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Factors Inducing Mushiness in Stored Prawns*

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ABSTRACT

After 1 week of storage on ice, little change was observed between the SDS-PAGE patterns of myofibrillar proteins extracted from fresh prawns with firm texture and mushy prawns. Trypsin and hepatopancreas homogenate incubated at 0°C with prawn segments, can cause mushiness at a very low level of added proteolytic activity. Chymotrypsin added at comparable amounts of activity does not cause mushiness, although fragmentation of high molecular weight components of myofibrillar proteins was demonstrated. Collagenase induces mushiness when assisted by low amounts of proteolytic activity. Purified collagenase, devoid of measurable amounts of proteolytic activity, did not cause mushiness but imparted a soft texture.

Mushiness in prawns stored on ice is not caused by an endogenous proteolytic system in the muscle. The main cause of mushiness following ice storage, appears to be due to diffusion of proteolytic and collagenolytic enzymes from the autolyzing hepatopancreas.

INTRODUCTION

Fresh water prawns (*Macrobrachium rosenbergii*), when stored on ice, have a short shelf life. After cooking prawns which have been stored for 4–8 days on ice, the portion of the tail adjacent to the head usually has a characteristic mushy texture. Several workers have reported mushiness following different

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periods of ice storage. Nip *et al.* (1985), Angel *et al.* (1985) and Waters & Hale (1981) reported the onset of mushiness after 4, 8 and 11 days of storage on ice, respectively. No connection was found between development of mushiness on ice storage, and total or proteolytic bacterial counts (Angel *et al.*, 1985, 1986*b*). Also, irradiation at 145 and 230 krad levels before ice storage, although significantly reducing the bacterial load, produced no changes in texture or time until the onset of mushiness (Angel *et al.*, 1986*b*).

In their histological studies, Rowland *et al.* (1982) produced evidence suggesting that mushiness was due to proteolytic enzymes diffusing from the digestive tracts, either from the hepatopancreas or from the gut running along the tail of the prawn.

Another possible mechanism for the development of mushiness could be proteolysis of myofibrillar proteins by an endogenous proteolytic system in the muscle. A Ca^{2+}-activated proteinase was described in crustacean claw muscles by Mykles & Skinner (1982, 1983). The proteolytic activity measured by these authors in muscle homogenates, was more than two-fold greater during molting than during the intermolt stage. The texture of cooked prawn muscle was also found to depend on the molting state of the fresh prawn (Angel *et al.*, 1986*a*). The frequency of mushy and soft texture in prawns cooked immediately after catching, was shown to be greater in molting than in intermolt animals. This suggests that mushiness may be related to the endogenous proteolytic activity in the prawn muscle.

The aim of this work was to examine the possibility that such a system is indeed operative in intact muscle tissue during cold storage and can therefore be responsible for the development of mushiness. Also examined were the extent of proteolysis in mushy prawns and the capability of proteases and collagenases to induce mushiness in prawn muscles.

MATERIALS AND METHODS

Adult prawns in the intermolt stage were used in all experiments.

Sensory evaluation of fresh and stored-on-ice prawns

From a lot of 60 fresh prawns on ice, 20 animals were selected at random for immediate evaluation of texture and for protein extraction from the tail segments. After thorough washing, the remaining prawns were stored (with heads on) under crushed ice in perforated containers to prevent accumulation of melted ice. The boxes were stored in a 2°C cold room and the ice was changed daily.

Following 7 days of storage the cephalothorax of the prawns was

removed, the exoskeleton was peeled off, and the tails were dissected longitudinally into two halves along all segments. One-half of a tail was cooked for 3 min in boiling water and its texture was evaluated as described previously (Angel *et al.*, 1986*a*). The cooked lobes and segments of each half-tail were classified as firm, soft or mushy. From the non-cooked halves, corresponding segments and lobes were selected for preparation of muscle homogenates and extraction of proteins (see below).

