tris buffer, pH 7·0) and then centrifuged at 35 000 × *g* to remove actomyosin and aggregated myosin. This fraction was labelled as the myosin-rich fraction (MR) and was tested for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The SDS-soluble protein was prepared at room temperature by stirring the remaining insoluble protein residue with 5% SDS in double-distilled deionized water (1:5 w/w) overnight. The suspension was centrifuged at 10 000 × *g* for 30 min and SDS in the supernate was removed by shaking with twice its volume of petroleum ether (Mao & Sterling, 1970*b*). This procedure was repeated ten times and subsequently the aqueous solution was dialyzed against double-distilled deionized water overnight in a cold room at 2°C.

Determination of protein concentration

The protein content of samples of the respective SAR, MR and SDS-soluble fractions was determined in duplicate by the biuret method (Snow, 1950) with crystalline BSA as the standard. Double-distilled deionized water, 0·3M KCl–20 mM Tris buffer (pH 7·0) and extracted, dialyzed 5% (w/v) SDS solution were the respective blanks for the three protein fractions analyzed. Spectrophotometric measurements were determined at 540 nm with a Pye-Unicam SP-800 spectrophotometer. The results for the various parameters are expressed per gram protein of the final preparation solution.

Determination of protein hydrophobicity and energy transfer coefficient

Protein hydrophocity was determined in duplicate by the method of Kato and Nakai (1980) using *cis*-parinaric acid (Molecular Probes, Inc.) as the fluorescence probe. Aliquots of the various replicate separated protein fractions were dissolved in 2 ml 0·1M phosphate buffer (pH 7·0) with or without 0·002% (w/v) SDS to make a series of protein solutions of various protein concentrations. An Aminco Bowman spectrofluorometer Model No. 4-8203 was used to measure the relative fluorescence intensities of the buffered protein solutions and of the blanks. The parinaric acid-protein conjugates were excited at 325 nm and the relative fluorescence intensity was measured at 420 nm. The slope (S_0) was calculated from the fluorescence intensity versus protein concentration plot. Energy transfer efficiency was calculated from fluorescence quenching of the tryptophan residues (Sklar *et al.*, 1977) using the equation:

$$T = [1 - (F/F_0)] \times 100 \quad (1)$$

where F is the fluorescence intensity at 340 nm, when excited at 280 nm, of parinaric acid-protein conjugates,

and F_0 is that of the protein alone. The intercept at the zero concentration of protein when T was plotted against protein concentration was designated as the energy transfer coefficient, T_0, using the linear equation:

$$T = T_0 + S_0 \times \% \text{ Protein} \quad (2)$$

Determination of SH— and SS— content

Each of the separated protein fractions was analyzed for total SH— (SH— + reduced SS—), available SH— (free and reactive) and unavailable SH— (buried SH—) by the methods of Habeeb (1972) and Beveridge *et al.* (1974) utilizing Ellman's reagent. The total SH— content of the three replicate fractions was determined in duplicate by dissolving a known amount of protein in 6 ml solution containing 2% (w/v) SDS, 0·08M sodium phosphate buffer (pH 8·0) and 0·5 mg/ml EDTA. Ellman's reagent (0·1 ml) was then added to 3·0 ml of this solution. After 15 min, the solution was read at 412 nm; a reagent blank also was run concurrently. The available SH— content of the various fractions was determined in replicate by mixing 5·0 ml distilled water, 2·0 ml Tris-glycine buffer (pH 8·0) containing 0·12% (w/v) EDTA (denoted as TRIS-Gly), 20 μl sample of known protein content, and 20 μl Ellman's reagent in a test tube. After 2 min, the solution was transferred to a cuvette and read at 412 nm concurrently with determination of a reagent blank. The unavailable SH— was determined as the difference between total and available SH— content.

The SS— content of the same fractions was determined in duplicate by mixing a 1·0 ml sample of known protein content and 20 μl 2-mercaptoethanol (ME). After this mixture had stood at room temperature for 1 h, 10 ml 12% (w/v) trichloroacetic acid was added and mixed. After an additional hour, samples were centrifuged at 5000 × g for 10 min to remove ME. The precipitate was dissolved in 10 ml 8M urea in TRIS-Gly and 40 μl Ellman's reagent and the solution was read at 412 nm.

