

Application of high hydrostatic pressure to control enzyme related fresh seafood texture deterioration

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Enzyme extracts from two fish species, bluefish and sheephead, were subjected to hydrostatic pressure treatments ranging between 1000–3000 atm for various durations and the residual activities of cathepsin C, collagenase, chymotrypsin- and trypsin-like enzymes compared with those of their bovine counterparts. The fish enzymes were generally more susceptible to hydrostatic pressure inactivation than their mammalian counterparts. Fresh fish muscle tissues were also subjected to similar pressures and the color and textural parameters determined. The color generally became lighter presenting a cooked appearance to the fresh fish flesh with increasing pressure as well as holding time. There was general toughening/hardening of the tissue when 1000 atm of pressure was applied. The upper limit for maintaining or enhancing tissue hardness was the application of 2000 atm for 10 min. Beyond this point, the tissue generally becomes softer with pressure and holding time. Copyright © 1996 Published by Elsevier Science Ltd on behalf of the Canadian Institute of Food Science and Technology

INTRODUCTION

Texture is considered to be one of the important quality attributes of seafoods (and muscle foods in general) which determines consumer acceptance and hence the marketability of such products. Unlike other muscle foods, however, seafoods generally undergo a more rapid resolution of rigor which culminates in extensive softening of the flesh. These observations have been attributed to (i) less connective tissue content in fish, and (ii) less extensive cross-link formation in fish collagenous tissue in comparison with their mammalian counterparts (Bracho & Haard, 1990). Furthermore, enzyme potentiated postmortem changes in muscle tissues also contribute to the rapid flesh softening and several fish muscle enzymes as well as digestive proteases including cathepsins, collagenases, Ca^{2+} -dependent proteases, alkaline proteases, trypsin and chymotrypsins have been variously implicated in the process (Bremner, 1992; Makinodan *et al.*, 1985; Yamashita & Konagaya, 1992;

Jiang *et al.*, 1992; Lindner *et al.*, 1988). While many preservation strategies have focussed on controlling microbial activity in seafoods during storage, the influence of enzymatic activity on spoilage appears to have been grossly underestimated. Recent evidence has shown that most of the deteriorative changes which reduce the shelf-life of such products may be due to enzyme-related biochemical activities rather than microbial (Fletcher & Statham, 1988; Fletcher & Hodgson, 1988).

In an earlier report, we noted the effects of high hydrostatic pressure on some texture-related enzymes, obtained commercially from bovine sources and further intimated on the possibility of hydrostatic pressure application to preserve seafoods (Ashie & Simpson, 1995). With about 95% of the habitat temperature of fish species being below 5°C throughout the year, seafoods have shown certain adaptive differences in the nature of the bonds that determine the higher orders of structure or conformations of their enzymes in comparison with their homologues from terrestrial or warm temperature habitats (Simpson & Haard, 1984a, b). Generally, enzymes from organisms adapted to cold habitats tend to be more thermolabile and have a more flexible structure than their warm temperature counterparts (Low & Somero, 1974).

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These differences have been attributed to (i) greater hydrogen bonding (Koffler, 1957); (ii) more extensive hydrophobic interactions (Singleton & Amelunxen, 1973); (iii) more pronounced disulfide linkages in the warm-adapted species (Komatsu & Feeney, 1970; Simpson & Haard, 1984a); and (iv) more organized structure as illustrated by degree of α -helicity in the warm-adapted species (Simpson & Haard, 1987). Hydrostatic pressure had been shown to inactivate enzymes by disrupting the bonds that determine the secondary, tertiary, and quaternary conformations without affecting the covalent bonds in the primary structure (Okamoto *et al.*, 1990). Notwithstanding any possible differences in the mechanisms of pressure- and heat-induced protein denaturation (Ohshima *et al.*, 1993), seafood enzymes would be expected to be more susceptible to high hydrostatic pressure inactivation than their terrestrial or warm-adapted counterparts based on the structural differences mentioned above. There is, however, no report of any such investigation to confirm or refute this view. Furthermore, even though there had been several studies on hydrostatic pressure application in surimi and other fish gel preparations, there is a relative paucity of information and research on the application of this technology in preserving fresh seafoods. Hence the objectives of the current study were (i) to assess the degree of susceptibility of some seafood enzymes (chymotrypsin-like, trypsin-like, collagenase, and cathepsin C) to hydrostatic pressure inactivation in comparison with their warm-adapted bovine counterparts; and (ii) to determine the efficacy of hydrostatic pressure as a tool for controlling fresh seafood texture deterioration.