Cold storage of separated segments

Tails from 30 animals were separated from the cephalothorax, peeled, and washed with water to remove any contamination with fluids from the hepatopancreas, in case the latter had been damaged. The vein and gut canal were removed from the tails. From each tail, the three segments in the proximity of the cephalothorax were separated, cut longitudinally into two symmetrical parts, and pooled together. The half-segments were washed thoroughly and dipped for 10 min in 0·03% NaN_3. Excess of liquid was removed by filter paper and the pooled half segments were distributed into 16 petri dishes. A low salt extract and myofibrillar protein extract were prepared separately from the content of each petri dish as follows: four homogenates were immediately prepared as nonstored controls; the rest were stored in a refrigerator at 1°C for 8, 15 and 22 days. Four petri dishes were then selected at random and from each, two half-segments were taken for evaluation of texture after cooking (see below) and the material remaining in each dish was homogenized separately. From each homogenate, sarcoplasmic and myofibrillar proteins extracts were prepared (see below).

Incubation of tail segments with enzymes

In 250-ml flasks, about 10 g of tail segments prepared as described above was suspended in 40 ml of solution composed of 10 mM tris buffer at pH 7·0, 50 mM NaCl, 2 mM $CaCl_2$ and 0·02% NaN_3. After 1 h of equilibration in an ice-water bath, the following proteolytic and collagenolytic preparations were added: trypsin (Sigma), hepatopancreas homogenates at final dilutions in the range of 1/10 to 1/140, chymotrypsin (Sigma), collagenase from *Clostridium histolyticum* (Worthington, containing protease activity, see below) and purified collagenase from *Cl. histolyticum* (Sigma) containing no measurable proteolytic activity on casein. The flasks with the ice bath were transferred to a cold room for 18 h. Proteolytic activity of these preparations on casein was measured at 30 and 0°C. Collagenase activity was measured on a specific substrate at 30°C (see below).

After 18 h the liquid was drained off and two segments from each flask were taken for evaluation of texture after cooking. Each segment was transferred to a test tube containing 5 ml of 50 mM NaCl and cooked for 4 min at 80°C with gentle stirring with a glass rod. The contents of the test tube were poured into a petri-dish for light microscope observation and for photographing. The texture of the largest remaining pieces was evaluated by trained panelists by lightly rubbing the pieces between the thumb and forefinger. Non-cooked segments were taken for homogenization and for extraction of sarcoplasmic and myofibrillar proteins.

Extraction of proteins from tail segments

Homogenization and extraction of sarcoplasmic proteins were carried out with a low salt solution consisting of: 50 mM NaCl, 1 mM $CaCl_2$ and 0·02% NaN_3. Extraction of myofibrillar proteins was done with a high salt solution consisting of 600 mM NaCl, 20 mM phosphate buffer at pH 7·5, and 0·02% NaN_3.

All extractions were carried out at 0 to 4°C. Samples were weighed and homogenized with ten volumes of low salt solution in an Omnimixer operated at maximum speed, applying eight strokes of 15 s with a 15 s interval. After 5 min of centrifugation at 250 *g* to break the foam, the homogenate was remixed and a duplicate sample of 15 g was placed into weighed centrifuge tubes. Following centrifugation for 15 min at 27 000 *g*, the supernatant containing the sarcoplasmic protein fraction was separated and the pellets were washed twice with 20 ml of low salt solution. A third wash was not necessary, since the amount of protein it removed was barely detectable. The volumes of the supernatants were determined from the difference between the weight of the tubes before centrifugation and after removal of the supernatants. To 6 ml of low salt extract, 3 ml of 18% trichloroacetic acid (TCA) was added. The protein-free supernatant obtained after centrifugation (15 min at 1100 *g*) was used to determine the nonprotein nitrogen (NPN) in the low salt extract.

In some cases 0·2% Triton X-100 was added to the extracting and washing low salt solution. The pellets were then washed two more times with 20 ml low salt solution without detergent, before the extraction of myofibrillar proteins.

To extract myofibrillar proteins, the pellets were dispersed in 30 ml high salt solution and, after standing for 30–60 min on ice with occasional stirring, they were centrifuged for 30 min at 32 000 *g*. The supernatant was separated, and the pellet was washed at least three times with 20 ml of high salt solution.

The full scheme of extraction was carried out only on samples of segments

incubated at 0°C in the refrigerator. Protein was then determined in all the supernatants, which enabled calculation of the amounts of sarcoplasmic and myofibrillar proteins per sample.

In the experiments with storage of whole prawns on ice or incubation of segments with enzymes, only the first sarcoplasmic and the first myofibrillar extracts were retained and used to prepare samples for electrophoresis.

The residue after the washings with high salt solution was transferred to a hydrolysis tube; 5 ml of 6N HCl was added and hydrolysis was carried out for 18 h at 108°C.

