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Effect of Antioxidants, Citrate, and Cryoprotectants on Protein Denaturation and Texture of Frozen Cod (*Gadus morhua*)

FARAH BADII AND NAZLIN K. HOWELL*

School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, United Kingdom GU2 7XH

To investigate the role of antioxidants and cryoprotectants in minimizing protein denaturation in frozen lean fish, cod fillets were treated with either antioxidants (vitamin C (500 mg kg^{-1}) or vitamin C (250 mg kg^{-1}) + vitamin E (250 mg kg^{-1})), antioxidants (vitamins C + E 250 mg kg^{-1}each) with citrate (100 mg kg^{-1}), cryoprotectants (4% (w/w) sucrose + 4% (w/w) sorbitol), or a mixture of antioxidants (vitamins C + E 250 mg kg^{1}), citrate (100 mg kg^{-1}), and cryoprotectants (sucrose 40 g kg^{-1} + sorbitol 40 g kg^{-1}). Untreated and treated fish samples were stored at −10 °C; cod fillets stored at −30 °C were used as a control. Stored frozen samples were analyzed at intervals for up to 210 days for changes in protein extractability, thermodynamic parameters (transition temperature T_m and enthalpy ΔH), structure by FT−Raman spectroscopy, and rheological properties by large and small deformation tests. Results indicated that protein denaturation and texture changes were minimized in the presence of cryoprotectants, as well as in the presence of antioxidants with citrate, antioxidants alone, or the mixture of antioxidants, citrate, and cryoprotectants. In the presence of increased formaldehyde levels in fish treated with vitamin C, toughening was still lower compared to that of the −10 °C control due to the antioxidant property of vitamin C. Thus, ice crystal formation and lipid oxidation products are the major factors that cause protein denaturation in lean frozen fish, and antioxidants in addition to cryoprotectants can be used to minimize toughness.

KEYWORDS: Frozen fish; protein denaturation; antioxidants; cryoprotectants; texture; formaldehyde formation; differential scanning calorimetry; Raman spectroscopy

INTRODUCTION

Although frozen storage prevents microbial spoilage of fish tissue, it is accompanied by changes in protein solubility, protein functionality, texture, and nutritional quality (*1*−*4*). Fish proteins, particularly myosin, are susceptible to aggregation at high storage temperatures and with time. Badii and Howell (*4*) compared frozen fillets of a formaldehyde-forming species (cod) with haddock, which forms negligible formaldehyde on frozen storage, and found little difference in the physical−chemical properties and texture; indicating that formaldehyde is not a major factor causing toughness in frozen cod fillets.

Factors that may cause biochemical changes in frozen lean gadoid fish, but not yet proven, are formaldehyde formation, ice crystal formation, and the effect of lipid oxidation products on proteins. Lipid oxidation causes rancidity and major changes in the color and flavor of fish (*5*). In the authors' laboratory, Saeed and Howell (*6*) showed the transfer of free radicals from fish oil to proteins (including Atlantic mackerel myosin as well as ovalbumin, lysozyme, and amino acids) by ESR spectroscopy, followed by aggregation of the proteins and amino acids as shown by fluorescence spectroscopy. Fatty fish were also susceptible to toughening on frozen storage which could be minimized by antioxidants. Therefore, to test the role of lipid oxidation products on lean fish texture, cod treated with natural antioxidants vitamins C and E were compared to untreated cod; the effect of antioxidants on lean fish texture has not been reported hitherto. There is a preference for using natural antioxidants, as synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are implicated in toxicological changes (*7*, *8*). In addition, because the lipid oxidation process is considered to be initiated by singlet oxygen, enzymes, or metal ions, a metal chelator (citrate) was added to the antioxidants to sequester iron, which is present in abundant supply in myoglobin (*9*).

Apart from lipid oxidation products, ice crystal formation can also damage fish proteins during freezing and storage (*10*, *11*). It is well-established that large ice crystals formed extracellularly by slow freezing can cause greater damage to cells than small intracellular ice crystals resulting from rapid freezing (*12*). In addition, freezing results in the concentration of solutes and restructuring of water molecules bound to proteins (*12*). Denaturation of proteins in this way can expose reactive groups which can interact to form aggregates (*13*). However, details of these reactions have not been reported for frozen fish muscle.

* Corresponding author. Telephone 44 1483 686448; fax 44 1483 576978; e-mail N.Howell@surrey.ac.uk.

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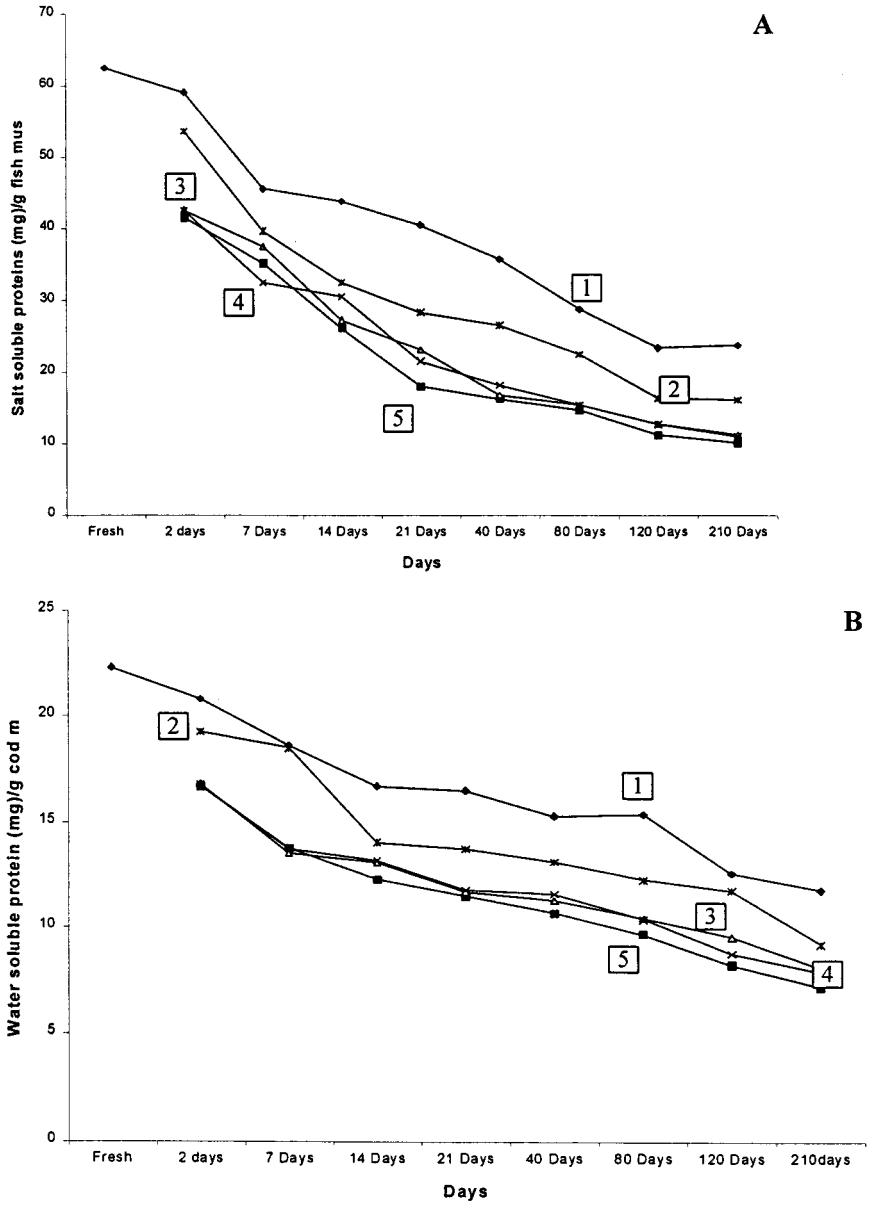

