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PII: S0308-8146(97)00234-3

Food Chemistry, Vol. 63, No. 1, pp. 39–50, 1998

Printed in Great Britain
0308-8146/98 $19.00+0.00

High pressure treatment effects on cod (*Gadus morhua*) muscle

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(Received 15 August 1997; revised version received and accepted 3 October 1997)

After treatment at pressures above 400 MPa, the oxidative stability of the lipids in cod (*Gadus morhua*) muscle were markedly decreased as measured by the thiobarbituric acid (TBA) number. This was thought to be due to the release of metal catalysts from complexes, since addition of EDTA effectively inhibited the increased rates of oxidation. As judged by differential scanning calorimetry and electrophoresis this effect did not appear to be related to denaturation of the major proteins in meat, since myosin was denatured at 100–200 MPa and actin and most of the sarcoplasmic proteins at 300 MPa. A few water soluble proteins survived pressures of 800 MPa including several of the proteases, but there was a marked decrease in activity of the neutral proteases (pH optima 6.6) above 200 MPa. In contrast to heat-treatment, pressure was shown to lead to the formation of structures that were stabilised, at least to some extent, by hydrogen bonds and texture profile analysis (TPA) showed that the structures produced by heat and pressure treatments had very different textures.

INTRODUCTION

High pressures, up to 1000 MPa, are being increasingly used in the Food Industry for the processing and preservation of a range of foods (Ledward, 1995). This technology offers the advantages of increased shelf-life, due to bacterial destruction, with minimum loss of quality since many of the undesirable changes associated with thermal processing are inhibited or prevented as covalent bonds are unaffected by pressure, in this temperature range. Commercially the process has been successful, to date, when applied to acid foods such as fruit juices and jams. However, there is increasing interest in the application of this technology to meat- and fish-based products (Culioli and Cheftel, 1997). We have recently reported on the effects of high pressure processing on meat and meat-like systems and found that one of the major disadvantages was that the application of high pressure, irrespective of the presence of oxygen during treatment, led to increased rates of lipid oxidation during subsequent aerobic storage (Cheah and Ledward, 1996). It was shown that such increased rates of lipid oxidation were primarily a result of the release of metal ions from complexes on pressure treatment (Cheah and Ledward, 1997). The lipids in fish are more susceptible to oxidation than those of most meat producing animals, since they have a high concentration of polyunsaturated fats and thus oxidative changes induced by pressure may be very significant.

In addition, pressure will modify the structure and function of many proteins. For example, myosin from both meat and fish will be denatured by pressure and subsequently form a gel-like texture (Culioli and Cheftel, 1997). In addition, proteases active in muscle may become more or less active on the application of pressure, depending on whether the substrate is made more available (Gomes and Ledward, 1996) or the pressure treatment *per se* leads to inactivation of the enzyme. All of these changes in protein structure and function will affect the texture of the muscle, and since the effect of pressure on protein structure is different from the effect of heat, the textures of heat-and pressure-treated meats may also be very different. In a heat-treated system, of the weak bonds maintaining the secondary, tertiary and quaternary structures, it is the hydrogen bonds that are most labile. In a pressure-treated system, it is the hydrophobic and electrostatic interactions which are most vulnerable, hydrogen bonds being largely unaffected by pressure (Galazka and Ledward, in press). Thus, the mode of unfolding and subsequent aggregation of the proteins will differ in heat-treated and pressure-treated systems and this may markedly affect the perceived eating and textural quality.

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To investigate the potential of high pressure processing to fish, the effects of pressure treatment on lipid stability were investigated as well as how changes in the proteins present modify the textural quality.

MATERIALS AND METHODS

All chemicals used were of analytical grade.

Pressurisation of samples

Cod (*Gadus morhua*) was obtained from a local market or fresh (1 day after catching) and kept on ice for 4 days before analysis. The fish was filleted, de-skinned and cut into pieces each weighing about 120 g. Samples of fish were sealed in Multivac bags (Bosley, International, NL) and pressure treated at 100 to 800 MPa at room temperature for 20 min in a prototype Stansted 'Food-Lab' high-pressure rig (Stansted Fluid Power Ltd, Stansted, UK). Some samples were packed in nitrogen gas before pressurisation. In others, fish was minced to allow incorporation of antioxidant (Cheah and Ledward, 1997). The pressurised samples were divided, some analysed immediately or after 1 day at 4°C and the rest stored in polyethylene bags at 4°C and analysed at intervals. In most cases, experiments were carried out on three different fish at different times. During pressurisation, the temperature gradually increased and reached a maximum when the maximum pressure was attained, it then decreased back to ambient values.

Maximum temperatures were normally reached after 1.5 to 2.5 min and were 42°C at 400 MPa and 49°C at 800 MPa. In some cases, pressure was increased in 50 to 100 MPa steps to maintain the temperature within 5°C of ambient and so minimise possible temperature effects.

Determination of pH and moisture content

The pH of each sample was determined by a combined glass electrode in suspensions of 10 g of sample in 100 ml of distilled water after mixing for 2 min. Moisture content was determined by the method of AOAC (1990).

Determination of thiobarbituric acid (TBA) number

TBA number was measured according to the method of Pearson (1976) and is expressed as mg malonaldehyde (MA) per kg sample.

Differential Scanning Calorimetry (DSC)

DSC was performed on a Perkin-Elmer DSC 7. The instrument was calibrated using indium; 10–20 mg of sample was weighed into an aluminium pan and heated from 10°C to 95°C at 10°C min^{-1}. An empty pan was used as reference. A rescan from 10–95°C was carried out on all samples to check if there was any reversible modification of the proteins.

Gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on gels of 7.5% polyacrylamide containing 1% SDS (Laemmli, 1970). 50 mg of minced fish sample was stirred with 10 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 8 M urea, 2% SDS and 2% 2-mercaptoethanol for 24 h, and filtered (Whatman No.5). The filtrate was mixed with an equal volume of 0.125 M Tris-HCl buffer (pH 6.8) containing 10% SDS, 2% 2-mercaptoethanol and 0.002% bromophenol blue, and boiled for 3 min; 10 ml of protein solution was applied to each well. Separation was at 150 V and 70 A. Fixation of the proteins was by 12% trichloroacetic acid for 1 h and staining by Coomassie Brilliant Blue (Neuhoff *et al.*, 1988). SDS-PAGE without 2-mercaptoethanol was performed by the same procedure.

Native-page

Ten grams of minced fish sample was mixed with 40 ml of 0.1 M phosphate buffer (pH 7.5), centrifuged at 2000 g for 20 min, and filtered through paper (Whatman No.5). Solutions were mixed with equal volumes of 0.125 M Tris-HCl buffer (pH 6.8) containing 10% glycerol and 0.002% bromophenol blue. Electrophoresis was carried out as outlined above.

Proteolytic activity

Cod fillets were chopped into small pieces and centrifuged at 5000 ***g*** for 30 min. The supernatant (fish juice) was used for the protease assay (An *et al.*, 1994).

The pH profile was determined to find the optimum pH for the cod proteases over the range 2.5 to 10. Proteolytic activity was determined as described by Erickson *et al.* (1983) using haemoglobin as a substrate with slight modification. Incubation was performed at 55°C for 3 h in McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M sodium citrate) for the pH range 2.5–8.0 and 0.1 M Tris-HCl buffer for the pH range 9.0–10.0.