Preparation of hepatopancreas homogenates

Hepatopancreas homogenates were prepared according to the method of Baranowski *et al.* (1984).

Electrophoresis

Electrophoresis was carried out by the method of Porzio & Pearson (1977) or of Laemmli (1970) with a resolving gel of 6% acrylamide. Samples for loading were prepared by mixing one volume of protein extract with two volumes of sample buffer and incubating 4 min in boiling water. The gels were stained with Coomassie blue R250.

Enzymatic and chemical assays

Proteolytic activity was measured on 0·6% casein at 0 and 30°C. To 1·5 ml solution at pH 7·2 containing 0·6% casein, 50 mM tris buffer and 1 mM $CaCl_2$, 0·015 ml of 0·3 mg/ml protease or 0·030 ml of hepatopancreas homogenate were added. The reaction mixture was incubated for 20 min at 30°C or 120 min at 0°C. The reaction was terminated by addition of 3 ml of 10% TCA. Samples to which TCA was added, prior to addition of the proteolytic activity, served as time zero controls. After standing overnight the tubes were centrifuged for 20 min at 1100 *g* in a clinical centrifuge. The material in the supernatant, which was reactive against Folin reagent, was measured by the method of Lowry *et al.* (1951) with tyrosine as standard. A unit of proteolytic activity was defined as the activity releasing an equivalent of 1 nmole of tyrosine/min.

Protein content was determined by the biuret method according to Gornal *et al.* (1949), with bovine serum albumin as the standard.

The nitrogen content in the low salt extracts and in their TCA supernatant (NPN) was determined by the Kjeldahl method.

Collagenase activity was measured at 30°C on a specific substrate, the

peptide derivative 4-phenylazobenzyl-oxycarbonyl-L-Pro–L-Leu–Gly–L-Pro–D-Arg (Boehringer Mannheim), according to the method of Wunsch & Heidrich (1963). One unit of collagenase activity was taken as the activity splitting 1 nmole/min of specific substrate.

Hydroxyproline in the hydrolysates of the residues after protein extraction was determined according to Neuman & Logan (1950).

Count of proteolytic bacteria

The count of proteolytic bacteria in the homogenates of prawn tail segments was performed on milk nutrient agar at 20°C, as described previously (Angel *et al.*, 1985). Results are given as colony-forming units (CFU) per gram of muscle.

RESULTS

The results of the sensory evaluation after cooking freshly caught prawns and following ice storage, are shown in Table 1. In fresh prawns, 90% of the lobes and first segments were firm. Only in isolated cases were the muscle lobes, protruding from the first segment into the cephalothorax, soft or mushy. After seven days on ice, all the lobes turned mushy as did most of the first segments, but only about one third of the second segments became mushy. The remaining portion of the tail was usually firm or soft. Only in a few cases were all the segments in the tail mushy. Usually, no mushy segments appeared after a firm one.

The electrophoretic patterns of proteins extracted from the tail segments, immediately after death and after one week of storage on ice, are shown in Fig. 1. Almost no difference is observed between firm and soft segments in fresh prawns. The only significant difference between the patterns of myofibrillar proteins extracted from mushy and firm samples, is one line just

TABLE 1
Sensory Evaluation of Cooked Prawns
(Percentages of total)

	Fresh prawns $n = 39$	*Stored 1 week on ice* $n = 28$		
	Lobe + Segment 1	*Lobe*	*Segment 1*	*Segment 2*
Firm	90	—	11	32
Soft	8	10	18	36
Mushy	2	90	71	32

