

Post mortem muscle protein degradation during ice-storage of Arctic (*Pandalus borealis*) and tropical (*Penaeus japonicus* and *Penaeus monodon*) shrimps: a comparative electrophoretic and immunological study

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Abstract: Water-, low-salt- and high-salt-soluble protein fractions from the abdominal muscles of *Pandalus borealis*, *Penaeus japonicus* and *Penaeus monodon* extracted immediately after death and after 5, 16, 24, 48, 72, 96 and 120 h (*P borealis*) or 16, 22, 43, 71 and 92 h (*Penaeus* spp) of ice-storage were analysed by one- and two-dimensional electrophoresis and immunological techniques. The most evident effect in *P borealis* was the decrease in the relative amount of myosin heavy chain (MHC) and a concomitant increase in the number and intensity of bands of molecular size about 100 kDa cross-reacting with anti-MHC antiserum. MHC degradation of *P borealis* was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of partially isolated native myosin. Other prominent features were the disappearance of bands of about 67 and 50 kDa after 24 h and the appearance of a band of slightly less than 50 kDa after 5 h of ice-storage. These last bands showed the potential to be used as freshness markers. One spot tentatively identified as desmin did not suffer significant changes in any of the three species. Two bands (about 100 and 96 kDa) gave a positive reaction with the α -actinin antibody in the zero-time extract of *P borealis*, but after 24 h only one faint 96 kDa band was detected. In contrast, the extracts of *P japonicus* and *P monodon* did not suffer significant alterations during the examined period, and even after 92 h of ice-storage only the 100 kDa anti- α -actinin cross-reacting band was clearly visible in the high-salt extract of *P japonicus*.

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INTRODUCTION

Pandalus borealis is a valuable fishery resource for Norway: 63 600 t were caught in 1999, constituting 2.4% of the total Norwegian landings of fish and shellfish.¹ Its value at landing, however, was 9.1% of the total. The export value of *P borealis* for the same year was 1312 million Norwegian kroner (4.4% of the total), although shrimp products were only 1.7% of the total exported volume of seafood, including cultured fish species.² *P borealis* has a circumboreal distribution where the ambient temperature oscillates between 3 and 7 °C.³ On the other hand, kuruma shrimp (*Penaeus japonicus*) and black tiger (or grass) shrimp (*Penaeus monodon*), the dominant cultured species throughout Asia,⁴ are tropical species reared at about 28 °C that can only tolerate cool conditions: in summer the survival rate after 36 h at 10 and 13 °C

was 3 and 83% respectively.⁴ Both species, especially kuruma, are highly valued.

Most of the published works on post mortem changes in shrimps and prawns refer to warm-water species. Summarised, the changes described include: decreased levels of ATP, with corresponding increases in ADP, AMP, IMP,^{5–8} adenine and hypoxanthine;⁹ and autolytic degradation starting in the hepatopancreas,¹⁰ with the consequent release of proteolytic and collagenolytic enzymes from this organ that induce the development of mushiness in the muscle.^{11,12} The structures most resistant to degradation are muscle, connective and cuticular tissues.¹⁰ Papadopoulos *et al.*,¹³ in freshwater prawns, and our group,¹⁴ in *P borealis*, reported the appearance and increase with storage time of fragments of molecular size 67–180 kDa which were attributed to increased solubilisa-

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tion and degradation of myofibrillar proteins. The *post mortem* pH value in Arctic *P borealis* was found to increase from 7.4 to 8.2.^{14,15}

Crustaceans have characteristic and highly appreciated tastes derived from their content of flavour compounds. These are low-molecular-weight water-soluble compounds such as free amino acids, peptides, nucleotides and related compounds.¹⁶ Freshness is always a major factor determining the sensory properties of the product, and the *post mortem* degradation would metabolise these compounds further and make them disappear, in addition to giving rise to other, less appealing compounds.

Since *P borealis* is an Arctic species, its endogenous enzymes as well as its associated microflora are highly psychrophilic and, consequently, spoilage should proceed faster than in tropical species during ice-storage. Spoilage is due to the action of the shrimp endogenous enzymes and of bacterial enzymes on the shrimp tissues and structures. Peptides, amino acids and nucleotides are known flavour components but are also good substrates for bacterial growth.

Given the economic value and perishability of these products, there is great interest in understanding the *post mortem* changes that decrease their shelf-life as well as in finding methods and markers to evaluate their freshness. The present work was undertaken to compare the *post mortem* degradation of muscle proteins in Arctic and tropical shrimps. One- and two-dimensional polyacrylamide gel electrophoresis of muscle extracts was used to tentatively identify the proteins that suffered the most dramatic alterations. The fate of myosin was studied in *P borealis* by SDS-PAGE analysis of the partially isolated native protein as well as by immunoblotting using anti-Arctic charr fast myosin heavy chain antiserum. Immunoblotting was also used to assess the degradation of α -actinin. We believe this to be the first comparative work addressing *post mortem* muscle protein degradation in Arctic and tropical shrimps.

MATERIALS AND METHODS

Animal samples

P borealis of about 5 g average weight had been caught by trawling off the Svalbard coast in June and immediately placed in ice. *P japonicus* and *P monodon* of about 20 and 10 g average weight respectively had been reared at 28°C and were killed in July by immersion in iced water. Immediately after death (0 h of ice-storage) the abdominal cuticles were removed and a piece of the ventral abdominal muscle was cut, as shown in Fig 1, to perform the water-, low-salt- and high-salt-soluble muscle protein extractions as described below. The extractions were performed on *P borealis* that had been stored in ice for 5, 16, 24, 48, 72, 96 and 120 h and on *P japonicus* and *P monodon* that had been ice-stored for 16, 22, 43, 71 and 92 h. Eight *P borealis*, three *P japonicus* and three *P monodon* were analysed for each storage period. In addition, the

diaphragm, soleus and gastrocnemius muscles of adult rats were used as standards.

Extraction of muscle proteins

The whole procedure was carried out on ice. After removal of the shell, about 100 mg of the ventral muscles were excised and finely minced in an Eppendorf tube with 500 μ l of doubly distilled H₂O. The contents were stirred for 10 min and the tubes were centrifuged for 5 min at 16 000 $\times g$. The supernatants containing the water extracts were transferred to new tubes, an approximately equal volume of ice-cold glycerol was added and the extracts were stored at -20°C. The pH of the water extracts was measured by spotting a drop onto pH indicator paper.

The low- and high-salt-soluble extracts were obtained following the protocol described by d'Albis *et al.*¹⁷ To the pellets left in the tubes after the water extraction, 500 μ l of a low-salt solution (20 mM NaCl, 1 mM EGTA, 15 mM MgCl₂, 5 mM dithiothreitol, 200 μ M phenylmethylsulphonyl fluoride, 3.4 mM NaPO₄H₂ and 1.6 mM Na₂PO₄H, pH 6.5) was added. The contents were stirred for 10 min and centrifuged for 5 min at 16 000 $\times g$. The supernatants containing the low-salt-soluble protein extracts were transferred to new tubes and an approximately equal volume of ice-cold glycerol was added. To the remaining pellets, 500 μ l of a high-salt solution (5 mM EGTA, 15 mM MgCl₂, 5 mM dithiothreitol, 200 μ M phenylmethylsulphonyl fluoride and 100 mM Na₄P₂O₇, pH 8.5) was added. The contents were again stirred for 10 min and centrifuged for 5 min at 16 000 $\times g$. The supernatants containing the high-salt-soluble protein extracts were transferred to new tubes and an approximately equal volume of ice-cold glycerol was added. All the extracts were stored at -20°C until analysed. The rat muscles were extracted as above but without the initial aqueous extraction.