Determination of aldehyde content

The method of Sawicki *et al.* (1961), modified for proteins by Blumenfeld and Gallop (1962) was used for duplicate analysis of the replicate samples of known protein content. The solutions were examined at 670 nm with a Pye-Unicam SP-800 spectrophotometer and compared with standards containing 0·02 to 0·1 μmoles/ml acetaldehyde.

Determination of free amino content

The method of Habeeb (1966) involving the reaction of trinitrobenzenesulfonic acid (TNBS) was used to quantitatively determine amino groups in duplicate on replicate samples of known protein content. The absorbance of the solution was read at 335 nm using a Pye-Unicam SP-800 spectrophotometer and compared to a blank treated similarly but containing 1 ml water instead of the protein solution. An extinction coefficient of $1{\cdot}4 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ was used to calculate the number of NH_2— groups present.

Determination of ester-link content

The method of Blumenfeld and Gallop (1962) based on simultaneous deesterification and formation of a hydroxamate was used to analyze duplicates of the separated replicate fractions of known protein content. Estimation of bound hydroxamate was made using the procedure of Seifter *et al.* (1960). Absorbance of the standard curves and sets of unknowns were determined at 520 nm using a Pye-Unicam SP-800 spectrophotometer.

RESULTS AND DISCUSSION

Protein concentration, protein hydrophobicity and energy transfer coefficient of sarcoplasmic, myosin-rich and SDS-soluble fractions

Protein concentration of the respective SAR, MR and SDS fractions of the cod (*Gadus morhua*) fillets are shown in Tables 1–3. The protein concentration decreased in all three fractions at the warmer frozen storage temperatures and varied inversely with hydrophobicity and reactive group content.

The S_0 of the respective SAR, MR and SDS fractions of the cod (*Gadus morhua*) fillets decreased in the SAR fraction at colder temperatures of −15°C to −30°C (Tables 1–3). The S_0 of both the MR and SDS fractions decreased from −12°C to −30°C and the fluctuating temperature condition exhibited a value intermediate to that of the −12°C and −15°C treatments. S_0 values were SDS fraction > MR > SAR fraction. The S_0 of both the MR and SDS fractions was lower for the fresh 48 h PMA control fillets than that of the various frozen stored treatments.

The T_0 index is a measure of the fluorescence efficiency of the tryptophan amino acids in the protein molecule. T_0 values varied with the fraction analyzed and with storage temperature (Tables 1–3). In the SAR and MR fractions, T_0 increased with colder storage temperatures. T_0 values of the −30°C treatment and the fresh 48 h PMA controls were similar for all fractions. The SDS fraction exhibited still higher T_0 values than the SAR and MR fractions but less overall change. The higher T_0 coefficient in this fraction was due to SDS disruption of both van der Waals and ionic bonds that caused the proteins to unfold, denaturate and expose their tryptophan groups.

Table 1. Protein Concentration, S_0, T_0, Total SH—, Available SH—, Unavailable SH—, SS—, Aldehydes, Free NH_2^- and Ester-link Content of the Sarcoplasmic Fraction of Cod (*Gadus morhua*) Fillets

Analysis	Treatment					
	Fresh 48h PMA[a]	Fluctuating temperature	−30°C	−22°C	−15°C	−12°C
Protein (mg/ml)	54·2	20·0	49·1	39·8	29·2	21·4
S_0	12·2	19·0	9·4	10·8	28·1	25·3
T_0	78·8	69·8	78·3	77·4	73·4	60·1
Total SH—, mmoles/g PRO	0·054	0·21	0·087	0·13	0·24	0·27
Avail. SH—, mmoles/g PRO	0·042	0·082	0·064	0·092	0·16	0·16
Unavail. SH—, mmoles/g PRO	0·012	0·13	0·024	0·038	0·077	0·11
SS—, mmoles/g PRO	0·10	0·26	0·11	0·11	0·16	0·34
Aldehydes, μmoles/g PRO	0·23	4·8	1·2	1·9	3·7	5·7
Free NH_2^- mmoles/g PRO	1·1	1·7	1·1	1·3	1·6	1·6
Ester-links, mmoles/g PRO	0·97	5·8	1·4	2·6	3·1	5·2

[a] Post-mortem age.

