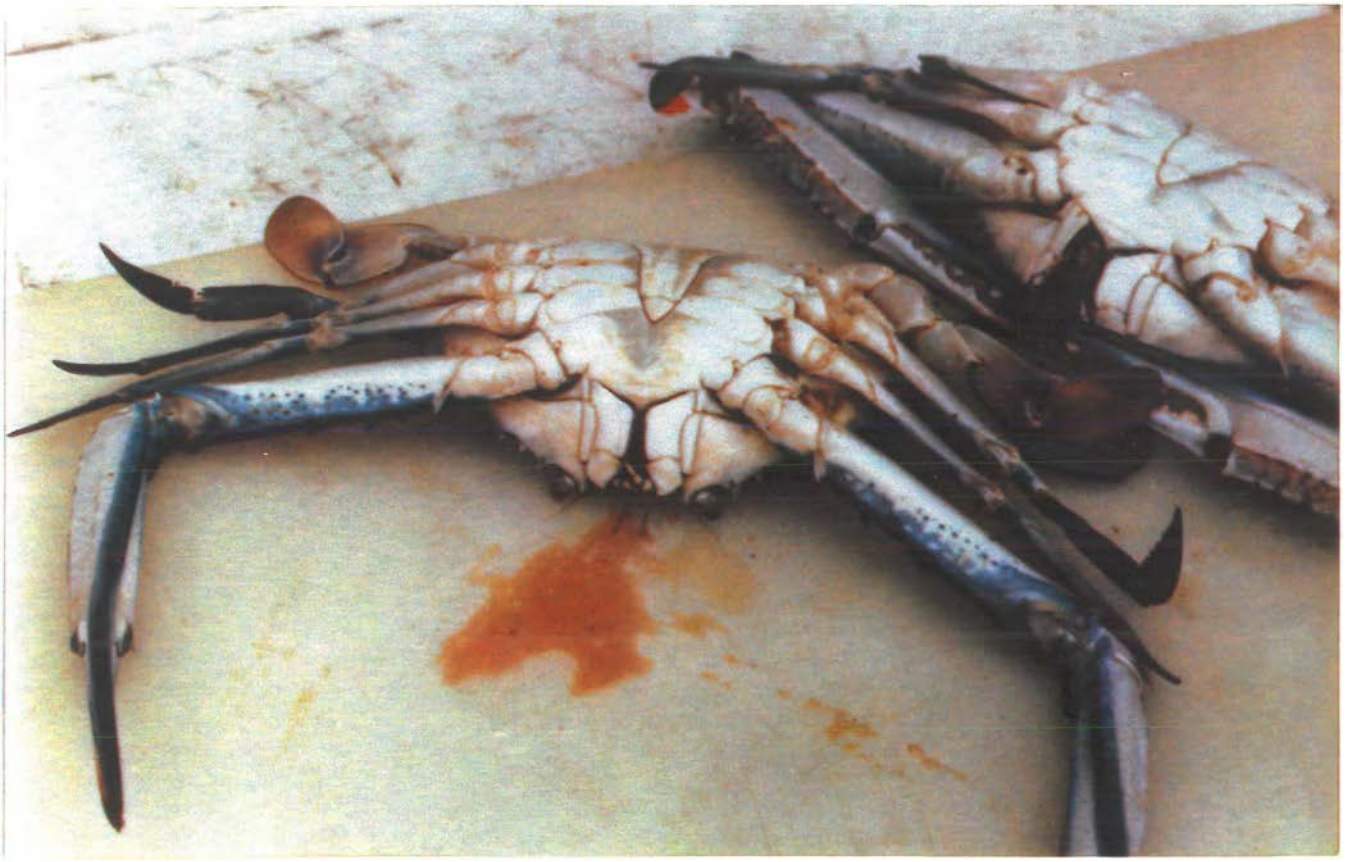
# FINAL REPORT

## FIRTA PROJECT 85/29

# "INVESTIGATION OF MUSHINESS IN CRABS."



Food Research and Technology Branch

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**FINAL REPORT - FIRTA 85/29**

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1. Title of Proposal

Investigation of mushiness in crabs.

2. Name of Applicant

Queensland Department of Primary Industries

3. Division, Department or Section

Food Research and Technology Branch,
Division of Dairying and Fisheries

4. Proposal

To investigate the major factors which contribute to the phenomenon of mushiness in the flesh of sand crabs (*Portunus pelagicus* Linnaeus) and to develop methods of prevention of mushiness thereby ensuring the availability of top quality product.

5. Person Responsible for Programme

J.R. Dulley, Acting Director,
Food Research and Technology Branch,
Queensland Food Research Laboratories,
19 Hercules Street, Hamilton, Q. 4007
Ph: (07) 2682421

6. Employed on the Programme

| | | |
|---|---|---|
| Project Leader | H.C. Deeth, BSc, BEcon, PhD | Principal Food Technologist (Research) |
| Other Staff | D.A. Dionysius, BSc, PhD | Biochemist |
| | S.L. Slattery, CBLT, BSc | Marine Biologist |
| | R.A.D. Smith, QDA | District Experimentalist |
| | G.B. Goodrick, BAppSc | Food Technologist |

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## SUMMARY


1. Mushiness in sand crabs is the result of breakdown of the flesh proteins by proteolytic enzymes from the hepatopancreas.

2. Only uncooked and undercooked crabs were found to develop mushiness.

3. The length of cooking time is important in the development of mushiness. A minimum of 10 minutes' cooking was found to be necessary to halt the proteolytic processes; this time had to be increased by several minutes when crabs were cold prior to cooking. Overcooking did not result in mushy flesh.

4. The practice of spiking crabs to render them inactive and prevent claw loss during cooking accelerated the deterioration in quality of uncooked crabs stored on ice. The rate of deterioration is dependent on the severity of the technique employed.

5. Backing of crabs did not increase flesh breakdown in uncooked crabs when caution was exercised in removing all traces of digestive organs from the meat.

6. No differences in quality were found for crabs caught by pots or trawling nets when they were stored cooked. However uncooked crabs caught by trawling methods were of poorer quality than pot-caught crabs after 3 days' storage on ice.

7. Cooked crabs stored on ice for 5 days retained acceptable texture, while uncooked crabs were rejected after 3 days' storage. Transfer of crabs stored in brine to ice resulted in rapid deterioration in flesh quality.

8. Uncooked crabs stored frozen at $-28^{o}C$ were of unacceptable quality after 1 week. Cooked crabs under the same conditions remained edible for at least 4 months.

9. Soft-shelled crabs which had recently moulted did not exhibit lower quality than full-bodied crabs provided they were cooked soon after capture. Uncooked post-moult crabs had lower indices of quality than inter-moult crabs held under similar conditions.

10. Crabs infected with the parasitic barnacle *Sacculina granifera* showed no defects in quality compared with healthy crabs.

11. Crabs which had been starved for 5 days had higher proteolytic activity but lower tissue weight in the hepatopancreas than fed crabs. The proteolytic potential of the two was considered to be similar.

12. Histological studies showed that major components of muscle tissue are destroyed during development of mushy flesh.

13. The proteolytic enzymes of hepatopancreatic extracts have a pH optimum of around 7.5. The heat stabilities of these enzymes at 50°, 60° and 70°C were determined.

14. The hepatopancreas contains seven major fractions with proteolytic activity separable by ion exchange chromatography. Enzyme characterisation studies revealed the presence of a range of specific enzymes eg. acid protease, carboxypeptidases, endopeptidases, trypsin, chymotrypsin, elastase and collagenase.

15. The major enzyme fractions which are active in hydrolysing raw crab flesh have been identified : three peaks from ion exchange chromatography exhibited high activity. One peak contained a relatively heat-stable, highly acidic enzyme with trypsin activity.

# PART 1

# INTRODUCTION

## General Background

## Plan of Operation

## GENERAL BACKGROUND

The fishery based on the sand crab or blue swimmer *Portunus pelagicus* (Linnaeus) is of substantial economic importance to the seafood industry in Australia. Approximately 900 tonnes are caught commercially each year (Potter *et al.*, 1987). Of these 140 tonnes are caught in South Australia, about 180 tonnes in New South Wales and most of the remainder in Queensland. The fishery varies considerably between States. For example the fishery in South Australia is at present largely "experimental" with only a small number of crabbers (10 or less), both male and female crabs are taken, no commercial sale of trawl-caught crabs is permitted and the crabs are largely sold by weight. By contrast, in Queensland a large number of professional crabbers are involved, only males can be taken, there is no restriction on the sale of trawl-caught crabs and the most common marketing mode is by body.

In addition to the commercial catch, that taken by recreational fishermen throughout Australia is also quite important. While individual amateurs catches may be small, their combined catch may represent a significant proportion of the total catch.

In recent years this fishery has increasingly experienced a problem of deterioration of the crab flesh, commonly called mushiness. The normally firm and resilient crab muscle loses integrity and becomes soft and friable. A mushy crab is externally indistinguishable from one with firm flesh and hence the defect cannot be detected at the point of sale. This causes severe disappointment for the consumer and produces buyer resistance to the product. For restaurateurs the problem is annoying and often very embarrassing. Ideally buyers would like assurance that the crabs they purchase will be firm and not mushy.

The problem of mushiness in the flesh of the crab has been widely known, and mostly ignored, for many years. However, with progressively larger catches and higher prices for crabs each year, the problem has become one which can no longer be ignored, as the economic losses which may be sustained are quite substantial.

Hence this project was undertaken to investigate the problem of mushiness, its incidence, causes and remedies. The findings are presented in this report.

**PLAN OF OPERATION**

The main objectives of the project were as follows:

**1. To document the incidence of the mushy flesh problem.**

**2. To establish the major cause(s) of the mushiness.**

**3. To develop fishing/handling procedures to prevent or minimise the occurrence of mushy flesh.**

**4. To disseminate all knowledge gained and techniques developed to appropriate sections of the industry.**

The project was carried out in four stages, roughly corresponding to these four objectives.

Stage one, documentation of the incidence of the problem was approached from several angles.

A 600 x 420mm colour poster describing the problem and inviting comments, information, theories and suggestions on causes was distributed to Queensland DPI offices, seafood wholesalers, fishermen's co-operatives, fish markets and some seafood retailers throughout the major crabbing areas of southern Queensland. A fact-finding tour of these centres was also undertaken by Dr Dionysius and Mr Slattery and included interviews with representatives from all sectors of the industry at the following locations: Rockhampton, Yeppoon, Gladstone, Tannum Sands, Bundaberg, Hervey Bay, Tin Can Bay, Maryborough, Burrum Heads and Brisbane. A questionnaire and press releases to local newspapers were also distributed. These, together with a reduced version of the poster are included in Appendix 1.

From the information gathered from these sources it was clearly evident that a major problem existed, and that a quick solution to the problem was needed. The theories put forward by people in the industry were numerous and varied. Most of the suggestions were related to poor handling and/or storage conditions prior to cooking. Many amateur fishermen held the belief that overcooking was the problem. Some causes nominated for the mushy flesh were:

(i) overcooking
(ii) overheating during post-capture holding e.g. in direct sunlight
(iii) storage of uncooked crabs near 0°C.
(iv) storage of uncooked crabs in refrigerated brine
(v) moult stage of the crab
(vi) undercooking of cold-stored crabs
(vii) harvesting of dead crabs by trawling methods
(viii) damage to digestive organs by crushing in trawl nets
(ix) accidental rupture of digestive organs during killing procedures e.g. 'spiking'
(x) freezing of green crabs
(xi) stress-induced changes during capture
(xii) "backing" of crabs, i.e. removal of carapace

A trip to Adelaide by Dr Deeth to observe the South Australian crab fishing industry also yielded valuable information. To coincide with this visit, a round-table meeting of crab fishermen, seafood processors, South Australian fisheries officers and research scientists from CSIRO and DSIR (New Zealand) was held. Pertinent points from this discussion were that there was considerable interest and concern about the problem in South Australia and that most of the fishermen seemed to have encountered it. They believed it was much more prevalent in the summer months, it was related to moulting, that freezing green crabs was a definite cause and that the time and handling between capture and cooking was paramount. Of interest was their concern about the optimal killing methods. It appeared that the South Australian crab was less sensitive to drowning in fresh water than is the Queensland crab. They also appear to discard their claws more readily when put on ice. It should be noted that the Queensland sand crab *Portunus pelagicus*, is known as the blue swimmer or bluey in South Australia. *Ovalipes australiensis* is commonly known as the sand crab in South Australia.

In stages two and three, the experimental sections of the project corresponding to objectives 2 and 3, many of the above factors were investigated to determine what role, if any, they played in the onset of mushiness. The results of this work are presented in Parts 2 and 3 of the report.

Part 2 describes the technological aspects: the experimental design, results and discussion of trials including frozen storage, storage in refrigerated brine, spiking, storage on ice, cooking conditions and textural analysis. The recommendations on correct storage and handling conditions presented in Part 5 of this report are based on these findings.

In Part 3, the biochemical basis of the mushiness problem was examined. The digestive enzymes involved in the process were partially purified and characterised, and other important parameters such as temperature stability, pH-activity profile, and each enzyme's ability to degrade crab flesh were determined.

Stage 4 in which objective 4, dissemination of findings, was addressed is discussed in detail in Part 6 of this report.

# PART 2

# TECHNOLOGICAL ASPECTS

Introduction

Materials and Methods

Results and Discussion

## INTRODUCTION

Post-mortem softening occurs in the flesh of several animal species. In beef it is related to the desirable increase in tenderness while in many other meats it is regarded as a defect. For example the well known defect in pork, referred to as PSE (pale, soft and exudative) is of major concern to the pig meat industry.

The flesh of several seafood species suffers from the softening phenomenon. In some cases the extent of softening renders the flesh inedible while in extreme cases extensive degradation of the flesh occurs with resultant serious losses for the industry. Mushiness, previously reported in crustacea such as the freshwater prawn *Macrobrachium rosenbergii* (Tillman and Finne, 1983), is an example of the former situation while the degradation caused by belly bursting in capelin (Gildberg and Raa, 1980) exemplifies the latter. The mushiness in the sand crab addressed in this project is of the former type.

Almost without exception, post-mortem softening has been attributed to proteolysis of flesh proteins by proteolytic enzymes. The increase in tenderness caused by aging of beef is caused by endogenous flesh enzymes while the softening in many other cases is due to the action of enzymes originating from digestive organs.

The organ associated with digestion in the decapods is the hepatopancreas. It is a divided organ situated laterodorsally from the stomach and containing simple, blind-ending diverticula which open into secretion ducts (van Weel, 1955). In the portunid crab hepatopancreas, the digestive enzymes are synthesized by "F-cells" which transform into "B-cells" (Barker and Gibson, 1978). The digestive juices are released by the B-cells via the hepatopancreas tubules and collecting ducts into the gut at the junction of the midgut and the pyloric stomach where they mix intimately with ingested food by the gastric mill (Johnson, 1980).

In the sand crab *Portunus pelagicus* the hepatopancreas has several distinct lobes on each side of the body and is in close contact with the cephalothoracic muscle. It is therefore strategically located to contaminate the surrounding muscle with its contents should the membrane surrounding the organ be damaged.

The effect of the hepatopancreas on the integrity of muscle tissue has been investigated for several crustacea. Wessels and Olley (1973) reported digestion of the meat in the carapace of the rock lobster *Jasus lalandii* by hepatopancreatic enzymes during freezing and frozen storage. They found that overnight starvation prior to freezing improved the quality of the meat and reduced the formation of proteolytic enzymes.

Studies on the freezing of mud crab *Scylla serrata* also revealed the importance of the digestive organs in the onset of mushy flesh. In experiments to determine optimum conditions for freezing, the viscera were removed by carapace removal or vacuum evisceration. The concomitant rupturing of the gut and release of digestive juices throughout the body cavity resulted in mushy flesh in these samples (Gillespie *et al.*, 1983). The results of blanching trials showed that mushiness will occur, even on frozen storage, if the cooking time is insufficient to inactivate the digestive enzymes. Similar advice was provided by Howgate (1984), who

recommended that mud crabs and sand crabs be cooked alive or very soon after death in order to obtain the best-flavoured meat of the highest yield for canning purposes.