Figure 1. Salt-soluble proteins (A) and water-soluble proteins (B) from cod mince stored for up to 210 days at (1) –30 °C; (2) –10 °C with cryoprotectant (sucrose 40 g kg^{-1} and sorbitol 40 g kg^{-1}); (3) –10 °C with vitamin C 500 mg kg^{-1}; (4) –10 °C with vitamins C and E (250 mg kg^{-1} each); and (5) –10 °C without antioxidants or cryoprotectant (control).

To investigate the effect of ice crystals on protein structure and function, the role of cryoprotectants that depress the freezing point and therefore minimize denaturation was tested. The application of cryoprotectants to mainly surimi products has been reported by several researchers (*14*−*17*). One combination of cryoprotectants reported to be effective in surimi, namely sucrose (4%) and sorbitol (4%) w/w in distilled water (*17*), was selected for the present study.

The merit and effect of natural antioxidants, citrate, and cryoprotectants on fish quality and protein aggregation during freezing storage of cod muscle were investigated in a model study using cod stored with or without protectants at −10 °C; a control sample was stored at −30 °C. Changes in protein extractability, thermodynamic parameters (transition temperature T_m and enthalpy ΔH), structure by FT−Raman spectroscopy, and texture properties by large and small deformation rheology were investigated. As formaldehyde has been implicated as a major cause of toughening in gadoid frozen fish like cod (*18*, *19*), formaldehyde levels were also monitored.

MATERIALS AND METHODS

Materials. Vitamin C, vitamin E, sucrose, sorbitol, and citric acid were obtained from Sigma-Aldrich Ltd, Poole, Dorset, UK. Cod fillets were purchased from J and M Sea Food, Guildford, UK, and delivered in ice to the laboratory.

Methods. Skinned cod fillets (6 kg) were lightly minced, and 1-kg portions were used for each treatment. Appropriate antioxidants − either Vitamin C (500 mg), Vitamin E + C (250 mg of each), citric acid (100 mg), or a mixture of Vitamin E + C (250 mg each) with citric acid (100 mg) − were dissolved in 50 mL of distilled water and mixed thoroughly with 1 kg of fish flesh at +4 °C. Vitamin E was not dispersed in water but was mixed directly into the fish mince. In addition, cryoprotectant containing sucrose (40 g kg^{-1} fish) + sorbitol (40 g kg^{-1} fish) in 50 mL of distilled water was added to 1 kg of fish, with or without Vitamins E + C (250 mg each) + citric acid (100 mg). Samples for each treatment were divided into 100-g portions and stored at −10 °C. For control samples, 50 mL of distilled water was added to 1 kg of fish flesh, and 100-g portions were stored at −10 and −30 °C for up to 210 days. The stored frozen samples were analyzed for the following: protein extractability in water and salt; texture

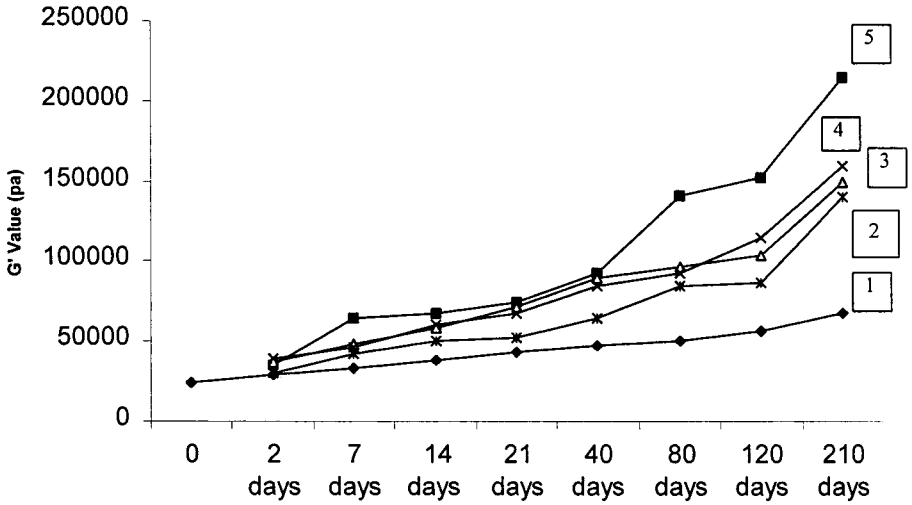

Figure 2. G′ values for cod muscle stored at (1) –30 °C; (2) –10 °C + cryoprotectant (sucrose 40 g kg^{-1} and sorbitol 40 g kg^{-1}); (3) –10 °C + antioxidants vitamin C 500 mg kg^{-1}; (4) –10 °C + vitamins C and E (250 mg kg^{-1} each); (5) –10 °C (control) for up to 210 days. Figures are mean values for three replicate samples.

changes by small deformation rheology; and structural changes by differential scanning calorimetry (DSC) and FT−Raman spectroscopy. For large deformation rheology, a limited experiment was undertaken using fillet pieces soaked in cryoprotectant. The number of replicates used for each test is described below. The tests were undertaken over a period of frozen storage up to 52 weeks.