Effects of pressure treatment on the protease activity of cod muscle were determined on the extruded juice as described above at the pH values of maximum activity (pH 3.3, 6.6 and 9.0).

Texture profile analysis (TPA)

Cod fillets were cut into rectangles measuring about 2 × 2 × 1.5 cm, sealed in polyethylene bags and heated in water baths at various temperatures (40, 50, 60, 70, and 80°C) for 10 min. The cooked samples were

immediately cooled in cold running water. The pressure treated samples (0, 200, 400, 600 and 800 MPa for 20 min) were cut to the sizes as indicated above and analysed. All samples were dried with filter paper and kept at 4°C before analysis. As before, all experiments were performed in triplicate, i.e. on three different fish, eight determinations being made in each experiment.

Texture profile analysis was carried out with a Stable Micro System Type (version 3.7G). All cod samples were compressed to 50% of their original height at 2 mm s^{-1} using a cylindrical-shaped piston, 38 mm in diameter. The texture profile was determined from these curves (Bourne, 1982).

RESULTS AND DISCUSSION

Appearance

The raw cod was slightly translucent with a reddish tinge and, when treated at 200 MPa, there was some loss of translucency. At higher pressures the redness was lost and it became white and opaque, i.e. similar to cooked samples.

Effect of pressure on pH

The pH of cod muscle increased slightly after pressure treatment (e.g. Tables 1–3). This is probably associated with the denaturation of some protein fractions. In cooked cod, the pH also increases, possibly due to a decrease in available acidic groups in the muscle, as a result of conformational changes associated with denaturation (Poulter *et al.*, 1985). After storage for 7 days at 4°C, the pH of untreated samples increased more than the pressure-treated samples (Tables 1 and 2). It is well established (Shoji and Saeki, 1989; Ledward, 1995) that high pressure treatment kills certain types of micro-organisms and some of these may be responsible for the degradation of fish muscle, resulting in the production of volatile substances such as acids, amines and bases (Spinelli *et al.*, 1964). During spoilage of meat and fish, pH invariably rises for these reasons and thus the relative stability of the pH in the pressure treated samples is not unexpected.

Table 1. Effect of pressure (20 min treatment time) on the TBA Number (mg malonaldehyde kg^{-1}) and pH of cod packed in air. All values are the means ± standard deviations of 4 replicates after storage at 4°C

Treatment	1 Day		7 Days	
MPa	TBA	pH	TBA	pH
0	0.30 ± 0.05	6.63 ± 0.05	0.18 ± 0.01	7.13 ± 0.00
200	0.26 ± 0.02	6.73 ± 0.03	0.24 ± 0.01	6.98 ± 0.04
400	0.74 ± 0.02	6.76 ± 0.05	1.67 ± 0.09	6.90 ± 0.08
600	1.16 ± 0.07	6.82 ± 0.03	2.07 ± 0.14	7.03 ± 0.01
800	1.30 ± 0.19	6.83 ± 0.05	2.84 ± 0.03	7.05 ± 0.03

Table 2. Effect of pressure (20 min treatment time) on the TBA Number (mg malonaldehyde kg^{-1}) and pH of cod packed in nitrogen. All values are the means ± standard deviations of 4 replicates after storage at 4°C

Treatment	1 Day		7 Days	
MPa	TBA	pH	TBA	pH
0	0.12 ± 0.01	6.98 ± 0.04	0.19 ± 0.01	7.31 ± 0.02
200	0.13 ± 0.02	6.98 ± 0.05	0.19 ± 0.02	7.05 ± 0.01
400	0.17 ± 0.01	7.01 ± 0.02	0.19 ± 0.03	7.25 ± 0.03
600	0.22 ± 0.05	7.14 ± 0.03	0.74 ± 0.01	7.25 ± 0.02
800	0.27 ± 0.07	7.15 ± 0.03	1.02 ± 0.03	7.18 ± 0.07

Effect of pressure on lipid oxidation

Table 1 illustrates the effect of high pressure (0 to 800 MPa for 20 min) on the TBA number of a sample of cod fillet packed in air. It is seen that the initial TBA number of the sample pressurised at 200 MPa for 20 min changes little when compared with the fresh sample. However, the TBA value increases considerably when the sample is treated at 400 MPa or higher for 20 min. After storage in air for 7 days at 4°C, the TBA number of the samples treated at 400, 600 and 800 MPa increased considerably. Table 2 shows the effect of high pressure on the TBA number of samples of a different fish packed in nitrogen. It is seen that the initial TBA number increases only slightly as a function of pressure. Storage of these samples at 4°C for 7 days results in a marginal increase in TBA number for samples treated at pressures of 400 MPa and less. However, the TBA number increases significantly for the samples treated at 600 and 800 MPa.

Table 3. Effect of 1% Na_2EDTA on TBA number (mg malonaldehyde kg^{-1}) and pH in minced cod following pressure treatment for 20 min and storage at 4°C. Values are the means ± standard deviations of 4 replicates

Sample	Storage (day)		
	0	4	8
0 MPa	0.30 ± 0.03	0.30 ± 0.04	0.27 ± 0.04
400 MPa	0.43 ± 0.06	0.47 ± 0.07	0.55 ± 0.06
800 MPa	0.72 ± 0.10	1.44 ± 0.08	2.28 ± 0.04
0 MPa + 1% Na_2EDTA	0.21 ± 0.02	0.22 ± 0.02	0.23 ± 0.01
400 MPa + 1% Na_2EDTA	0.20 ± 0.02	0.19 ± 0.01	0.20 ± 0.00
800 MPa + 1% Na_2EDTA	0.28 ± 0.02	0.29 ± 0.01	0.33 ± 0.01
pH			
0 MPa	7.29 ± 0.05	7.52 ± 0.05	7.83 ± 0.02
400 MPa	7.33 ± 0.00	7.36 ± 0.01	7.23 ± 0.03
800 MPa	7.33 ± 0.03	7.37 ± 0.02	7.22 ± 0.01
0 MPa + 1% Na_2EDTA	6.74 ± 0.02	6.78 ± 0.00	6.67 ± 0.01
400 MPa + 1% Na_2EDTA	6.79 ± 0.06	6.85 ± 0.02	6.74 ± 0.01
800 MPa + 1% Na_2EDTA	6.77 ± 0.04	6.85 ± 0.02	6.73 ± 0.02

The above experiments show that pressures below about 400 MPa have little effect on lipid oxidation in cod muscle but a significant effect is seen at higher pressures. These results are in agreement with Tanaka *et al.* (1991) and Ohshima *et al.* (1992) who suggest that accelerated oxidation may be due to denaturation of haem protein by pressure which releases metal ions which promote auto-oxidation of lipid in pressurised fish meat. Other studies (Wada, 1992) suggest that the denaturation of proteins may be important in catalysing lipid oxidation in pressure treated meat. Overall, we find no significant difference in TBA number in the unpressurised and pressurised samples packed in nitrogen. However, during subsequent storage in air for 7 days at 4°C, the TBA number increases rapidly in samples treated at pressures above 400 MPa. This suggests that the catalytic effect does not depend on the presence of oxygen during pressure treatment. This agrees with studies carried out by Cheah and Ledward (1996) on minced pork. In addition, these authors found that an increase in free metal ion (Fe and Cu) content on pressure treatment was the major reason for the acceleration of lipid oxidation (Cheah and Ledward, 1997). To confirm that a similar mechanism acts in cod, minced samples containing 1% (w/w) of ethylenediamine tetraacetic acid (EDTA) were subjected, in air, to pressures in the range ambient to 800 MPa for 20 min. Typical results are shown in Table 3 where it is seen that the addition of EDTA effectively inhibits the pressure induced catalysis of lipid oxidation in cod. This is in agreement with the work of Cheah and Ledward (1997) on pork meat and confirms that the acceleration of lipid oxidation is due to the release of free metal ions (Fe and Cu) from complexes at around 400 MPa. EDTA does, as expected, lower the pH of the fish (Table 3, Cheah and Ledward, 1997) but, even at the lower pH, it effectively inhibits the catalysis.