MATERIALS AND METHODS

Bovine enzymes (trypsin, chymotrypsin, and cathepsin C) and the synthetic substrates glycyl-L-phenylalanine β -naphthylamide (gly-phe NA), 4-phenylazobenzyloxycarbonyl-pro-leu-gly-pro-D-arg (PZ-peptide), N-benzoyl-L-tyrosine ethyl ester (BTEE), and N α -benzoyl-DL-arginine p-nitroanilide (BAPNA), were purchased from Sigma Chemical Co (St. Louis, MO, USA). Fresh bluefish (*Pomatomus saltatrix*) and sheephead samples were obtained from a local fish market (Waldman Plus, Montreal, Quebec) and kept under ice until ready for use.

Enzyme extraction

Crude fish enzymes were extracted according to the method of Baranowski *et al.* (1984) in the following manner. Fresh fish flesh taken from the upper dorsal region of the sample was chopped into pieces and suspended in 0.05 M Tris-HCl buffer (pH 7.4 with 5.0 mM CaCl₂; 1:3 ratio). The suspension was then homogenized at low speed for 5 min using a polytron blender

(Brinkman model PT 3000, Littau, Switzerland) and centrifuged at 12 000 g for 30 min at 4°C. The supernatant was filtered using glasswool and subjected to further centrifugation at 39 000 g for 30 min at 4°C and the extract filtered through 0.22 μ m sterile filter under vacuum. This crude enzyme extract was kept on ice and aliquots taken for pressure treatment and subsequent enzyme assays. Crude enzyme extracts from fish tissues pre-treated with pressure were also assayed. Protein concentration was determined by the Hartree method (Hartree, 1972).

Enzyme assay

Chymotrypsin and trypsin activities were spectrophotometrically determined according to the methods of Hummel (1959) and Simpson & Haard (1984b), using BTEE and BAPNA as substrates, respectively. Cathepsin C activity was assayed using gly-phe-NA as substrate and the amount of 2-naphthylamine released was measured from its extinction coefficient at 340 nm (Lee *et al.*, 1971). For collagenase activity, the method of Wunsch and Heidrich (1963) was used as follows: 0.5 ml of the crude enzyme extract and 2 ml of PZ-peptide (0.5 mg ml⁻¹), both of which had been pre-incubated at 37°C for 5 min were mixed and incubated at this temperature (in a water bath) for 15 min. After this period, 0.5 ml aliquot was transferred into another tube containing 1.0 ml of 0.5% (w/v) citric acid and 5 ml ethyl acetate. After vigorous shaking with a vortex mixer, the aqueous and non-aqueous layers were allowed to separate and 4 ml of the upper acetate layer transferred to a separate tube and quickly dried with 0.3 g anhydrous Na₂SO₄. The amount of hydrolytic product was read at 320 nm.

Pressure treatment

Crude fish enzyme extracts as well as 1 mg ml⁻¹ solutions of commercially obtained enzymes in 2.5"/5" whirl-pak bags (VWR Scientific, Montreal, Canada) were subjected to hydrostatic pressures ranging from 1 000–3 000 atm using an isostatic pressure unit (Autoclave Engineers, Columbus, Ohio). Similarly, samples of fish muscle tissue taken from the dorsal region were placed in 4.5"/9" whirl-pak bags, vacuum sealed, and subjected to pressure treatment. Period required for pressurization ranged between 20–120 s while depressurization was within 10 s. Temperature within the pressure chamber was ambient.

Measurement of textural properties

Pressurized and control samples were taken from the whirl-pak bags and cut into cubes of dimensions (mm) 30 × 20 × 15. The textural properties were measured with an Instron (Lloyd Instruments — Omnitronix, Mississauga, Ontario) at room temperature using a