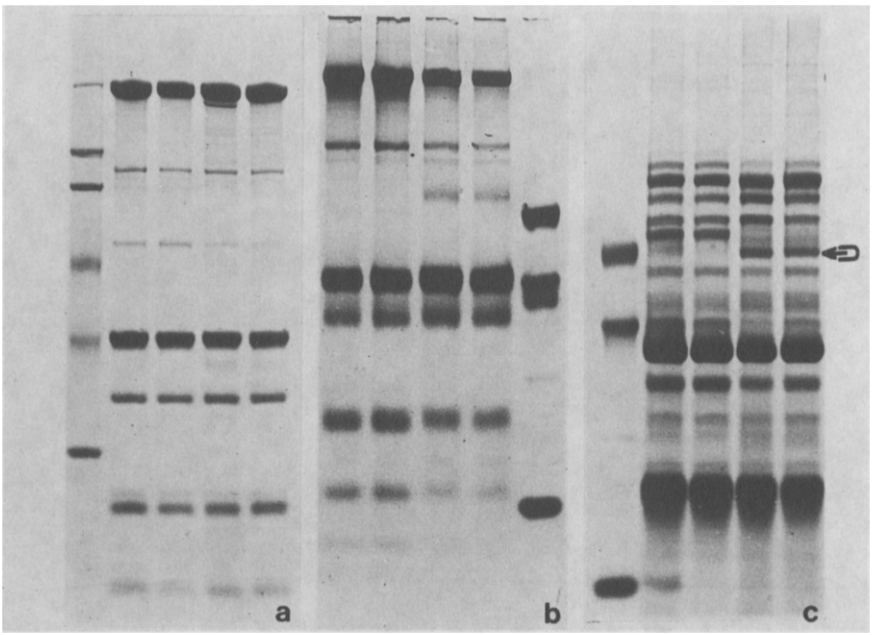

Fig. 1. SDS-PAGE of proteins extracted from firm and mushy tail segments, Porzio & Pearson's system (1977).

(a) Myofibrillar proteins. Lane 1, molecular weight markers: myosin, 205 000; β-galactosidase, 116 000; phosphorylase B, 97 400; bovine serum albumin, 68 000; egg albumin 43 000; carbonic anhydrase, 29 000. Lanes 2 and 3: myofibrillar proteins of fresh prawns extracted from firm and soft segments, respectively. Lanes 4 and 5: myofibrillar proteins extracted from mushy and non-mushy segments, respectively, of prawns stored for 1 week on ice.

(b) Effect of homogenization and washing in the presence of 0·2% Triton X-100 on the pattern of myofibrillar proteins. Fresh prawns and firm segments. Lanes 6 and 7: homogenization and washing with Triton X-100. Lanes 8 and 9: homogenization without Triton X-100. Lane 10, molecular weight markers: bovine serum albumin, 68 000; egg albumin, 43 000; myoglobin, 17 600.

(c) Sarcoplasmic proteins. Lane 11, molecular weight markers as in Lane 6. Lanes 12 and 13: sarcoplasmic proteins extracted from firm and soft segments, respectively, of fresh prawns. Lanes 14 and 15: sarcoplasmic proteins extracted from mushy and non-mushy segments of prawns stored 1 week on ice.

below myosin. A diffuse band between actin and tropomyosin appears in mushy as well as in non-mushy segments after one week of storage of prawns on ice. The bands of actin and Troponin-T are not resolved (Fig. 1a).

Extraction of sarcoplasmic proteins and washing the insoluble material with low salt solution in the presence of 0·2% Triton X-100, partially removes a line of about 105 000 daltons and almost completely removes a line of about 75 00 daltons (Fig. 1b). These lines, which are observed also in the patterns of sarcoplasmic proteins (Fig. 1c), probably consist of adsorbed