Protein Extractability. Proteins were extracted from three different portions of thawed minced muscle with either a solution of 50 mM phosphate buffer pH 7.5 (water-soluble proteins, WSP) or 50 mM phosphate buffer + 0. 8 M NaCl pH 7.5 (salt-soluble proteins, SSP) (*4*). Protein concentration was determined by the Bradford method using the Coomassie Blue reagent in triplicate (*4*).

Rheology and Texture Analysis. *Small Deformation Testing.* Small deformation rheological tests of minced fish paste, with or without either antioxidants, citrate, or cryoprotectants, were undertaken on a Rheometrics constant stress 100 rheometer using a temperature sweep from 25 to 90 °C, and cooling back to 25 °C at a heating rate of 2 °C/min. The applied stress was 1 Pa to keep the oscillatory strain at about 1%, sufficiently low to ensure the measurements taken were within the linear viscoelastic region (*20*). The frequency of oscillation of 1 rad/sec was chosen so that sufficient data were obtained without compromising the measurement of entanglements. A 40-mm parallel plate geometry was used with a gap of 2 mm, and the sample was surrounded by silicone oil to prevent evaporation of solvent. The elastic modulus G′ values were recorded. Samples were tested at 0, 2, 7, 14, 21, 40, 80, 120, and 210 days in triplicate.

Large Deformation Testing. Cod fillets were cut into pieces (2 × 2.5 × 1.5 cm) and soaked in the cryoprotectant mixture (sucrose 40 g kg^{-1} fish + sorbitol 40 g kg^{-1} fish) for 1 h at 4 °C, followed by storage at −10 °C for 10 weeks. Samples (four replicates), with or without the cryoprotectant were subjected to compression testing on a TAX-T2 texture analyzer (Stable Microsystems, Godalming, UK), using a cylindrical probe of 35-mm diameter according to the manufacturer's instructions. The force (kg) required for compression of the muscle to a distance of 10 mm was measured.

Differential Scanning Calorimetry. Changes in thermodynamic parameters of frozen minced cod with or without either antioxidants or cryoprotectants stored for 120 days at −10 or −30 °C were examined in duplicate using a DSC VII calorimeter (Setaram, Lyon, France). Thawed cod muscle (0.750 g) and reference (water) were heated at 0.5 °C/min from 10 °C to 80 °C. Heat absorbed or released by the denatured sample results in an endothermic or exothermic peak as a function of temperature. The temperature reached when half of the protein is denatured is referred to as the transition temperature (T_m) and was measured at the tip of the peak. The total energy required to denature the protein, the enthalpy change (ΔH), was measured by integrating the area under the peak (Setaram DSC handbook and software). DSC analysis was performed in duplicate. For some samples the experiments were repeated, and the coefficient of variation of the method was found to be less than 5%.

FT−Raman Spectroscopy. Cod samples in the presence of either antioxidant, antioxidant with citrate, cryoprotectants, or a mixture of antioxidant/citrate/cryoprotectant were stored at −10 °C for 120 days. Control untreated samples were stored at −10 and −30 °C. Frozen fish samples were thawed and characterized in 7-mL glass containers (FBG-Anchor, Cricklewood, London) on a Perkin-Elmer System 2000 FT−Raman spectrophotometer with excitation from a Nd:YAG laser at 1064 nm. Frequency calibration of the instrument was performed using the sulfur line at 217 cm^{-1}. Duplicate sets of the samples were prepared and analyzed at 4 °C on two different occasions using laser power of 1785 mW. The spectra were an average of 64 scans which were baseline corrected, smoothed, and normalized to the intensity of the phenylalanine band at 1004 cm^{-1} (*21*, *22*). The recorded spectra were analyzed using Grams 32 (Galactic Industries Corp., Salem, NH). Assignments of the bands in the spectra to protein vibrational modes were made based on the literature (*21*, *23*, *24*).

Formaldehyde Concentration. Formaldehyde was extracted by acid distillation (*25*) in triplicate from three different portions of treated and untreated minced cod fillets, and each of the three extracts were measured in duplicate by the Nash (*26*) method, yielding free plus bound formaldehyde. The samples were tested after storage at −10 and −30 °C for 120 and 210 days.

Statistical Analysis. For most experiments, the mean and standard deviation were calculated and *t*-tests were performed to compare the different treatments. In the case of DSC and Raman spectroscopy, duplicate samples were used, with the exception of a few experiments which were repeated, and for both methods the coefficient of variation was found to be less than 5%. To discriminate between treatments, the standard deviation has been assumed from the coefficient of variation of 5% for the method, and upper and lower 95% confidence bands were calculated for each measurement.

RESULTS AND DISCUSSION

Protein solubility of cod mince in buffer or salt solution decreased more rapidly with time of storage at −10 °C compared with those stored at −30 °C ($p < 0.0005$) (**Figure 1A**,**B**). The alteration of protein extractability is a useful factor which may be used to determine the textural quality of frozen fish muscle, as protein aggregation is accompanied by a significant decrease in their solubility (*4*, *27*−*30*). In this study, the presence of antioxidants, and particularly cryoprotectants, enhanced the solubility compared with that of the untreated control sample