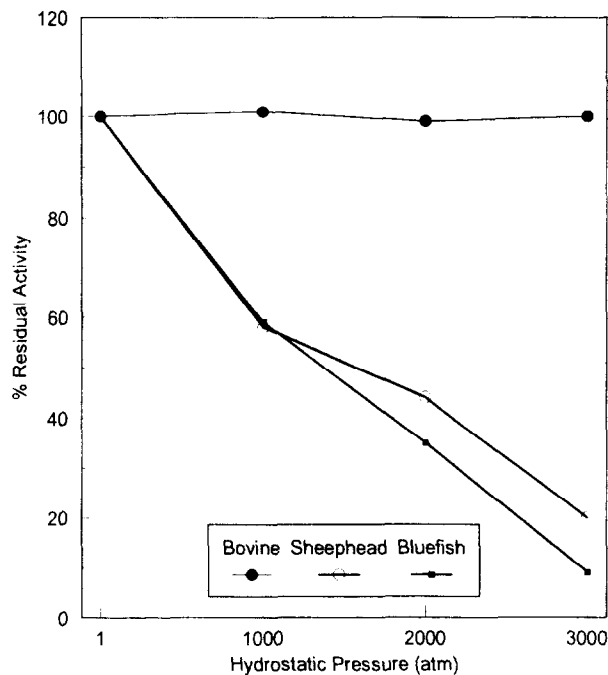


Fig. 1. Effect of hydrostatic pressure on bovine and seafood cathepsin C. Data is based on mean values for triplicate pressure treatments for 30 min durations of crude enzyme extracts from fish samples and commercially obtained bovine enzymes.

compressive probe at a speed of 20 mm/min^{-1} . A steadily increasing load was applied to a maximum of 45 Newtons and the corresponding deformations measured. Values of textural parameters were expressed as the mean of three measurements.

Color measurement

L, a, and b values of the pressurized and control fish samples were determined with Minolta Chromameter II reflectance equipment. Differences between mean values were analyzed using the least significant difference (LSD) multiple comparison test (Steel & Torrie, 1960). The general linear model (GLM) procedure of the Statistical Analysis System (SAS) on McGill University mainframe was used.

RESULTS AND DISCUSSION

Figures 1–3 show the relative effects of hydrostatic pressure on some enzymes derived from bovine and the fish species, sheephead and bluefish. With regards to cathepsin C, whereas application of 3 000 atm pressure for 30 min had little or no effect on the bovine enzyme, sheephead and bluefish enzymes lost as much as 80% and 91% of their original activities, respectively. Similarly, while bovine trypsin retained about 49% of its original activity after similar treatment, sheephead and bluefish trypsins retained only 34% and 26%, respectively. These results indicate that the seafood enzymes

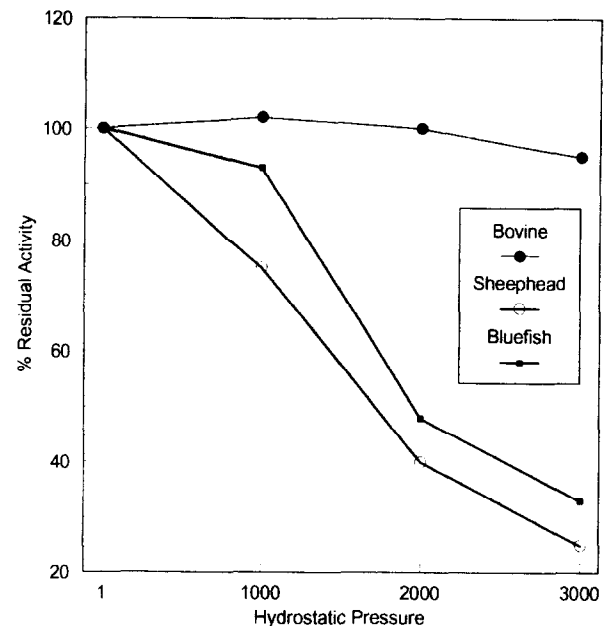


Fig. 2. Effect of hydrostatic pressure on bovine and seafood chymotrypsins. Data is based on mean values for triplicate pressure treatments for 30 min durations of crude enzyme extracts from fish samples and commercially obtained bovine enzymes.