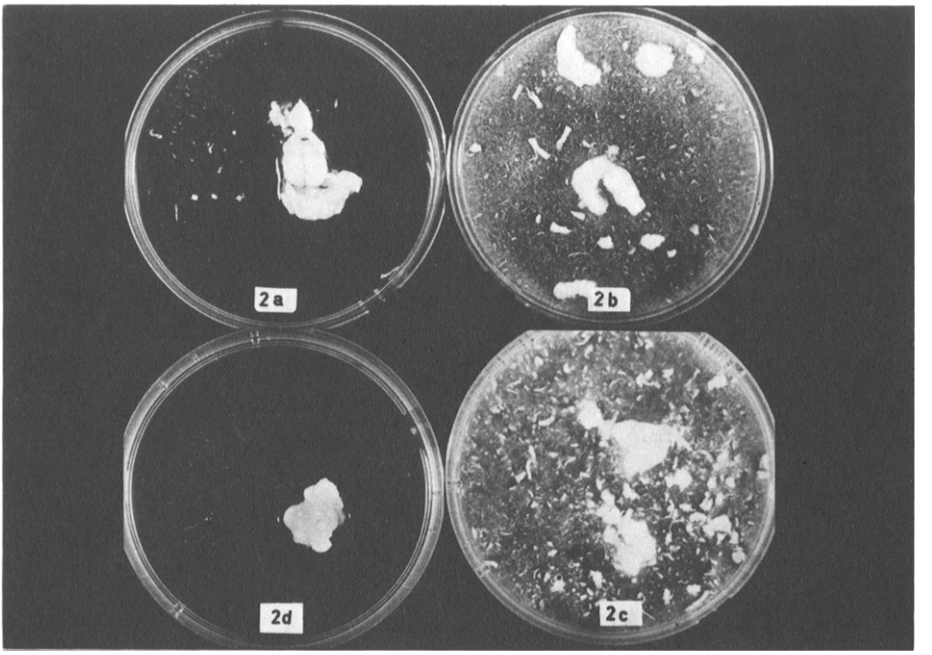

Fig. 2. Effect of overnight incubation at 0°C of muscle segments with enzymes on integrity after cooking. (a) Control, no enzyme; (b) 0·010 mg/ml trypsin; (c) hepatopancreas extract diluted × 10, 1·0 mg/ml protein; (d) 0·010 mg/ml chymotrypsin.

non-myofibrillar proteins. The 105 000 dalton component probably consists of sarcoplasmic reticular membrane protein strongly adhering to myofibrillar proteins. In a review on muscle proteins, Goll *et al.* (1977) described the removal of this component from porcine myofibrillar protein preparations by washing with Triton X-100.

The patterns of sarcoplasmic proteins are shown in Fig. 1c. In fresh, non-stored prawns, no difference was observed in the patterns between soft and firm segments. Likewise, no difference was observed between mushy and soft segments after one week of storage on ice. The main change observed after one week of ice storage was in the position of one line of about 70 000 daltons (marked by an arrow in Fig. 1c).

The effects of incubation of tail segments with diluted hepatopancreas homogenate or proteolytic or collagenolytic enzymes for 18 h at 0°C on texture after cooking, are shown in Fig. 2 and Table 2. While control segments remained intact and firm (Fig. 2a), the segments incubated with diluted hepatopancreas homogenate or 10 ppm trypsin disintegrated on cooking (heating for 4 min at 80°C) into a few large pieces and numerous fibres (Figs. 2b and 2c). The pieces which remained partially intact gave a mushy feeling when pressed between the index finger and the thumb, similar

TABLE 2
Effect of Incubation of Tail Segments with Enzymes

Enzyme	*Protein added (mg/ml)*	*Activity/ml*		*Disintegration of muscle*
		0°C	*30°C*	
Trypsin	0·010	7 PU[a]	70 PU[a]	+ + +
	0·002	1·8 PU[a]	15 PU[a]	+
	0·0005	0·4 PU[a]	3 PU[a]	—
Chymotrypsin	0·010	4 PU[a]	50 PU[a]	—
Hepatopancreas homogenate	1·0	18 PU[a]	120 PU[a] 1 CU[b]	+ + +
	0·25	4 PU[a]	30 PU[a]	+
	0·06	1 PU[a]	8 PU[a]	—
Collagenase (Worthington)	0·02	0·5 PU[a]	13 PU[a] 5 CU[b]	+ + +
Collagenase (Sigma)	0·0075	0·0 PU[a]	0 PU[a] 82 CU[b]	—[c]

[a] Proteolytic units: nmoles tyrosine/min.
[b] Collagenase units: nmoles specific substrate/min.
[c] Soft texture.

to the mushiness obtained after cooking prawns following prolonged storage on ice.

The lowest amount of added proteolytic activity of trypsin or hepatopancreas homogenate observed to induce mushiness after 18 h incubation at 0°C was about 1 unit/ml (measured on casein at 0°C and pH 7·2). Segments incubated with lower amounts of added proteolytic activity and stored further at 1°C without liquid for 8 days did not turn mushy. Chymotrypsin at an activity level fourfold the minimum for trypsin did not induce mushiness (Fig. 2d).

Collagenase from *Cl. histolyticum*, contaminated with protease activity, induced mushiness at low protease and collagenase activities. Purified collagenase from the same species, containing no measurable amount of proteolytic activity on casein, did not induce mushiness. The added collagenase activity (measured on a specific substrate) was more than tenfold higher than in the experiment with the non-purified collagenase (Table 2).