The protein content in the extracts diluted with glycerol was estimated by their optical density at 280 nm using an absorptivity (a_{280}) value of 1 (empirically determined at our laboratory). For SDS-PAGE the extracts were diluted with Laemmli buffer¹⁸ (200 ml l⁻¹ glycerol, 50 ml l⁻¹ β -mercaptoethanol, 48 g l⁻¹ SDS and 125 mM Tris-HCl, 1 mM EDTA,

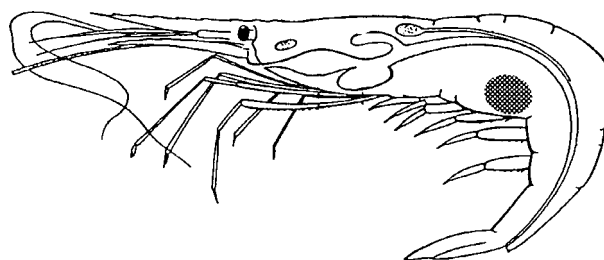


Figure 1. Schematic representation of *Pandalus borealis* showing the area (cross-hatched) where samples were taken. Muscle samples from *Penaeus japonicus* and *Penaeus monodon* were taken at the same location.

pH 6.8) to a concentration of $0.2 \mu\text{g protein } \mu\text{l}^{-1}$, boiled for 5 min and stored frozen.

SDS-PAGE

Electrophoresis was performed according to Laemmli¹⁸ in either $14 \text{ cm} \times 16 \text{ cm}$ or $8 \text{ cm} \times 10 \text{ cm}$ (mini gels), 0.5 mm thick slab gels. The stacking gel contained 50 g l^{-1} acrylamide and 1.3 g l^{-1} piperazine diacrylamide, whilst the separating gel consisted of 150 g l^{-1} acrylamide and 0.87 g l^{-1} piperazine diacrylamide.^{19,20} About $1 \mu\text{g}$ of protein was loaded into the wells and electrophoresis was carried out at 20 mA per slab ($14 \text{ cm} \times 16 \text{ cm}$) or at a constant 150 V ($8 \text{ cm} \times 10 \text{ cm}$). The gels were silver-stained²¹ and dried between two sheets of cellophane. The analyses were made on individual as well as pooled samples. The results shown correspond to pooled extracts of eight *P borealis*, three *P japonicus* and three *P monodon* for each storage period.

Two-dimensional electrophoretic analysis

Two-dimensional (2D) electrophoretic analysis was carried out on the pooled high-salt-soluble extracts. The first dimension, isoelectric focusing (IEF), was performed according to O'Farrell²² in $10 \text{ cm} \times 1.5 \text{ mm}$ or $6 \text{ cm} \times 1 \text{ mm}$ rod gels containing 30 g l^{-1} acrylamide, 0.8 g l^{-1} piperazine diacrylamide²⁰ and 20 ml l^{-1} Servalyt IsoDalt (range 3–10 spread in the 5–7 range). A $5 \mu\text{g}$ amount of total protein was loaded onto the rods. IEF was performed for about 7200 Vh . The rods were extruded, briefly equilibrated in 62.5 mM Tris-HCl (pH 6.8), 23 g l^{-1} SDS, 50 ml l^{-1} β -mercaptoethanol and 300 ml l^{-1} glycerol containing bromophenol blue, then either immediately loaded onto the second-dimension slab gels or stored frozen at -20°C for later analysis.

The second dimension (SDS-PAGE) was performed as described above in $14 \text{ cm} \times 16 \text{ cm}$ gels. A mixture of rat soleus, diaphragm and gastrocnemius muscles was also analysed by 2D electrophoresis for comparison purposes.

Immunoblotting

The protein content of each extract was separated electrophoretically as described above in BioRad mini slab gels ($7 \text{ cm} \times 8 \text{ cm}$, 0.5 mm thick) at a constant 150 V for 1 h . A $1 \mu\text{g}$ amount of total protein was loaded in the slots to test anti-MHC cross-reactivity only, and $10 \mu\text{g}$ to test both anti-MHC and anti- α -actinin cross-reactivities. Two gels were run for each analysis. After electrophoresis, one gel was stained with Coomassie Brilliant Blue R-250, whilst the proteins of the second gel were transferred onto nitrocellulose according to Towbin *et al.*²³ using a BioRad mini transfer unit at 100 V for 50 min . The transfer buffer contained 1 g l^{-1} SDS, 50 mM Tris, 380 mM glycine and 200 ml l^{-1} ethanol. The nitrocellulose sheets were blocked with phosphate buffer saline (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4) containing 50 g l^{-1} powdered

skimmed milk for 1 h . The blots were incubated for 1 h in about 5 mg l^{-1} of a primary polyclonal antiserum anti-Arctic charr fast skeletal MHC produced as described by Martinez and Pettersen.²⁴ Monoclonal anti- α -actinin (Sigma A-5044) was used according to the instructions of the manufacturer. Secondary antibodies were peroxidase-labelled goat anti-rabbit IgG (Sigma A-6154) and rabbit anti-mouse IgG (Sigma A-9044) respectively. Detection was achieved using IBI Enzygraphic Web (Kodak International Biotechnologies Inc).

Electrophoresis of native myosin isoforms

Electrophoresis was performed according to d'Albis *et al.*²⁵ and Hoh *et al.*²⁶ with the modifications described by Martinez *et al.*²⁷ The gel running buffer comprised 20 mM $\text{Na}_4\text{P}_2\text{O}_7$ (pH 8.5), 5 mM MgCl_2 , 100 ml l^{-1} glycerol and 0.5 ml l^{-1} β -mercaptoethanol. The cylindrical ($6 \text{ cm} \times 0.5 \text{ cm}$) gels contained 40 g l^{-1} polyacrylamide (39 g l^{-1} acrylamide and 1 g l^{-1} piperazine diacrylamide). About $7 \mu\text{g}$ of total protein was loaded on each rod. Myosin from rat diaphragm muscle was used as an internal standard. Electrophoresis was carried out at a constant voltage of 90 V for 9 h at a temperature between 0 and -2°C .²⁷

After electrophoresis the gels were briefly stained for 2 min with Coomassie Brilliant Blue R-250, then destained with distilled water until the protein bands containing the native myosin became visible. The bands were cut, equilibrated for 10 min in Laemmli buffer¹⁸ and loaded in the sample wells of 0.75 mm thick mini gels. The gels had the same composition as those used for the second dimension (SDS-PAGE) described above; they were run at a constant 175 V for about 1 h and silver-stained.²¹

RESULTS

The pH values of the water extracts of *P japonicus* and *P monodon* are shown in Fig 2. Since the pH indicator paper was destroyed by an accident on board the boat, we were unable to measure the pH values of *P borealis*