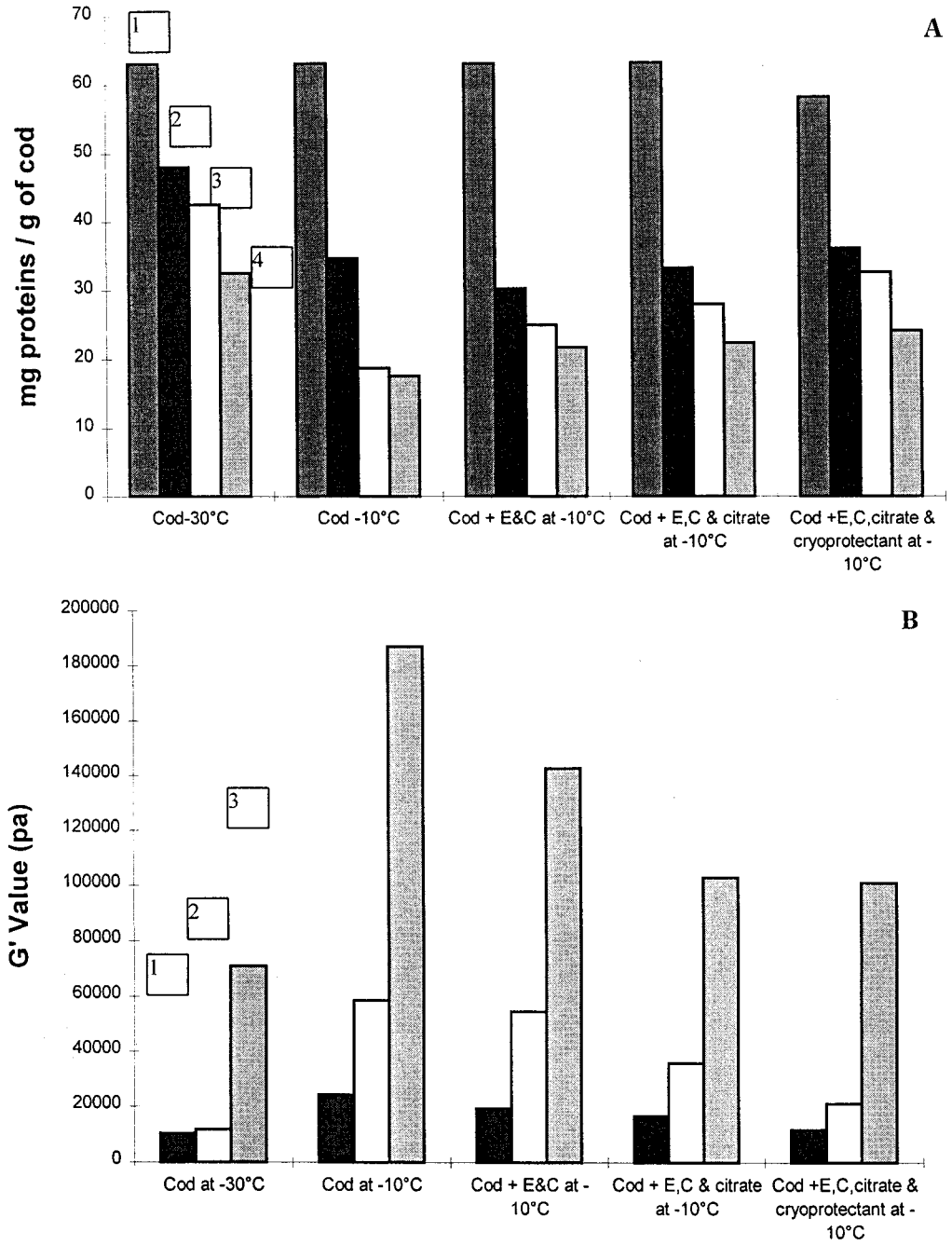

Figure 3. (A) Salt soluble protein concentration (mg/g) in cod muscle stored at –30 or –10 °C with or without antioxidant [(vitamins E + C 250 mg kg^{-1} each), (vitamins E + C 250 mg kg^{-1} each and citrate 100 mg kg^{-1}), or (vitamins C + E (250 mg kg^{-1} each) + cryoprotectant (sucrose 40 g kg^{-1} and sorbitol 40 g kg^{-1}))] for (1) 0 days; (2) 14 days; (3) 35 days; and (4) 120 days. (B) G′ values for cod muscle stored at –30 and –10 °C with or without antioxidant [(vitamins E + C 250 mg kg^{-1} each), (vitamins E + C 250 mg kg^{-1} each and citrate 100 mg kg^{-1}), or (vitamins C + E (250 mg kg^{-1} each) + cryoprotectant (sucrose 40 g kg^{-1} and sorbitol 40 g kg^{-1}))] for (1) 14 days; (2) 35 days; and (3) 120 days. Values are mean values for three replicates.

stored at −10 °C (**Figure 1A**,**B**). In particular, salt-soluble proteins (SSP) obtained from samples treated with cryoprotectants and kept at −10 °C for 120 days decreased by about 32%, while SSPs from untreated samples were reduced by 57%, indicating significantly enhanced solubility in the presence of cryoprotectants ($p < 0.002$). In addition, antioxidants vitamin C or vitamins C+E gave significantly higher SSP values compared with those of control samples at −10 °C ($p < 0.01$). However, there was no significant difference between the two antioxidant systems; that is, vitamin C and vitamin C+E ($p < 0.1$).

Water-soluble proteins decreased by 21 and 39%, respectively, for samples treated with cryoprotecant and untreated samples stored at −10 °C for 120 days, indicating a significant increase in the presence of cryoprotectant ($p < 0.01$). Sych et al (*16*) reported a protective effect of sucrose and sorbitol when added to cod surimi. In addition, water-soluble protein concentration also increased significantly in the presence of antioxidants vitamin C and for vitamin C+E ($p < 0.01$). However, there was no significant difference between the vitamin C and vitamin C+E treatments ($p < 0.1$).