In experiments carried out at 400 MPa, the increase in oxidation rate in additive free samples was either quite marked, e.g. Table 1, or very limited, e.g. Tables 2 and 3, suggesting that the key pressure to trigger the oxidation is around this value. In eight fish analysed after treatment at 400 MPa, significantly increased ($P < 0.001$) rates of lipid oxidation were seen in six of them during storage at 4°C. Pressures of 600 and 800 MPa always markedly increased lipid oxidation as measured by TBA Number. The effect is not pH-dependent, since there was no correlation with initial pH. To obtain further insight into this phenomena and to evaluate textural changes, the effect of pressure on the proteins in cod was studied.

Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) data for untreated and pressurised cod muscle are presented in Fig. 1. The thermogram (Fig. 1) for the untreated sample is similar to that obtained by Davies *et al.* (1988, 1994) and Hastings *et al.* (1985). The double peak centred around 42°C (Peak 1) is thought to be due to myosin denaturation (Fig. 1). The peaks at about 52°C (Peak 2), 58°C (Peak 3) and 72°C (Peak 4) correspond to sarcoplasmic proteins and perhaps collagen (2 and 3) and actin (4), respectively (Davies *et al.*, 1988). It is seen from Fig. 1 that samples treated at 100 MPa for 20 min show a significant loss of the myosin peak and at 200 MPa almost all the myosin peak has disappeared. As the pressure is increased further, to 300 MPa, it is apparent that many of the sarcoplasmic proteins and actin denature. It is also apparent that, after pressure-treatment, a new low melting transition is seen around 32°C (Peak N on Fig. 1). This new structure appears to form at pressures of 100 MPa or more and to be little affected by the degree of pressure (to 800 MPa) to which the fish is subjected. It is also apparent that at pressures of 300 MPa and higher, further new structures are formed which appear to melt in the range 40–60°C (Peak B on Fig. 1). There is some suggestion that Peak 2 is not affected by pressure but this new transition makes definition difficult. The results of the variation in peak temperatures and the associated enthalpies are shown in Tables 4 and 5. Assuming that ΔH between 40 and 60°C in Table 5 is due to both myosin denaturation and a new pressure-induced structure, it would appear that additional pressures above 300 MPa have no effect on the enthalpy of unfolding of the new structure. Thus the new structures once formed do not appear to be additionally modified by pressures above 200 MPa (Peak N) or 300 MPa (Peak B).

On rescanning the untreated sample or any of the pressure-treated ones, there was little evidence of the new structures seen in the pressure-treated samples being retained or reformed on cooling (Fig. 1). In addition, scanning a heat-treated sample yielded a flat line with no evidence of any new or reformed structure.

Thus, the DSC thermograms (Fig. 1) show that high pressure treatment at 400 MPa denatures both actin and most, but not all, of the sarcoplasmic proteins in cod but the cod myosin is fully denatured at 200 MPa. Research on the effect of heat on croaker surimi containing 3% salt (Wu *et al.*, 1985) and beef (Parson and Patterson, 1986) showed that myosin denatures readily with heat whereas actin is less affected. Recent research (Cheah and Ledward, 1996) has shown that, in pork, both actin and myosin are denatured after pressure-treatment at 300 to 400 MPa, but not at 200 MPa. Thus, cod myosin is far more labile to pressure than myosin from meat, although the actins appear to have similar stabilities to pressure. This is in agreement with the known thermal stabilities of the myosins, those from warm blooded animals and tropical fish being far more stable than those from cold water fish (Davies *et al.*, 1988). In addition, it is known that the pressure denaturation of proteins favours the formation of hydrogen-bonded gels and precipitates (Defaye *et al.*, 1995) and the new structures seen in the pressure-treated