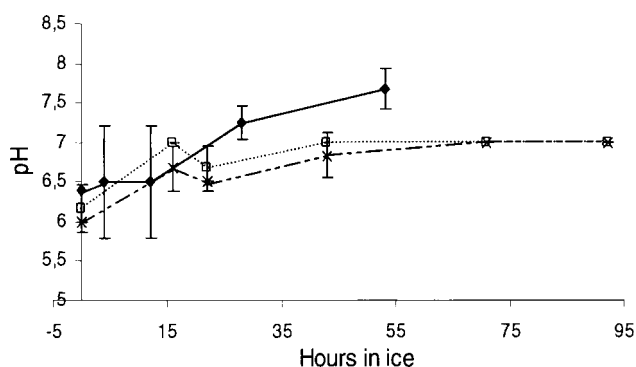


Figure 2. pH values of water extracts from muscle of *Pandalus borealis* (full diamonds), *Penaeus japonicus* (open squares) and *Penaeus monodon* (crosses) immediately after death and until 92 h of ice-storage. Each point is the average $\pm$ standard deviation of individual determinations in three shrimps.

extracts. Therefore the data shown correspond to a previous experiment during which *P borealis* had been ice-stored for 53h. The *P borealis* used in that experiment differed from the present ones in size and in the fact that the muscle samples were taken from the dorsal instead of the ventral region.¹⁴ *P monodon* had the lowest initial pH value, followed by *P japonicus* and *P borealis*, and after 28h of ice-storage, *P borealis* had the highest pH value of all. While the water extracts of the two tropical species seemed to reach a maximum of pH 7.0 after about 48h of ice-storage and remain constant thereafter, the pH value of the water extracts from ice-stored *P borealis* continued to increase from 48 to 53h.

The results of SDS-PAGE of the water-, low-salt- and high-salt-soluble fractions are shown in Fig 3. For *P borealis* (Fig 3A) the water- and low-salt-soluble extracts presented a very similar qualitative protein composition. One of the most prominent features was the presence of a series of bands of molecular size about 100kDa in all the extracts, even in newly killed shrimps (labelled with a bracket in Fig 3A). These bands gave a positive reaction with anti-myosin antiserum, as shown in Fig 4 and labelled with a bracket. Only the upper parts of the blots are shown in Fig 4, because there were no other reacting bands in them. One band of about 67kDa observed by SDS-PAGE analysis (labelled with an arrowhead in Fig 3), which was present at the time of death in all the fractions, disappeared progressively with increasing storage time and was undetectable after 24h of ice-storage. Two other protein bands of about 50kDa (labelled with an asterisk in Fig 3A) also suffered changes during storage. The upper band was detectable only up to 24h. Concomitantly with the disappearance of this band, the intensity of the lower band increased: it was present in minor amounts during the first 5h of ice-storage, it seemed to reach a maximum at about 24h and then its intensity started to decrease.

The water- and low-salt-soluble fractions of *P japonicus* and *P monodon* differed in more bands than the corresponding extracts of *P borealis*. In the tropical species, little MHC degradation was observed even after 92h of ice-storage, as confirmed by the immunoblots with anti-fast myosin heavy chain antiserum (Fig 4).

The degradation of myosin was confirmed in *P borealis* by partial purification of this molecule by electrophoresis under native conditions for 9h and subsequent SDS-PAGE analysis of the bands of gel containing native isomyosins (Fig 5). Fig 5A is a schematic representation of the native gels after a 9h run. Fig 5B shows the upper part of the SDS-PAGE gel where the excised bands M, a and b of Fig 5A were loaded. Band M contained native myosin made up of intact MHC, while bands a and b contained myosin molecules with degraded MHC, supported by the fact that bands a and b appeared only after 12 and 24h of ice-storage respectively and their intensity increased

with time. This analysis was not performed on the warm-water shrimps, because no severe MHC degradation was observed in these species by SDS-PAGE.

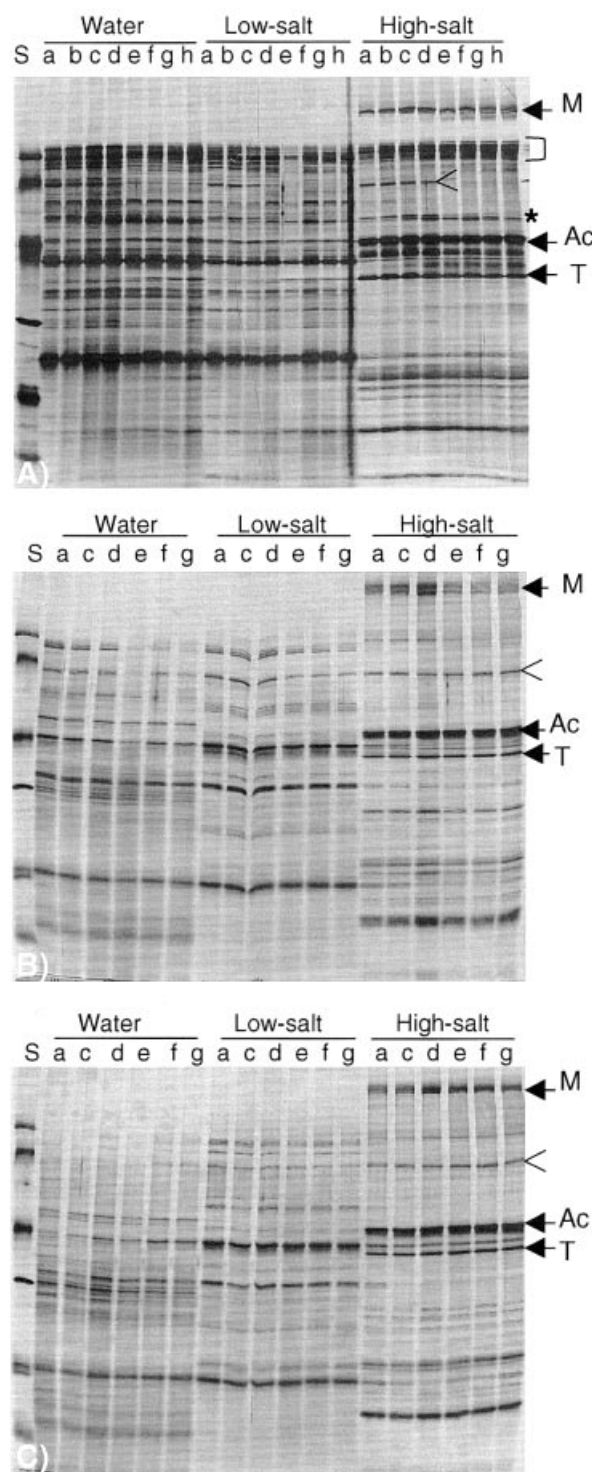


Figure 3. SDS-150g l⁻¹ PAGE of water extracts, low-salt extracts and high-salt extracts of (A) *Pandalus borealis*, (B) *Penaeus japonicus* and (C) *Penaeus monodon*. *P borealis* had been stored in ice for (a) 0, (b) 5, (c) 16, (d) 24, (e) 48, (f) 72, (g) 96 and (h) 120h before extraction. *P japonicus* and *P monodon* had been stored in ice for (a) 0, (c) 16, (d) 22, (e) 43, (f) 71 and (g) 92h before extraction. Lane S: Pharmacia's low-molecular-weight calibration kit, with bands of (from top to bottom) 94, 67, 43, 30, 20.1 and 14.4kDa. M, MHC; Ac, actin; T, tropomyosin. The arrowheads, asterisk and bracket indicate bands referred to in the text.