The electrophoretic patterns of myofibrillar proteins extracted from segments treated with enzymes are shown in Fig. 3. The 6% acrylamide gel used as resolving gel in the Laemmli system (Laemmli, 1970) resolves the high-molecular weight fragments of myosin which appear as discrete lines distributed between myosin and actin. Although more proteolytic activity

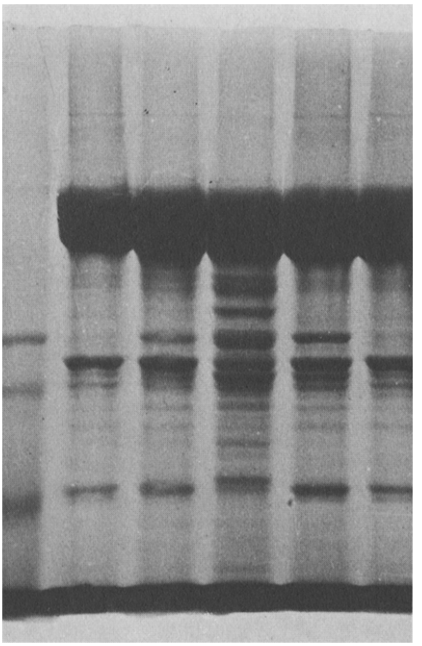

Fig. 3. SDS-PAGE of myofibrillar proteins extracted from segments incubated with enzymes. Laemmli's system (1970) with 6% acrylamide in the resolving gel. Lane 1, molecular weight markers: β-galactosidase, 116 000; phosphorylase B, 97 400; bovine serum albumin, 68 000. Lane 2: Control, no enzyme. Lane 3: incubation with hepatopancreas homogenate. Lane 4: incubation with 0·010 mg/ml trypsin. Lane 5: incubation with 0·010 mg/ml chymotrypsin. Lane 6: incubation with 0·0075 mg/ml of purified *Cl. histolyticum* collagenase (Sigma).

TABLE 3
Results of Cold Storage Experiments with Prawn Tail Segments

Days at 1°C	*Extracted proteins (mg per gram muscle)*			*Kjeldahl N (mg per gram muscle)*		*Hydroxyproline (mg per gram muscle)*	*Proteolytic bacteria (log CFU[a]/g)*
	Myofibrillar ±7	*Sarcoplasmic* ±5	*Ratio[b]* ±0·2	*Sarcoplasmic* ±0·5	*TCA-soluble* ±0·5	*Insoluble residue[c]* ±0·05	
0	101	51	2·0	10·6	6·3	0·43	2·9
8	97	43	2·3	10·2	4·3	0·53	3·9
15	94	41	2·3	9·0	4·4	0·47	4·5
22	98	44	2·3	10·6	4·8	0·52	5·0

[a] Colony-forming units.
[b] Myofibrillar/sarcoplasmic proteins.
[c] After all protein extractions.