In a second set of experiments (**Figure 3**), the addition of vitamins C + E or vitamins C + E + citrate also resulted in higher SSP values compared with those of untreated samples stored at −10 °C ($p < 0.001$). The addition of citrate increased the SSP levels significantly ($p < 0.05$). The addition of

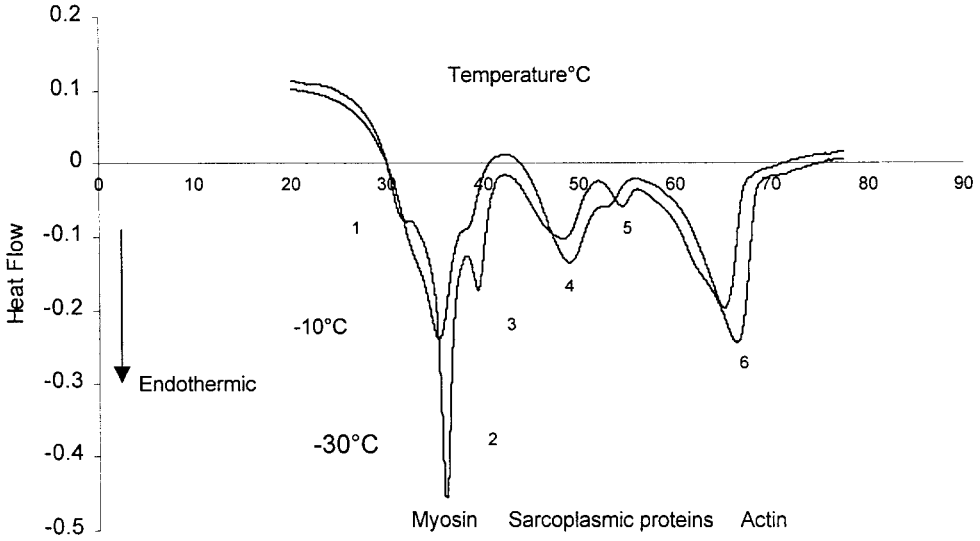

Figure 4. Differential scanning calorimetry of cod muscle stored frozen for 120 days at –10 and –30 °C (control) indicating the transitions for myosin and actin.

Table 1. Transition Temperature, T_m (°C), and Enthalpy, ΔH (J/g), Values for Myosin and Actin in Cod Fillets Stored for 120 days at –30 (control) or –10 °C with or without Cryoprotectants (Sucrose 40 g kg^{-1} and Sorbitol 40 g kg^{-1}) or Antioxidants Vitamin C (500 mg kg^{-1}) or Vitamins E + C (250 mg kg^{-1} each)[a]

	myosin T_{max} °C	myosin enthalpy J/g	actin T_{max} °C	actin enthalpy J/g
–30 °C	36.03 (32.50, 39.56)	0.67 (0.60, 0.74)	65.11 (58.73, 71.49)	0.29 (0.26, 0.32)
–10 °C	35.31 (31.85, 38.77)	0.54 (0.49, 0.59)	66.41 (59.90, 72.92)	0.37 (0.33, 0.41)
–10 °C + cryoprotectants	35.67 (32.17, 39.17)	0.64 (0.58, 0.70)	66.49 (59.97, 73.01)	0.34 (0.31, 0.37)
–10 °C + vitamins E + C	36.33 (32.77, 39.89)	0.45 (0.41, 0.49)	66.02 (59.55, 72.49)	0.29 (0.26, 0.32)
–10 °C + vitamin C	35.60 (32.11, 39.09)	0.47 (0.42, 0.52)	65.98 (59.51, 72.45)	0.30 (0.27, 0.33)

[a] To discriminate between treatments of duplicate samples the standard deviation has been assumed from the coefficient of variation of 5% for the method, and upper and lower 95% confidence bands are shown for each measurement.

cryoprotectants + citrate + antioxidants also increased the SSP values significantly ($p < 0.001$) compared to those of the control fish stored at −10 °C. Moreover, there was a significant difference between the antioxidant + citrate treatment compared with the cryoprotectant + antioxidant + citrate treatment ($p < 0.05$), the latter being more effective in increasing SSP. In our laboratory we have shown that in fatty fish antioxidants vitamins C and E exhibited a protective effect against lipid oxidation and reduced protein aggregation (*31*, *32*). However, this effect has not been reported hitherto for lean fish which contains only 1% lipid. Oxidation of lipids in frozen, stored cod and haddock has been undertaken in the authors' laboratory and is reported elsewhere (*33*).

Rheological Changes. Rheological measurements indicated that the elastic modulus G′ increased with time and temperature of storage, being significantly higher for samples stored at −10 compared with those stored at −30 °C ($p < 0.001$) (**Figure 2**). Compared with the control sample (−10 °C), fish treated with both antioxidants ($p < 0.005$) and cryoprotectants ($p < 0.002$) showed lower G′ values during frozen storage at −10 °C indicating a protective effect on fish texture. The cryoprotectant treatment showed lower G′ values compared to those of the antioxidant treatments ($p < 0.05$). The increase in G′ accompanied the decrease in solubility of proteins extracted during frozen storage which indicates a relationship between protein denaturation by ice crystal formation or lipid oxidation products and toughness of frozen fish (*34*). Other investigators have reported changes in the water-holding properties of fish muscle due to surface dehydration of proteins and in the relationship between protein solubility and toughening of muscle during frozen storage (*10*, *18*). The production of aggregates and subsequent increase in the viscoelastic properties leads to unpalatable fish fillets or mince resulting in the loss of a valuable protein source. Although an increase in the elastic modulus on heating is often considered a useful functional property of proteins (for example in gelled products (*35*)), an increase in the elastic modulus of raw materials such as fish fillets is related to protein−protein and protein−lipid interactions leading to aggregation into undesirable, tough products. In this study, the deleterious effect of ice crystal formation and lipid oxidation products was confirmed by the addition of cryoprotectants or antioxidants on their own which resulted in higher SSP (actomyosin) solubility, and also improved the texture of fish muscle compared to that of the control fish stored at −10 °C.

When the antioxidant or cryoprotectant was blended with citric acid, the protein extractability increased (**Figure 3A**), and the G′ values were lower compared with those of samples without citric acid (**Figure 3B**) ($p < 0.005$). Citrate chelates metal ions and therefore helps to minimize lipid oxidation and protein damage (*9*). Compared with the −10 °C control, the addition of citrate, antioxidant, and cryoprotectant also decreased protein aggregation, thereby reducing G′ values and toughness ($p < 0.001$). This combination is hitherto unreported for improving the quality of frozen lean fish. Clearly, both lipid oxidation products and protein denaturation by ice crystals during frozen storage are important for maintaining the structure and sensory properties of fish muscle.