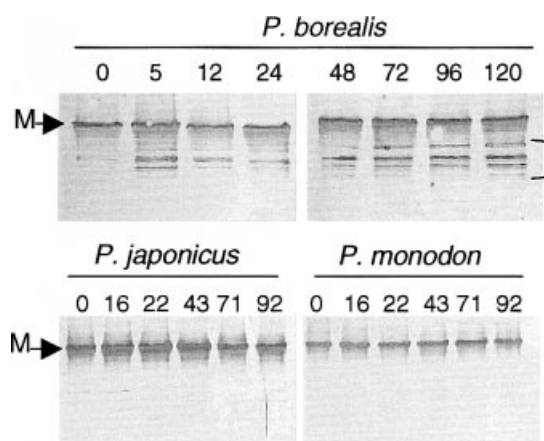


Figure 4. Immunoreactivities of high-salt-soluble extracts of *Pandalus borealis*, *Penaeus japonicus* and *Penaeus monodon* resolved by SDS-150g l⁻¹ PAGE with rabbit anti-Arctic charr fast MHC antiserum. The number at the top of each lane indicates the hours of ice-storage. M, MHC band. The bracket indicates degradation fragments from MHC (see text).

2D electrophoresis of the high-salt-soluble extracts from *P. borealis* (Fig 6) revealed the presence of only one tropomyosin isoform and several actin spots. The 67kDa protein band (encircled in Fig 6) had a pI of

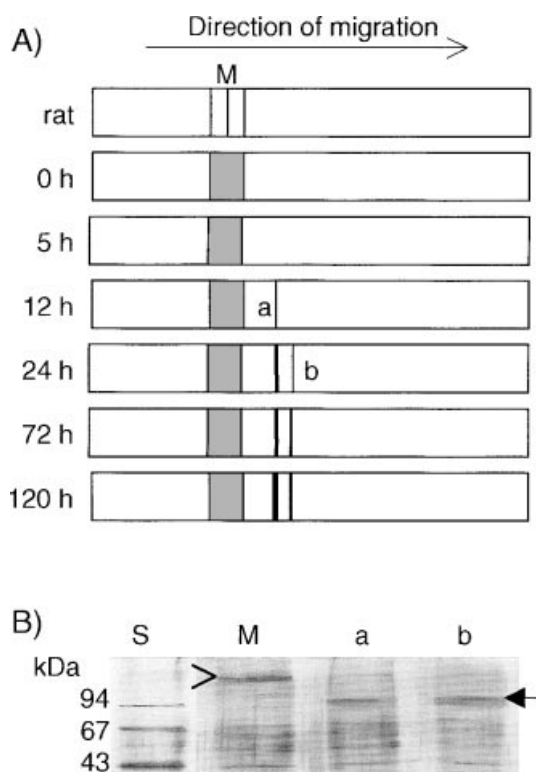


Figure 5. (A) Schematic representation of native myosin isoforms after a 9h run. From top to bottom the samples were rat diaphragm (used as standard) and high-salt-soluble extracts of *Pandalus borealis* after 0, 5, 12, 24, 72 and 120h of ice-storage respectively. M, bands corresponding to the native myosin isoforms of *P. borealis*; a and b, additional, faster-migrating bands noticed only after 12 and 24h respectively of ice-storage. (B) SDS-150g l⁻¹ PAGE of electrophoretically isolated bands M, a and b, as indicated on top of the lanes. The arrowhead indicates the MHC band. The arrow indicates proteolytic fragments. Lane S: Pharmacia's low-molecular-weight standards. Only the upper part of the gel is shown.

about 5.1, more acidic than actin. It was prominent at both 0 and 5h of ice-storage, its amount decreased with increasing time and it disappeared between 24 and 48h of ice-storage. The three spots labelled with arrows and an arrowhead in the 2D gels shown in Fig 6 seem to be the main constituents of the two bands labelled with an asterisk in Fig 3. The spot of higher apparent molecular mass (labelled with an arrow in Fig 6) disappeared with increasing storage time, while the lower band in SDS-PAGE gels corresponds to at least two spots in the 2D gels: the intensity of both spots increased with increasing storage time, especially that of the most acidic one (labelled with a thin arrow).

The 2D patterns of *P. japonicus* and *P. monodon* (Fig 7) were very similar to each other and different from *P. borealis*: the intensity of the 67kDa protein spot (encircled in Fig 7) did not show substantial changes with increasing storage time. One spot, labelled with an arrowhead in Fig 7, may correspond to the smaller and more alkaline of the three spots of about 50kDa of *P. borealis* shown in Fig 6 owing to its position (ie molecular mass and pI) in the 2D gels. That spot, weak in all gels, is detectable in the gel corresponding to Fig 7c, but hardly visible in the figure, probably owing to differences in the amount of protein loaded. As in *P. borealis*, there were several actin spots, but tropomyosin seemed to resolve into two spots differing in pI in the warm-water shrimps.

The presence of α -actinin in the high-salt-soluble extracts was identified by its immunoreactivity. To be able to locate it, two types of immunoblots were performed: first with both anti-MHC antiserum and anti- α -actinin antibodies (Fig 8b), by which bands reacting to both antibody preparations were identified, and then only with anti- α -actinin antibodies to locate α -actinin only (Fig 8c). Bands reacting with anti-MHC antiserum are labelled with a bracket, and bands corresponding to α -actinin with an arrowhead (approximate molecular mass 97kDa). At the time of death, two anti- α -actinin cross-reacting bands were observed in *P. borealis*, only one band was weakly detected after 24h, and the same band could hardly be appreciated in the blots (but not in the figure) after 72h of ice-storage. In contrast, only one anti- α -actinin cross-reacting band was detected in the high-salt-soluble extracts of *P. japonicus* even after 92h of ice-storage. The electrophoretic mobility of this band was similar to that of the higher-molecular-mass cross-reacting band in *P. borealis*.

DISCUSSION

The samples used in the present work were taken from the ventral abdominal region in order to avoid contamination with digestive enzymes, since the gastrointestinal tract in shrimps runs in the dorsal region.