With the emergence of the aquaculture industry, much attention has been paid to the rearing, handling and storage of the freshwater prawn, *Macrobrachium rosenbergii*. In 1980, the (USA) National Aquaculture Plan recognised mushiness as a problem requiring attention and one which was giving US wholesalers a negative opinion of this shrimp (Rowland *et al.*, 1982). Research reports have appeared on many aspects of its handling and quality, including the effects of freezing (Nip and Moy, 1979; Miyajima and Cobb, 1977), thawing and subsequent chilling (Nip and Moy, 1981), storage on ice (Angel *et al.*, 1981 and 1985; Waters *et al.*, 1981), and purging of ice-chilled prawns (Nip *et al.*, 1985b). The onset of mushiness has been attributed to the migration of proteolytic enzymes from the cephalothorax (Rowland *et al.*, 1982).

Another recognized factor in the onset of mushiness in some species is parasitic infection. A myxosporidian parasite (*Kudoa* sp.) has been associated with severe textural defects and poor keeping quality of the Pacific whiting, *Merluccius productus*, which have limited the commercial exploitation of the species (Dassow *et al.*, 1970; Erickson *et al.*, 1983). Similarly a myxosporidium has been associated with the extremely soft flesh encountered in Australian yellowtail kingfish when cooked (Lester, 1982b). While sand crabs are known to be hosts for some parasites (Phillips and Cannon, 1978; Potter *et al.*, 1987), no investigation of their association with mushiness has been carried out.

The stage of ecdysis can affect the texture of flesh from crustaceans. It has been suggested the the meat of post-moult crabs may be more susceptible to proteolysis by leaking hepatopancreas enzymes than would the meat of inter-moult crabs (Gillespie *et al.*, 1983). The effect of moulting on the flesh of the sand crab is not known.

Quantification of textural changes in foods is usually approached in two ways : subjectively through the use of sensory panels and objectively with the aid of texture measuring devices. Both methods have been successfully used for measuring the texture of the prawn *M. rosenbergii* (Angel *et al.*, 1985; Nip *et al.*, 1985b). While the correlations between the two measures can be low, both can be valuable tools for evaluating textural changes in prawns (Angel *et al.*, 1985). The use of these assessments of the texture of sand crab meat has not previously been reported.

In addition to assessment of the macrotextural changes in the development of mushiness, it is also instructive to assess the micro effects. Because of the apparent proteolytic breakdown occurring during mushiness development, an index of the extent of proteolysis would appear to be valuable. Such an index has not been used in reported studies on post-harvest softening in crustaceans. A second method of observing changes at the micro level is through histology. This approach was adopted by Rowland *et al.*(1982) in a study of proteolysis in *M. rosenbergii* tails. They were able to observe breakdown of the Z lines and increased gaping between myofibres indicative of breakdown of proteins in the sarcoplasm. Considerable histological change was observed during the progress of proteolysis.

The effects of various handling procedures on the development of mushiness have been addressed in this project. Because flesh breakdown occurs after rupture of the hepatopancreas, some types of physical trauma likely to have this effect have been examined.

1. The practice of spiking to destroy the autonomic nervous system and thus prevent claw loss could lead to physical rupture of the hepatopancreas.

2. Some crabbers remove the carapace of the crab before cooking. This usually occurs at sea, and is carried out on crabs with damaged shells.

There are two methods of measuring the size of a crab in Queensland according to the Fisheries Act, 1976. "Crabs shall be measured across the greatest dimension of the carapace but in some cases where the carapace is missing or damaged shall be measured from the anterior extremity of the fourth abdominal segment thereof". Some fishermen remove the carapace of smaller crabs in order to exploit a discrepancy in the correlation between these two measurements; i.e. some crabs illegal by the first measuring method may be legal by the second.

3. Crabs are caught in pots or by trawling. Those caught in trawl nets are subjected to more trauma as they can suffer crushing in the net by the weight of the rest of the haul and can be damaged when dropped out of the net onto the sorting tray.

An important aspect addressed in this part is that of cooking conditions. During the surveys in stage one, the most common causes of mushiness given by various people were undercooking and overcooking. This has now been investigated in relation to the heat-stability of the hepatopancreatic enzymes and the acceptability of the cooked meat.

## MATERIALS AND METHODS

### Crabs

Pot-caught. Crabs were mostly caught in pots or dillies. Pots were baited with fish frames, set and retrieved after 1-2 hours. On removal of crabs from the pots, they were placed in ice in the dinghy and taken ashore 1-5 hours after capture. Subsequent handling and storage conditions were as stated in the various trials described below.

Trawl-caught. Some crabs were caught in trawl nets. The nets were usually trawled for 1 hour before being hauled aboard and emptied onto the sorting tray. Crabs caught were placed in ice as soon as possible or treated in various ways as outlined below.

Randomly purchased. For some experiments crabs were purchased at random from the Queensland Fish Board. In general the history of these crabs was not known although the time since capture could be assumed to be 1-2 days.

Special samples. On occasions, special batches of crabs were examined. These were usually obtained following information that they were suspected of being mushy or others from the same batch (or fisherman) were mushy.

### Physical analyses

Textural analysis. The meat used for textural analyses was the muscle associated with the swimming appendage located in the posterior cephalothoracic segment. This muscle provided a relatively large sample of even consistency.

Sensory assessment was carried out by either an expert panel of three or an untrained panel of 35 depending on the number of crabs and the amount of meat available for evaluation. The texture was graded in half-unit intervals according to the following scheme.

| Texture description | Grade |
|---|---|
| Very firm | 5 |
| Firm | 4 |
| Slightly firm | 3 |
| Weak | 2 |
| Mushy | 1 |

Textural deterioration was quite evident but not of serious concern in meat with grades between 4 and 3. However any crab meat scoring 3 or less was considered to be seriously affected and would be expected to cause consumer dissatisfaction. Hence in this work, a grade of 3.25 was chosen as the limit of acceptability.

Flavour of the crab meat was also assessed by the untrained sensory panel. A five-point scale was used, with a range of scores from 5 for sweet-

tasting meat, to 1 for bitter or other detectable off-flavours. Overall acceptability, scored from 5 to 1 in decreasing order of preference, was also recorded by the panel.

Mechanical assessment of texture was performed using a modified L.E.E.-Kramer shear cell attached to a Model 1130 Instron Universal Testing Machine. Due to the limited amount of meat present in the crab cephalothorax a mounting block was inserted into the shear cell to hold a plug of meat 16 mm x 25 mm x 25 mm. Two 3 mm shear blades penetrated the meat perpendicular to the muscle fibres and across the fibre grain, and were driven by a 1 Newton force at 20 mm per minute. The recorder chart speed was 100 mm per minute and the scale was x 1. The area under the shear force curve was measured using a Tamaya Digital Planimeter and the data adjusted for weight of meat loaded to give work done in N cm/g.

Temperature measurement. During cooking trials and storage trials, temperature of crabs was monitored using a thermocouple inserted under the shell into a central position within the flesh. The thermocouple was connected to a digital thermometer.

## Chemical analyses

Preparation of samples. The meat was separated from the shell and homogenised with an equal weight of water in a Waring blendor (40 s, high speed). Ten g of homogenate was centrifuged at 15000 *g* for 30 min, and 1 mL of the supernatant removed for determination of protease activity. One g of homogenate was used to determine total nitrogen content and a further 20 g of homogenate was blended with 20 g of 24% trichloroacetic acid (TCA) and filtered through Whatman's No. 542 filter paper. The filtrate (0.2 mL corresponding to 0.017 g of muscle) was utilised in the Folin-Ciocalteu assay, while 10 mL (0.83 g of muscle) was analysed for non-protein nitrogen.

In trials where both proteolytic activity and texture of the meat were measured, the crabs were broken in half and one half cooked in a plastic bag using standard cooking conditions. Proteolytic activity was determined on the uncooked half, while texture and P.I. measurements were done on the cooked half.

Hepatopancreas preparations for protease activity were obtained by firstly homogenising the tissue with an equal volume of water in a Waring blendor (60 s, high speed), and then centrifuging for 15 min at 45000 *g*. The supernatant was assayed by the standard method (see below).

Protease assay. Protease activity was measured using 0.17% azocasein as substrate. The procedure followed that of Jensen *et al.* (1980), and one unit of activity was defined as the amount of enzyme required to produce an increase in absorbance at 366 nm of 0.01 per h under the standard assay conditions.

Total protein. Total protein (TP) was determined by the Kjeldahl method (AOAC, 1984) which evaluated total nitrogen (TN) and total non-protein nitrogen (TNPN). Total protein was thus determined by the formula:

$$TP\ (\%) = (TN\% - TNPN\%) \times 6.25$$

Folin-Ciocalteu assay. The Dulley and Grieve (1975) modification of the Lowry (1951) method for protein determination was applied to the TCA extract to determine the amino acid content expressed as p.p.m. tyrosine equivalents.

Proteolysis index. The proteolysis index (P.I.) was used to give an indication of the degree of protein breakdown within the meat. It was determined by the following formula:

$$P.I. = \frac{\text{p.p.m. tyrosine equivalents}}{\text{TP\%}}$$

## Standard cooking methods

Crabs were cooked in seawater or salt water (approx. 3%) in a conventional prawn cooker (140 L capacity) heated by a gas burner fitted with a 4 cm I.D. nozzle. Those cooked on board the trawler had an internal temperature prior to cooking the same as seawater, while crabs removed from storage were defrosted and/or warmed up to an internal temperature of 10°C to 15°C prior to cooking. For iced or refrigerated crabs this took on average 30 minutes, while frozen crabs required several hours. The cooking time referred to in these investigations is the time from when the water returned to the boil after introduction of the crabs until crabs were removed. It should be noted that crabs kept in storage take longer to return to the boil than those at sea temperature, and that their internal temperature at this time is also lower. Cooked crabs were cooled by flushing with cold water after removal from the cooker.

The standard cooking time for these studies was 10 minutes. In some trials the cooking time was varied, and these variations are noted in the studies concerned.

## Handling trials

Spiking. Live freshly caught crabs were killed by inserting a tapered stainless steel spike through the oral aperture in a ventral direction, quickly moving it laterally until a breaking of internal skeletal structures is felt. This is intended to destroy the supraoesophageal ganglion (Gillespie *et al.*, 1983).

In one trial, crabs were spiked using a vigorous technique, or left intact and stored on ice for up to 4 days. Batches of ten from both treatments were removed at days 0, 2 and 4, cooked by the standard method, and then analysed for P.I. and texture.

In a second trial, crabs were spiked using a gentler technique. Again, crabs were stored on ice and batches of ten removed after 0, 1, 2 and 4 days' storage. In addition to sensory texture evaluations on the cooked flesh, the mechanical assessment of texture using the Instron was performed. P.I. values were also determined.

Cooking. The effects of cooking time on the chemical and physical parameters of crab flesh were studied in a trial where crabs were cooked

for times ranging from 4 to 12 minutes, and then stored on ice for 2 days. Ten crabs were used for each time point. Data for internal temperature of the crab during cooking, sensory grade, shear force and P.I. of the meat, and protease activity in the hepatopancreas were measured.

In another cooking trial, freshly caught crabs were cooked for times of 5, 10, 15 or 20 minutes, and after overnight storage at 5°C the meat was presented to an untrained taste panel for assessment of texture, flavour and overall acceptability.

Backing. Freshly caught crabs had their carapaces removed i.e. "backed" after they became inactive. These crabs (10 in total) were stored on ice for 3 h, cooked by the standard method, and then analysed for texture and P.I.

Pot- *vs* trawl-caught crabs. Crabs caught by pot and trawl techniques on the same day and in the same area were kept at 5°C in both cooked and uncooked states for periods of 3 or 7 days. On removal from cold storage, the uncooked crabs were cooked by the standard method, and together with the cooked crabs, were analysed for P.I. and texture. A total of 80 crabs (10 per treatment) was used in the trial.

## Storage trials

Brine/ice storage. Several trials were conducted on the effect of storage of uncooked crabs in brine or ice, or a combination of brine storage followed by ice. The brine consisted of refrigerated seawater held at a temperature of 0.2° to 1.8°C. Batches of 10 uncooked crabs were kept in brine or on ice for appropriate times (up to 3 days for brine, 5 days for ice). They were then cooked and analysed for P.I. and texture. For brine/ice combinations, crabs were removed from brine after 1, 2 or 3 days and subsequently held on ice for a further 2 days before cooking and analysis. For comparison, freshly caught crabs were cooked and stored for up to 5 days on ice prior to analysis. A total of 418 crabs was used in these trials.

Frozen storage. Frozen storage trials were conducted on trawl-caught crabs, both cooked and uncooked. Within 12 h of capture the crabs were frozen by placing them in a blast freezer overnight, at a temperature of -37°C. The following morning the crabs were glazed by dipping them in fresh water. They were then stored at -28°C in cartons sealed with plastic sheets. At selected times batches of 10 crabs from both cooked and uncooked lots were removed, thawed, and kept for 0 or 2 days on ice. Crabs with no ice storage time were cooked (if uncooked), and analysed for P.I. and texture within a few hours of thawing. Crabs which were stored on ice for 2 days following thawing were subsequently cooked (if uncooked) and analysed.

## Physiological studies

During the course of this project it was possible to examine the effect of some physiological characteristics on the quality of the flesh. While processing crabs for other trials, any which were in the pre- or post-

moult stages of ecdysis, or any which were infected with the parasitic barnacle, *Sacculina granifera*, were noted for closer examination.

Ecdysis. Crabs in pre-moult or post-moult stages of the cycle were identified by their characteristic carapace appearance. Such crabs were found in batches of uncooked crabs in brine/ice storage trials, and in batches of cooked crabs in freezing and pot *vs* trawl trials. A total of 30 post-moult crabs (16 cooked, 14 uncooked), and 3 pre-moult crabs (all cooked) were identified. Z-score analysis (see statistical methods) was used to compare the physical and chemical parameters of these crabs with others in the same treatment.

Parasitic infection. Twelve crabs parasitised with *Sacculina granifera* were cooked and then stored on ice for 2 days prior to analysis. Unparasitised crabs, treated in the same way, were used as controls.

Feeding/starving trial. Ten crabs were held in two ponds and fed for two days to allow them to stabilise following capture. Five crabs in one pond were then starved for 5 days while the others were still fed. All crabs were then killed, hepatopancreases removed, weighed and homogenised, and the protease activities of the homogenates measured.

## Histology

The muscle associated with the posterior cephalothoracic segment was removed from uncooked crabs stored on ice for 1 or 5 days. Samples of tissue were kept for fresh frozen sectioning or placed in fixative (Bouin's or 10% buffered formalin). Ten µm fresh frozen sections were fixed in alcohol and stained using haematoxylin and eosin. Tissue fixed in Bouin's fluid or formalin was embedded in paraffin, sectioned at 7 µm, and stained; the former with phosphotungstic acid haematoxylin, PTAH (Bancroft, 1977) and the latter with Heidenhain's iron haematoxylin and counterstained with eosin. Photomicrographs were obtained using the Olympia "Vanox" microscope fitted with Olympus Photomicrographic Equipment C-35A attachments.

## Statistical methods

Analysis of variance. Statistical treatment of the data in most of the trials described in this section was carried out using analysis of variance. This enabled the data to be tested for the significance of the difference between treatment means. Least significant differences (LSD) were used to identify which treatments differed significantly.

Z-score. Where numbers of crabs in a particular study were limited , eg. in pre- and post-moult crab studies, the individual value for a parameter was compared with the mean for that group of data, assuming the distribution of data to be normal. The number of standard deviations which that value lies below or above the mean of the distribution is known as the Z-score. The average Z-score for all the individual values gives an indication of how much that characteristic differs from normal values.

## RESULTS AND DISCUSSION

### Crab temperature during cooking

The internal temperature profile of crabs cooked for 10 min is shown in Figure 1. This profile is the average of 33 time-temperature measurements on crabs warmed in air for approximately 30 min after removal from ice storage. The temperature gradient is about 5.5°C per min, and while the internal temperature of the crab on immersion varied with storage conditions and duration of warming-up time, the gradients were always similar to that shown in Figure 1. This suggests that iced crabs, if cooked immediately, should have their cooking time extended by several minutes to approximate the standard procedure. Frozen crabs would need their cooking time even further extended, possibly by another 5 minutes.

### Spiking

Figure 2 shows the data for P.I. and grade of vigorously spiked crabs and undamaged crabs stored uncooked on ice for up to 4 days. There is a significant difference ($P<0.01$) between P.I. values for spiked and intact crabs over the trial period. While the grade of spiked crabs was significantly lower at day 0 ($P<0.01$), the rate of deterioration was no greater than for undamaged crabs after that time. The presence of significant changes in flesh quality at day 0 indicate that the spiking technique was effective in producing flesh damage in the couple of hours between spiking and analysis.