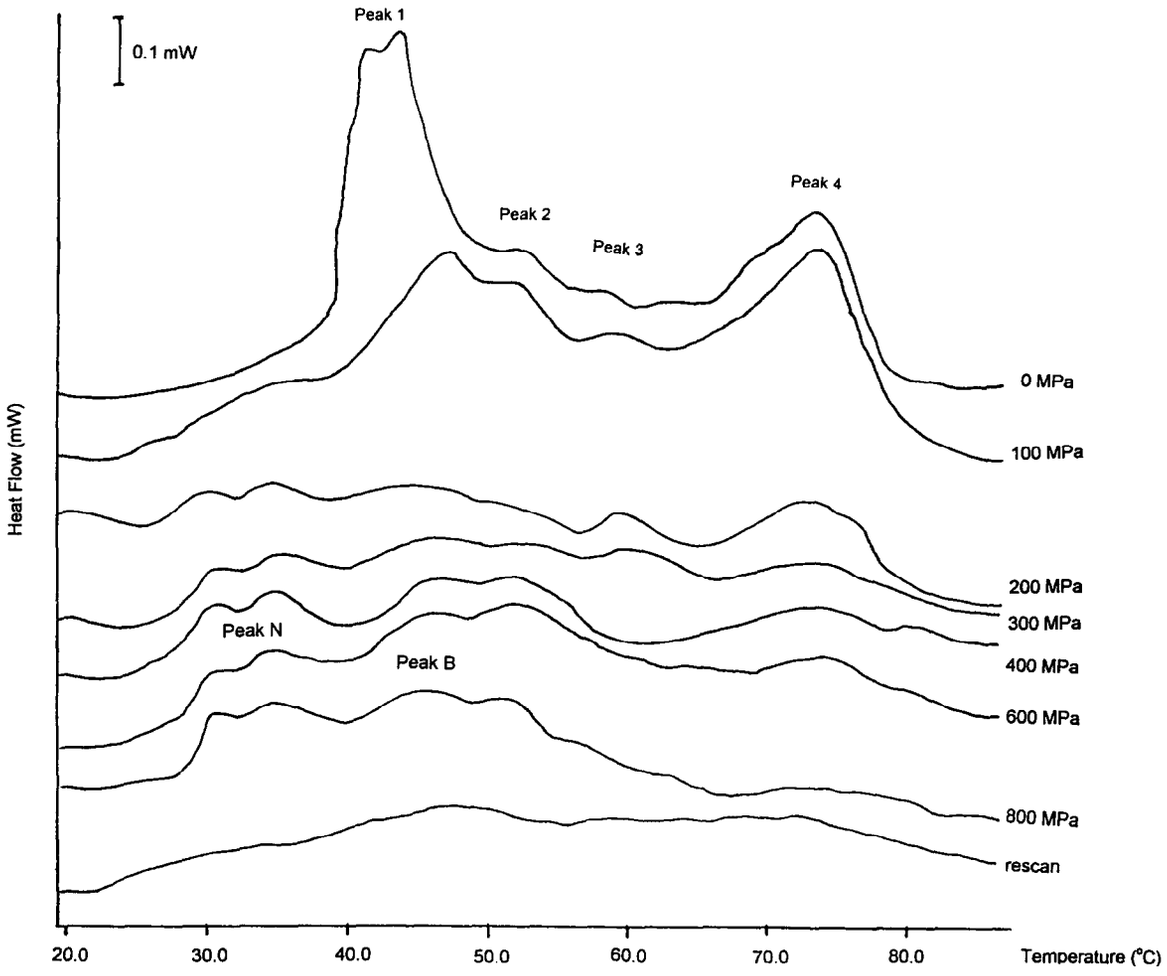

Fig. 1. Thermograms of cod muscle, heated at 10°C min^{-1} after treatment at different pressures in air for 20 min at ambient temperature. Peak 1 corresponds to myosin; Peaks 2 and 3 to sarcoplasmic proteins and Peak 4 to actin. Peaks N and B represent new structures produced after pressure treatment.

Table 4. Peak transition temperature (T_{max}) for the proteins present in pressurised cod muscle. All values are the means ± standard deviation of 4 determinations

Treatment	T_{max} (°C)					
	Peak N	Peak 1	Peak B	Peak 2	Peak 3	Peak 4
0 MPa	Not seen	43.1 ± 1.2	Not seen	52.3 ± 1.6	57.4 ± 1.4	72.8 ± 1.4
100 MPa	31.9 ± 0.3	45.8 ± 0.9	Not seen	51.7 ± 0.5	59.9 ± 2.1	72.5 ± 0.2
200 MPa	32.9 ± 0.9	Indistinct	Not seen	50.0 ± 0.4	57.3 ± 1.7	71.7 ± 0.4
300 MPa	30.6 ± 0.8	Indistinct	45.7 ± 0.1	51.6 ± 1.0	59.8 ± 0.5	71.7 ± 1.6
400 MPa	30.8 ± 0.6	Indistinct	44.5 ± 0.4	50.8 ± 0.3	Indistinct	Indistinct
600 MPa	30.5 ± 0.3	Indistinct	44.4 ± 0.4	50.8 ± 0.7	Indistinct	Indistinct
800 MPa	30.1 ± 0.5	Indistinct	43.4 ± 0.5	50.4 ± 0.3	Indistinct	Indistinct

Peak N is the first peak of the new doublet assumed to be associated with a structure formed following myosin denaturation.
Peak 1 corresponds to the first peak of the myosin denaturation.
Peak B is the first peak of the new structure formed at higher pressures.
Peaks 2 and 3 correspond to sarcoplasmic protein denaturation.
Peak 4 corresponds to actin denaturation.
At pressures of 300 MPa and above a 'new' structure may well contribute to Peak 2.

samples presumably reflect the presence of such networks, since hydrogen bonds break endothermally. It would appear that denaturation of myosin alone (e.g. at pressures of 100 and 200 MPa gives rise to the low melting structure (T_N) which is not modified by further denaturation of sarcoplasmic protein and actin. However, denaturation of these (actin and sarcoplasmic) proteins gives rise to the higher melting structures (40–60°C) seen in Fig. 1. Thermal denaturation, gives rise to structures in which hydrophobic interactions rather

Table 5. The effect of high pressure treatment on the relative enthalpy of protein denaturation in cod muscle (joules g^{-1} wet sample). All values are the means ± standard deviation of 4 determinations

Treatment	ΔH_N	ΔH_1	ΔH_4
0 MPa	0	1.56 ± 0.14	0.69 ± 0.08
100 MPa	0.15 ± 0.06	0.67 ± 0.15	0.69 ± 0.09
200 MPa	0.13 ± 0.01	0.61 ± 0.06	0.47 ± 0.03
300 MPa	0.15 ± 0.01	0.35 ± 0.08	0.10 ± 0.01
400 MPa	0.21 ± 0.01	0.42 ± 0.07	Indistinct
600 MPa	0.14 ± 0.03	0.37 ± 0.07	Indistinct
800 MPa	0.18 ± 0.02	0.32 ± 0.10	Indistinct

ΔH_N: enthalpy of denaturation for the new peak (28–40°C). ΔH_1: enthalpy of denaturation for myosin, sarcoplasmic protein and a new structure (40–60°C). ΔH_4: enthalpy of denaturation for actin (65–80°C).

than hydrogen bonds are the dominant stabilising forces (Galazka and Ledward, in press) and thus, on scanning heated fish muscle, or rescanning pressure-treated samples, no endothermic peaks will be seen.

The rationale for the above is as follows: as pressure is progressively exerted on a protein, electrostatic and hydrophobic interactions will readily break (Galazka and Ledward, in press) and, as pressure is increased still further, hydrogen bonds may ultimately rupture. However, as pressure is released, hydrogen bonds will form initially and only subsequently will any hydrophobic or electrostatic forces be superimposed on this structure. Thus the network will be dominated by the formation of a hydrogen bonded gel (Fig. 1). On the application of heat to a protein, hydrogen bonds will initially rupture and subsequently hydrophobic and perhaps electrostatic bonds may also rupture. On subsequent cooling, hydrophobic and electrostatic bonds will rapidly form and any contribution of hydrogen bonds to the network will have to be superimposed on this network as the temperature decreases further. Thus, few hydrogen bonds will form and the structure will not display an endotherm on reheating. For the same reason, the pressure-treated gels after heating will not generate a distinct thermogram (Fig. 1). Thus the network formed on pressure-and heat-treatment of cod muscle, will generate very different networks and presumably exhibit very different rheological properties.

Since cod myosin is so heat- (and pressure-) labile, this work was repeated using a 'stepped' pressure increase so that the temperature never rose more than 5°C. The results obtained were very similar, indicating that the effects were pressure-, not temperature-, induced. Similar results to those reported above were obtained for fish stored on ice for a further 7 days and on two further samples of 'fresh' cod.

Electrophoretic studies

Polyacrylamide gel electrophoresis (Fig. 2) was carried out to determine the effect of high pressure treatment on the sarcoplasmic proteins in cod. From the densitometric profile (Fig. 3), we see that the untreated sample has six major protein fractions ('A', 'B', 'C', 'D', 'E' and 'F'). The pressure-treated sample at 200 MPa has a similar profile to the fresh sample, with a slight decrease in area of most peaks. However, at higher pressures, fractions ('B', 'C' and 'D') decrease with increasing applied pressure but fractions 'A' and 'F' are little affected by pressure-treatment. Thus some sarcoplasmic proteins in cod are resistant to pressure-denaturation and these may correspond to those found in Peak 2 of the thermograms. In addition, in samples treated at 400 MPa and higher, fraction 'E' increases. This suggests that a protein, perhaps in fraction 'D', is degraded by pressure to the lower molecular weight fraction 'E'. This may be due to enzyme action or due to the rupture of intermolecular hydrophobic interactions which are known to be pressure-labile (Galazka and Ledward, in press). When cod is cooked at 80°C for 10 min, there is a marked decrease in fraction 'F' and fractions 'A', 'B', 'C', 'D' and 'E' are not seen (Fig. 2).