The *post mortem* pH values in shrimp muscles have been reported previously^{6,14,15} and are confirmed here to reach values close to or higher than 7.0. Fish muscle, on the other hand, seldom reaches values

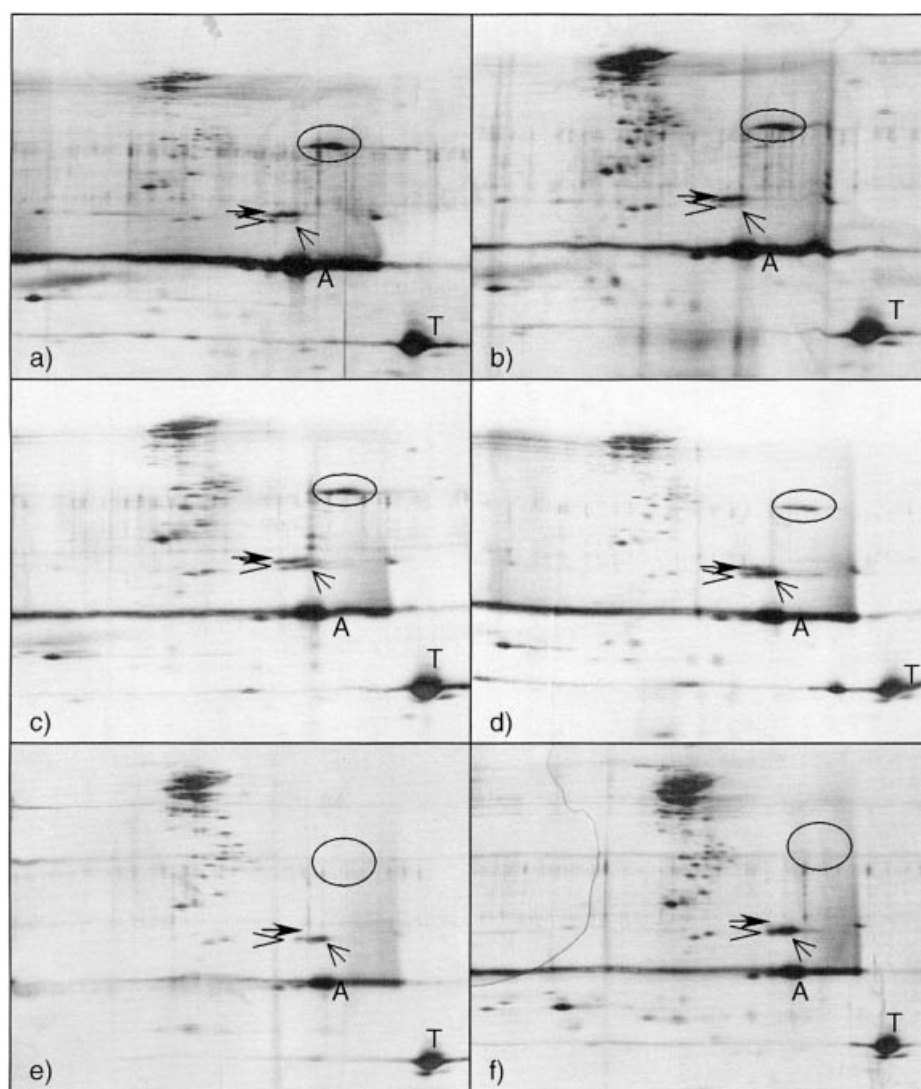


Figure 6. Two-dimensional electrophoresis of high-salt-soluble muscle proteins of *Pandalus borealis*. First dimension, IEF, from left (basic) to right (acidic). Second dimension, SDS-150 g l⁻¹ PAGE, from top to bottom. The shrimps had been stored on ice for (a) 0, (b) 5, (c) 16, (d) 24, (e) 48 and (f) 72 h. A, actin; T, tropomyosin. The arrows, arrowheads and circles indicate spots referred to in the text.

above pH 7.0^{28–30} during *rigor mortis*. The low pH values of fish muscle usually delay *post mortem* autolytic and bacterial degradation. Lacking the protection provided by low pH values, muscle deterioration in shrimps will proceed faster than in fish. Since *P borealis* seemed to reach higher pH values faster than the other two tropical species, its shelf-life in ice-storage should be expected to be correspondingly shorter.

We detected two MHC bands in the high-salt-soluble extracts of the three species, even in newly killed individuals. Only the two bands of *P japonicus*, which were the two that differed most in molecular mass, are noticeable in Fig 3. The others cannot be seen in the gels owing to overloading of the MHC band and the high percentage of polyacrylamide used. However, the two bands were clearly visible during the developing step of the silver-staining procedure. It is possible that the faster-migrating band may originate from proteolytic degradation of the slower-migrating form, but we did not detect a decrease in the amount of the upper band concomitant with an increase in the amount of the lower band. We rather believe that these two bands represent two different MHC isoforms, as

has been shown to be the case in insect muscles,³¹ and that both isoforms suffered a similar degree of proteolytic degradation during ice-storage, as shown by the increase in the intensity of bands of molecular mass between 80 and 100 kDa in *P borealis*. Some of these bands, however, may represent paramyosin. Paramyosin is a rod-shaped α -helical molecule that forms the central core of the thick filament in most invertebrate muscles,^{32–34} including crustaceans,^{35–37} in which it has a molecular size of 105, 110 or 130 kDa, depending on the isoform.^{35,37} Two of the regions in the paramyosin molecule important for assembly have been reported to be homologous to regions of MHC.³⁸ Therefore cross-reactivity with anti-MHC antiserum is a possibility. However, the great abundance of these bands, together with the simultaneous decrease in intensity of the MHC band, as well as the lack of cross-reactivity of a hypothetical paramyosin band with anti-MHC antiserum in *P japonicus* and *P monodon*, suggests that they are mainly degradation fragments of MHC, in agreement with previous works on shrimps.^{13,14}

The 67 kDa band also seemed to be subject to

proteolysis during ice-storage of *P borealis* but not of *P japonicus* or *P monodon*. In the absence, to our knowledge, of published works on 2D patterns of crustacean muscles, we have tried to identify this band by comparison with our own analysis of minke whale muscles (unpublished), rat diaphragm, soleus and gastrocnemius (Fig 6) and red and white muscles of lean and fatty fish species,³⁹ and with published 2D patterns of muscle extracts from chicken,⁴⁰ human deltoid muscle⁴¹ and mouse gastrocnemius.⁴² Only in the 2D analysis of Arctic charr embryos (*Salvelinus*

alpinus) was a spot with similar molecular mass and pI noticed.⁴³ This unidentified spot progressively disappeared after hatching and it was no longer noticeable in 1-year-old fish. The molecular mass of this spot rather precisely matches that of albumin, but not its pI: while serum albumin has a pI of approximately 5.4⁴² and is more basic than actin, the present spot had an estimated pI of about 5.1, more acidic than actin. This spot may represent a degradation fragment of a higher-molecular-weight protein or a novel protein. The fact that it was present in all three species at the time of