The protective effect of cryoprotectants on the texture of cod fillets was confirmed by large deformation compression testing in intact fish muscle pieces on a Stable Microsystems TAX-T2 texture analyzer. For cod fillet samples stored at −10 °C, the force required to compress the muscle treated with cryopro-

Table 2. Formaldehyde Production and pH Values for Cod Fillets Treated with Antioxidants (Vitamin C 500 mg kg^{-1} or Vitamins E + C 250 mg kg^{-1} each) or Cryoprotectant (Sucrose 40 g kg^{-1} and 40 g kg^{-1} Sorbitol) Stored at –10 and –30 °C for 120 and 210 Days

		–30 °C	–10 °C	–10 °C + vit C	–10 °C + vit C + E	–10 °C + cryoprotectant
120 days	formaldehyde (μmol/g fillets)	0.19 ± 0.021	2.18 ± 0.28	2.8 ± 0.18	2.03 ± 0.067	2.37 ± 0.21
	pH	6.34	6.42	6.55	6.52	6.44
210 days	formaldehyde (μmol/g fillets)	0.7 ± 0.042	2.582 ± 0.34	5.548 ± 0.28	3.752 ± 0.053	3.842 ± 0.33
	pH	6.73	6.58	6.62	6.67	6.54

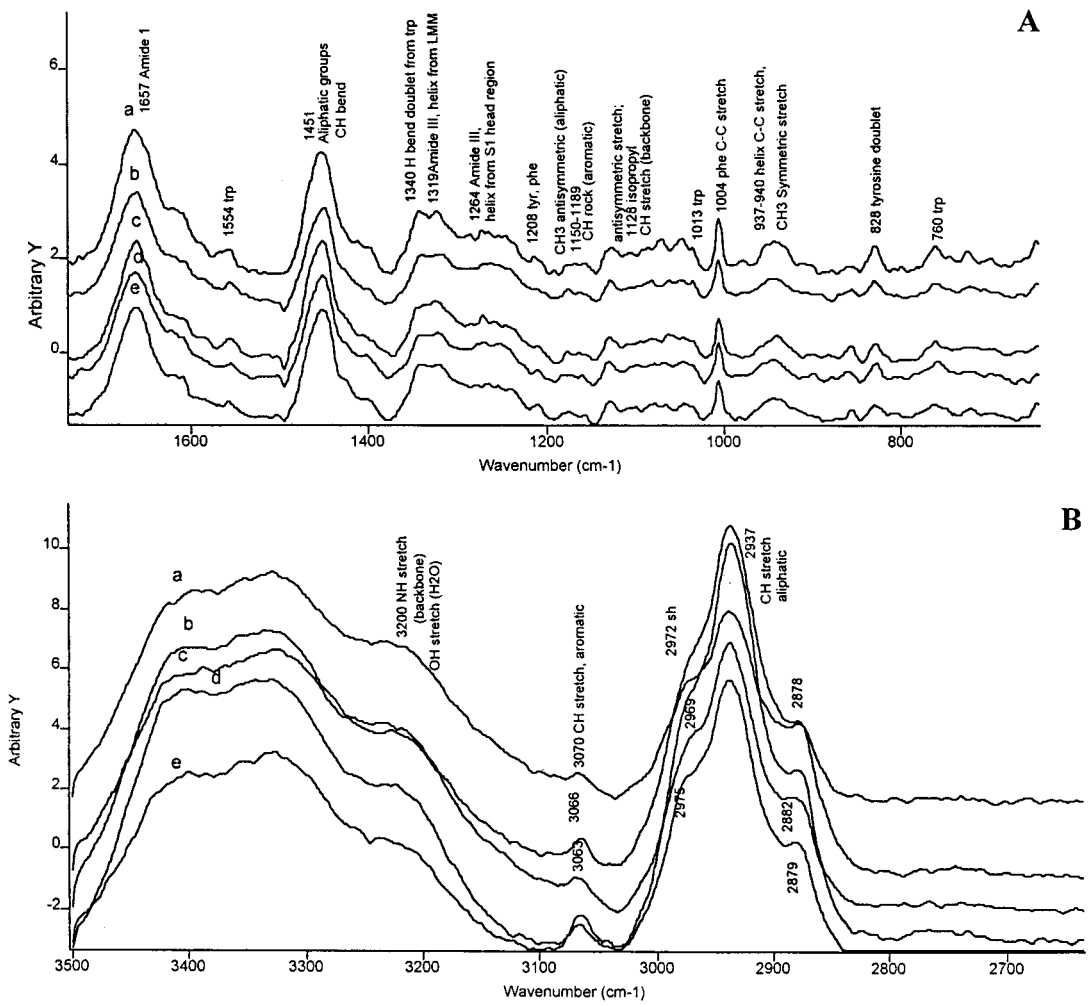

Figure 5. Raman spectra (A) 1700–600 cm^{-1}, and (B) 3500–2700 cm^{-1}, for cod fillets stored for 120 days at (a) –30 °C; (b) –10 °C; (c) –10 °C with cryoprotectant (sucrose 40 g kg^{-1} and sorbitol 40 g kg^{-1}); (d) –10 °C with vitamins E + C, 250 mg kg^{-1} each; and (e) –10 °C with antioxidants (vitamin C 500 mg kg^{-1}).

tectants (1.50 ± 0.5 kg) was less than that required for the control fish (2.8 ± 1.2 kg) ($p < 0.05$).

Differential Scanning Calorimetry. DSC thermograms of untreated cod fillets and of those treated with antioxidants and cryoprotectants showed six transitions. The thermograms for muscle stored at −30 °C (control) and −10 °C are presented to indicate peak assignments (**Figure 4**) (*35*). There were three transitions assigned to myosin: Peaks 1, 2, and 3 with the main transition Peak 2 at T_m 36.0 °C). Two transitions, Peaks 4 and 5, were assigned to the water-soluble sarcoplasmic proteins (T_m 48−52 °C), and a single transition, Peak 6, was assigned to actin (T_m 65 °C). The T_m and enthalpy for the myosin and actin transitions for all treatments are presented in **Table 1**. As stated in the methods section, to discriminate between treatments the standard deviation has been assumed from the coefficient of variation of 5% for the method and upper and lower 95% confidence bands were calculated for each measurement. Notwithstanding multiple comparisons, it is evident that for myosin stored at −10 °C compared with that stored at −30 °C, the T_m changes were not significantly changed, but ΔH decreased from 0.67 to 0.54 J/g indicating protein denaturation and exposure of nonpolar groups (*36*). In contrast, ΔH for actin increased from 0.29 J/g at −30 °C to 0.37 J/g at −10 °C; the increase may be indicative of actin polymerization or dehydration effects, in which case the denaturing effect on myosin is underestimated (*33*, *37*, *38*).