Figure 3 shows the P.I., grade and shear force data for crabs spiked using a gentler technique. This treatment still resulted in a deterioration in quality but at a slower rate than the above trial. The grades of crabs were again significantly lower ($P<0.01$) than undamaged crabs, while the P.I. did not show such a clear separation. The meat from this trial was also analysed for shear force, which showed a good correlation with the sensory grading ($r = 0.73$, $P<0.001$). Szczesniak and Torgeson (1965) reported correlations between the Kramer shear press and sensory tenderness of beef, pork and chicken meat ranging from -0.42 to -0.98, but for the most part highly significant correlations have been found by different laboratories.

### Cooking

The effects of cooking time on the chemical and physical parameters of crab flesh are shown in Figure 4. With increasing cooking time there was an improvement in the measured parameters, i.e. P.I. values decreased, and textural indices (grade and shear force) increased in value. Significant differences occurred between 6 and 8 minutes for shear force data ($P<0.05$), and between 8 and 10 minutes for P.I. data ($P<0.05$). The correlation between shear force and grade ($r = 0.69$, $P<0.001$) is similar to the value obtained in the spiking trial. The increase in shear force values associated with increased cooking time agrees with data obtained by Ahmed *et al.* (1972) for shrimp, and Findlay and Stanley (1984) for scallop meat.

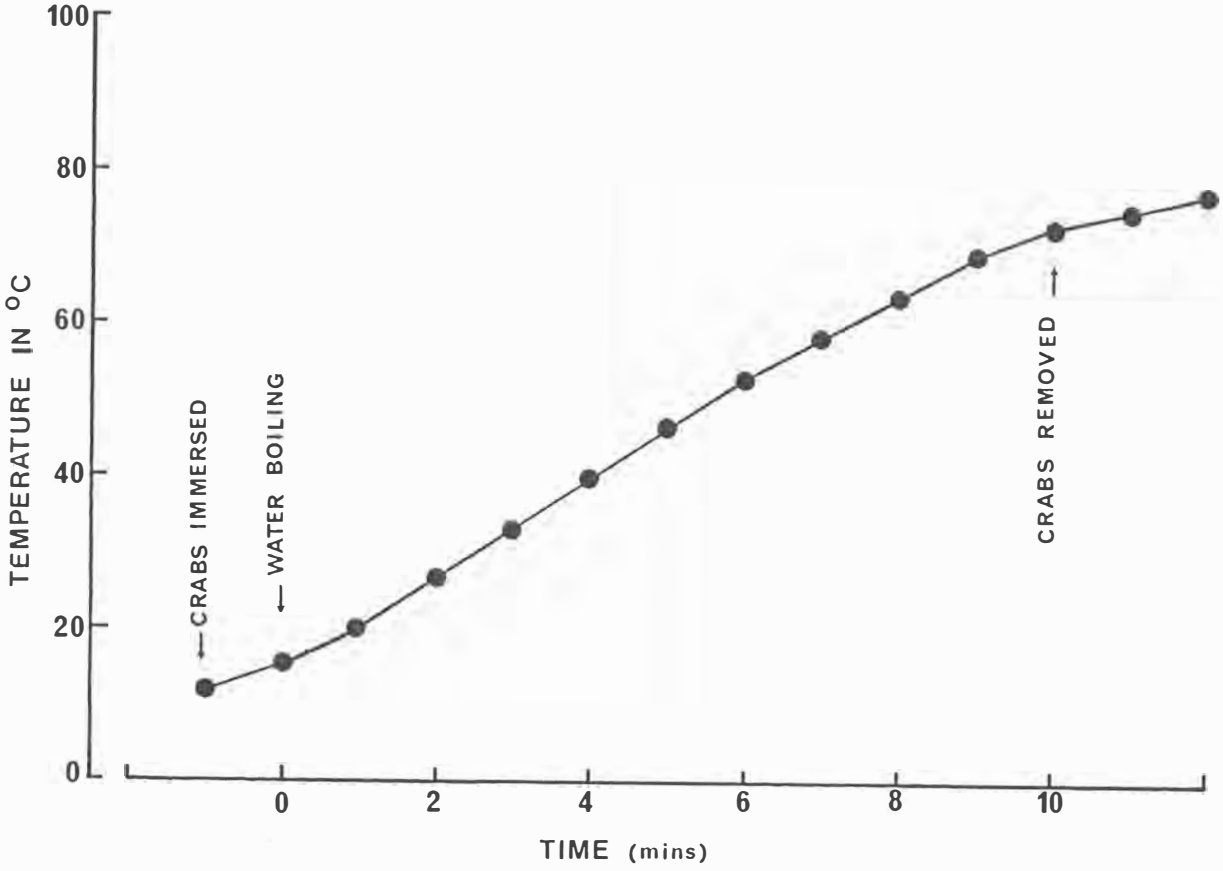


FIGURE 1. Internal temperature profile for crabs cooked for 10 minutes (mean of 33 determinations)

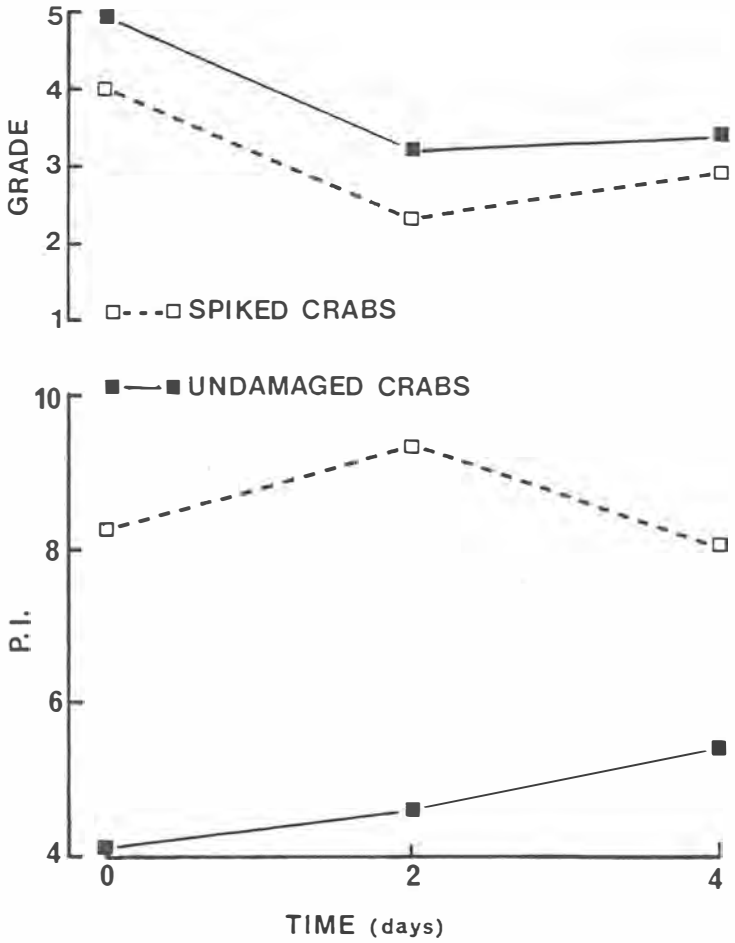


FIGURE 2. Effect of spiking on the P.I. and grade of ice-stored crabs (each point represents the mean of 10 determinations)

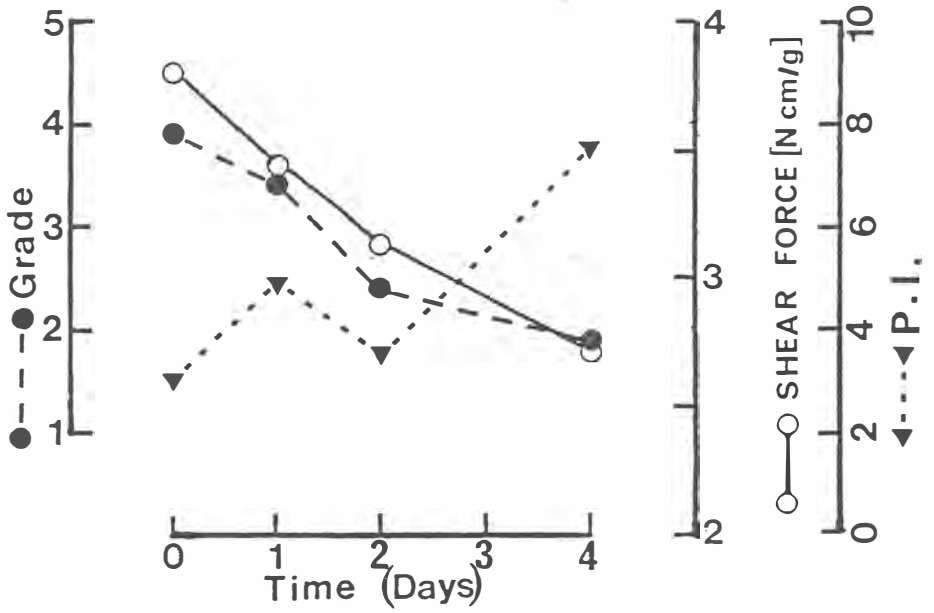


FIGURE 3. Changes in P.I. and texture of spiked crabs during storage on ice (each point represents the mean of 10 determinations)

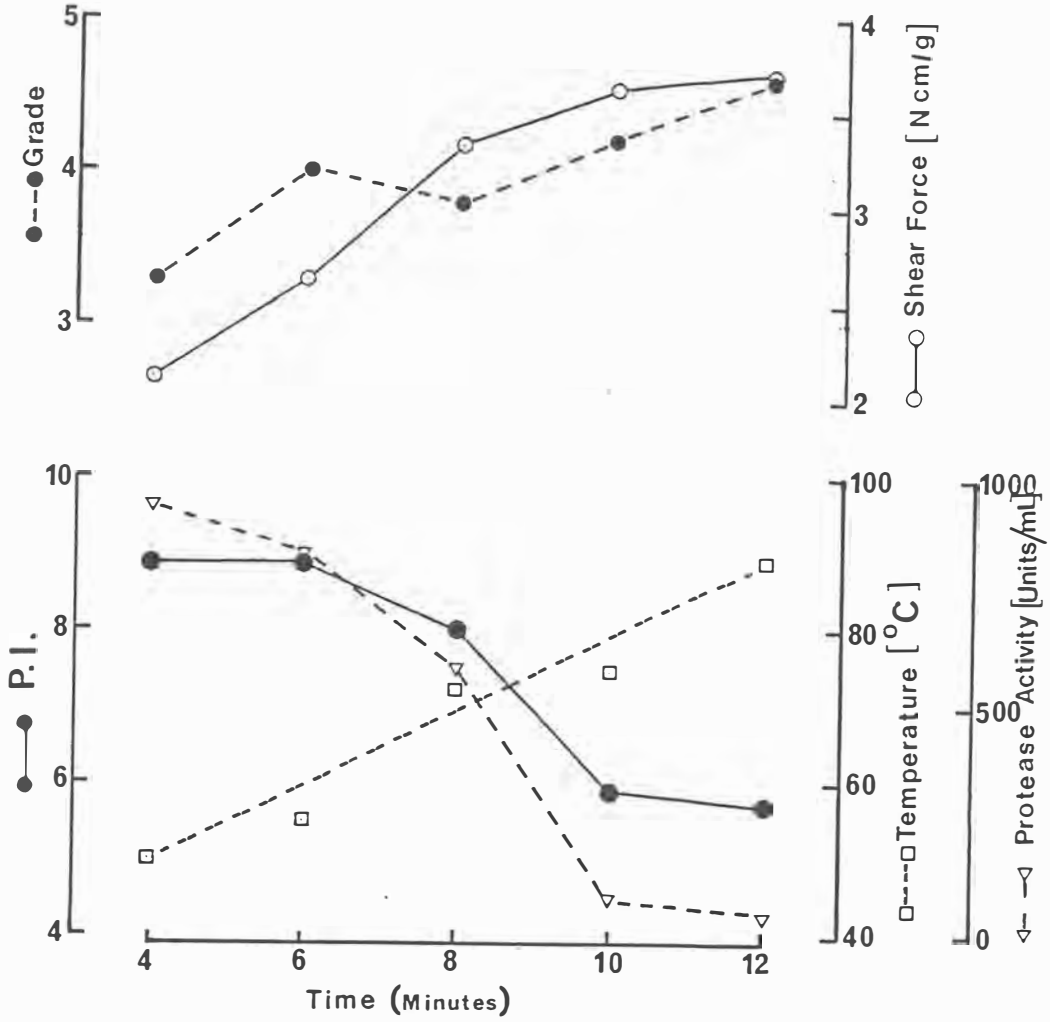


FIGURE 4. Effect of cooking time on the P.I. and texture of crab meat, and protease activity of hepatopancreas (each point represents the mean of 10 determinations)

Figure 4 also shows the effect of cooking time on the protease activity in the crab's hepatopancreas. There was an overall loss of activity with increased cooking, with a significant drop ($P<0.01$) from 8 to 10 minutes. Nearly all proteolytic activity was lost after 10 minutes' cooking, which corresponds to an internal temperature in the crab of about 80°C. This dramatic drop in activity occurring at 8 to 10 minutes was paralleled by the significant fall in P.I. of the meat. The inference from this is that the hepatopancreas enzymes are responsible for proteolysis in the flesh. These findings provide an excellent basis for formulating recommendations on correct cooking procedures.

The results of sensory evaluation of crab meat cooked for various times are shown in Table 1.

**TABLE 1**

**Sensory evaluation scores of cooked crab meat**

| Trait | Cooking time (min) 5 | 10 | 15 | 20 |
|---|---|---|---|---|
| texture | $3.15^{a}$ | $3.74^{b}$ | $4.12^{b}$ | $3.79^{b}$ |
| flavour | 4.06 | 4.29 | 4.21 | 3.85 |
| overall acceptability | $3.53^{x}$ | $4.29^{xy}$ | $4.53^{yz}$ | $4.00^{z}$ |

(Mean scores followed by a different letter are significantly different at the 5% level)

There was a significant difference in texture score ($P<0.01$) when the cooking time was extended from 5 to 10 minutes. Further increases in cooking time did not affect the texture, and certainly did not lead to mushiness. The panel did not detect any flavour changes, but did record a gradual change in overall acceptability which, like texture, had an optimum score at 15 minutes' cooking time.

## Backing

Table 2 below shows the effect of backing crabs on P.I. and grade. The results are compared with those for crabs which had been stored on ice for 24 h, or spiked and stored on ice for 3 h before cooking and analysis.

**TABLE 2**

**P.I. and grade of backed, spiked and ice-stored crabs**

| Treatment | P.I. | Grade |
|---|---|---|
| backed | $2.86^{a}$ | $4.42^{xy}$ |
| spiked | $8.23^{b}$ | $4.02^{y}$ |
| ice storage | $4.37^{a}$ | $4.63^{x}$ |

(Mean values followed by a different letter are significantly different at the 1% level)

The results show that removing the carapace from freshly caught crabs did not cause as much damage as spiking. Ward (1982) and Ward *et al.* (1985) found that blue crabs, *Callinectes sapidus*, which were backed and eviscerated prior to boiling showed no differences in bacteriological, Instron or taste panel data, or total yield, compared with whole crabs. If the backing procedure is to be carried out, diligent removal of the viscera and effective washing must be performed to remove all traces of digestive enzymes.

## Pot- *vs* trawl-caught crabs

A comparison of the crabs caught by different techniques was carried out after batches from both pot and trawl methods, stored cooked and uncooked, were kept on ice for 3 and 7 days. The results of P.I. and texture determinations are presented in Table 3.

**TABLE 3**

**Grade and P.I. of pot- and trawl-caught crabs stored on ice**

| Treatment | | Storage time (days) | | | |
|---|---|---|---|---|---|
| | | 3 | 7 | 3 | 7 |
| | | Grade | | P.I. | |
| cooked | pot-caught | $4.62^{a}$ | $4.29^{a}$ | $7.00^{xy}$ | $7.98^{xyz}$ |
| | trawl-caught | $4.48^{a}$ | $4.55^{a}$ | $5.67^{x}$ | $5.31^{x}$ |
| uncooked | pot-caught | $3.24^{b}$ | $2.68^{c}$ | $8.27^{xyz}$ | $9.11^{yz}$ |
| | trawl-caught | $2.35^{d}$ | $2.62^{c}$ | $10.42^{z}$ | $10.56^{z}$ |

(Mean scores followed by a different letter are significantly different at the 5% level)

There were no significant differences in the texture or chemical quality of pot- and trawl-caught crabs which had been cooked and stored for 3 or 7 days on ice. However for uncooked crabs, the grades of trawl-caught crabs after 3-d storage were significantly lower than pot-caught specimens, and P.I. values were higher. This poorer keeping quality of uncooked trawler crabs cannot be explained by differences in location or time of capture, as all crabs were caught on the same day and in the same area. The possibility exists that the crabs are damaged during the trawling operation, especially during retrieval and sorting. Effects such as crushing may damage internal organs, releasing digestive enzymes which can then act on the flesh during the storage period. The shock of capture may also lead to the mobilisation of enzyme systems.