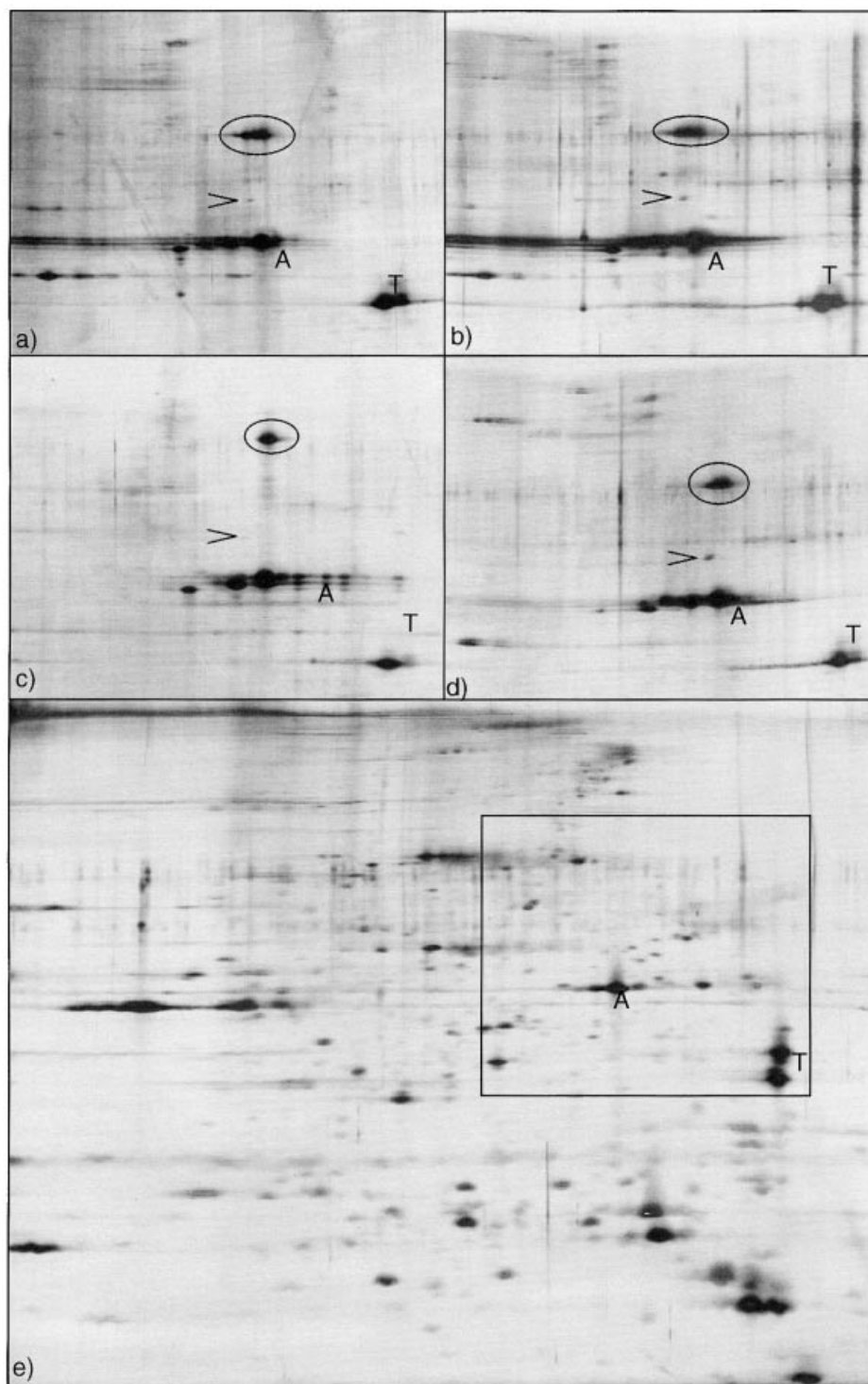


Figure 7. Two-dimensional electrophoresis of high-salt-soluble muscle proteins of (a, b) *Penaeus japonicus*, (c, d) *Penaeus monodon* and (e) mixture of high-salt-soluble extracts from rat diaphragm, soleus and gastrocnemius muscles. First dimension, IEF, from left (basic) to right (acidic). Second dimension, SDS-150g l⁻¹ PAGE, from top to bottom. The shrimps had been stored on ice for (a, c) 0 and (b, d) 92h. A, actin; T, tropomyosin. The arrowheads and circles indicate spots referred to in the text. Part (e) is a full-size gel, and the square indicates the part of the gel shown in Figs 6a–6f and 7a–7d.

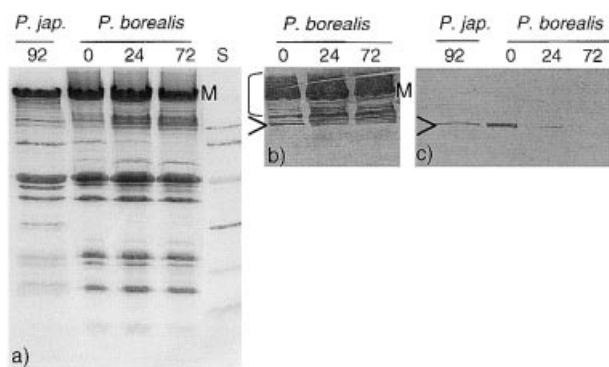


Figure 8. (a) SDS-150g l⁻¹ PAGE and Coomassie Brilliant Blue R-250 staining of high-salt-soluble protein extracts of muscle from *Penaeus japonicus* and *Pandalus borealis* stored on ice for various periods (indicated in hours at the top of each lane). Lane S: Pharmacia's low-molecular-weight standards. (b, c) Immunoreactivities of the same extracts with (b) both rabbit anti-Arctic charr fast MHC antiserum and mouse anti- α -actinin IgM and (c) mouse anti- α -actinin IgM only. M, MHC. The bracket indicates bands reacting with anti-fast MHC antiserum. The arrowhead indicates bands reacting with anti- α -actinin IgM.

death and disappeared only in *P. borealis* leads us to suggest that it may represent a protein characteristic of shrimp muscles.

As to the identity of the other three spots of molecular mass of about 50 kDa (Fig 6), the smallest and most alkaline might be desmin, by comparison with published 2D patterns of muscle extracts of human,⁴¹ mouse⁴² and Atlantic salmon (*Salmo salar*).⁴⁴ If this identification is correct, then desmin would not be severely affected by *post mortem* deterioration during ice-storage in any of the three shrimp species, as seems to be the case in fish but not in bovine muscles.⁴⁵

We have not been able to identify the other two 50 kDa (Fig 6) spots that occur in *P. borealis*. It seemed in any case that the lower spot might be a degradation fragment of the higher one owing to their similar pI values and their concomitant increase and decrease in intensity respectively. The time for the appearance and disappearance of these bands during ice-storage was remarkably similar to that found in a previous work,¹⁴ although the individuals used differed in size and genetic stock⁴⁶ and the previous samples were taken from the dorsal region of the tail. This means that these bands may be used as markers to estimate the freshness of *P. borealis* as 'days on ice'. Increased storage temperature will accelerate the changes in the protein pattern,¹⁴ and therefore these protein markers would reflect the quality of the shrimps better than the more conventional 'days after capture'.

α -Actinin has been shown to be removed from the Z-disc during *post mortem* aging in fish muscles⁴⁷⁻⁴⁹ owing to the high intracellular levels of Ca²⁺ after death⁵⁰ and the action of calpain.⁵¹ Tsuchiya and Seki⁵¹ found that carp calpain could release α -actinin, with a molecular mass of 100 kDa, without proteolysis from the myofibrils and then digest it instantly to render a fragment of 96 kDa. Their results help to explain the presence of two anti- α -actinin cross-

reacting bands, of molecular masses close to 100 and 96 kDa respectively, in our newly killed samples of *P. borealis*. α -Actinin resistance to proteolysis has been shown to be species-dependent,⁴⁸ which is confirmed here: while Arctic *P. borealis* proved to be a very susceptible species (two bands were detected immediately after death, and only the 96 kDa anti- α -actinin cross-reacting band remained weakly detectable after 24 h of ice-storage and hardly detectable after 72 h), tropical *P. japonicus* was not (the higher-molecular-mass (100 kDa) anti- α -actinin cross-reacting band could be clearly detected after 92 h of ice-storage).