Total SH—, available SH—, unavailable SH— and SS— content of sarco-plasmic, myosin-rich and SDS soluble fractions

The total SH—, available SH—, unavailable SH— and SS— content of the respective SAR, MR and SDS fractions of the cod (*Gadus morhua*) fillets are shown in Tables 1–3. The total SH— content in the SAR increased with warmer frozen storage temperatures. Little change occurred in the MR and SDS fractions between −30°C and −15°C whereas at −12°C there was a large increase. The total SH— content of the fluctuating storage treatment for all three fractions resembled the results found at the warmest storage condition of −12°C. The total SH— content for the MR fraction of the frozen fillets stored between −30°C and −15°C parallelled the values found for the fresh 48h PMA controls.

Table 2. Protein Concentration, S_0, T_0, Total SH—, Available SH—, Unavailable SH—, SS—, Aldehydes, Free NH_2^- and Ester-link Content of the Myosin-rich Fraction of Cod (*Gadus morhua*) Fillets

Analysis	Treatment					
	Fresh 48h PMA[a]	Fluctuating temperature	−30°C	−22°C	−15°C	−12°C
Protein (mg/ml)	27.9	15.2	22.8	21.6	16.6	7.60
S_0	10.2	49.1	11.5	16.1	26.2	69.9
T_0	84.5	77.1	84.1	83.3	79.2	70.2
Total SH—, mmoles/g PRO	0.070	0.11	0.070	0.070	0.074	0.14
Avail. SH—, mmoles/g PRO	0.028	0.052	0.038	0.035	0.030	0.056
Unavail. SH—, mmoles/g PRO	0.032	0.058	0.033	0.034	0.043	0.084
SS—, mmoles/g PRO	1.4	2.3	1.3	2.2	2.2	2.8
Aldehydes, μmoles/g PRO	0.40	5.5	0.58	1.4	3.1	5.3
Free NH_2^- mmoles/g PRO	0.43	0.58	0.43	0.49	0.65	1.0
Ester-links mmoles/g PRO	0.23	1.6	0.28	0.37	0.88	3·0

[a] Post-mortem age.

Table 3. Protein Concentration, S_0, T_0, Total SH-, Available SH-, Unavailable SH-, SS-, Aldehydes, Free NH_2^- and Ester-link Content of the SDS-soluble Fraction of Cod (*Gadus Morhua*) Fillets

Analysis	Treatment					
	Fresh 48h PMA[a]	Fluctuating temperature	−30°C	−22°C	−15°C	−12°C
Protein (mg/ml)	28·8	10·5	20·6	19·2	13·4	9·56
S_0	28·1	138·0	29·7	58·5	64·4	143·0
T_0	94·5	97·6	93·1	92·6	96·0	95·6
Total SH—, mmoles/g PRO	0·042	0·10	0·058	0·064	0·065	0·090
Avail. SH—, mmoles/g PRO	—	—	—	—	—	—
Unavail. SH—, mmoles/g PRO	—	—	—	—	—	—
SS—, mmoles/g PRO	1·4	3·3	1·5	1·8	1·9	2·4
Aldehydes, μmoles/g PRO	0·95	7·6	1·5	1·8	3·6	9·5
Free NH_2^-, mmoles/g PRO	0·58	0·78	0·65	0·63	0·70	0·92
Ester-links, mmoles/g PRO	0·78	12·1	2·7	3·6	8·1	17·2

[a] Post-mortem age.

The free available or unreacted SH— content of the SAR fraction increased at the warmer storage temperatures whereas the change in the MR fraction for the available SH— content was similar to the findings for total SH— content. The available SH— content of both the SAR and MR fractions was lowest in the control fillets. The available SH— content of the fluctuating temperature treatment for the MR fraction had values similar to those found at −12°C.