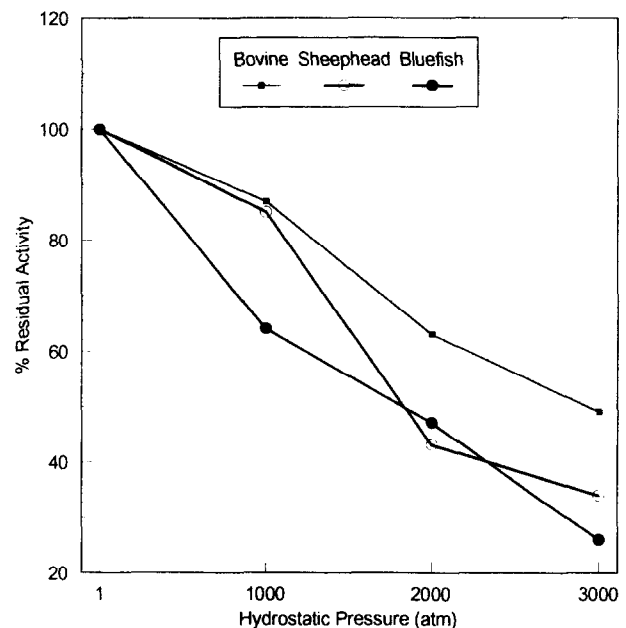


Fig. 3. Effect of hydrostatic pressure on bovine and seafood trypsins. Data is based on mean values for triplicate pressure treatments for 30 min durations of crude enzyme extracts from fish samples and commercially obtained bovine enzymes.

were generally more susceptible to hydrostatic pressure inactivation than their bovine counterparts and correlate well with the adaptive differences in the nature of enzymes derived from warm- and cold-adapted organisms (Simpson & Haard, 1987; Low & Somero, 1974). Furthermore, the results suggest that despite the reported differences between the mechanism(s) of thermal

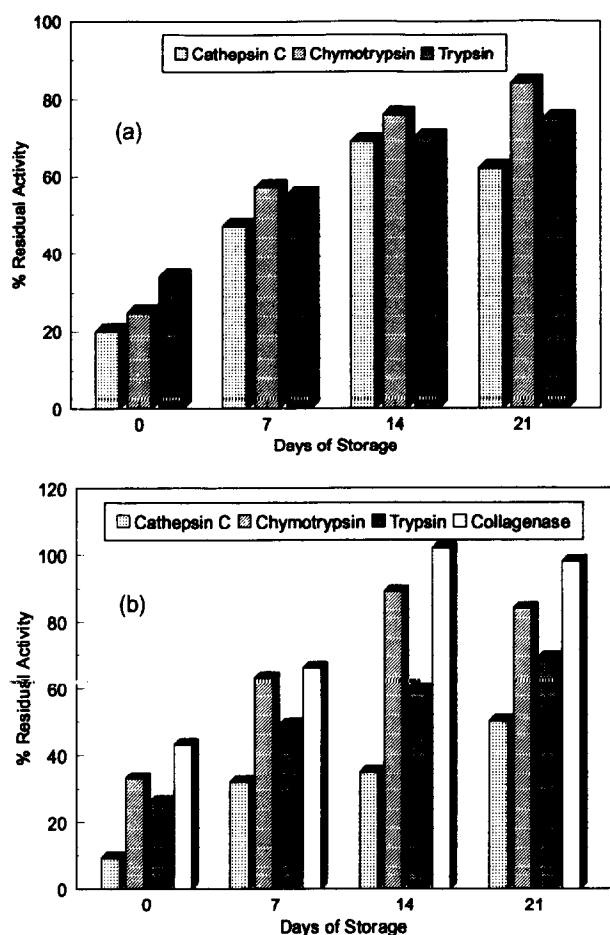


Fig. 4. Effect of refrigerated storage on some pressure-treated enzymes from (a) sheephead, and (b) bluefish. Data is based on triplicate treatments of fish samples with 3000 atm pressure for 30 min durations.

and pressure-induced protein denaturation (Okamoto *et al.*, 1990; Ohshima *et al.*, 1993), the process may involve the disruption of similar bonds.

In agreement with our previous report (Ashie & Simpson, 1995), the enzymes from both fish species also showed various levels of reactivation when stored at refrigerated temperatures (4–7°C). This is shown in Fig. 4 for enzymes from the two fish species after being subjected to 3000 atm pressure for 30 min durations. However, unlike our previously reported data which showed significant and consistent differences in the susceptibility of the enzymes to hydrostatic pressure treatment, there was no such consistency in the susceptibilities shown by the seafood enzymes. For instance, when 2000 atm pressure was applied to the crude enzyme from sheephead for 30 min, the residual activities for cathepsin C, chymotrypsin- and trypsin-like enzymes were 44%, 40% and 43%, respectively. However, when subjected to 1000 atm pressure for the same period, the three enzymes retained 58%, 75% and 85% of their respective original activities. Hence no clear order of susceptibility could be deduced for the seafood enzymes as for the bovine enzymes.