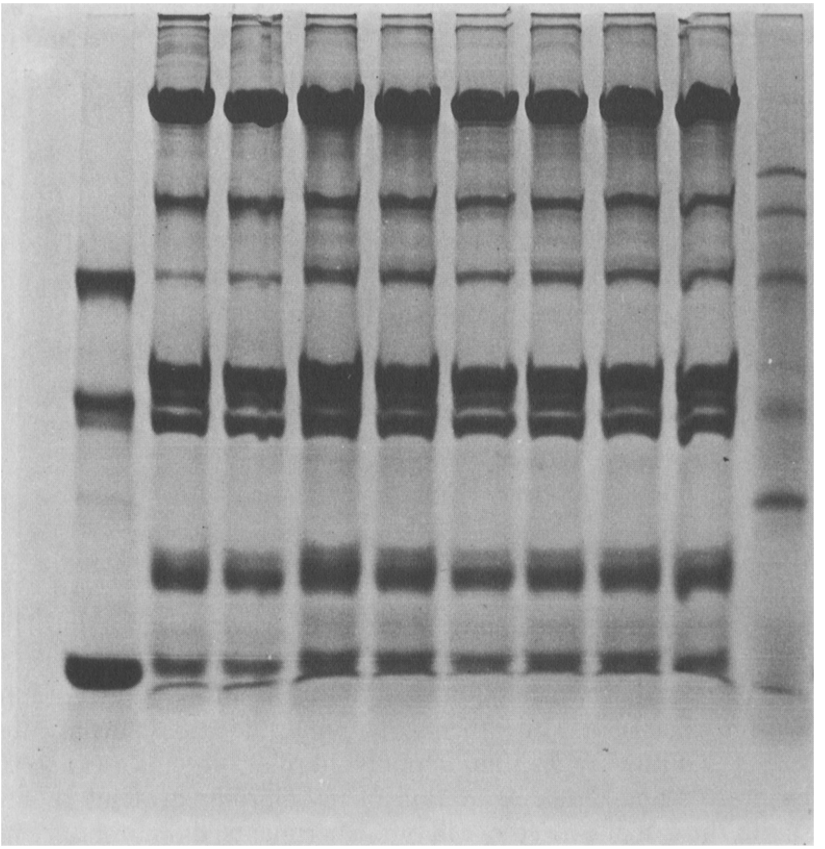

Fig. 4. SDS-PAGE of myofibrillar proteins extracted from segments stored at 1°C, Porzio & Pearson's (1977) system. Lane 1: Molecular weight markers as in Fig. 1, Lane 6. Lanes 2 and 3: controls, fresh prawns. Lanes 4 and 5: 8 days of storage. Lanes 6 and 7: 15 days of storage. Lanes 8 and 9: 22 days of storage. Lane 10: molecular weight markers as in Fig. 1, Lane 1 (myosin is missing).

was added to the sample treated with diluted hepatopancreas homogenate than to the one treated with trypsin, the latter suffered more extensive breakdown of its myosin.

With chymotrypsin, although no mushiness was induced at the added level of activity (Table 2), fragmentation of myofibrillar proteins is clearly indicated (Fig. 3, lane 5).

The results of cold storage at 1°C are given in Table 3 and Fig. 4. After 3 weeks of cold storage the number of proteolytic bacteria was up to 10^5 CFU/g. However, the stored segments developed no off-flavor, and the texture after cooking was soft but not mushy. This suggests that the microorganisms did not penetrate into the stored segments, or the

proteolytic activity demonstrated by a test at 20°C on casein was ineffective on intact tissue at 1°C. Angel *et al.* (1985) reported that no correlation was found between the onset of mushiness and bacterial count. The total amount of extractable proteins and their myofibrillar/sarcoplasmic ratio did not significantly change. Also, no decrease in the hydroxyproline remaining in the insoluble residue after extraction with low salt and high salt solutions was observed. After one week of cold storage, the level of low salt-soluble N did not change significantly, while the level of the TCA-soluble N decreased but remained stable during the remainder of the storage period. The electrophoretic patterns of the extracted myofibrillar proteins are shown in Fig. 4. The data in Table 3 and the patterns in Fig. 4 do not indicate that breakdown of myofibrillar proteins occurred during cold storage.

DISCUSSION

The results of the cold storage experiments suggest that the cause of mushiness does not reside in the muscle, or the segments stored at 1°C for an extended period of time would have turned mushy after cooking. The fact that mushiness always appeared first at segments adjacent to the hepatopancreas, suggests that the factor inducing mushiness on cold storage comes from this organ, which then softens and undergoes partial autolysis.

The fact that little fragmentation of myofibrillar proteins was observed in mushy prawns and that degradation of myofibrillar proteins by chymotrypsin (to the extent carried out in our experiments) did not induce mushiness, suggests that extensive degradation of myofibrillar proteins is not a necessary condition for development of a mushy texture. Mushiness could result from the degradation of a specific component necessary for the preservation of the integrity of the tissue on cooking. This component does not seem to be attacked by low levels of chymotrypsin.

The low degree of proteolysis observed in mushy segments can also be explained if mushiness is ascribed to degradation of connective tissue in the muscle by collagenolytic activity diffusing from the disintegrating hepatopancreas during storage (Baranowski *et al.*, 1984). In order to act effectively, the collagenase has to be assisted by a low level of protease. It appears that hepatopancreas collagenase which is known to differ from that obtained from *Cl. histolyticum* (Eisen *et al.*, 1973) is only slightly active on the specific substrate for collagenase. The properties of the proteolytic and collagenolytic activities of the hepatopancreas homogenates will be reported separately.

In conclusion, the data suggest that mushiness is accompanied only by a limited extent of proteolysis of myofibrillar proteins. If this limited

proteolysis is a necessary condition for development of mushiness, then it must involve a specific component. This proteolysis was shown not to be due to a proteolytic system endogenous to the muscle. Collagenolytic activity, assisted by a low level of proteolytic activity, both diffusing from the hepatopancreas, seems to be a plausible cause of mushiness.

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