The hepatopancreas and digestive tract of crustaceans are very rich in proteolytic and collagenolytic enzymes.⁵²⁻⁵⁵ However, we do not attribute the proteolytic degradation observed in this work to contamination from intestinal contents or digestive enzymes. It cannot be explained either by assuming that the shrimps were preparing to undergo moulting, since the sampling took place in summer and moulting occurs in spring. An alternative explanation may lie in the fact that the muscles of crustaceans are rich in both Ca²⁺-dependent^{56,57} (some of which, in addition, specifically degrade myofibrillar proteins⁵⁸) and Ca²⁺-independent⁵⁹⁻⁶⁴ proteolytic enzymes and that those enzymes are typically adapted to perform at the natural living temperature of the organism: at about 0 °C in Arctic species and at about 28 °C in tropical species. Therefore, while ice-storage may significantly inhibit the activity of thermophilic enzymes, it hardly has any inhibitory effect on psychrophilic ones. Accordingly, proteolytic degradation can take place at a faster rate during cold storage of Arctic species than during cold storage of temperate or tropical species. Muscle proteases are usually active within the pH range measured in the water extracts. Since both the low- and high-salt extracts contained EGTA, an inhibitor of Ca²⁺-activated proteases,^{47,57} it is likely that most of the degradation observed in the zero-time samples of *P. borealis* (bands labelled with a bracket in Fig 3A) occurred during the time necessary to carry out the water extraction.

In summary, the *post mortem* degradation during ice-storage seemed to be somehow different in the Arctic than in the tropical species: while in the Arctic species important protein degradation (giving rise to fragments with the potential to be used as markers for freshness) suggests active muscle proteases, proteolytic degradation in the tropical species seemed to be of much less relevance and probably inhibited by the greater difference between the normal living environmental (28 °C) and storage (0 °C) temperatures. The shelf-life during ice-storage of tropical species might therefore be better reflected by changes in other molecules.

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REFERENCES

- Directorate of Fisheries, Landing statistics—Norwegian catches 1996–1999. *Fiskets Gang* 1:15 (2000).
- Norwegian Seafood Export Council, Årsstatistikk Desember 1999. *Eksportutvalget for Fisk Desember*, p 17 (1999).
- Sætre R, Miljørapport. *Fisken og Havet, Særnummer 2*, Institute of Marine Research, Bergen (1997).
- Bremner HA, Paterson BD and Goodrick B, Live transport of crabs and shrimp from Australia—kuruma shrimp and spanner crabs as physiological case studies, in *Seafood from Producer to Consumer*, Ed by Luten JB, Børresen T and Oehlenschläger J, Elsevier Science Publishers, Amsterdam, pp 71–85 (1997).
- Ban M, Ushio H and Yamanaka H, Postmortem biochemical changes in muscle of *Buccinum striatissimum* during storage. *Fisheries Sci* 64:482–486 (1998).
- Flick GJ and Lovell RT, Post-mortem biochemical changes in the muscle of the Gulf shrimp, *Penaeus aztecus*. *J Food Sci* 37:609–611 (1972).
- Matsumoto M and Yamanaka H, Studies on rigor-mortis of kuruma prawn muscle. *Nippon Suisan Gakkaishi* 57:2121–2126 (1991).
- Yamanaka H and Shimida R, Post-mortem biochemical changes in the muscle of the Japanese spiny lobster during storage. *Fisheries Sci* 62:821–824 (1996).
- Lou SN, Purine content in grass shrimp during storage as related to freshness. *J Food Sci* 63:442–444 (1998).
- Lightner DV, Normal post mortem changes in the brown shrimp, *Penaeus aztecus*. *Fish Bull* 72:223–236 (1973).
- Baranowski ES, Nip WK and Moy JH, Partial characterization of a crude enzyme extract from the freshwater prawn *Macrobrachium rosenbergii*. *J Food Sci* 49:1494–1495 (1984).
- Nip WK, Lan CY and Moy JH, Partial characterization of a collagenolytic enzyme fraction from the hepatopancreas of the freshwater prawn, *Macrobrachium rosenbergii*. *J Food Sci* 50:1187–1188 (1985).
- Papadopoulos LS, Smith SB, Wheeler TL and Finne G, Muscle ultrastructural changes in freshwater prawns, *Macrobrachium rosenbergii* during iced storage. *J Food Sci* 54:1125–1128, 1157 (1989).
- Martinez I and Lauritzen K, Effect of storage conditions on the protein content of the shrimp *Pandalus borealis*, in *Making the Most out of the Catch. Proceedings from an International Post-harvest Seafood Symposium*, Ed by Bremner A, Davis C and Austin B, The National Seafood Centre, Australia, Brisbane, pp 121–128 (1997).
- Viggoson H, Alternativ pilling av reker. *FTFI Rep U47* (1988).
- Konosu S and Yamaguchi K, The flavour components in fish and shellfish, in *Chemistry and Biochemistry of Marine Food Products*, Ed by Martin RE, Flick GJ, Hebard CE and Ward DR, AVI Publishing, Westport, CT, pp 367–404 (1982).
- D'Albis A, Janmot C and Béchet JJ, Comparison of myosins from the masseter muscle of adult rat, mouse and guinea pig. Persistence of neonatal-type isoforms in the murine muscle. *Eur J Biochem* 156:291–296 (1986).
- Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685 (1970).
- Anderson CW, Baum PR and Gesteland RF, Processing of adenovirus 2-induced proteins. *J Virol* 12:412–423 (1973).
- Hochstrasser D, Patchornik A and Merrill C, Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining. *Anal Biochem* 173:412–423 (1988).
- Ansorge W, Fast visualization of protein bands by impregnation in potassium permanganate and silver nitrate, in *Electrophoresis '82*, Ed by Stathakos D, Walter de Gruyter, Berlin, pp 235–242 (1983).
- O'Farrell PH, High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007–4021 (1975).
- Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354 (1979).
- Martinez I and Pettersen GW, Temperature-induced precocious transitions of myosin heavy chain isoforms in the white muscle of Arctic charr, *Salvelinus alpinus*. *Basic and Applied Myology* 2:89–95 (1992).
- D'Albis A, Pantaloni C and Béchet JJ, An electrophoretic study of native myosin isoenzymes and their subunit content. *Eur J Biochem* 99:261–272 (1979).
- Hoh JFY, McGrath PA and White RI, Electrophoretic analysis of multiple forms of myosin in fast-twitch and slow-twitch muscles of the chick. *Biochem J* 157:87–95 (1976).
- Martinez I, Ofstad R and Olsen RL, Electrophoretic study of myosin isoforms in white muscles of some teleost fishes. *Comp Biochem Physiol* 96B:221–227 (1990).
- Dunasjski E, Texture of fish muscle. *J Texture Stud* 10:301–318 (1979).
- Martinez I, Ofstad R and Eide O, Correlation of chemical parameters in cod mince and surimi with the surimi quality, in *Seafood Quality Determination*, Ed by Kramer DE and Liston J, Elsevier Science Publishers, Amsterdam, pp 389–401 (1987).
- Rustad T, Muscle chemistry and quality of wild and farmed cod, in *Quality Assurance in the Fish Industry*, Ed by Huss HH, Jacobsen M and Liston J, Elsevier Science Publishers, Amsterdam, pp 19–27 (1992).
- Ziegler C, Ader G and Beinbrech G, Evidence for two myosin types in indirect insect flight muscles. *Comp Biochem Physiol* 107B:91–98 (1994).
- Bullard B, Luke B and Winkelman L, The paramyosin of insect flight muscle. *J Mol Biol* 75:359–367 (1973).
- Cohen C, Szent-Györgyi AG and Kendrick-Jones J, Paramyosin and the filament of molluscan 'catch' muscles. I. Paramyosin structure and assembly. *J Mol Biol* 56:223–237 (1971).
- Schmitz H, Ashton FT, Pepe FA and Beinbrech G, Substructures in the core of the thick filaments: arrangement and number in relation to the paramyosin content of insect flight muscles. *Tissue Cell* 26:83–100 (1994).
- Maroto M, Arredondo JJ, San Román M, Marco R and Cervera M, Analysis of paramyosin/miniparamyosin gene. *J Biol Chem* 270:4375–4382 (1995).
- Mykles DL, Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: variants of troponin, paramyosin, and myosin light chains comprise four distinct protein assemblages. *J Exp Zool* 234:23–32 (1985).
- Sakurai Y, Kanzawa N and Maruyama K, Characterization of myosin and paramyosin from crayfish fast and slow muscles. *Comp Biochem Physiol* 113B:105–111 (1996).
- Hoppe PE and Waterston RH, Regions of the rod important for myosin and paramyosin in body wall muscle. *Biophys J* 70:SUAM5 (1996).
- Martinez I, Ofstad R and Olsen RL, Myosin isoforms in red and white muscles of some marine teleost fishes. *J Muscle Res Cell Motil* 11:489–495 (1990).
- Hirabayashi T, Two-dimensional gel electrophoresis of chicken