In the presence of antioxidants vitamin C or vitamins E + C, ΔH values for the main myosin peak were 0.47 and 0.45 J/g, respectively, compared with 0.54 J/g for the −10 °C untreated control; there were no differences in the T_m (**Table**

Table 3. Relative Peak Intensity of the Raman Bands in the Region 3100–700 cm^{-1} for Cod Muscle Stored for 120 days at –30 (control) and –10 °C with and without Antioxidants (vitamin C 500 mg kg^{-1}) or Vitamins E + C (250 mg kg^{-1} each) or Cryoprotectants (Sucrose 40 g kg^{-1} and Sorbitol 40 g kg^{-1})[a]

peak assignment (wavenumber ± 2 cm^{-1})	relative peak intensity				
	cod –30 °C	cod –10 °C	cod –10 °C + vit C	cod –10 °C + vit C +E	cod –10 °C + cryoprotectant
Trp (760)	0.41 (0.37, 0.45)	0.30 (757) (0.27, 0.33)	0.31 (0.28, 0.34)	0.32 (0.29, 0.35)	0.42 (0.38, 0.46)
CC ring stretch and CH_2 residue rock (Tyr) (830, 850)	0.33 (830) 0.31 (850)	0.41 0.23	0.34 0.30 (855)	0.37 0.21 (856)	0.40 0.35 (854)
α-helix, CC stretch, CH_3 symmetric stretch (940)	0.47 (0.42, 0.52)	0.36 (0.32, 0.40)	0.38 (0.34, 0.42)	0.42 (0.38, 0.46)	0.46 (0.41, 0.51)
β-sheet structure (990)	0.08 (0.07, 0.09)	0.14 (0.13, 0.15)	0.11 (0.10, 0.12)	0.08 (0.07, 0.09)	0.08 (0.07, 0.09)
CC ring stretch Phe (1004)	1.0	1.0	1.0	1.0	1.0
Trp (1013)	0.19 (0.17, 0.21)	0.12 (0.11, 0.13)	0.11 (0.1, 0.12)	0.11 (0.1, 0.12)	0.18 (0.16, 0.20)
Isopropyl antisymmetric stretch CN stretch (backbone) (1127)	0.36 0.32, 0.40)	0.40 (0.36, 0.44)	0.41 (0.37, 0.45)	0.39 (0.35, 0.43)	0.38 (0.34, 0.42)
CH_3 antisymmetric rock (aliphatic), CH rock (aromatic) (1155)	0.13 (0.12, 0.14)	0.20 (0.18, 0.22)	0.14 (0.13, 0.15)	0.12 (0.11, 0.13)	0.12 (0.11, 0.13)
β-sheet type structure (1239)	0.26 (0.23, 0.29)	0.55 (0.50, 0.60)	0.53 (0.48, 0.58)	0.52 (0.47, 0.57)	0.48 (0.43, 0.53)
Amide III, helix, S_1 globular region (1264)	0.63 (0.57, 0.69)	0.59 (0.53, 0.65)	0.69 (0.62, 0.76)	0.70 (0.63, 0.77)	0.70 (0.63, 0.77)
Amide III, helix from LMM (1319)	0.93 (0.84, 1.02)	0.81 (0.73, 0.89)	0.92 (0.83, 1.01)	0.93 (0.84, 1.02)	0.92 (0.83, 1.01)
H bend, Trp (1340)	0.93 (0.84, 1.02)	0.80 (0.72, 0.88)	0.89 (0.80, 0.98)	0.90 (0.81, 0.99)	0.90
Aliphatic groups CH bend (1450)	1.94 (1.75, 2.13)	1.6 (1.44, 1.76)	2.0 (1.80, 2.20)	2.0	2.0
Amide II, COO^- antisymmetric stretch (Asp), Trp (1554)	0.4 (0.36, 0.44)	0.13 (0.12, 0.14)	0.31 (0.28, 0.34)	0.39 (0.35, 0.43)	0.42 (0.38, 0.46)
Amide I (1657)	1.90 (1.71, 2.09)	1.6 (1662) (1.44, 1.76)	1.98 (1.79, 2.17)	1.98 (1.79, 2.17)	1.90 (1.71, 2.09)
CH stretch, aliphatic (2937)	5.50 (4.96, 6.04)	3.70 (3.34, 4.06)	4.77 (4.30, 5.24)	4.57 (4.12, 5.02)	4.70 (4.24, 5.16)
Shoulder (2878)	2.30 (2.07, 2.53)	1.46 (2885) (1.32, 1.60)	1.87 (1.69, 2.05)	1.90 (1.71, 2.09)	2.40 (2.16, 2.64)
Shoulder (2972)	3.40 (3.07, 3.73)	2.30 (2975) (2.07, 2.53)	2.90 (2.62, 3.18)	4.50 (4.06, 4.94)	3.50 (3.16, 3.84)
CH stretch, aromatic (3067)	0.50 (0.45, 0.55)	0.39 (0.35, 0.43)	0.50 (0.45, 0.55)	0.50	0.50

[a] Figures in parentheses next to the assignments (or next to the intensity values if altered) refer to wavenumbers ± 2 cm^{-1}. The spectra were an average of 64 scans which were baseline corrected, smoothed, and normalized to the intensity of the phenylalanine band at 1004 cm^{-1}. To discriminate between treatments the standard deviation has been assumed from the coefficient of variation of 5% for the method, and upper and lower 95% confidence bands are shown for each measurement in parentheses below the intensity value.

1). In the presence of cryoprotectants, ΔH for the treated sample (myosin) was 0.64 J/g, similar to that for the −30 °C control (0.67 J/g) and in contrast to that for the −10 °C control (0.54 J/g). This indicated stabilization of the myosin molecules by the cryoprotectant. Similar stabilization of myosin and other molecules by sugar molecules is reported (*12*, *16*, *17*, *39*). There was no change in T_m of actin in treated samples compared to the −10 °C control (*16*, *39*).