**Brine/ice storage**

The results of several trials involving storage in brine, on ice, and in brine/ice combinations, are shown in Figure 5. Cooked crabs kept on ice retain good quality over the 5-d storage period, while uncooked crabs kept on ice were unacceptably mushy if stored for longer than 3 days. Crabs stored uncooked in brine were of similar quality to those in ice storage. However upon removal from brine and transferral to ice storage, the rate of deterioration was faster than for ice alone. While the mechanism for this is unknown, the change in osmotic pressure accompanying the transferral to ice may result in membrane damage and release of hepatopancreatic enzymes. The flesh would therefore deteriorate at a faster rate than that of crabs stored only on ice. As a consequence of these findings it is recommended that crabs which have been stored in brine should be cooked immediately upon removal.

The results of the statistical treatment of the data for brine and/or ice storage of uncooked crabs are shown in Table 4.

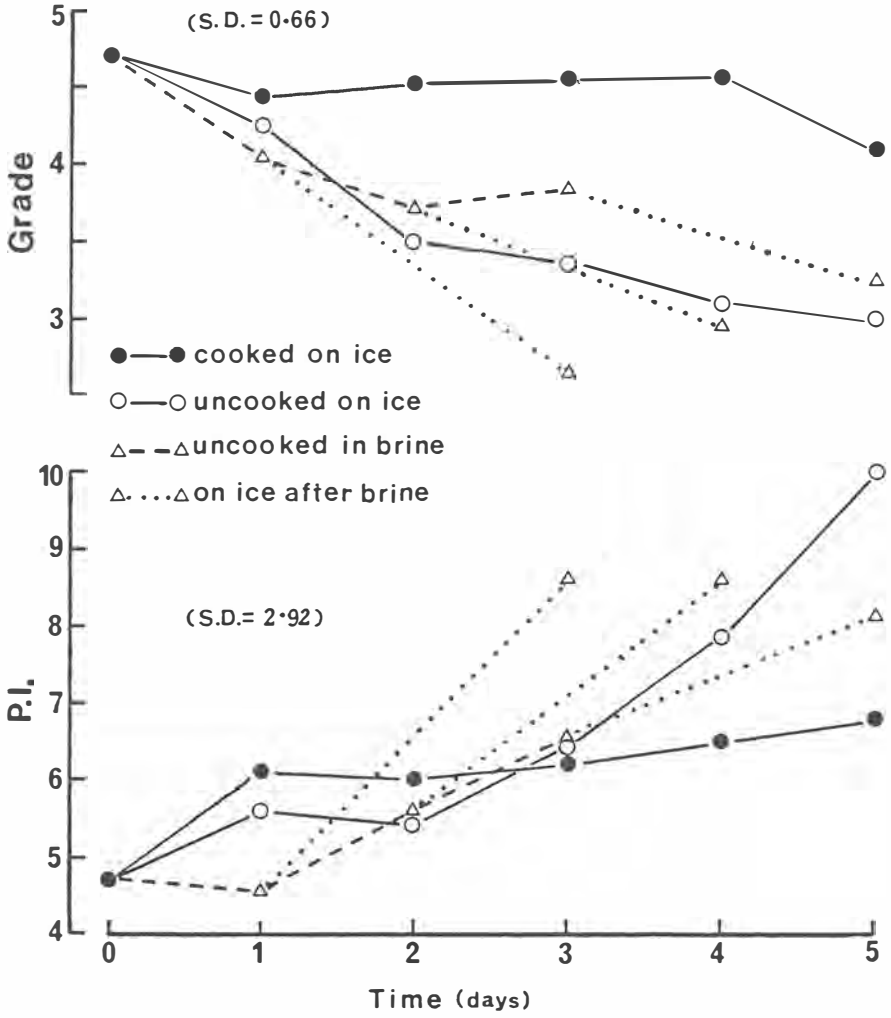


FIGURE 5. Grade and P.I. for crabs stored on ice, in brine and on ice after brine (each point represents the mean of 35 determinations)

**TABLE 4**

**Differences between means of texture scores and P.I. values for brine and/or ice storage of uncooked crabs**

(Treatments followed by different letters have significantly different means at the 5% level)

| Treatment (days in brine, days on ice) arranged in descending order of **texture score** | Treatment (days in brine, days on ice) arranged in ascending order of **P.I. value** |
|---|---|
| 0, $0^{a}$ | 1, $0^{v}$ |
| 0, $1^{b}$ | 0, $0^{v}$ |
| 1, $0^{bc}$ | 0, $2^{vw}$ |
| 3, $0^{cd}$ | 2, $0^{vw}$ |
| 2, $0^{cd}$ | 0, $1^{vw}$ |
| 0, $2^{de}$ | 0, $3^{w}$ |
| 0, $3^{ef}$ | 3, $0^{wx}$ |
| limit of acceptability | |
| 3, $2^{eg}$ | 0, $4^{xy}$ |
| 0, $4^{fg}$ | 3, $2^{xy}$ |
| 0, $5^{fgh}$ } rejected | 2, $2^{yz}$ |
| 2, $2^{gh}$ | 1, $2^{yz}$ |
| 1, $2^{h}$ | 0, $5^{z}$ |

It can be seen that all uncooked crabs which have had brine/ice storage were rejected on the basis of texture. As well, uncooked crabs kept for more than 3 days on ice, treatments 0, 4 and 0, 5, were unacceptably mushy. Table 4 also shows that the treatments rejected on texture score correspond to the treatments with the highest P.I. values. Hence crabs with P.I. values above 7.8 will be rejected (see Figure 5).

A batch of twelve uncooked, brine-stored crabs purchased from the Queensland Fish Board was analysed for P.I. and protease activity of the flesh. While the exact history of these crabs was not known, they had been stored for 1 or 2 days in brine on board a trawler. The results of chemical analyses are shown in Table 5.

**TABLE 5**

**P.I. and protease activity of uncooked crabs stored in brine**

| Sample | Storage time (days) 1 | | 2 | |
|---|---|---|---|---|
| | P.I. | Protease activity (units/g) | P.I. | Protease activity (units/g) |
| 1 | 2.2 | 8.4 | 10.2 | 77.0 |
| 2 | 3.6 | 11.8 | 6.8 | 32.4 |
| 3 | 2.9 | 9.6 | 14.3 | 99.4 |
| 4 | 2.9 | 6.0 | 14.1 | 140.0 |
| 5 | 2.1 | 2.6 | 6.6 | 87.2 |
| 6 | 3.5 | 8.8 | 4.8 | 39.6 |
| mean | 2.9 | 7.9 | 9.5 | 79.3 |

The results indicate a dramatic increase in P.I. and protease activity when the brine storage time is increased from 1 to 2 days. Whatever the process involved, large amounts of protease must enter the flesh in a short period of time and produce extensive degradation of muscle proteins. The correlation between P.I. and protease activity was very high ($r = 0.93$).

## Frozen storage

The effect of frozen storage on the quality of cooked and uncooked crabs is shown in Figure 6. Uncooked crabs were rejectable on the basis of texture after any time in frozen storage. The quality deteriorated further if the crabs were stored on ice for 2 days following thawing; this was particularly evident in the P.I. data. Cooked crabs retained an acceptable level of texture throughout the 16 weeks of the trial, and P.I. values were significantly lower ($P<0.01$) than for uncooked crabs at all time points. Storage of cooked crabs on ice after frozen storage resulted in further slight decreases in textural and chemical quality.

The process of freezing may damage the internal organs, eg. by rupturing membranes through ice crystal formation. In uncooked crabs proteolysis would then proceed, as evidenced by the effect of the 2-d storage after thawing. Cooking should inactivate the enzymes and effect longer shelf life after thawing. However it must be stressed that correct cooking is necessary, especially for crabs which have just been removed from storage and may be still frozen at the time of cooking.

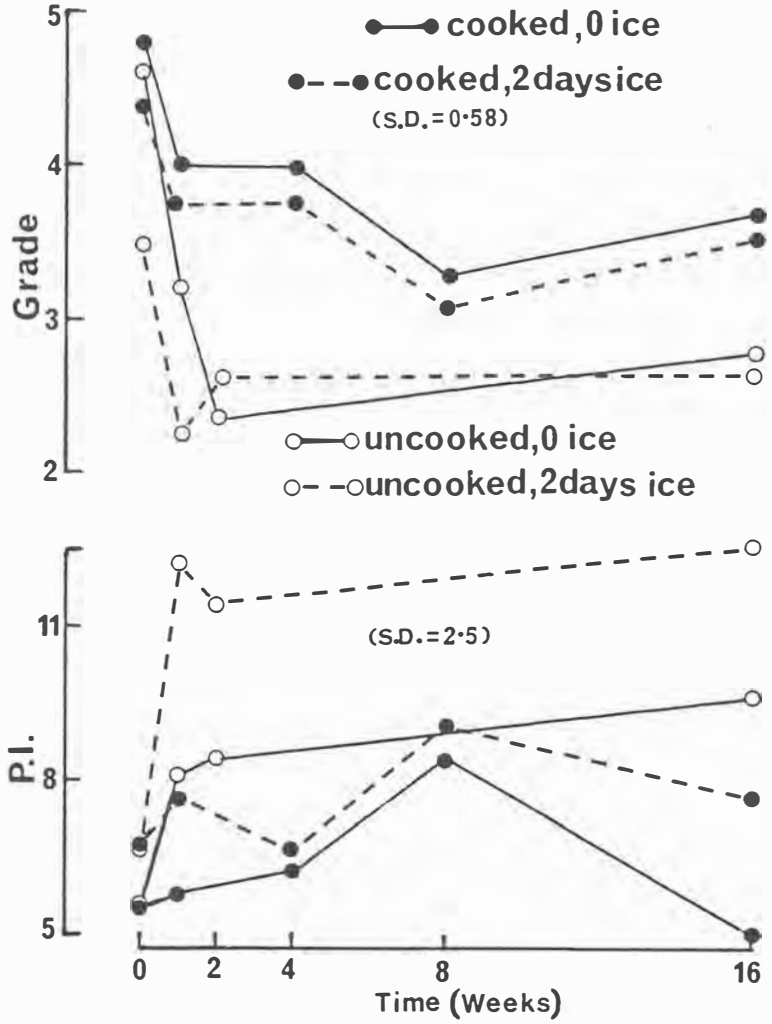


FIGURE 6. Grade and P.I. of cooked and uncooked crabs after frozen storage (each point represents the mean of 10 determinations)

## Ecdysis

Post-moult and pre-moult crabs included in brine/ice storage trials, freezing trials and pot *vs* trawl trials were compared with the total pool of crabs which had been subjected to the same treatment eg. uncooked post-moult crabs which had been stored in brine for 3 days were compared with all uncooked crabs stored in brine for 3 days. The results are presented in Table 6.

**TABLE 6**

**Data for post- and pre-moult crabs from various trials**

| | Grade | | P.I. | |
|---|---|---|---|---|
| condition | mean | averaged Z-score | mean | averaged Z-score |
| Cooked | | | | |
| post-moult (n=16) | 4.19 | -0.11 | 8.33 | -0.35 |
| pre-moult (n=3) | 4.67 | 0.52 | 5.77 | -0.24 |
| Uncooked | | | | |
| post-moult (n=14) | 2.20 | -1.89 | 10.55 | 0.98 |

The low Z-scores for cooked post-moult crabs indicate that these crabs show no significant differences in quality when compared with cooked inter-moult crabs stored under the same conditions in the trials. The findings for cooked pre-moult crabs were similar. Uncooked post-moult crabs however had much lower grades and higher P.I. values than uncooked inter-moult crabs stored under the same conditions. This means that the rate of deterioration in post-moult crabs is even greater than expected, i.e. the maximum storage time on ice before unacceptable limits are reached would be 1 or 2 days, rather than the 3 days for inter-moult crabs (Figure 5). The lower meat content in the cephalothorax of recently moulted crabs would allow for easier penetration of hepatopancreas enzymes into the flesh (Gillespie *et al.*, 1983). Uncooked post-moult crabs would therefore have greater proteolysis than corresponding uncooked inter-moult crabs, while effective cooking would stop any potential for accelerated degradation of the flesh of cooked post-moult crabs compared with cooked inter-moult crabs.

## Parasitic infection

Data for crabs parasitised with *Sacculina granifera*, and unparasitised crabs, all stored for 2 days on ice after cooking, were compared using the Student's t-test. The results are shown in Table 7.

**TABLE 7**

**Grade and P.I. of parasitised and healthy crabs**

| | Grade | P.I. |
|---|---|---|
| parasitised crabs (n=12) | 4.70 | 5.84 |
| healthy crabs (n=12) | 4.39 | 6.65 |
| t value | 1.52 (NS) | 1.06 (NS) |

(NS means that the t value is not significant)

The results show that there were no significant differences in quality between parasitised and unparasitised crabs under the handling conditions used.

**Feeding/starvation**

Table 8 below shows the mean protease activity in the hepatopancreas of crabs which have been fed or starved for 5 days.

**TABLE 8**

**Protease activity in the hepatopancreas of fed and starved crabs**

| Treatment | Protease activity (units/mL) |
|---|---|
| starved (n=5) | 29920[a] |
| fed (n=5) | 17360[b] |

(Mean values followed by a different letter are significantly different at the 1% level)

Crabs which had been starved contained significantly more protease activity than fed crabs. However the weight of hepatopancreas was reduced (20 g *vs* 30 g), and the juices were darker in colour. The net result is that both groups of crabs had an equivalent potential for proteolysis. The possible weight loss during starvation would argue against this practice during holding of live crabs.

**Histology**

A high-power (x 1000) micrograph of good quality muscle from a crab stored for 1 day on ice and stained with Heidenhain's iron haematoxylin and eosin (Figure 7) shows the ultrastructure of undigested muscle, with the sarcomeres displaying features such as Z line, A band and M line (Lester Davey, 1983). The lower power (x 400) view (Figure 8) shows the low connective tissue content of sand crab muscle as noted by Mykles and Skinner (1983). In Figure 9, normal muscle tissue (a), together with highly digested tissue (b) is illustrated (magnif. x 40). This tissue, which was stained with haematoxylin and eosin, was difficult to display as it tended to wash off the specimen during fixing procedures, and off the slide during dewaxing and staining. In tightly packed muscle, proteolytic enzymes act on the surface of the exposed meat, penetrating inwards. Figure 9 shows a decreasing level of digestion progressing along the muscle fibre (b to c). It is also likely that the enzymes move along areas of less dense tissue such as the sarcoplasm between fibres, causing a weakening in connective tissue. This would explain the tearing (d) seen between the partially digested fibre and the intact one adjacent. Further into the tissue this site is unaffected (e). A collagenolytic enzyme such as that found in the hepatopancreas of the fiddler crab, *Uca pugilator* (Eisen *et al.*, 1973), or the freshwater prawn, *Macrobrachium rosenbergii* (Nip *et al.*, 1985a) could be responsible for this gaping between fibres. Collagenase and elastase activities were detected in crab hepatopancreas extracts in this project (see Part 3). Figure 10 shows a micrograph of degraded muscle after 5 days' storage on ice (x 400, Heidenhain's iron haematoxylin and eosin). The A bands have not stained, and the Z lines are thicker and darker.

Figure 11 is a high-power view (x 1000) of undamaged tissue (1-day storage) stained with PTAH, showing the different staining pattern with this reagent. The A bands are evident but the Z lines and M lines are not clearly discernible. The connective tissue between muscle fibres is intact. Damaged tissue shown at the same power and with the same stain can be seen in Figure 12. There is incomplete staining of the A bands which towards the end of the fibre allows a view of the Z lines. Gaping between fibres has also occurred. Collagen is still present as the darker staining material lining the muscle fibre and amongst the connective tissue, but some weakening must have occurred to result in the gaping. In the later stages of mushiness seen in Figure 13, the A band has not stained while stain has collected in the region of the Z lines. The cytoskeletal latticework is still evident. The connective tissue originally present between fibres has also been removed. Rowland *et al.* (1982) found similar changes with mushiness in *M. rosenbergii*, and related it to hepatopancreas damage. They concluded that the darkening of the Z lines was due to their being no longer intact; the space left in the tissue traps stain, while gaping between myofibres indicates degradation of proteins in the sarcoplasm.