The unavailable SH— content represents SH— groups buried in the protein molecule. Both the SAR and MR fractions showed an increase in unavailable SH— with increasingly warmer frozen storage temperatures. The SAR fraction of the fresh 48 h PMA controls had the lowest unavailable SH— content. Conversely, the fluctuating treatment exhibited the highest unavailable SH— content for the SAR fraction whereas values for the MR fraction were intermediate to those of the −15°C and −12°C storage conditions.

Disulfide links can be either inter- or intramolecular. The SS- content of both the SAR and SDS fractions from the fillets held at −30°C, −22°C and −15°C was similar to that of the fresh 48 h PMA controls. In addition, the SAR and MR fractions extracted from the fillets held at −12°C exhibited a higher SS— content whereas the SDS-soluble fraction extracted from the fillets held under the fluctuating temperature conditions showed the highest SS— content.

The literature abounds with conflicting results for changes in total SH—, available (free or reactive) SH— and SS— content of proteins in relation to frozen storage temperature and time. Numerous researchers (Seagran, 1956; Connell, 1960*a*; Buttkus, 1970; Mao & Sterling, 1970*b*; Poulter & Lawrie, 1978) have reported no change in the SH— content of blackfish (SDS-soluble fraction), rockfish, whiting, herring, lemon sole, skate, cod, rabbit and trout samples. It is noteworthy that some of these results were obtained in the presence of blocking agents such as sulfites, ME and *N*-ethylmaleimide that prevented formation of both SS— and non-reducible covalent bonds (Buttkus, 1970; Matthews *et al.*, 1980).

Conversely, results of the present study concur with those of Dzinleski *et al.* on beef (1969) and Lim and Haard's (1984) findings on Greenland halibut. Mao and Sterling (1970*b*) observed a slight decrease in SH— content of the myosin fraction for blackfish whereas Lim and Haard (1984) reported a large decrease in free SH— (total SH—) for both the water- and salt-soluble fractions of halibut, and Hofmann and Hamm's (1978) results showed a net decrease in available SH— content for cod. A decrease in SH— content was also found in frozen, stored chicken (Khan, 1966) and in iced fish in the absence of protein aggregation (Buttkus, 1970). Levitt (1966) reported that the enzymes of the SAR fraction which are inactivated by freezing may be SH— proteins.

Aldehyde, free amino and ester-link content of sarcoplasmic, myosin-rich and SDS-soluble fractions

The results for the aldehyde, free NH_2^- and ester-link content of the respective SAR, MR and SDS fractions of the cod (*Gadus morhua*) fillets are also shown in

Tables 1–3. The aldehyde content of the respective SAR, MR and SDS fractions increased with warmer storage temperatures. All fractions of the fresh 48 h PMA controls were lower in aldehyde content than the frozen stored samples whereas the aldehyde content of the fluctuating treatment was similar to that of the fillets stored at −12°C. It is noteworthy that the aldehyde content was much lower than that of the other reactive groups analyzed.