Electrophoretic patterns (SDS-PAGE) in the absence of 2-mercaptoethanol showed no significant difference between fresh, pressure treated and cooked samples (Fig. 4), whereas the electrophoretic pattern (SDS-PAGE) in the presence of 2-mercaptoethanol (Fig. 5) showed that a 67 K Da protein increase in concentration after pressure treatment at 200 to 600 MPa. However, there are no differences between the control and samples pressure-treated at 800 MPa.

Thus it would appear that few, if any, covalent bonds, including disulphide ones, form on pressure- (or heat-treatment). The appearance of the 67 K Da band, in the presence of 2-mercoptoethanol, in the 200–600 MPa treated samples, was unexpected. The result was reproducible, being found in five different cod samples.

Storage of the fish at 4°C for 7 days had no apparent affect on any of the electrophoretograms.

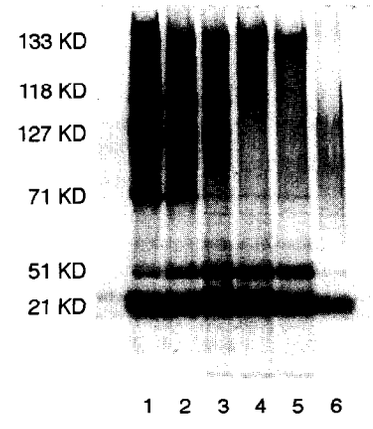

Fig. 2. Native-PAGE of the water soluble extracts of cooked or pressure treated (20 min) cod muscle. Lane 1, untreated; Lane 2, 200 MPa; Lane 3, 400 MPa; Lane 4, 600 MPa; Lane 5, 800 MPa and Lane 6, cooked at 80°C for 10 min.

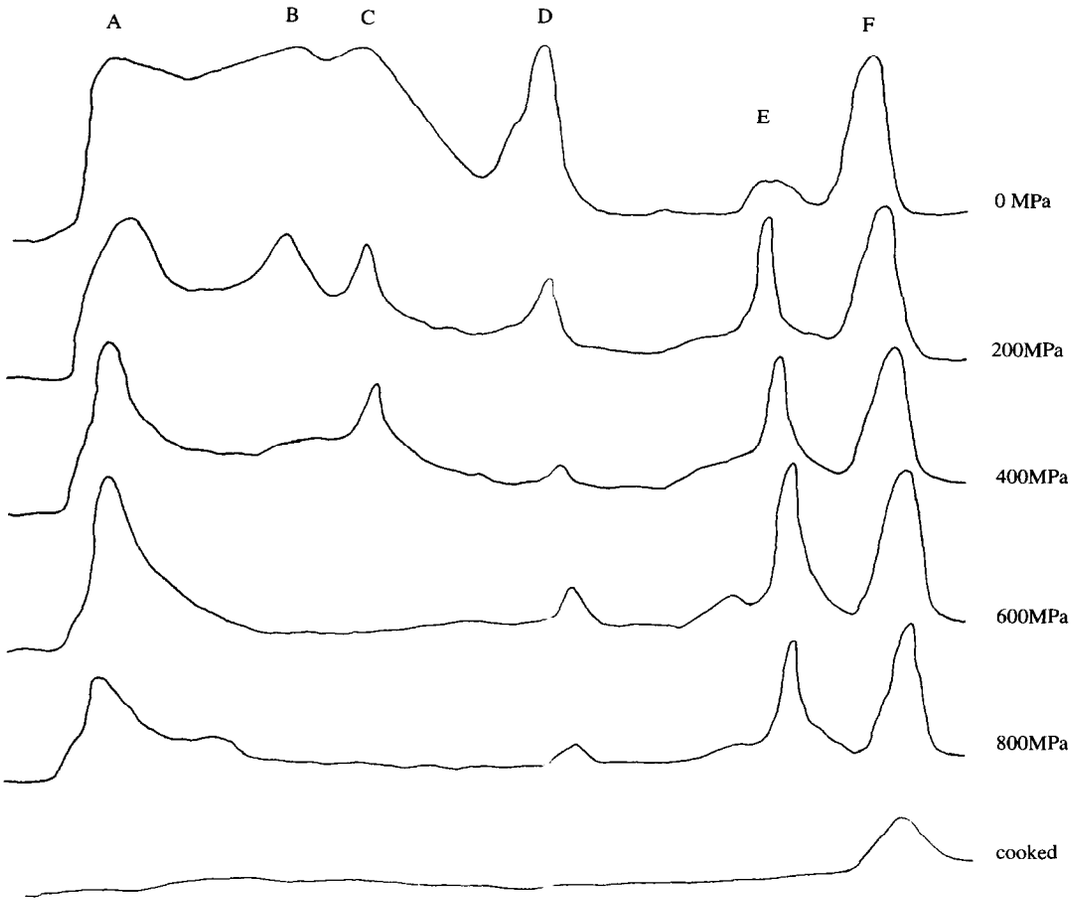

Fig. 3. Densitograms of the electrophoretic gels shown in Fig. 2. Fractions A, B, C, D, E and F correspond to marker proteins of approximate molecular weight, 133, 118, 127, 71, 51 and 21 kd, respectively.

Proteolytic activity

pH profile of cod proteases

The effects of pH on the proteolytic activity extracted from cod muscle, against haemoglobin is shown in Fig. 6. Optimum activity is seen to be at pH 6.6 and second and the third optima appear at pH 9.0 and 3.3, respectively.

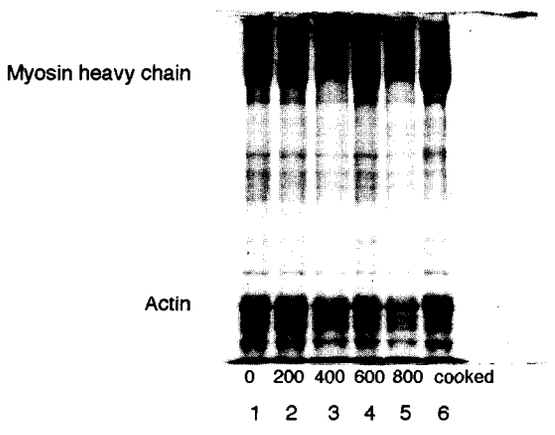

Fig. 4. SDS PAGE analysis of solubilised protein in the absence of 2-mercaptoethanol for cod muscle after treatment at different pressures for 20 min or heating at 80°C for 10 min. Lane 1, untreated; Lane 2, 200 MPa; Lane 3, 400 MPa; Lane 4, 600 MPa; Lane 5, 800 MPa and Lane 6, cooked.

The effects of pressure treatment on the proteolytic activity of cod muscle is presented in Fig. 7. We see that the activity at pH 6.6 decreases rapidly at pressures above 200 MPa, while the activity at pH 3.3 and 9.0 appears to increase slightly after pressure treatment at 200 MPa. These activities gradually decrease at higher pressures. For the cooked (80°C for 10 min) samples, the enzyme activities at pH 3.3, 6.6 and 9.0 were low (Fig. 7).