$Ca^{2+}$-activated proteases in pork (Dayton *et al.*, 1976 a and b) and crab (Mykles and Skinner, 1983) muscle were found to dissolve myofilaments and release α-actinin from Z lines, giving a pattern of breakdown similar to that observed in the sand crab. However the finding of low protease activity endogenous to crab muscle (see Part 3) indicates that the majority of proteolysis has origins in the digestive organs.

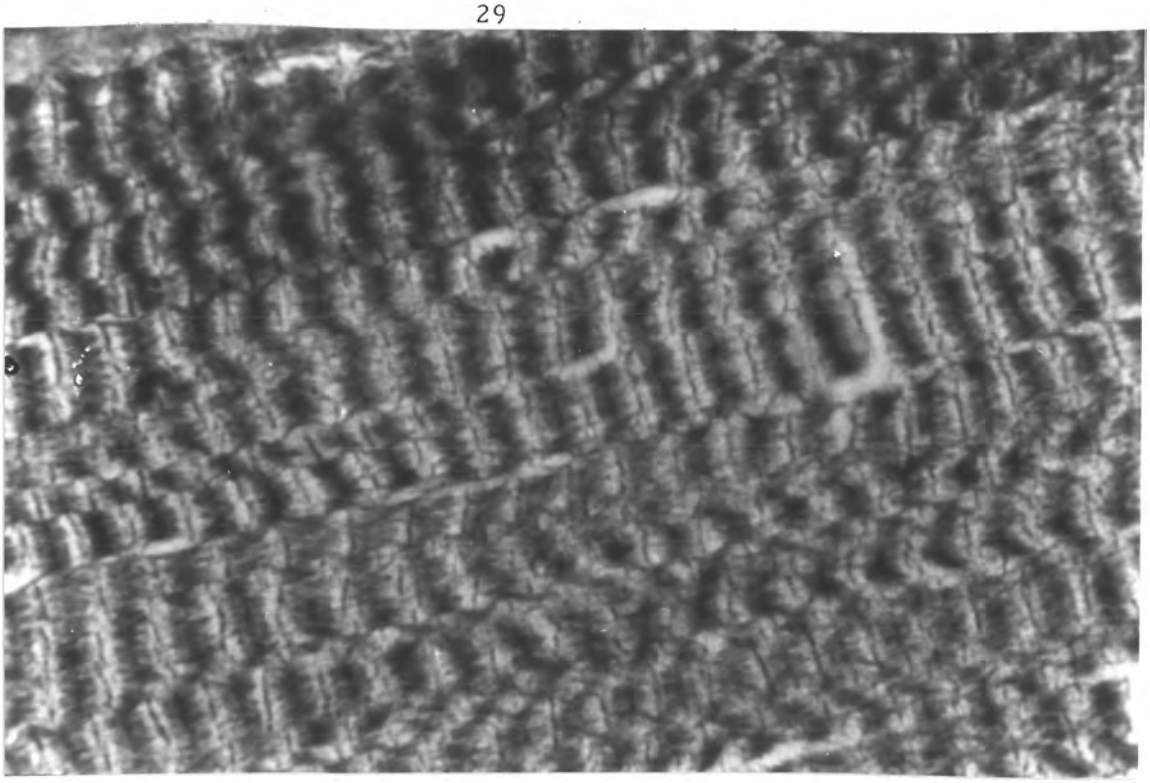

**FIGURE 7.** Micrograph of crab muscle after 1-d storage on ice (x 1000; Heidenhain's iron haematoxylin and eosin stain)

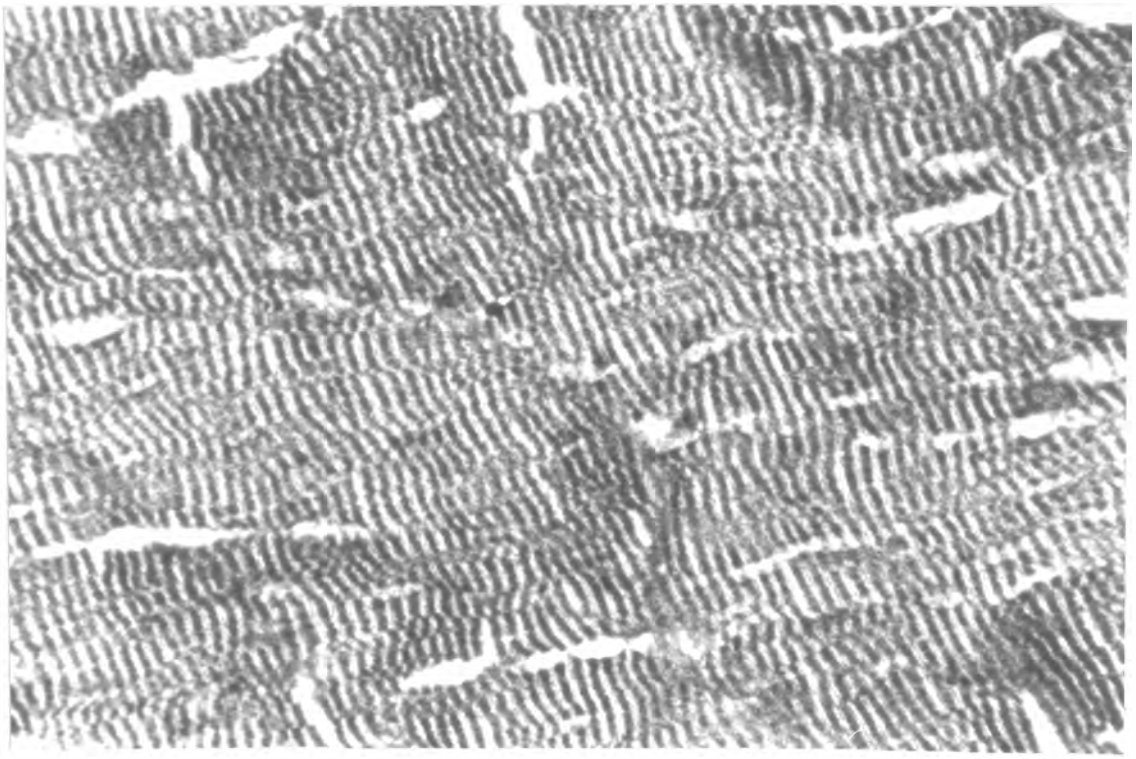

**FIGURE 8.** Micrograph of crab muscle after 1-d storage on ice (x 400; Heidenhain's iron haematoxylin and eosin stain)

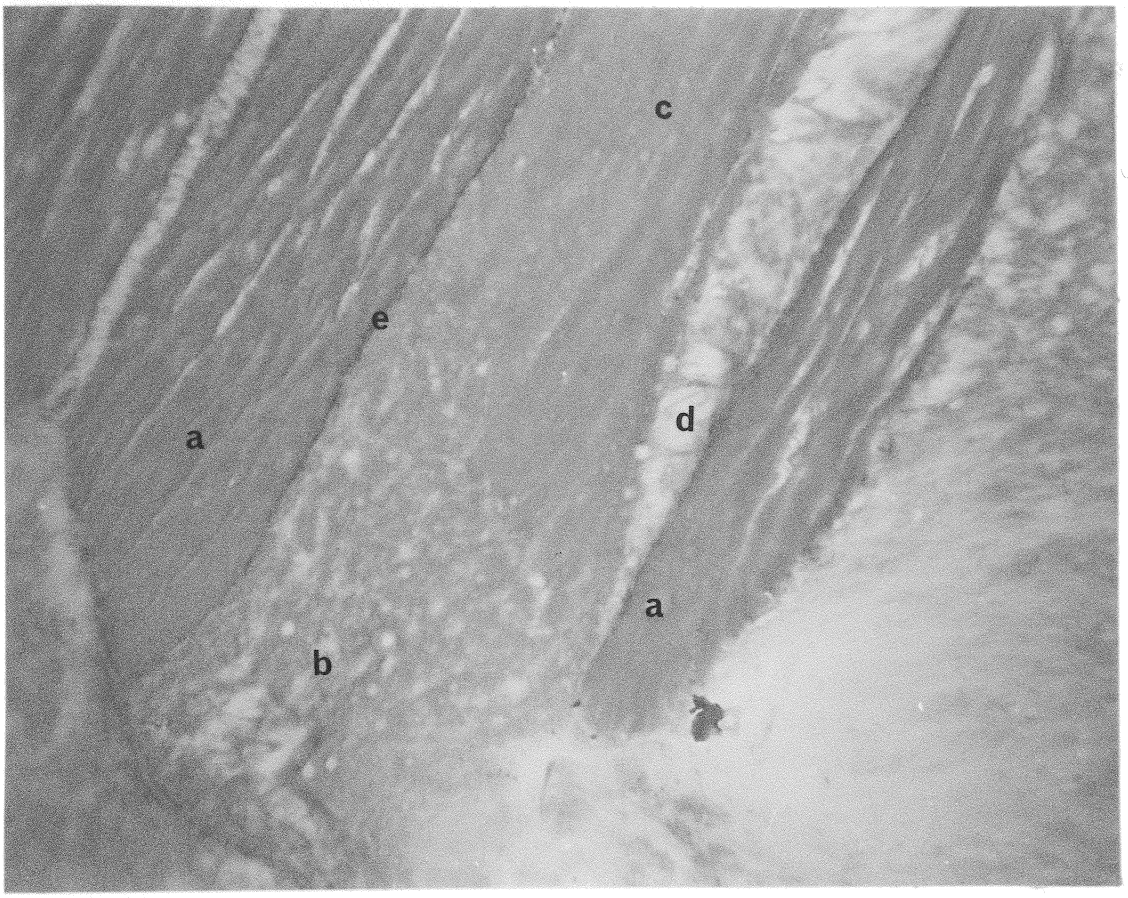


**FIGURE 9.** Micrograph of crab muscle after 5-d storage on ice (x 40; haematoxylin and eosin stain)

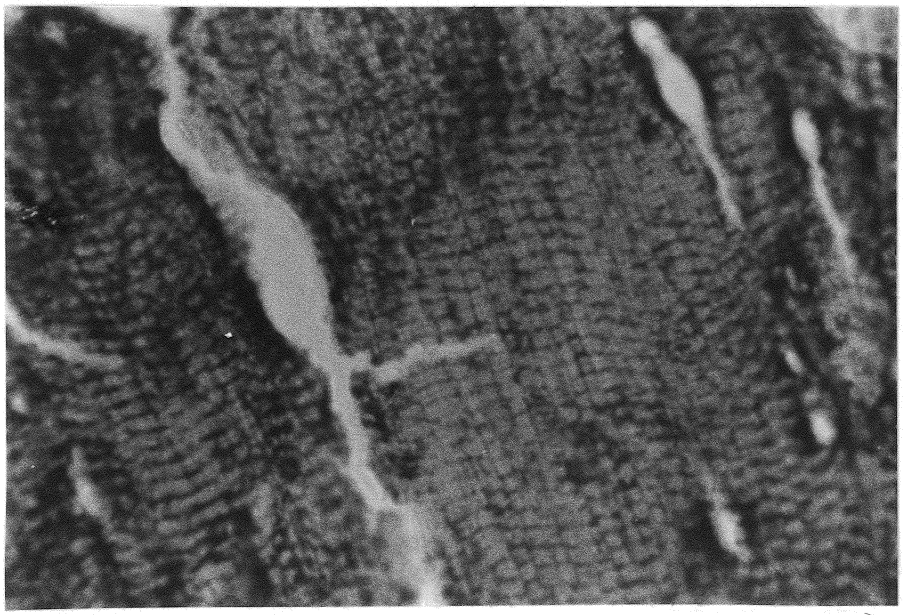

**FIGURE 10.** Micrograph of crab muscle after 5-d storage on ice (x 400; Heidenhain's iron haematoxylin and eosin stain)