Effect of Antioxidants on Formaldehyde Production. Formaldehyde production in all cod samples stored at −10 °C increased compared to those stored at −30 °C (*4*), especially for samples containing vitamin C as antioxidant ($p < 0.01$) (**Table 2**). Vitamin C may accelerate or act as a cofactor in the breakdown of trimethylamine oxide (TMAO) into dimethylamine and formaldehyde (*40*). It is interesting to note that although vitamin C increased the formation of formaldehyde in fish muscle on frozen storage, the results indicated that this increase did not enhance protein insolubility or the G′ values or toughening of fish fillets. As the pH values of all the samples were similar (pH range 6.54−6.73), the effect of vitamin C was not due to the lowering of pH, which is reported to affect the texture of food gels (*41*). The main effect of vitamin C, like that of vitamin E, is due to its antioxidant property to minimize lipid oxidation (*35*) and subsequent protein denaturation; this role of antioxidants in controlling toughness is more important than the role of formaldehyde formation in promoting toughness.

FT-Raman Spectroscopy. Raman spectra obtained for minced cod fillet samples, both untreated and treated with antioxidants or cryoprotectants, are shown in **Figure 5** and detailed in **Table 3**. As stated in the methods section, duplicate sets of experiments were undertaken. To discriminate between treatments the standard deviation has been assumed from the coefficient of variation of 5% (found to be the maximum for this method), and upper and lower 95% confidence bands were calculated for each measurement. Intensity values above or below these bands were considered significantly different. A shift in the wavelength of ±2 is considered significantly different (*21*, *22*). Comparison of spectra in the 500 to 1800 cm^{-1} region showed differences in the frequency, intensity, and shapes of the peaks (**Figure 5A**, **Table 3**). The α-helix content (940 cm^{-1}) decreased, and β-sheet structure (bands at 990 and 1239 cm^{-1}) increased, especially with samples stored at −10 °C without any antioxidants or cryoprotectants. A decrease in α-helix and increase in β-sheet are indicative of protein−protein interactions

which have been reported for heat-denatured proteins (*21*) and for frozen stored hake fillets (*24*). In particular, in the Amide I region, the band centered at 1657 cm^{-1} decreased in intensity and shifted to a higher wavenumber (1662 cm^{-1}) for samples stored at −10 °C compared with those stored at −30 °C. The band in the Amide III region (1264 cm^{-1}) also decreased in intensity for samples stored at −10 °C. However, the samples treated with cryoprotectants were similar to those stored at −30 °C; this confirms changes in the secondary structure which may occur as molecules unfold under the pressure of ice crystals and denature due to water restructuring at the protein surface as well as due to freeze concentration of salts.

The tryptophan bands intensity, particularly at 760 and 1013 cm^{-1}, decreased for samples with or without antioxidant at −10 °C but was similar for the sample in the presence of cryoprotectant at −10 °C, compared with samples at −30 °C; this decrease indicates exposure of hydrophobic tryptophan residues. Similarly, tryptophan bands at 1340 and 1554 cm^{-1} also decreased at −10 compared to −30 °C, but in the presence of both antioxidants and cryoprotectants, the values were similar to those of the −30 °C sample. An increase in exposed hydrophobic groups in frozen cod fillets was also shown using fluorescent probes in a previous study (*4*).

The intensity ratio I_{850}/I_{830} corresponding to the tyrosine doublet at 850 and 830 cm^{-1}, increased in the samples stored at −10 °C (0.56) compared with those at −30 °C (0.37). The I_{850}/I_{830} ratios for samples stored at −10 °C in the presence of cryoprotectants (0.87) and antioxidants vitamin C (0.88) were higher than those of samples stored at −10 and −30 °C. This increase indicates tyrosine residues exposed on the protein surface which can interact with water molecules as a hydrogen bond donor or acceptor (*23*, *42*−*44*). Changes were observed in the band centered at 1208 cm^{-1} (Tyr, Phe) which was absent in samples stored at −10 °C; however, this band was observed in the samples stored at −10 °C in the presence of antioxidants or cryoprotectants, similar to the control stored at −30 °C.

The band in the CH stretching region, centered at 2937 cm^{-1}, did not shift in samples stored at −10 °C compared with −30°; however, the shoulders at 2878 and 2972 cm^{-1} shifted to 2885 and 2975 cm^{-1}, respectively (**Figure 5B**). For these peaks, the intensity was lower at −10 °C in the absence of antioxidants or cryoprotectants than it was in the −30 °C sample. In the CH stretching region centered at 3067 cm^{-1} (aromatic groups), the peak intensity for samples stored at −10 °C decreased compared with those of samples at −30 °C or those with antioxidants and cryoprotectants stored at −10 °C. These changes, resulting from the freezing of fish muscle, may indicate alterations both in the hydrophobic groups and in other polar amino acids, as the CH stretch region has been reported by Howell et al (*22*) to incorporate signals from aromatic and aliphatic groups as well as from polar amino acids. Further changes in the aliphatic groups were noted by a decrease in the CH bend at 1450 cm^{-1} in samples at −10 compared to those at −30 °C; in the presence of antioxidants and cryoprotectants the intensity was similar to samples stored at −30 °C, indicating the protection of proteins.

CONCLUSIONS

Preliminary investigations using antioxidants (natural vitamins C and E), citric acid as sequestrant, and cryoprotectants (sucrose and sorbitol) to control lipid oxidation and ice crystal formation, indicated that the cryoprotectant/antioxidant/citrate mixture as well as the cryoprotectants and antioxidants on their own are effective treatments for minimizing protein denaturation and toughening in lean fish. The importance of antioxidants in the protection of proteins in lean fish has not been reported hitherto. Thus, both lipid oxidation and ice crystal growth are major factors that contribute to the toughening of lean fish in frozen storage; formaldehyde is less important than previously considered. Overall, the results indicate that the denaturation of proteins caused mainly by ice crystal formation and/or resultant solute concentration, and also by lipid oxidation products, involves hydrophobic groups and exposure of other polar groups due to the unfolding of the molecules, accompanied by changes in the secondary structure. Further studies are needed to evaluate different combinations of antioxidants and cryoprotectants in terms of concentration, synergism, and methods of application to fillets to give optimum results for preventing protein aggregation and texture deterioration in frozen storage.

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