Erickson *et al.* (1983) found that the optimum pH values of true cod (*Gadus macrocephalus*) proteases against haemoglobin were at 3.2–3.3 and 7.8–8.0, and for pacific whiting (*Merluccius productus*), were at 3.5–3.9 and 7.1–7.2. Protease activities vary among different species of fish and often differ even within the same species (Wasson, 1992). Thus our results are in reasonable agreement with previously reported ones. In Fig. 7, it is seen that protease activity decreases with increasing pressure beyond 200 MPa. This is in fair agreement with Native-PAGE electrophoresis data (Fig. 3) which shows a loss of some sarcoplasmic protein fractions as pressure is increased. Ohmori *et al.* (1991) found that the neutral protease activity from beef round was decreased by pressure treatment above 400 MPa. In

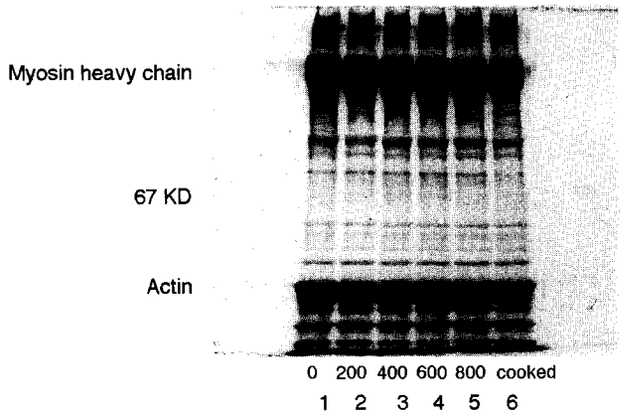

Fig. 5. SDS-PAGE analysis of solubilised protein in the presence of 2-mercaptoethanol for cod muscle after treatment at different pressures for 20 min or heating at 80°C for 10 min. Lane 1, untreated; Lane 2, 200 MPa; Lane 3, 400 MPa; Lane 4, 600 MPa; Lane 5, 800 MPa and Lane 6, cooked.

addition, aminopeptidase and carboxypeptidase were completely inactivated at 500 and 400 MPa, respectively. However, Fig. 7 shows that the alkaline protease activity was not lost on pressure treatment up to 500 MPa and acid protease activity was only slightly decreased at 400 MPa and above. The slight increase in protease activity at pH 3.3 and 9.0 for the 200 MPa treated samples may be due to the treatment leading to destruction of the lysosomal membrane and subsequent leakage of the protease and thus enhanced activity (Ohmori *et al.*, 1991). Kurth (1986) has also reported that pressure treatment at 100–150 MPa enhances cathepsin B1 activity. The results for the cooked samples (80°C for 10 min) show that the proteases can tolerate this temperature treatment (Fig. 7). Cheng *et al.* (1979) reported that the proteolytic protease activity in a crude fraction extracted from croaker was optimum at 60°C and was not lost at 85°C. That the neutral protease is most affected by pressure is of interest since,

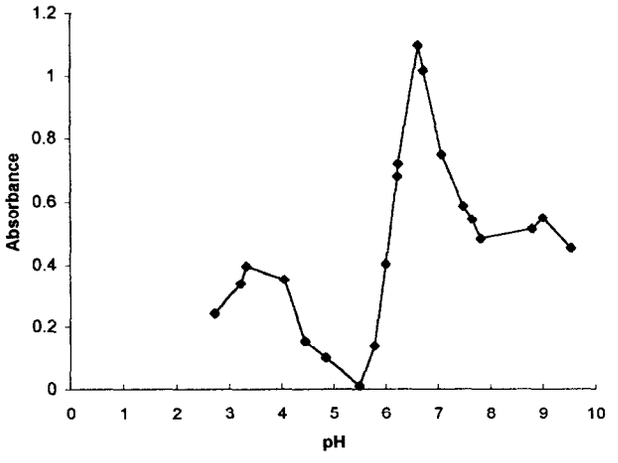

Fig. 6. Proteolytic activity of cod muscle, judged by its activity against haemoglobin at 55°C, as a function of pH. The absorbance (at 280 nm), is directly proportional to the activity.

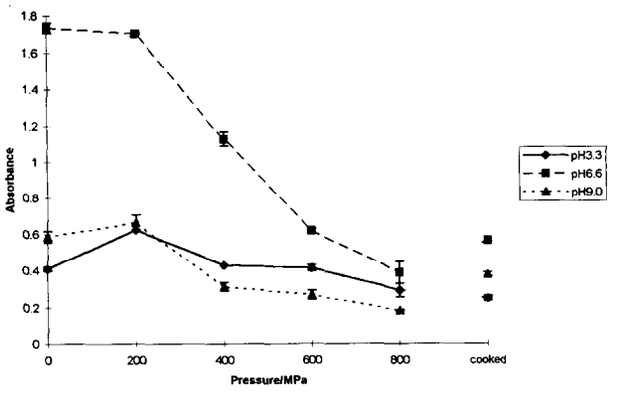

Fig. 7. Influence of high pressure treatment on the protease activity (proportional to absorbance) of cod muscle after treatment at the stated pressure for 20 min. Cooked sample was at 80°C for 10 min. All values are mean + standard deviation of triplicates.

at the pH of the muscle, this is likely to be the most active and thus have most effect on texture.

Effect of heat and pressure on fish texture

Texture profile analysis (TPA) was carried out to determine the effect of thermal and pressure treatment on the texture of fish fillets. Typical results are shown in Figs 8–13. An increase in temperature had only a small effect on the springiness and cohesiveness of the cod fillets, but there was a significant decrease in gumminess,

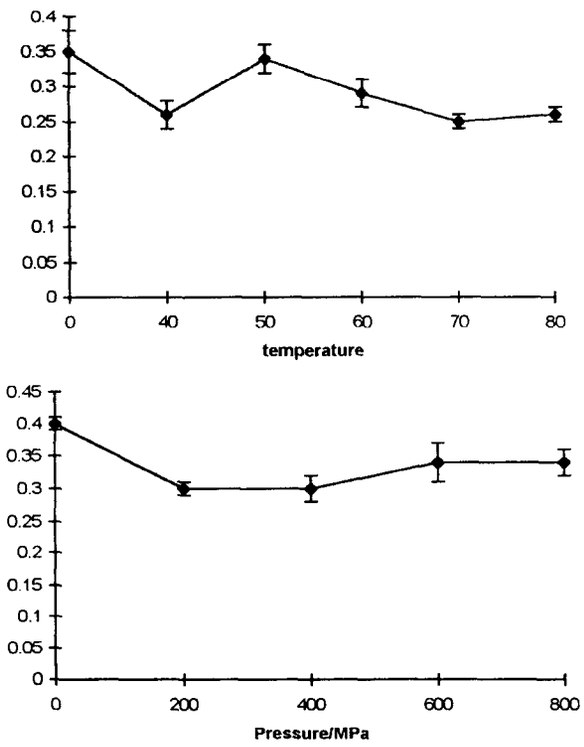

Fig. 8. The effect of temperature (40–80°C for 10 min) or pressure (200–800 MPa for 20 min) on the cohesiveness of cod muscle. All values are the mean ± standard deviation of 8 determinations.