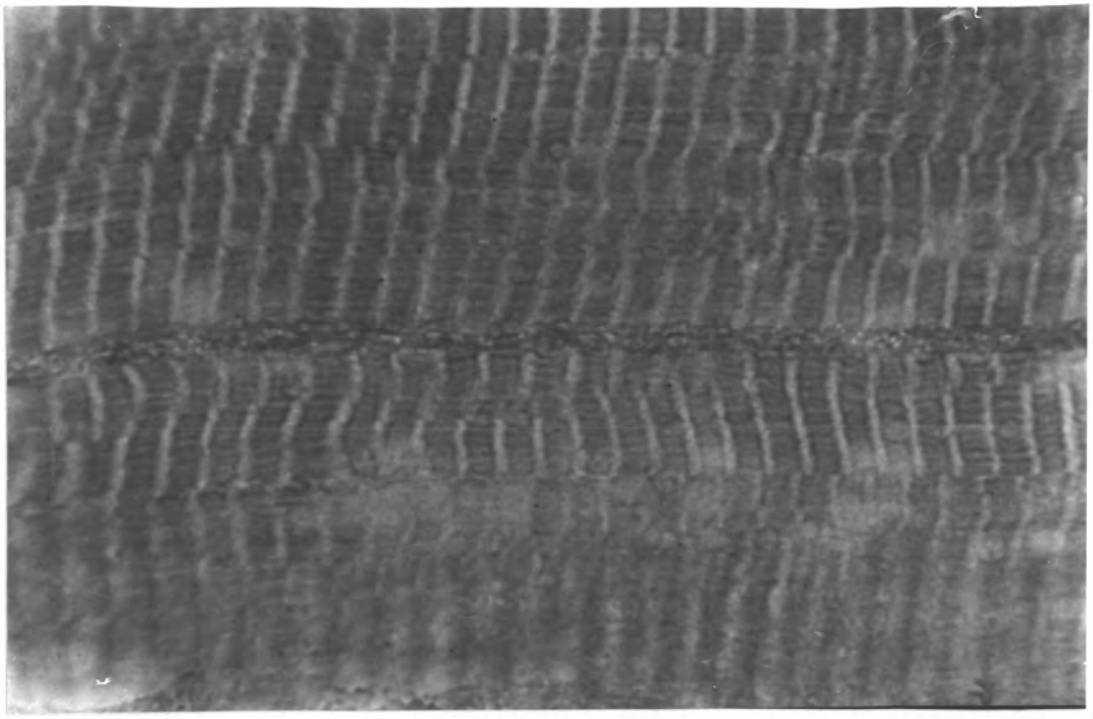

**FIGURE 11.** Micrograph of crab muscle after 1-d storage on ice (x 1000; PTAH stain)

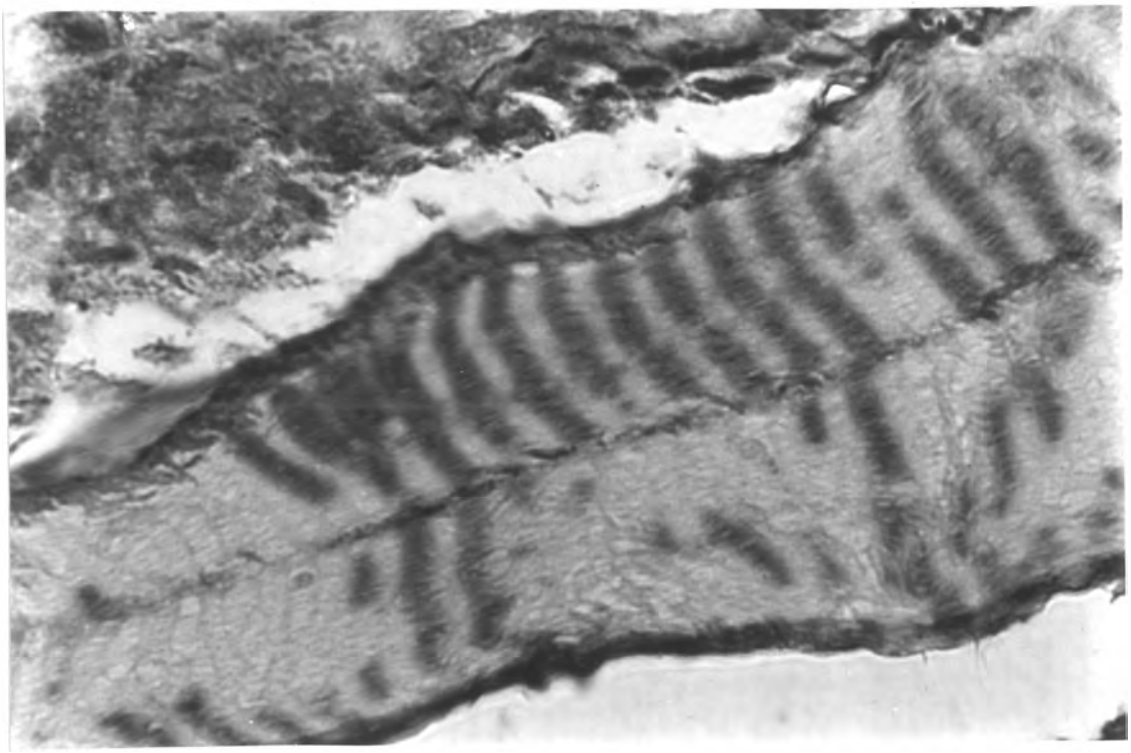

**FIGURE 12.** Micrograph of crab muscle after 5-d storage on ice (x 1000; PTAH stain)

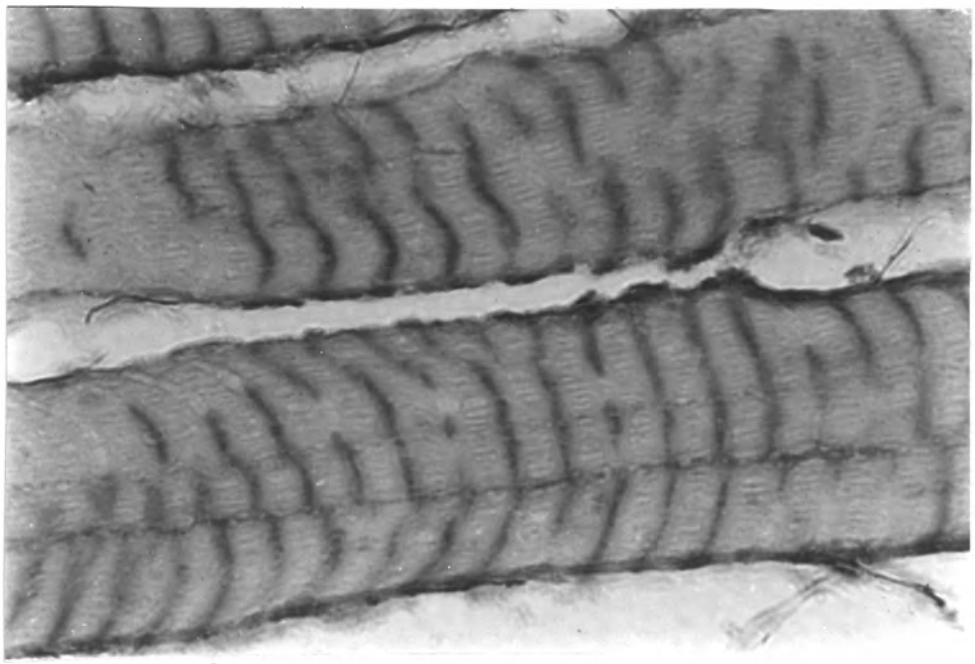

**FIGURE 13.** Micrograph of crab muscle after 5-d storage on ice (x 1000; PTAH stain)

# PART 3

# BIOCHEMICAL ASPECTS

Introduction

Materials and Methods

Results and Discussion

## INTRODUCTION

A large number of studies have investigated the role that proteolytic enzymes play in the spoilage of fish and crustacea. In a review on autolysis in fish, Mukundan *et al.* (1986) stressed that gut enzymes, and proteases in particular, are an integral part of the degradative process, and noted that the prevention of autolysis in fresh fish is not an easy task.

Most of the work on crustacea has centred on species such as Antarctic krill, *Euphausia superba*, various members of the lobster family, and the freshwater prawn, *Macrobrachium rosenbergii*. Because of the economic importance of these species, the maintenance of top quality is essential to the well-being of the industry.

Kawamura *et al.* (1981), in studies on Antarctic krill, found that cephalothoracic proteases were responsible for the high degree of autolysis in whole-bodied animals. In another study, contamination of fresh krill muscle with hepatopancreas or stomach was shown to greatly increase the amount of water-soluble protein, with subsequent loss of gel-forming ability of the muscle (Shibata and Ozaki, 1983). Ellingsen and Mohr (1979), when reporting a new process for the large-scale production of free amino acids from Antarctic krill, concluded that autolytic processes, and in particular proteolysis and lipolysis, are predominant during the first week of storage of fresh krill at chill temperatures. The efficiency of the digestive system in this crustacean has led to the finding by Mohr's group that the proteases may provide a new system for the effective enzymatic debridement of necrotic ulcerations (Hellgren *et al.*, 1986).

The specific proteases in krill have been characterised by several groups. Using homogenates of whole krill, Nishimura *et al.* (1983) found that carboxypeptidases A and B, aminopeptidase, trypsin and cathepsin A were present. Specific inhibitors and the use of gel filtration for molecular weight determinations aided the characterisation of protease types. Kimoto and others (1981, 1983) undertook enzyme characterisation studies in order to determine the proteolytic mechanisms acting during storage or food production. They found not only neutral and alkaline proteinases, including three serine enzymes, but two major acid proteinases maximally active at pH 3 using haemoglobin as substrate.

Olley *et al.* (1973) examined the hepatopancreatic proteases responsible for deterioration of the flesh of the rock lobster *Jasus lalandii*. Electrophoretic analysis revealed the presence of at least nine fractions with proteolytic activity.

In a comprehensive study of the digestive enzymes of the American lobster, *Homarus americanus*, Brockerhoff *et al.* (1970) separated seven proteolytic enzymes by ion exchange chromatography : two acid proteinases, carboxypeptidase A, and four neutral proteinases, including a highly acidic enzyme with trypsin activity. Earlier studies on crayfish were also aimed at determining the various proteolytic enzymes present in the gastric juices and hepatopancreas (de Villez, 1965; Kleine, 1967; Pfleiderer *et al.*, 1967).

A group of researchers working at the University of Hawaii have partially characterised the enzymes thought to be responsible for the autolysis in *M. rosenbergii*. Collagenase, together with trypsin and chymotrypsin-like enzymes, are primarily involved (Baranowski *et al.*, 1984; Nip *et al.*, 1985a). These enzymes are all thought to originate from the hepatopancreas and that endogenous flesh proteases play no part in the degradation (Angel *et al.*, 1985).

The degradative effects of gut proteases on muscle tissue have also been observed in some species of fish. The phenomenon of belly bursting in capelin, *Mallotus villosus*, was found to be most frequent in fish with high protease activity, although other factors such as post-mortem pH and the type of food ingested may also be important (Gildberg and Raa, 1980). A trypsin-like enzyme isolated from the digestive tract of capelin was found to hydrolyse myofibrillar components of fish muscle (Hjelmeland and Raa, 1980). These authors suggest that the belly bursting is caused by leaching of digestive enzymes such as trypsin, with subsequent hydrolysis of the surrounding belly tissues. Raa and Gildberg (1976) described the production of an acid silage from the viscera of cod, *Gadus morhua*, utilising the proteases and lipases present in the starting material. The soluble fraction from this silage is rich in protein and has possible uses as a food additive. Autolysis in silages prepared from whole fish is primarily due to gut enzymes which are distributed throughout the fish mass after the grinding process (Meinke and Mattil, 1973). Proteolytic enzymes from cod viscera have also been applied to deskinning of other seafoods such as squid (Raa, 1984).

In addition to the numerous reports of quality changes in fish and crustacea effected by gut enzymes, there is some evidence in the literature that endogenous muscle enzymes could also lead to problems. Konagaya (1985 a and b) has reported that the softening in the flesh of chum salmon, *Oncorhyncus keta*, caught during spawning migration is due to acid proteases present in the muscle. The level of proteolytic enzymes such as cathepsin D is several-fold higher in spawning salmon than that in fish caught during feeding migration. Cathepsins have also been partially purified from Chinook salmon, *Oncorhyncus tshawytscha* (Ting *et al.*, 1968). The enzymes thought to be responsible for autolysis in the muscle of carp, *Cyprinus carpio*, have been studied by Makinodan and co-workers (Makinodan *et al.*, 1979, 1982). Although a number of enzymes including cathepsin D and a neutral proteinase have been isolated, their possible role in the autolytic process is still unclear.

The mushiness of Pacific whiting, *Merluccius productus*, has often been associated with a myxosporidian parasite (Dassow *et al.*, 1970), and despite the detection of cathepsins B and C in the muscle, it is uncertain whether these enzymes' activities reflect an immunological response to the parasite or their natural occurrence. In addition, the parasite may be responsible for the high levels of acid protease activity (Erickson *et al.*, 1983).

In crustacean systems, some studies have looked at the proteases responsible for muscle atrophy during the pre-moult period of crabs and lobsters. Mykles and Skinner (1983, 1986) have found a number of calcium-dependent proteinases in the claw muscle which are active in myofibrillar protein turnover. These enzymes may play a role in both moulting and non-moulting animals, and possibly in autolytic digestion post-mortem.

The results in Part 2 of this report, on the technological aspects of storage and handling procedures, have strongly implicated the enzymes of the hepatopancreas in the onset of mushiness in the flesh of the sand crab. In particular, trials on spiking and cooking times and temperatures stress the need to fully inactivate these digestive enzymes before significant proteolysis occurs. The literature has many examples of the damage that may arise from the infiltration of such enzymes into the surrounding flesh. However, little is known of the biochemistry of proteases in crabs, and in particular the sand crab, *Portunus pelagicus*. Knowledge in this area was considered to be an integral part of the project, and the studies described herein have been undertaken with this objective.

## MATERIALS AND METHODS

### Chemicals

The following chemicals were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A. : L-alanine, p-nitroanilide azocasein, N-benzoyl-L-tyrosine ethyl ester (BTEE), α-chymotrypsin (type II), collagen (type I, insoluble), hydroxy-L-proline, L-leucine p-nitroanilide, piperazine, N-succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanilide, N-α-p-tosyl-L-arginine methyl ester (TAME) and trypsin (type XII - S). Haemoglobin and Folin-Ciocalteu's phenol reagent were from Merck, Darmstadt, West Germany; furylacryloyl-L-alanyl-L-lysine (FAAL) and furylacryloyl-L-phenylalanyl-L-phenylalanine (FAPP) from Calbiochem, La Jolla, Ca., U.S.A.; chloramine T (technical grade) from Ajax Chemicals, Sydney, Australia; and 4-dimethylaminobenzaldehyde from BDH Chemicals Ltd, Poole, England. DEAE-Sepharose CL-6B, PBE 94 resin, Polybuffer 74 and Pharmalyte 2.5-5.0 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other laboratory chemicals were of analytical grade or the highest purity available.

### Enzyme extract

The hepatopancreas from fresh sand crabs was excised and contaminating viscera removed. The organ was then homogenised in a Waring blendor for 1 min at high speed, and the homogenate centrifuged at 48000 *g* and 5°C for 30 min. The supernatant was used immediately or stored frozen at -20°C.

### Enzyme assays

All enzyme assays were performed at 37°C. Except where otherwise stated, one unit of enzyme activity is defined as the amount of enzyme required to produce a change in absorbance of 0.01 per minute at the appropriate wavelength.

Trypsin. The standard assay used tosyl-L-arginine methyl ester (TAME) as substrate at 1 mM final concentration and 0.1 M Tris-HCl buffer, pH 7.5 containing 20 mM $CaCl_2$, according to Walsh (1970).

Chymotrypsin. Benzoyl-L-tyrosine ethyl ester (BTEE, 1 mM final concentration) was used as substrate in chymotrypsin assays employing 0.1 M Tris-HCl buffer, pH 7.8 containing 20 mM $CaCl_2$, according to Walsh and Wilcox (1970).

Leucine aminopeptidase. To the assay cuvette was added 2 mL of 0.1 M Tris-HCl buffer, pH 7.50, and 50 µL of 118 mM L-leucine p-nitroanilide, prepared in methanol. After equilibration in the spectrophotometer for several minutes, the reaction was initiated by the addition of an aliquot (usually 100 µL) of enzyme. The rate of change in absorbance at 405 nm was measured for several minutes.

α-Aminoacyl-peptide hydrolase. The assay conditions used follow those for leucine aminopeptidase, except that L-alanine p-nitroanilide was used as substrate at a final concentration of 1.5 mM. A 200 µL aliquot of enzyme was routinely used to start the reaction.

Carboxypeptidase A. The assay was based on the method of Riordan and Holmquist (1984) : 1 mM furylacryloyl-L-phenylalanyl-L-phenylanine (FAPP) substrate was dissolved in assay buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.5), and the decrease in absorbance at 350 mm followed after the addition of 50 µL of enzyme to the cuvette containing 0.6 mL of substrate.

Carboxypeptidase N. Carboxypeptidase N activity was measured with furylacryloyl-L-alanyl-L-lysine (FAAL, 0.5 mM) as substrate, using the method of Skidgel and Erdos (1984). A wavelength of 336 nm was used to follow the reaction.

Elastase. The measurement of elastase activity employed a modification of the Geiger method (1984). The substrate, succinyl-L-alanyl-L-alanyl-L-alanine (1 mM), was equilibrated at 37°C in 2.5 mL of 0.2 M ethanolamine buffer, pH 7.8, containing 0.05% w/v Triton X-100. Enzyme solution (200 µL) was then added to start the reaction, which was followed at 405 nm.

Collagenase. Collagenase determinations were based on the measurement of hydroxyproline release from collagen during a 4-h incubation at 37°C. A 0.2% collagen solution was prepared by dissolving 200 mg collagen in 2N NaOH, adjusting the pH to 7.5 with HCl, and diluting to 100 mL with 0.1 M Tris-HCl, 20 mM $CaCl_2$, pH 7.5. Any undissolved collagen was removed by low-speed centrifugation for 10 minutes. The incubation mixture contained 2.0 mL of substrate and 0.5 mL of enzyme solution. The hydroxyproline released during the reaction was measured using the method of Woessner (1961). One unit of activity produces a change in absorbance at 557 nm of 0.001 under the assay conditions.

Acid protease. The method of Lenney (1975) was modified to measure acid protease activity. To 0.4 mL of 1% acid-denatured haemoglobin in 0.05 M citrate buffer, pH 3.4, was added 0.25 mL of sample. Following incubation at 37°C for 3 h, the reaction was stopped by the addition of 0.65 mL of 24% trichloroacetic acid. The supernatant obtained after centrifugation at 1600 *g* for 10 min was read at 280 nm. One unit of acid protease activity produces an increase in $A_{280}$ of 0.01 per h at 37°C.

General proteolytic activity. Details of the general protease assay using azocasein as substrate are given in Part 2 of this report.

Proteolytic activity against crab flesh. Raw crab flesh from the main swimming muscle was thoroughly homogenised in a Waring blendor. To 10 g of flesh was added 3 mL of concentrated enzyme (ion exchange chromatography, peaks 1-7), and after mixing, each flask was incubated at 5°C for 2 days. TCA-soluble material was obtained by filtration after

11.25 g of the mixture was homogenised with 30 mL of 16.5% trichloroacetic acid. Analysis with Folin-Ciocalteu's reagent was performed on 200 µL of the filtrate. One unit of activity releases one µg of tyrosine equivalents per g of flesh in 48 h at 5°C. Buffer (0.1 M Tris-HCl, pH 7.5) was included as a control.

In another experiment, the effects of commercial trypsin and collagenase preparations, and hepatopancreas homogenate, on the release of peptide material from homogenised crab flesh were studied. To 20 g of flesh, 1 g of one of the following solutions was added : trypsin (0.3 mg/mL); collagenase (0.05 mg/mL); hepatopancreas extract; or water in the presence and absence of 0.8% sodium azide. Each mixture was stored at 5°C, and samples removed for P.I. determinations on days 0, 1, 4 and 7. Total viable counts (TVC) were determined on control samples on days 1 and 7.

Proteolytic activity of crab muscle and hepatopancreas. Crab muscle was excised and all contaminating tissues, including discoloured muscle, was thoroughly removed from the sample. The tissue was homogenised in a blendor with three parts of water, and then centrifuged for 30 min at 48000 *g*. A 1-mL sample of supernatant was used in the azocasein enzyme assay. $CaCl_2$ (5 mM), or EDTA (5 mM) was added in some assays to test their effects on activity.