Table 1. Tristimulus color values of pressure treated fish tissue

Pressure Applied	Holding Time	L	a	b	ΔE
1	0	40.5	1.5	-2.6	40.562 ^f
1000	5	43.3	1.5	-2.6	43.405 ^{f,c}
	10	46.3	1.5	-2.7	46.407 ^{d,e}
	15	46.9	1.6	-2.3	46.988 ^{d,e}
	30	49.7	1.4	-2.5	49.784 ^{d,c}
2000	5	48.7	1.8	-2.2	48.784 ^{d,c}
	10	52.3	1.5	-1.4	52.290 ^c
	15	56.2	1.3	-1.2	56.179 ^b
	30	59.3	0.6	-0.8	59.259 ^b
3000	5	67.7	1.1	-0.5	67.661 ^a
	10	68.8	0.9	0.2	68.807 ^a
	15	69.0	0.6	-0.3	69.004 ^a
	30	69.1	0.2	0.8	69.105 ^a

Table showing changes in tristimulus color values of pressure-treated fish tissue. Data represents the mean of at least nine measurements. Mean ΔE values sharing the same superscript are not significantly different ($P > 0.05$). Least significant difference (LSD) = 3.6341.

Certain time–pressure combination treatments of the fish muscle resulted in significant ($P \leq 0.05$) changes in color as shown by the tristimulus values in Table 1. The color generally became lighter with increasing pressure presenting an increasingly cooked appearance to the flesh. The L-values, which give an indication of the brightness or lightness of the sample, showed progressive increase with both the amount of pressure applied and the duration of pressure application. The yellow/blue color indicator (b-values), though generally negative, also showed a similar trend with both the amount of pressure applied and holding time. On the other hand, the green/redness indicator (a-values) was reduced with amount of pressure and holding time. Further statistical analysis of the ΔE values (Francis & Clydesdale, 1975), an indicator of total color difference ($\Delta E^2 = \Delta L^2 + \Delta a^2 + \Delta b^2$), showed that there was no significant difference ($P > 0.05$) in color between sample treatments up to 2000 atm for 10 min. Beyond this limit, however, the changes were quite significant (Table 1). These observations are further illustrated in Fig. 5. The color changes noted here are in agreement with data reported elsewhere with pressure treatment of squid mantle, cod and mackerel muscle, as well as Alaska pollock surimi (Nagashima *et al.*, 1993; Ohshima *et al.*, 1993; Shoji *et al.*, 1990). The mechanism(s) involved in color change is, however, still unclear even though pressure-induced denaturation of heme compounds like metmyoglobin have been shown to cause spectral shifts in such compounds (Gibson & Carey, 1977; Zipp & Kauzmann, 1973).

Measurement of textural parameters showed the sensitivity of fresh fish flesh to hydrostatic pressure depends on both the amount of pressure applied as well as the holding time. As illustrated in Fig. 6a, the strength which

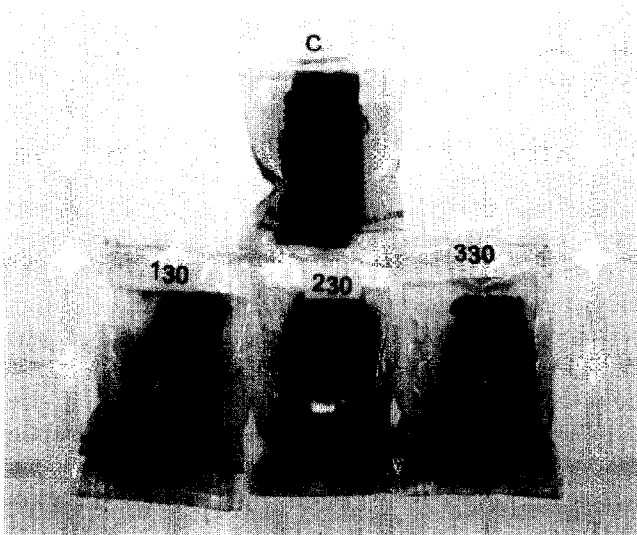


Fig. 5. A photograph showing changes in appearance of fish samples subjected to pressures of 1 000–3 000 atm for 30 min durations in comparison with untreated control (C) samples. Superscript indicates amount of pressure $\times 1\,000$ atm while subscript indicates duration of pressure application.