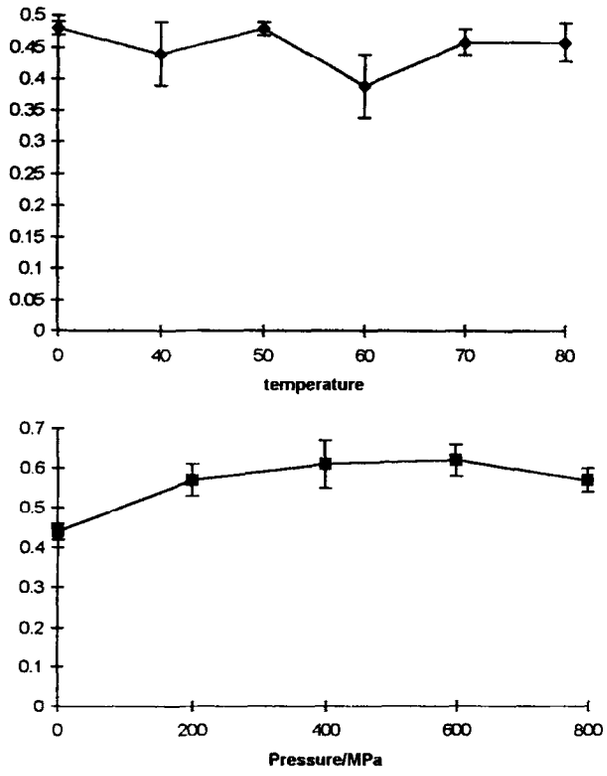

Fig. 9. The effect of temperature (40–80°C for 10 min) or pressure (200–800 MPa for 20 min) on the springiness of cod muscle. All values are the mean ± standard deviation of 8 determinations.

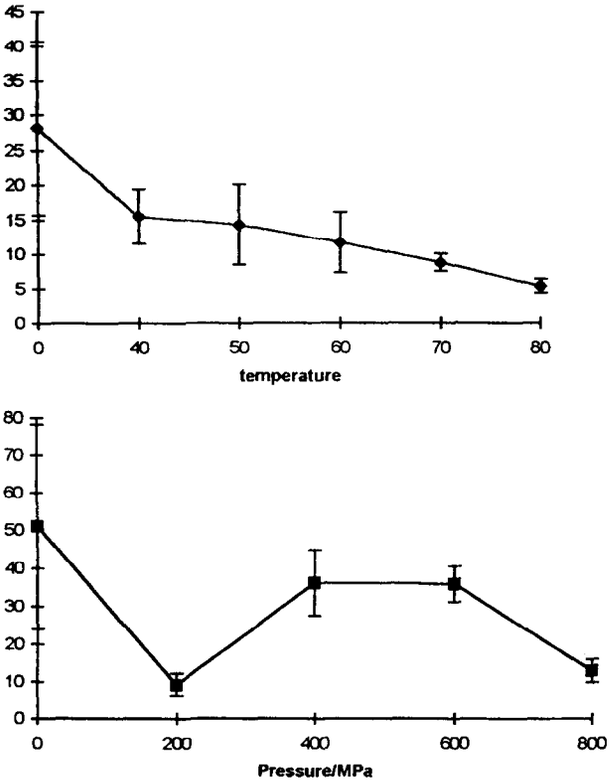

Fig. 10. The effect of temperature (40–80°C for 10 min) or pressure (200–800 MPa for 20 min) on the adhesiveness of cod muscle. All values are the mean ± standard deviation of 8 determinations.

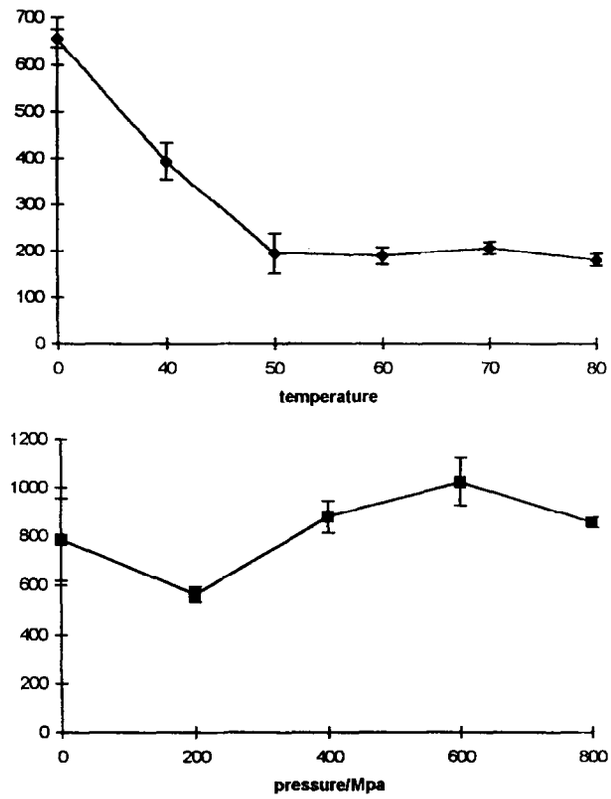

Fig. 11. The effect of temperature (40–80°C for 10 min) or pressure (200–800 MPa for 20 min) on the gumminess of cod muscle. All values are the mean ± standard deviation of 8 determinations.

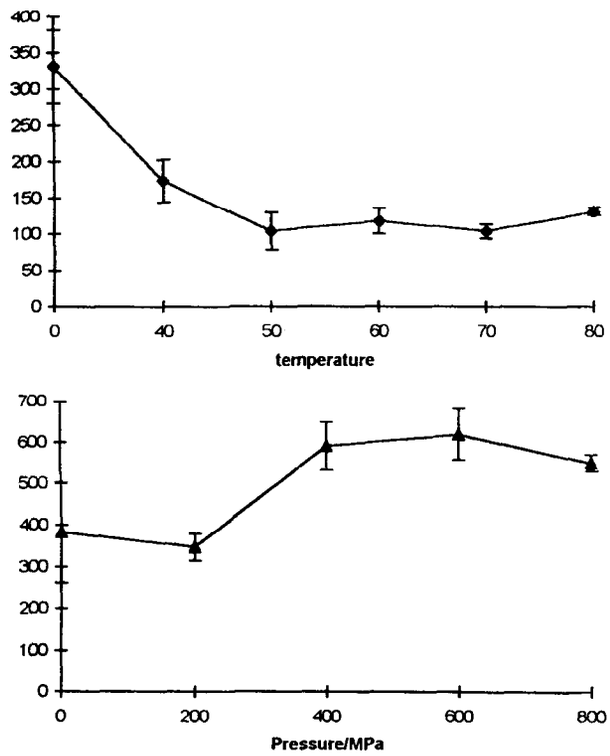

Fig. 12. The effect of temperature (40–80°C for 10 min) or pressure (200–800 MPa for 20 min) on the chewiness of cod muscle. All values are the mean ± standard deviation of 8 determinations.