- skeletal muscle with agarose gels in the first dimension. *Anal Biochem* **117**:443–451 (1981).
- 41 Giometti CS, Danon MJ and Anderson NG, Human muscle proteins: analysis by two-dimensional electrophoresis. *Neurology* **33**:1152–1156 (1983).
- 42 SWISS-2D-PAGE Database. Available (23 July, 2001): http://www.expasy.ch/cgi-bin/map2/def?/MUSCLE_MOUSE.
- 43 Martinez I and Christiansen JS, Myofibrillar proteins in developing white muscle of Arctic charr, *Salvelinus alpinus* (L.). *Comp Biochem Physiol* **107B**:11–20 (1994).
- 44 Morzel M, Verrez-Bagnis V, Arendt EK and Fleurence J, Use of two-dimensional electrophoresis to evaluate proteolysis in salmon (*Salmo salar*) muscle as affected by lactic acid fermentation. *J Agric Food Chem* **48**:239–244 (2000).
- 45 Verrez-Bagnis V, Post-mortem denaturation of fish muscle proteins—changes in some myofibrillar, intermediate filament and costameric proteins, in *Proceedings of Final Meeting Concerted Action 'Evaluation of Fish Freshness', AIR3 CT94 2283*, Ed by Olafsdottir G, Luten J, Dalggaard P, Careche M, Verrez-Bagnis V, Martinsdottir E and Heia K, International Institute of Refrigeration, Paris, pp 229–237 (1997).
- 46 Martinez I, Skjerdal T, Dreyer B and Aljanabi S, Genetic structuring of *Pandalus borealis* in the Northeast Atlantic. II. RAPD analysis. 1997 *Ann Sci Conf of the International Council for the Exploration of the Sea (ICES)*, paper presented at the ICES Conference 1997.
- 47 Astier C, Labbe JP, Roustan C and Benjamin Y, Sarcomeric disorganization in postmortem fish muscles. *Comp Biochem Physiol* **100B**:459–465 (1991).
- 48 Papa I, Alvarez C, Verrez-Bagnis V, Fleurence J and Benyamin Y, Post mortem release of fish white muscle α -actinin as a marker of disorganization. *J Sci Food Agric* **72**:63–70 (1996).
- 49 Tsuchiya H, Kita S and Seki N, Postmortem changes in α -actinin and connectin in carp and rainbow trout muscles. *Nippon Suisan Gakkaishi* **58**:793–798 (1992).
- 50 Busch WA, Stromer MH, Goll DE and Suzuki A, Ca^{2+} -specific removal of Z lines from rabbit skeletal muscle. *J Cell Biol* **52**:367–381 (1972).
- 51 Tsuchiya H and Seki N, Action of calpain on α -actinin within and isolated from carp myofibrils. *Nippon Suisan Gakkaishi* **57**:1133–1139 (1991).
- 52 Gates BJ and Travis J, Isolation and properties of shrimp trypsin. *Biochemistry* **8**:4483–4489 (1969).
- 53 Kimoto K, Kusama S and Marukami K, Purification and characterization of serine proteinases from *Euphasia superba*. *Agric Biol Chem* **47**:529–534 (1983).
- 54 Klimova OA, Borukhov SI, Solovyeva NI, Balaevskaya TO and Strongin AY, The isolation and properties of collagenolytic proteases from crab hepatopancreas. *Biochem Biophys Res Commun* **166**:1411–1420 (1990).
- 55 Tsai IH, Chuang KL and Chuang JL, Chymotrypsins in digestive tracts of crustacean decapods (shrimps). *Comp Biochem Physiol* **85B**:235–239 (1986).
- 56 Beyette JR, Ma JS and Mykles DL, Purification and autolytic degradation of a calpain-like calcium-dependent proteinase from lobster (*Homarus americanus*) striated muscle. *Comp Biochem Physiol* **104**:95–99 (1993).
- 57 Mykles DL and Skinner DM, Four Ca^{2+} -dependent proteinase activities isolated from crustacean muscle differing in size, net charge and sensitivity to Ca^{2+} and inhibitors. *J Biol Chem* **261**:9865–9871 (1986).
- 58 Mykles DL and Skinner DM, Molt-cycle associated changes in calcium-dependent proteinase activity that degrades actin and myosin in crustacean muscle. *Dev Biol* **92**:386–397 (1982).
- 59 Doke SN and Ninjoor V, Characteristics of an alkaline proteinase and exopeptidase from shrimp (*Penaeus indicus*) muscle. *J Food Sci* **52**:1203–1208, 1211 (1987).
- 60 Karani MN, Gore MS and Nair PM, High and low molecular weight proteolytic activities in prawn (*Metapenaeus brevicornis*) muscle. *Indian J Biochem Biophys* **36**:272–285 (1999).
- 61 Mykles DL, High-molecular-weight serine proteinase from lobster muscle that degrades myofibrillar proteins. *J Exp Zool* **250**:244–252 (1989).
- 62 Mykles DL, Purification and characterization of a multicatalytic proteinase from crustacean muscle: comparison of latent and heat-activated forms. *Arch Biochem Biophys* **274**:2126–2128 (1989).
- 63 Sherekar SV, Doke SN, Gore MS and Ninjoor V, Proteinases of tropical fish and their role in autolysis. *Indian J Exp Biol* **24**:440–444 (1986).
- 64 Sherekar SV, Gore MS and Ninjoor V, SH activated metallo-proteinases in prawns (*Penaeus indicus*). *Indian J Exp Biol* **29**:460–462 (1991).