There is a dearth of information on frozen storage changes in aldehyde content of proteins due to storage temperature or time or both. Mao and Sterling (1970*b*) observed slightly increased aldehyde content in the myosin fraction of blackfish whereas the SDS-soluble fraction remained unchanged. Present results showed increases in aldehyde content at the warmer frozen storage temperatures in all three fractions from the cod fillets. Researchers in the 1960s (Bornstein & Piez, 1966; Rojkind *et al.*, 1968; Paz *et al.*, 1969) postulated that aldehydes are formed by the oxidation of the ϵ-NH_2^- group of lysine. Displacement of an ϵ-NH_2^- group by oxidation to aldehydes or ketones (Siegel *et al.*, 1970; Siegel, 1974; Stimler & Tanzer, 1977) results in the loss of water hydrates from the terminal group. A number of these new hydrophobic groups, coupled with the increase in other hydrophobic parameters, could twist the protein, increase hydrophobic interactions and maximize nonaqueous interactions. As well, this oxidation to aldehydes favours the release of ammonia (NH_3) which results in a new reactive group that may have a major role in crosslinking and mutarotation of proteins during frozen storage. The loss of ϵ-NH_2^- groups of lysine by deamination to aldehydes may partially explain previous findings from our laboratory where exponential increases in NH_3 were found in samples analyzed from the same lot of fish during frozen storage under these various temperature conditions (LeBlanc *et al.*, 1988). Aldehydes, resulting from this reaction, may partake in an aldol condensation at the acidic conditions found in frozen stored fish (MacCallum *et al.*, 1968; Botta *et al.*, 1973). This reaction causes a localized pH change between ice and water that favours exclusion of protons from the ice and inclusion of anions by the ice. Frozen storage preservation fosters these salting out effects (Hultin, 1986). In addition to oxidation of the ϵ-NH_2^- group of lysine, aldehydes in the protein moiety may result from autoxidation of polyunsaturated lipids that covalently, ionically and hydrophobically bond to the protein. Unpublished observations from our laboratory showed that fillets stored at fluctuating and −12°C treatments were *ca.* three times higher in free radical content than those stored at −30°C.

Similar to aldehyde formation, free NH_2^- content increased gradually at the warmer storage treatments (Tables 1–3). The fresh 48 h PMA controls were generlly lower in free NH_2^- content than the frozen stored samples. The free NH_2^- content was highest for the fluctuating temperature treatment of the SAR fraction whereas values were intermediate to the −15°C and −12°C data for the MR and SDS fractions. Overall, the changes in the free NH_2^- content were much less than those found for the other reactive groups analyzed.

The small change in the free NH_2^- content in the present study can be explained by the disappearance of the ϵ-NH_2^- group of lysine (Mihalyi, 1963), the concomitant increases in fragmented proteins (LeBlanc & LeBlanc, 1989) and the changes in nonprotein nitrogen and amino acids reported by Khan (1966) and Jiang and Lee (1985). Jiang and Lee's (1985) findings indicated an increase in the total free amino acid content of frozen fish muscle with storage time which correlated with decreased protein stability in the muscle. Similarly, Khan (1966) found an increase in the NPN fraction of frozen stored chicken muscle. Conversely, Lim and Haard (1984) found no appreciable overall change in the amino acid composition for fresh, frozen minced or SDS-insoluble fractions of Greenland halibut. This finding was explained as either due to limited participation by amino acids in protein crosslinking or the hydrolysis procedure used to prepare the sample. Microstructural alterations viewed by light, scanning and transmission electron microscopy on samples from this lot of fish (LeBlanc, 1988) further support the molecular changes found by these chemical analyses. Research by Poulter and Lawrie (1977) used the lysine-specific 'fluoro-dinitro-benzene' to measure the increase in free NH_2^- content of frozen stored whiting, lemon sole and skate muscle. Changes as found in their study may be caused by rotation of the protein from filamentous to globular during denaturation or as proteolytic hydrolysis of the various proteins into lower molecular weight polypeptides (Love, 1966; Shenouda, 1980; LeBlanc & LeBlanc, 1989) or both. Proteolytic hydrolysis was observed in the SDS-PAGE of the myofibrillar protein profiles (LeBlanc & LeBlanc, 1989). The new position for the NH_2^- group may not permit crosslinking with formaldehyde or other aldehydes into Schiff bases. Both Buttkus (1967) and Poulsen and Lindelov (1981) observed the disappearance of amino acids with the addition of aldehydes. In particular, Buttkus demonstrated that the loss of the ϵ-NH_2^- group of lysine in the presence of malonaldehyde was temperature-dependent, viz. +20°C > −20°C > 0°C. Only NH_3 and secondary amines, not amides, have been reported to react in the presence of aldehyde (HCHO) and protein to form the Prot—NH—CH_2—NH—CH_2—Prot crosslinks (Fraenkel-Conrat & Mecham, 1949). Thus the free NH_2^- content in the SAR fraction of the −12°C and fluctuating treatments (Table 1) may decrease because of chemical modification, covalent crosslinking, denaturation or oxidation during frozen storage caused by changes in the solubility gradient.