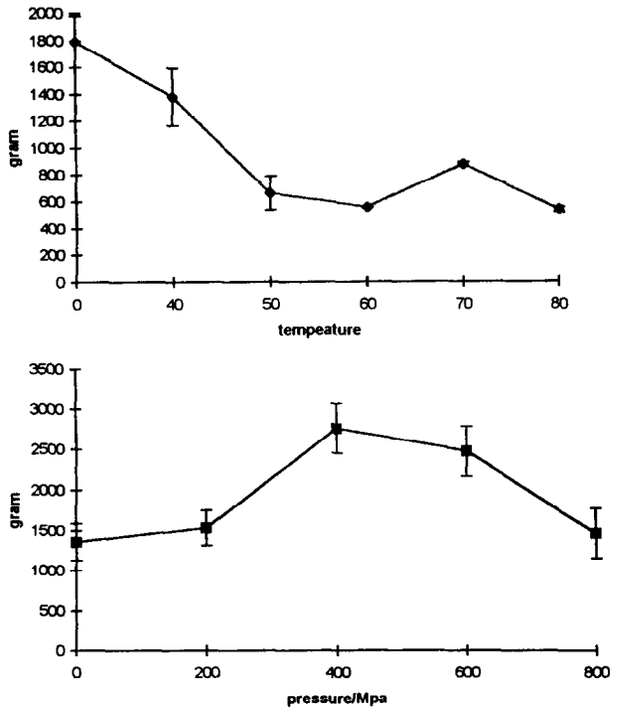

Fig. 13. The effect of temperature (40–80°C for 10 min) or pressure (200–800 MPa for 20 min) on the hardness of cod muscle. All values are the mean ± standard deviation of 8 determinations.

hardness, adhesiveness and chewiness when heated to 50°C. Small decreases in adhesiveness were observed at higher temperatures but little change was seen in the other parameters. It should be noted that cod myosin (and collagen) denatures at about 40–45°C (Fig. 1; Davies *et al.*, 1988) and these transitions must contribute to the significant changes noted in this temperature range. It would appear that the subsequent denaturation of actin (≈70°C) has little effect on the textural characteristics. It is also likely that proteolysis continues at these temperatures and contributes to the observed decreases.

The results of heating are in agreement with research by Deng (1981) who reported that, although the shear force value (toughening) of mullet flesh increased sharply on heating, due to increased protein–protein interactions, on prolonged heating they decreased. The decrease in toughness could be associated with two possible mechanisms; one is the thermal dissociation of muscle protein and the other is the enzymatic hydrolysis of muscle protein. Considering the stability of the proteases to heat, the latter seems most likely. In addition, the texture characteristics correlate, to some extent, with the moisture content of the samples, which decreases with increases in temperature (Table 6). This is in line with reports by Cheng *et al.* (1979) who found a close relationship between firmness and springiness and water holding capacity of gels from croaker, mullet, ribbon and sand trout. Similar results have been reported by other researchers (Lee and Toledo, 1976; Lanier *et al.*, 1981).

Table 6. The effect of pressure (200–800 MPa for 20 min) and thermal treatment (40–80°C for 10 min) on moisture content and pH of cod muscle. Values are the means ± standard deviations of 4 determinations

Treatment	Moisture content (%)	pH
0	80.69 ± 0.05	6.55 ± 0.03
200 MPa	80.59 ± 0.09	6.81 ± 0.02
400 MPa	80.50 ± 0.09	6.81 ± 0.01
600 MPa	80.08 ± 0.14	6.78 ± 0.01
800 MPa	79.40 ± 0.29	6.87 ± 0.01
40°C	80.62 ± 0.05	6.63 ± 0.01
50°C	80.08 ± 0.03	6.69 ± 0.01
60°C	79.44 ± 0.08	6.85 ± 0.0
70°C	78.82 ± 0.09	6.72 ± 0.01
80°C	78.87 ± 0.19	6.76 ± 0.01

As with temperature, the effect of pressure on both cohesiveness (Fig. 8) and springiness (Fig. 9) was small, although cohesiveness decreased from 0 to 300 MPa and springiness increased over this range. There was also a sharp decrease in adhesiveness of cod fillet when treated at 200 MPa (Fig. 10) and some loss of gumminess (Fig. 11) while the values increased when treated at 400 and 600 MPa. Chewiness and hardness of samples treated at 400 and 600 MPa increased significantly when compared with the fresh samples and samples treated at 200 MPa. However, these values dropped after treatment at 800 MPa (Figs 12 and 13). Ashie and Simpson (1996) have recently reported on the effects of pressures, up to 300 MPa on the texture of blue fish and found that although samples treated at 300 MPa appeared harder by sensory examination, Instron compressive probe measurements indicated they were softer. Their results were rather variable. Comparisons of the texture profiles of the pressure-treated samples with the thermograms, suggest very strongly that the loss of myosin structure leads to the decrease in adhesiveness, gumminess and cohesiveness. In contrast, an increase in springiness, and subsequent unfolding of the actin (and sarcoplasmic proteins) at 400 and 600 MPa leads to the increases in gumminess, hardness and adhesiveness. The formation of the hydrogen bonded networks (Fig. 1) must contribute to these observations.

When the textural properties of heat- and pressure-treated fish fillets are compared, it is seen that the gumminess, adhesiveness, hardness and chewiness of heated samples decrease as temperature increases to 50°C, presumably due to myosin denaturation (and collagen), since actin and many sarcoplasmic proteins do not denature below 50°C. Decreases in hardness and chewiness were not found on increasing pressure to 200 MPa, even though myosin readily denatures, confirming the difference in the nature of the gel networks formed.

For samples subjected to 800 MPa, the properties such as gumminess, hardness, chewiness and adhesiveness decreased slightly when compared with samples treated at 400–600 MPa, although the enzyme activity

Table 7. Pressure-induced changes in cod muscle

Pressure range (MPa)	Lipid stability	Protein stability	Protease activity	Texture
0–200	No effect	Myosin, denatured, New gel formed	Slight increase in acid proteases	Decreased adhesiveness, gumminess, cohesiveness
200–400	No effect	Actin plus some sarcoplasmic denatured. New structure formed.	Marked decrease in acid, neutral and alkaline proteases	Increased hardness, adhesiveness, chewiness, gumminess
400–800	Marked decrease	Little further change	Further decreases especially with alkaline protease.	Slight changes including decrease in adhesiveness and hardness

was relatively low (Fig. 7). The decreases might be the influence of the decreasing moisture content in the muscle seen after treatment at 600 and 800 MPa (Table 6).

CONCLUSIONS

The major effects of pressure on cod muscle are summarised in Table 7. Pressure treatment of cold water fish bears many similarities to the effects on red meats. Thus at pressures above 400 MPa, lipid oxidation is increased due to the release of free metal ions. Cod contains about 0.2 mg ion and 22.4 μg of copper per 100 g (Jhaveri *et al.*, 1984). Since haem is itself a powerful catalyst (Johns *et al.*, 1989) and in beef we have failed to detect a significant loss in haem ion on pressure treatment (Defaye, unpublished) it seems likely that the iron is liberated primarily from the non-haem complexes, such as ferritin and haemosiderin. Since most of the protein changes occur below 400 MPa, the liberation of the ions is not related to denaturation of the structural proteins.

If pressure-treatment is to become a viable processing technology for fish, the instability of the lipids to oxidation needs to be addressed when pressures above 400 MPa are used. However, the effects on texture are primarily observed at pressures below 400 MPa. Such effects on texture are of interest since they suggest that the characteristic pressure-treated texture is different from that seen in both raw or cooked fish, being harder, chewier and gummier than the cooked product. This would appear to be primarily due to the different response of the myofibrillar proteins to heat and pressure, plus, to a limited extent, the survival of proteolytic enzymes in both the heated and pressure-treated samples.

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