indicates toughness/softness of the tissue, was generally increased on application of 1 000 atm pressure for the entire period used in this study (5–30 min). At 2 000 atm, however, there was no significant change in strength until the holding time had exceeded 10 min. Beyond this point, there was general reduction in strength. When subjected to 3 000 atm pressure, even for 5 min, the fish tissue generally became softer than the control sample. We found the textural parameters of samples treated at 3 000 atm rather unexpected since sensory examination of the texture seemed to indicate these to be harder than the untreated as well as those treated with lower pressures. However, comparison of Fig. 6b shows some similarities between responses of tissue strength and elasticity to pressure treatment. Since elasticity indicates springiness or cohesiveness of the tissue (Bourne, 1976), it is our view that the 'apparent' drop in tissue strength or toughness at higher pressures may be due to loss of elasticity possibly as a result of disruption of cohesive forces within the tissue. In the initial stages of pressure application when the toughness was increased (1 000 atm), the decrease in volume as a result of compaction could enhance protein–protein interactions as had been reported for some proteins in cod and mackerel (Ohshima *et al.*, 1993). Such interactions would lead to an increase in bond formation causing an increase in tissue elasticity and toughness. However, when the pressure exceeds a certain critical point (2 000 atm for 10 min in this case), these and other bonds are rather disrupted causing a reduction in elasticity with the concomitant reduction in toughness. Thus even though the tissue may seem tough/hard to touch, the reduced elasticity causes it to yield abruptly and very easily under compression.

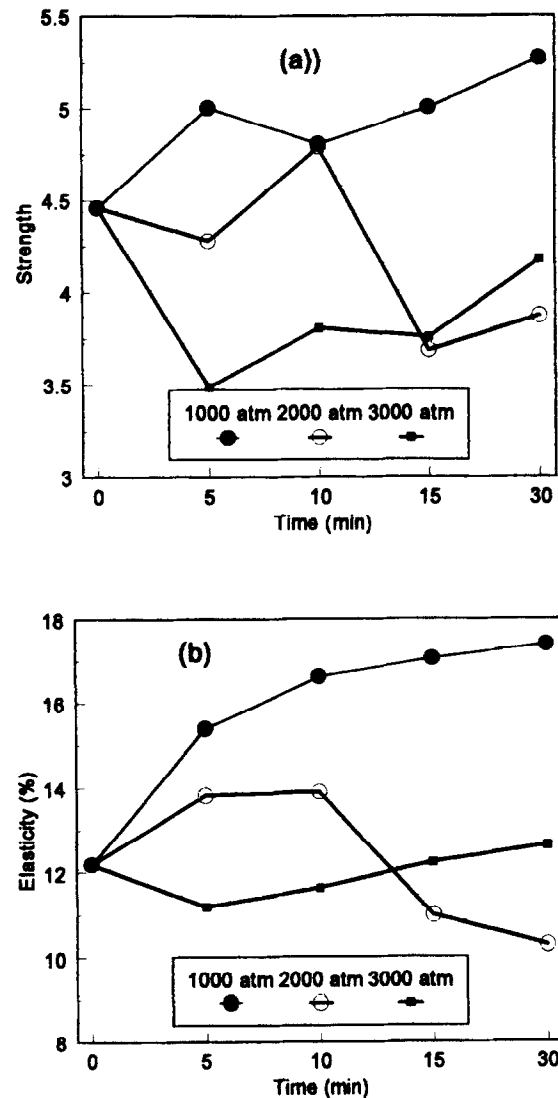


Fig. 6. Effects of hydrostatic pressure on strength and elasticity of bluefish tissue. Data is based on the mean of 9 replicates.

The softening or loss of tissue strength as a result of high hydrostatic pressure application had been the principle underlying the current interest in exploiting this technology in meat tenderization. It is reported to cause loss of structural continuity due to rupture of I-filaments, loss of M-line protein and cleavage of A-filaments in the myofibrils (Suzuki *et al.*, 1990; MacFarlane, 1985). However, the problem in the seafood industry, unlike other muscle foods, is the rapidity with which the flesh softens and it is therefore required to maintain firm/tough texture. The results reported here indicate that the upper limit of pressure application for maintaining or enhancing fresh bluefish toughness could not exceed 2 000 atm for 10 min since higher pressures would lead to loss of tissue elasticity resulting in 'apparent' flesh softening. It is therefore reasonable to assume that the reported changes in myofibrillar proteins (Suzuki *et al.*, 1990; MacFarlane, 1985) do not occur below 2 000 atm pressure for 10 min. These