For the measurement of activity in hepatopancreas, a 50-fold dilution of extract in water was prepared, and 200 µL of this used in the standard protease assay.

## Ion exchange chromatography

The enzyme extract was dialysed against column starting buffer, 0.02 M Tris-HCl, pH 7.0, and then centrifuged at 48000 *g* for 30 min to remove any precipitate. The dialysed extract, 10 mL in volume, was loaded onto a column (dimensions 30 cm x 1.6 cm) of DEAE-Sepharose CL-6B equilibrated with starting buffer. Protein was eluted with a 0-0.8 M NaCl gradient in 0.02 M Tris-HCl buffer, pH 7.0, using a flow rate of 30 mL per hour. Total gradient volume was 600 mL, and fraction size 5 mL. Following completion of the gradient, a residual peak of activity was eluted with 0.8 M or 1.0 M NaCl in starting buffer. All chromatographic procedures were performed at 5°C. Salt concentrations of column fractions were determined with the aid of a TPS conductivity meter, and activity measurements with the standard azocasein assay.

For one purification, the digestive juices which collected overnight from storage of crabs at 5°C were used. The juices were pooled and centrifuged (48000 *g*, 30 min), and 5.5 mL of supernatant loaded on the DEAE-Sepharose column. All other chromatographic procedures were unchanged.

## Pooling of enzyme fractions

Following the measurement of enzyme activity, fractions from ion exchange chromatography were pooled according to the activity profile. The resultant seven peaks, numbered 1 to 7 in order of elution from the column, were concentrated to 10 mL using an Amicon ultrafiltration unit and PM-10 ultrafiltration membrane. The concentrated peaks were dialysed

against 0.1 M Tris-HCl buffer, pH 7.5, and stored at 5°C for use in subsequent biochemical investigations.

## pH-activity profile

The enzymic activity of crude hepatopancreatic extract in the pH range 2 to 10 was tested using 0.3% casein substrate in an hour-long incubation at 37°C. The following buffers, all 0.1 M in concentration were used : pH 2, citric acid-HCl; pH 2.5-6.0, citrate; pH 6.5 and 7.0, phosphate; pH 7.5-9, Tris-HCl; pH 10.0, carbonate. Substrate was prepared by slowly dissolving casein in hot 0.02 N HCl and diluting to give a final concentration of 1.0%. The assay method was as follows : to 1 mL of substrate was added 0.2 to 0.8 mL of enzyme, followed by buffer to give 3.0 mL final volume. After 1 h the reaction was stopped by the addition of 3 mL of 10% TCA, and the mixture filtered through Whatman's No. 1 filter paper. The absorbance of the filtrate at 280 nm was then determined. One assay unit is defined as an increase in absorbance of 1.0 per h under the assay conditions.

## Temperature stability studies

Temperature stability was determined by preincubating enzyme extract or column peaks at temperatures of 50°, 60°, 65° and 70°C for time periods of up to 1 h prior to activity measurements using the azocasein assay. For accuracy, tubes were pre-heated before addition of enzyme in the preincubation step, and immediately placed on ice upon removal from the water bath. A 50-fold dilution of enzyme extract was used in these experiments, with 200 µL needed for the assay. Aliquots of 100 µL were adequate for studies on the column-purified enzyme peaks.

In experiments to determine the enzymic activity of hepatopancreas proteases at cold room temperatures, the standard azocasein assay conditions were varied so that the incubation was carried out at 2° and 6°C, as well as at 20° and 37°C.

## Chromatofocusing of ion exchange chromatography peak 6

The isoelectric point of the trypsin-like enzyme of peak 6 from DEAE-Sepharose chromatography was determined on a chromatofocusing column employing PBE 94 exchange resin, piperazine-HCl, 25 mM, pH 5.50 as starting buffer, and a ten-fold dilution of Polybuffer 74, pH 3.50, as eluting buffer. The enzyme solution was dialysed against starting buffer and a 5 mL sample loaded onto the ion exchange column (dimensions 17 cm x 1.6 cm) which was equilibrated with starting buffer. Eluting buffer, total volume 330 mL, was then used to establish the pH gradient and 10-min fractions, approximately 5 mL each in volume, were collected. At the completion of the gradient, 1 M NaCl was used to elute protein still bound to the column. Measurements of pH, absorbance at 280 nm and trypsin activity were then made on column fractions.

## RESULTS AND DISCUSSION

### pH-activity profile

The pH-activity profile of a crude extract of hepatopancreas is shown in Figure 14. Maximal activity occurs around neutral pH; however significant activity is still present in the alkaline range, and a small but discernable peak of acid protease activity also occurs at pH 3.5. Based on these findings, routine assaying employed a buffer system at pH 7.5 and for measurement of enzymes active in the acid range a buffer at pH 3.4 was used.

The pH of flesh in the sand crab is normally around pH 7.5 to 8.0 and rises during storage. The neutral and alkaline proteases would therefore be expected to play a major role in any degradative processes in the flesh. In mammalian systems the pancreatic enzymes trypsin and chymotrypsin are most active in this pH range. Acid proteases such as cathepsins, while having an important function in the acid environment of the stomach and other digestive organs, would not appear to be significant in flesh digestion. The pH of hepatopancreatic extract was found to be 6.2.

### Ion exchange chromatography

Separation of the proteolytic enzymes present in crude extracts of hepatopancreas was effected by chromatography on the anion exchange resin, DEAE-Sepharose CL-6B. Earlier attempts using gel filtration on Sephadex G-150, ACA 44 and ACA 54 in 0.1 M Tris-HCl buffer at pH 7.5 proved unsuccessful, with most of the activity eluting in one large peak. With the ion exchange resin however, good separation of activity peaks was obtained. A typical elution profile is shown in Figure 15. Seven major peaks of activity were eluted at salt concentrations varying from 0.15 M (peak 1) to 0.8 M (peak 7). The elution volumes of the pooled peaks and their absorbances at 280 nm are shown in Table 9.

**TABLE 9**

**Pooled peaks from DEAE-Sepharose chromatography**

| Activity peak | Elution volume (mL) | $A_{280}$ |
|---|---|---|
| 1 | 130-160 | 0.70 |
| 2 | 161-205 | 1.29 |
| 3 | 215-295 | 1.89 |
| 4 | 340-380 | 0.75 |
| 5 | 390-450 | 0.08 |
| 6 | 465-540 | 0.06 |
| 7 | 575-670 | 0.03 |

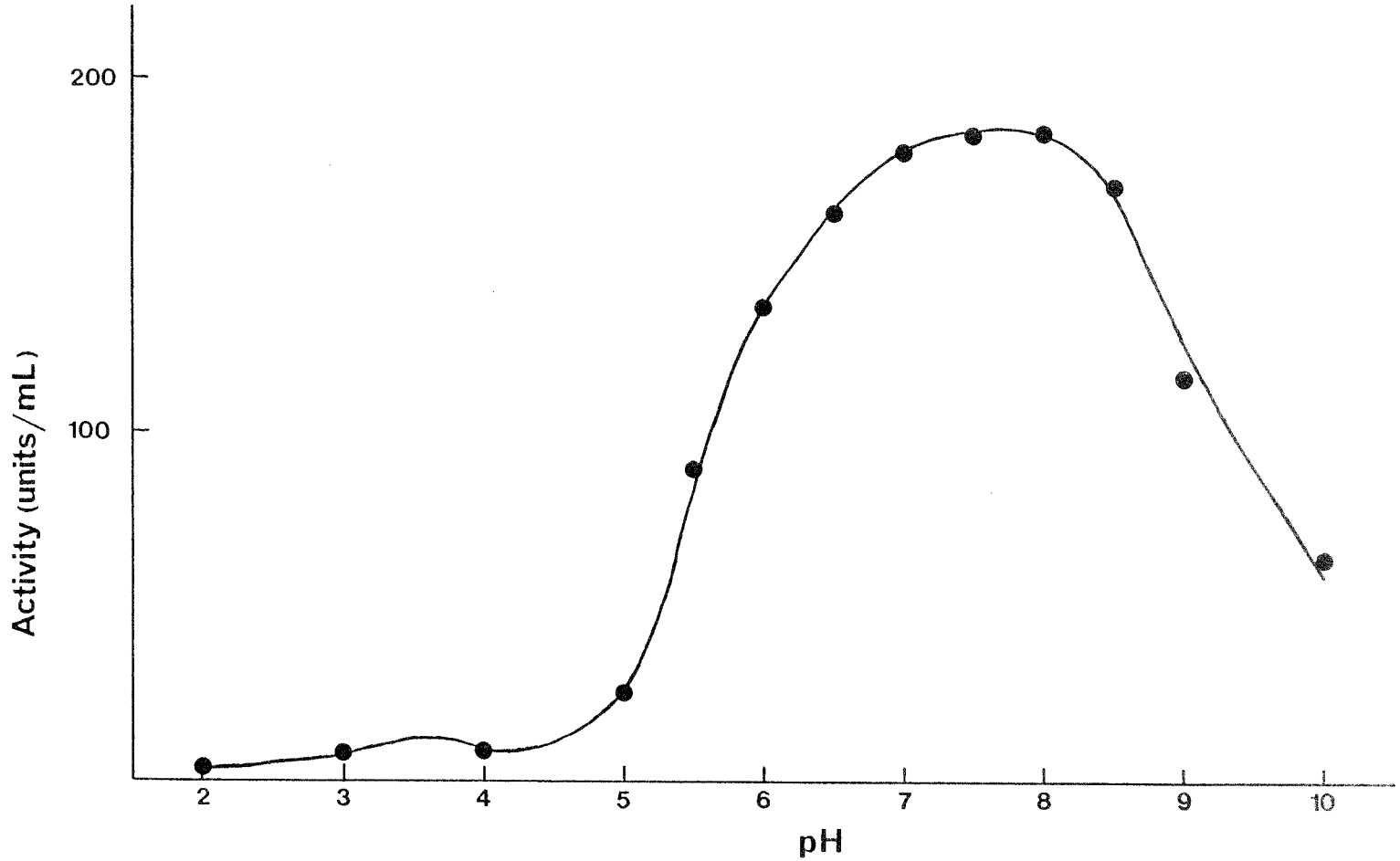


Figure 14. pH-activity profile of crude hepatopancreas extract

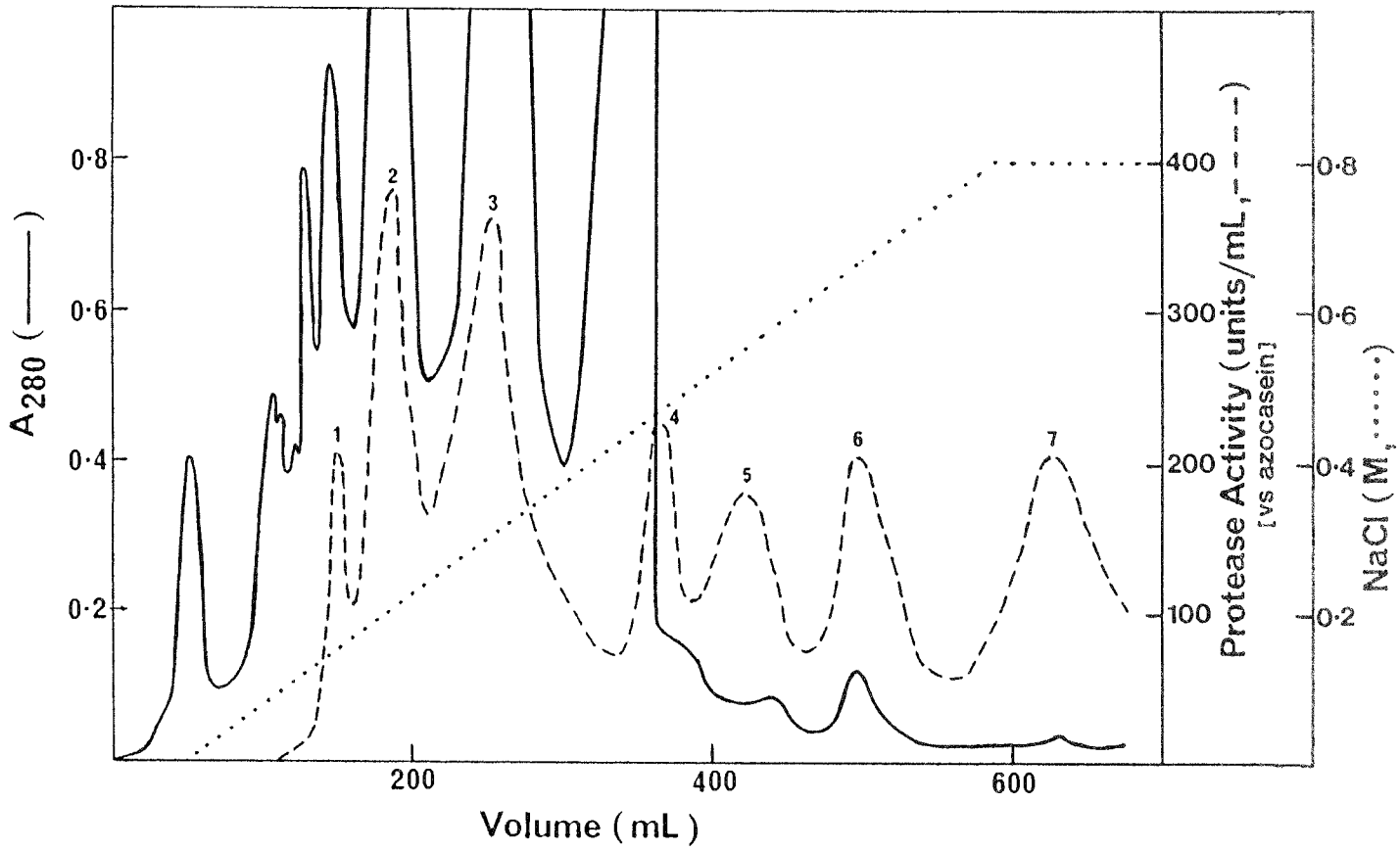


Figure 15. DEAE-Sepharose CL-6B chromatography of hepatopancreas extract at pH 7.0

It is noteworthy that peaks 6 and 7 required high salt concentrations to effect their elution, indicating that the enzymes have a highly acidic character. The activity in peak 6 was obviously associated with a protein peak, as indicated by the 280 nm absorbance profile. In contrast, peak 7 has a very low absorbance associated with its very high protease activity. Several indications are present however, which show that a protein is involved in this fraction; ultrafiltration resulted in an increase in $A_{280}$ from 0.03 to 0.20, and an ultraviolet absorption spectrum of the concentrate revealed the typical profile, with a maximum absorbance at 280 nm.

The pooled activity peaks were concentrated by ultrafiltration for further use in enzyme characterisation studies. Some losses were incurred during this step, but the majority of the enzyme activity was retained in the concentrate. Because of the possibility of autolysis during storage the subsequent studies on a particular enzyme preparation were performed as quickly as possible.

In later preparations the chromatography step was performed with a longer bed of resin (38 cm cf. 30 cm) and smaller fraction size (3 mL instead of 5 mL). These changes resulted in a slightly different activity profile (Figure 16). Peak 2 was now partially separated into two parts (2A and 2B), and peak 5 surprisingly eluted closer to peak 6. The protein profile was also altered; a number of peaks eluting early in the profile were absent, as was a large peak which preceded activity peak 4. However the number and relative activities of the enzyme peaks remain unchanged, suggesting that these proteins are not associated with any major proteolytic fractions. The differences may reflect changes in hepatopancreas preparations, eg. the inclusion in earlier extracts of some other digestive organs, or seasonal changes in protein composition. The preparation used to give the results of Figure 16 was from crabs caught in July, the "off" season, when it could be expected that lower activity would result in a reduction or loss of some proteins related to metabolic function.

Ion exchange chromatography of digestive juices revealed no major differences in the protein elution profile, or the activity profile, compared with hepatopancreas extract. The levels of enzyme activity were also similar.

## Temperature stability studies

Enzyme extract. The stability of extract proteases to incubation temperatures of 50°, 60° and 70°C is shown in Figure 17. Treatment for 1 h at 50°C resulted in only a 5% reduction in activity, and at 60°C only 40% activity was lost over the same time period. Incubation at 70°C resulted in much more rapid loss of activity. However, almost 40% of the original activity was still present after 5 minutes, and 15% after 20 minutes. The retention of this amount of activity is critical when it is remembered that in normal cooking practice the internal temperature of the crab only reaches 70°C towards the end of the cooking process (see Part 2 of this report).