Changes in ester-links for the three fractions (Tables 1–3) corresponded with those found for aldehydes, viz., SDS > SAR > MR fraction. Regardless of fraction analyzed, the fresh 48 h PMA controls were lower in ester-links than the various frozen stored fillets. In the SAR fraction the fluctuating temperature treatment exhibited the highest ester-link content while in the MR and SDS fractions the values were intermediate to those of the −12°C and −15°C treatments. Mao and Sterling (1970*b*) found increased ester links in −5°C frozen (30 h) and −5°C frozen-stored (30 days) myosin and SDS fractions of blackfish compared to fresh controls. Ratios calculated from Mao and Sterling's study were similar to those presently found for the MR fraction of cod stored at <−22°C for *ca.* 300 days. The SDS fraction also had increased ester-links in the frozen stored samples but ratios determined from Mao and Sterling's (1970*b*) data were much lower than those found in the present study. Large increases occurred in ester-links at elevated frozen storage temperatures, particularly in the SDS fraction (Tables 1–3).

Evaluation of hydrophobicity and reactive groups of the various storage treatments showed distinct changes in the SAR, MR and SDS-soluble fractions extracted from the fillets. This may be explained by exposure of hydrophobic sites due to protein unfolding at the warmer frozen storage temperatures. As a result, protein solubility decreases and hydrophobic interactions are activated between the exposed hydrophobic sites that cause protein molecule aggregation—a mechanism of frozen storage denaturation. In addition, SS— formation has been implicated as a causative agent in protein aggregation during frozen storage (Buttkus, 1970; Matsumoto, 1980; LeBlanc, 1988; LeBlanc and LeBlanc, 1989). Consequently, SS— changes should parallel the pattern shown for the SH— content in Tables 1–3.

The denaturation process that leads to increased hydrophobicity, concomitant decreased solubility (Nakai, 1983) and increased muscle toughness is a combination of the physical frozen storage changes and the chemical protein deterioration. During the frozen storage process both proteins and enzymes are solubilized because of the localized increases in salt concentrations, the acidic environment and a decrease in charge frequency. The tissue water described as 'free' and available as a solvent becomes frozen; that water described as 'bound' on specific sites remains fluid and is not available as a solvent (Dyer & Dingle, 1961; Charm & Moody, 1966; Pham, 1987). The degree of binding of 'bound' water probably reflects the difference in molecular structure of the solids of various food products. Kent (1975) reported temperature effects on 'bound' water to be more drastic than the length of frozen storage. Because more than 90% of the tissue moisture freezes at common freezing temperatures, the concentration of soluble solutes increases *ca.* tenfold (Riedel, 1956; Duckworth 1971; Pham, 1987). Such an increase in salt concentration has been reported to affect both cell permeability and protein properties by causing competition and some breakdown of existing electrostatic bonds. The increased salt concentration also has been found to affect other secondary forces *viz.* van der Waals, hydrogen and hydrophobic bonds. The amount of dissociational, aggregational and conformational proteinaceous change depends on the type of salt and tissue.

As a result, enzymatic and biochemical reactions are accelerated inter- and intramolecularly. These reactions lead to SH— crosslinking as well as deamination and oxidation of free NH_2^- groups as seen in the present study. In addition, a number of reactions occur that change the chemical nature of the proteins *viz.* increases in SS—, aldehydes, ester-links and free NH_2^- groups (Tables 1–3). These changes increase protein hydrophobicity and cause dehydration at these localized sites because of the loss of hydrophilic groups. Increased hydrophobicity decreases the intramolecular distances between the protein reactive groups giving a higher protein muscle density. The crosslinks, hydrophobic groups and loss of internal H_2O result in muscle fibrousness that is detected as 'toughness' during extended frozen storage, particularly at the elevated or fluctuating temperature conditions. Present findings suggest that changes in reactive groups of SAR may have greater implications in frozen storage denaturation of fish muscle than previous reports have noted.

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