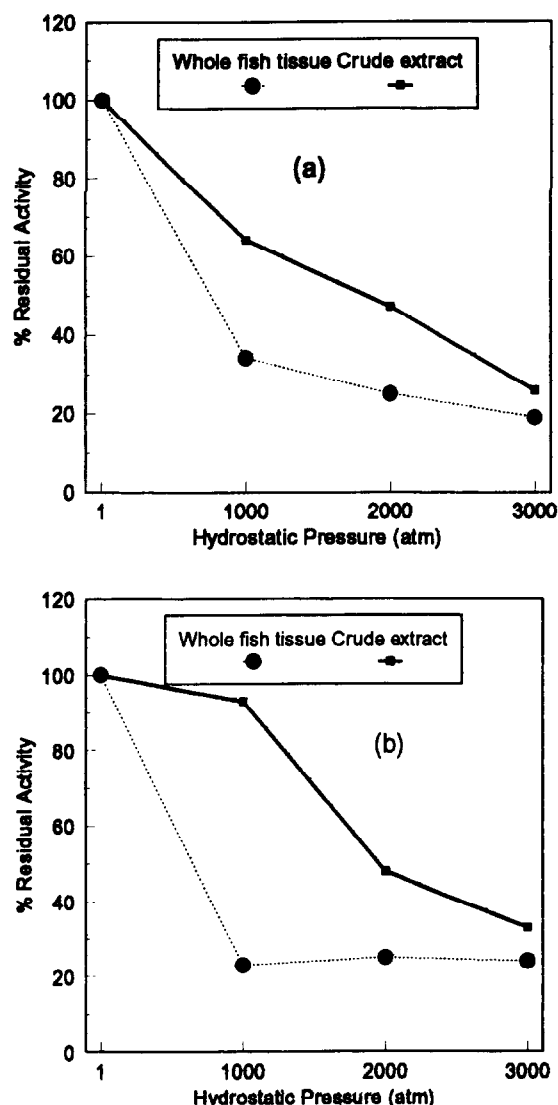


Fig. 7. Comparison of the effects of hydrostatic pressure on residual enzyme activities in fish tissues subjected to pressure treatment prior to enzyme extraction and crude enzyme extracts obtained prior to pressure treatment. (a) and (b) represent residual activities of trypsin- and chymotrypsin-like enzymes, respectively.

critical conditions may vary for the treatment of other fish species since tissue toughness varies among fish species. However, enzymes implicated in seafood texture deterioration have been shown to be susceptible to inactivation under such conditions. For instance, when we subjected the crude enzyme extract from bluefish to 1 000 atm pressure for 30 min, the residual activities of cathepsin C, chymotrypsin- and trypsin-like enzymes were 59%, 93% and 64%, respectively while collagenase was seemingly unaffected (Figs 1–3). At 2 000 atm for 10 min, they retained 48%, 65% and 45% of their respective original activities while collagenase retained 88% activity (data not shown). Assay of crude enzyme extracts obtained after pressure-treatment of whole fish tissue seems to indicate that the extent of inactivation of some of these proteases is even higher than pressure-

treated crude enzyme extracts. As shown in Fig. 7, about 66% and 77% of trypsin- and chymotrypsin-like activities were lost, respectively, when subjected to 1 000 atm pressure for 30 min. In contrast, only 36% and 7% of their respective activities were destroyed for the same treatment on the crude enzyme extract. Even though similar high pressure inactivation of seafood proteases have been reported, much higher pressures were required (Nagashima *et al.*, 1993). The reason for the difference in extent of inactivation of the proteases from pressure-treated whole fish tissue and crude enzyme extract would require further studies. We speculate, however, that while intramolecular changes in enzyme structure/conformation is the main cause of inactivation in solution, the possible enhancement of protein–protein interactions in the whole fish tissue would also result in intermolecular changes being involved in the process of inactivation.

CONCLUSION

The results presented here give further support for the enormous potential of hydrostatic pressure application as a food processing technique. It may be used to control the texture of seafoods and other muscle foods due to its ability to inactivate the endogenous proteases implicated in texture deterioration. Furthermore, the higher susceptibility of seafood enzymes to inactivation by hydrostatic pressure treatment would ensure an effective control of enzyme activity while maintaining overall quality (Steel & Torrie, 1980).

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