The activity of extract proteases at different temperatures is shown in Table 10.

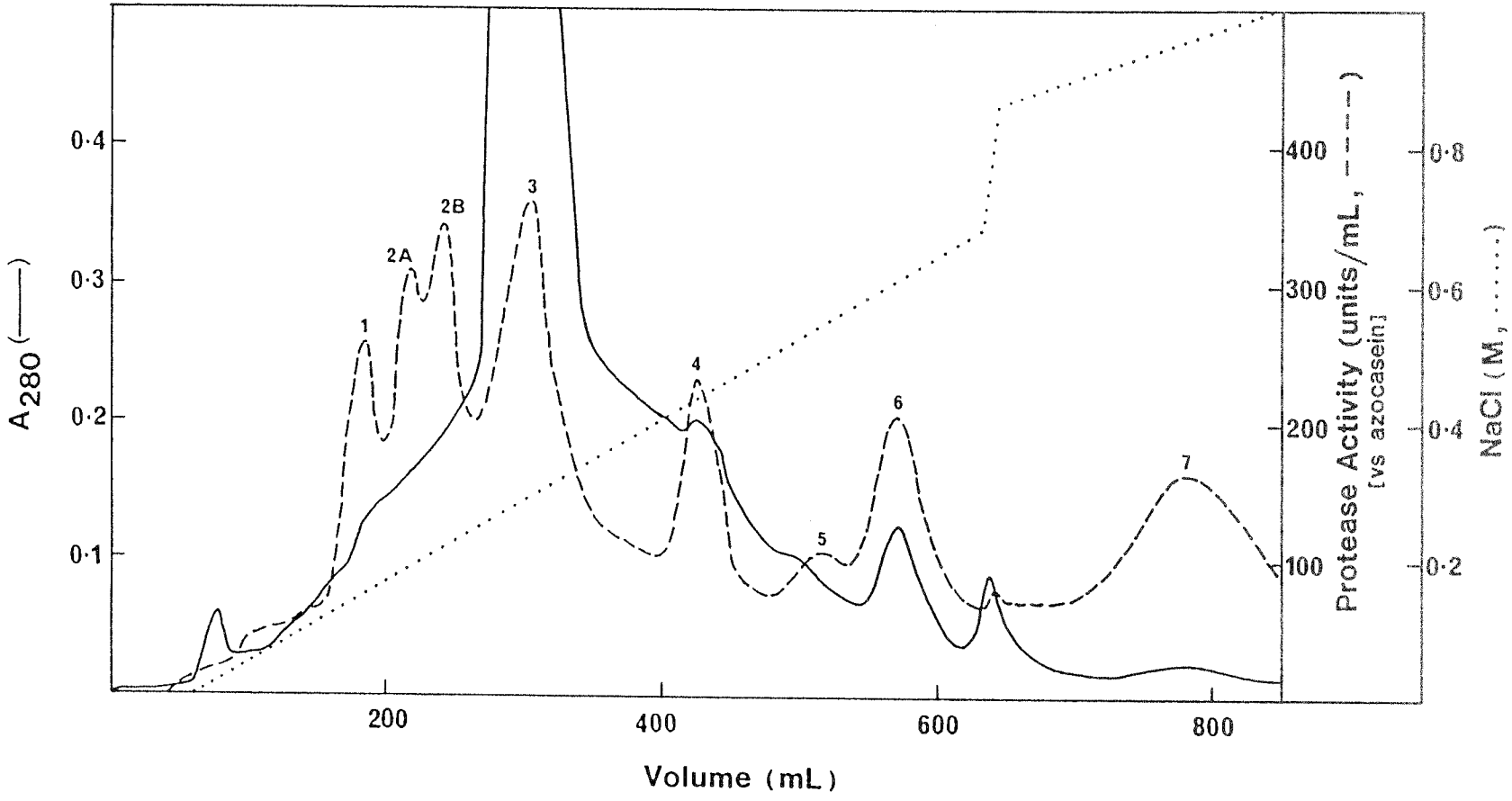


Figure 16. DEAE-Sepharose CL-6B chromatography of hepatopancreas extract at pH 7.0 (modified elution conditions)

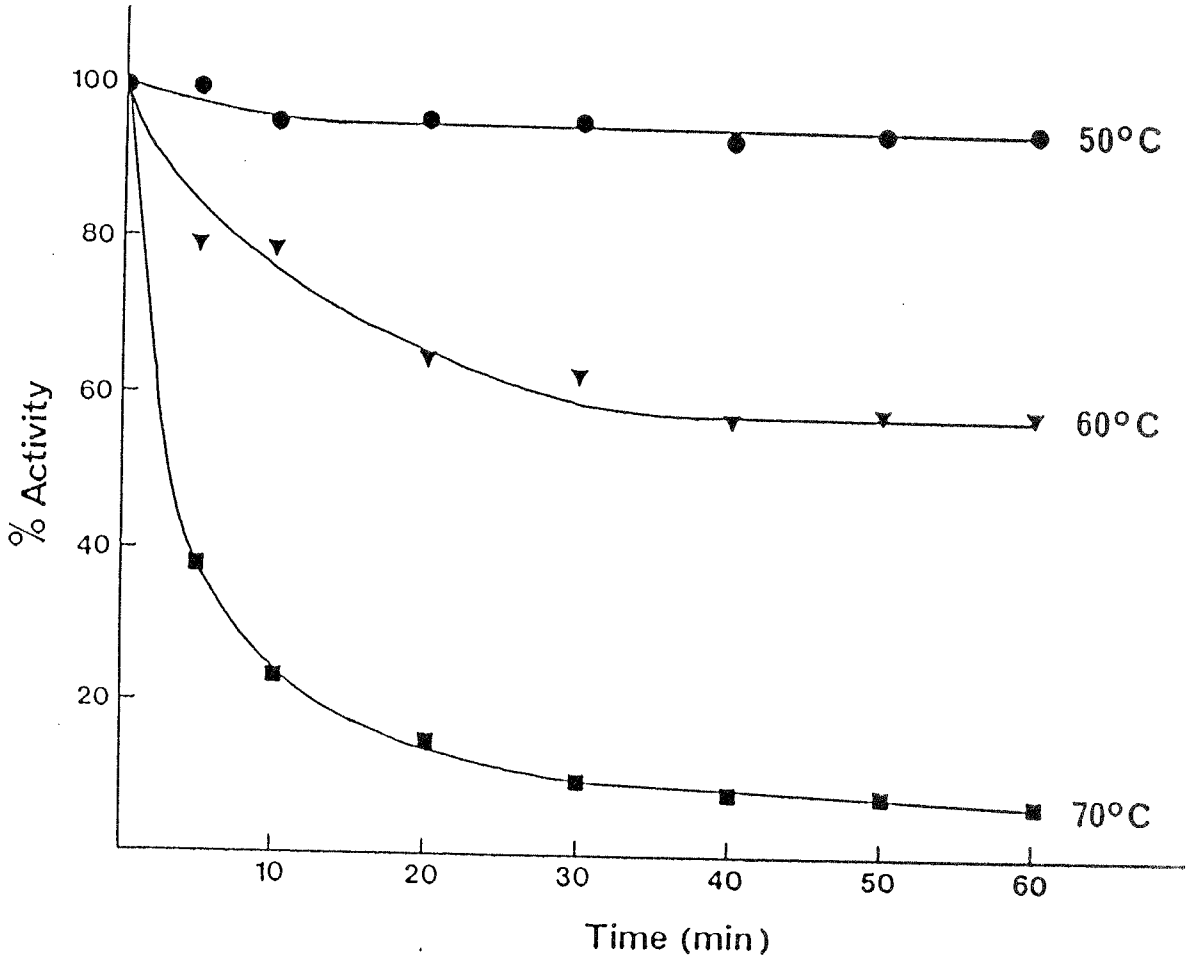


Figure 17. Thermal stability of hepatopancreas extract

**TABLE 10**

**Activity of hepatopancreas extract at various temperatures**

| Temperature (°C) | % Activity |
|---|---|
| 37 | 100 |
| 20 | 47.1 |
| 6 | 27.6 |
| 2 | 15.4 |

The results indicate that appreciable activity is present at 6° and 2°C, temperatures approximating cold room or ice conditions.

Pooled activity peaks. The existence of one or more heat-stable proteases was investigated by studying the heat stability of concentrated peaks from DEAE-Sepharose chromatography. Samples of peaks 1 to 7 (Figure 16) were incubated for 15 and 30 min at 60°, 65° and 70°C. The results are shown in Table 11.

**TABLE 11**

**Heat stability of DEAE-Sepharose chromatography peaks**

| | % Original activity | | | | |
|---|---|---|---|---|---|
| Peak | 60°C, 15 min | 60°C, 30 min | 65°C, 15 min | 65°C, 30 min | 70°C, 15 min |
| 1 | 17.0 | 15.3 | 7.0 | 6.1 | 4.7 |
| 2A | 22.9 | 19.1 | 9.5 | 7.4 | 2.1 |
| 2B | 42.6 | 31.4 | 18.1 | 13.3 | 4.7 |
| 3 | 43.6 | 37.0 | 22.6 | 19.3 | 9.1 |
| 4 | 29.2 | 24.9 | 13.7 | 12.2 | 2.8 |
| 5 | 41.9 | 32.4 | 8.1 | 5.6 | 4.1 |
| 6 | 80.9 | 77.9 | 17.7 | 8.5 | 6.0 |
| 7 | 59.7 | 40.3 | 8.8 | 4.4 | 7.8 |

For all peaks, greater than 90% of the activity was lost during the 70°, 15 min heat treatment. This contrasts with the results of extract incubations (see Figure 17). The increased stability shown by crude extract proteases probably arises from the protection against denaturation afforded by the presence of high concentration of protein. The results for treatments at 60°C show that several enzymes retain significant activity, even after 30 minutes. Of particular interest is peak 6, which still had 78% of its original activity after 30 minutes. However at 65°C, only 18% activity remained after 15 minutes. The inactivation temperature for this enzyme would appear to lie between 60° and 65°C. The importance of this enzyme will be discussed in a later section in relation to its activity towards crab flesh.

## Enzyme characterisation

The identification of specific enzymes was carried out using artificial substrates characteristic of the various protease enzymes described in the literature. Each activity peak from DEAE-Sepharose chromatography was tested for all of the enzyme activities described in the Materials and Methods section. The enzymes found to be present are listed in Table 12.

**TABLE 12**

**Proteolytic enzyme activities of activity peaks from DEAE-Sepharose chromatography**

| Activity peak | Total activity *vs* azocasein (units) | Specific enzymes | |
|---|---|---|---|
| | | Enzyme | Activity (units/mL) |
| 1 | 4120 | acid protease | 55 |
| | | amino acid arylamidase | 144 |
| 2 | 10760 | acid protease | 90 |
| | | elastase | 310 |
| | | collagenase | 158 |
| 3 | 17200 | acid protease | 68 |
| | | chymotrypsin | 110 |
| | | carboxypeptidase A | 166 |
| | | carboxypeptidase N | 1140 |
| | | leucine aminopeptidase | 14 |
| 4 | 7620 | trypsin | 184 |
| 5 | 5600 | (no specific enzyme activities detected) | |
| 6 | 6520 | trypsin | 2780 |
| 7 | 6240 | trypsin | 397 |

The number of enzymes detected is indicative of the role the hepatopancreas plays in the digestion process, and although the list is not exhaustive, representative enzymes from the major groupings of proteases, i.e. exopeptidases, endopeptidases, acid proteases and connective tissue proteases were identified in the peaks. Peak 3, the fraction which had the greatest activity towards the protein substrate azocasein, also contained the largest number of specific enzymes. Acid proteases were identified in peaks 1, 2 and 3, and while time did not permit further characterisation, it would be expected that the major classes of cathepsins are present in hepatopancreas preparations. As

mentioned earlier these enzymes probably are not involved in the degradative processes in the flesh.

Enzymes with tryptic activity were detected in peaks 4, 6 and 7. The good separation of these activity peaks on the column (see Figure 15 and Table 9) suggests that cross-contamination of enzymes is not responsible for the presence of three separate activities. More likely is that autolysis products which are still active towards the substrate TAME, and possess different ionic charges at pH 7.0, are eluted at different times from the column. Reports of the existence of autolysed derivatives of trypsin appear in the literature (Maroux and Desnuelle, 1969; Schroeder and Shaw, 1968). A third explanation concerns the relative activities of these three peaks towards azocasein and TAME (Table 12). While peak 6 has very high activity towards TAME, the other peaks have less than 15% of that level towards the artificial substrate. In contrast, all peaks have similar activities towards azocasein, a general protease substrate (7620, 6520 and 6240 units for peaks 4, 6 and 7, respectively). The tryptic activity may therefore be secondary to the major functional activity, as yet unidentified, in these peaks. Peak 6, however, may contain only a homogeneous solution of trypsin, for its very high activity against TAME is coupled with a low $A_{280}$ (Table 9). The resulting high specific activity is indicative of, and a prerequisite for, a pure enzyme preparation.

**Proteolytic activity against crab flesh**

The activity of each column peak towards raw homogenised crab flesh is shown in Table 13.

**TABLE 13**

**Activity of DEAE-Sepharose column peaks towards raw crab flesh**

| Activity peak | Total activity *vs* azocasein (units) | Total activity *vs* crab flesh (units) |
|---|---|---|
| 1 | 4120 | 510 |
| 2 | 10760 | 4230 |
| 3 | 17200 | 4110 |
| 4 | 7620 | 900 |
| 5 | 5600 | 690 |
| 6 | 6520 | 2530 |
| 7 | 6240 | 1200 |

While all enzyme peaks exhibited some activity, the enzymes of peaks 2, 3 and 6 showed very high levels. The results reveal that while the standard azocasein assay gives an indication of activity towards protein substrates, it does not accurately reflect the activity that each enzyme has in degrading crab flesh. As an example, peaks 5 and 6 have similar activities using azocasein (5600 and 6250 units, respectively), yet markedly different activities towards crab flesh (690 and 2530 units, respectively).

Peak 2 contains acid protease, collagenase and elastase activities. None of these activities would produce extensive proteolysis in post-mortem flesh (see General Discussion), and it therefore appears probable that a non-specific protease, capable of hydrolysing azocasein and crab flesh, is present in this fraction.

Peak 3, as well as exhibiting acid protease activity, has a number of exo- and endopeptidase activities, and their combined action on a protein substrate would produce extensive hydrolysis. Such a result is seen in the activity of this peak towards azocasein and crab flesh (Table 13).

Peak 6, which has high trypsin activity, is of particular interest as it is also relatively stable to high temperatures. This thermal stability, coupled with its ability to degrade the crab flesh makes it a prime candidate for causing the mushiness. The enzymes of peaks 2 and 3 cannot be ignored either, for although they are more labile (Table 11), their flesh-degrading ability with even minimal residual activity may be significant.

The effects on P.I. of hepatopancreas, trypsin and collagenase action on raw crab flesh are shown in Figure 18. The controls containing water showed only a slight rise in P.I. over the 7-d storage time. In the absence of sodium azide, the TVC rose from $7 \times 10^4$ per g of flesh to $>3 \times 10^8$ per g over 7 days, while in its presence the count increased to only $5 \times 10^6$ per g. The P.I.-time curves were identical for the two controls, indicating that contamination with psychrotrophic bacteria did not affect the level of degradation in the flesh. Collagenase also did not alter significantly the P.I. values compared with controls. This does not exclude the possibility that collagenase is at least partly responsible for mushiness *in vivo* however, since the degradation products of its action could be further attacked by other proteases in the hepatopancreas.

The presence of trypsin or hepatopancreas homogenate had a dramatic effect on the chemical quality of the flesh. Three enzymes with trypsin-like activity have been found in the hepatopancreas (see earlier results), and peak 6 enzyme in particular had high activity towards crab flesh (Table 13). These results implicate the trypsins as being at least partly responsible for the degradative ability of hepatopancreas.

## Proteolytic activity of crab muscle and hepatopancreas

The protease activities of muscle and hepatopancreas are shown in Table 14.

**TABLE 14**

**Proteolytic activity of crab tissues**

| Sample | Additions (mM) | Activity |
|---|---|---|
| muscle | none | 16.8 units/g |
| | $CaCl_2$ (5 mM) | 20.8 |
| | EDTA (5 mM) | 8.0 |
| hepatopancreas | none | 31750 units/mL |

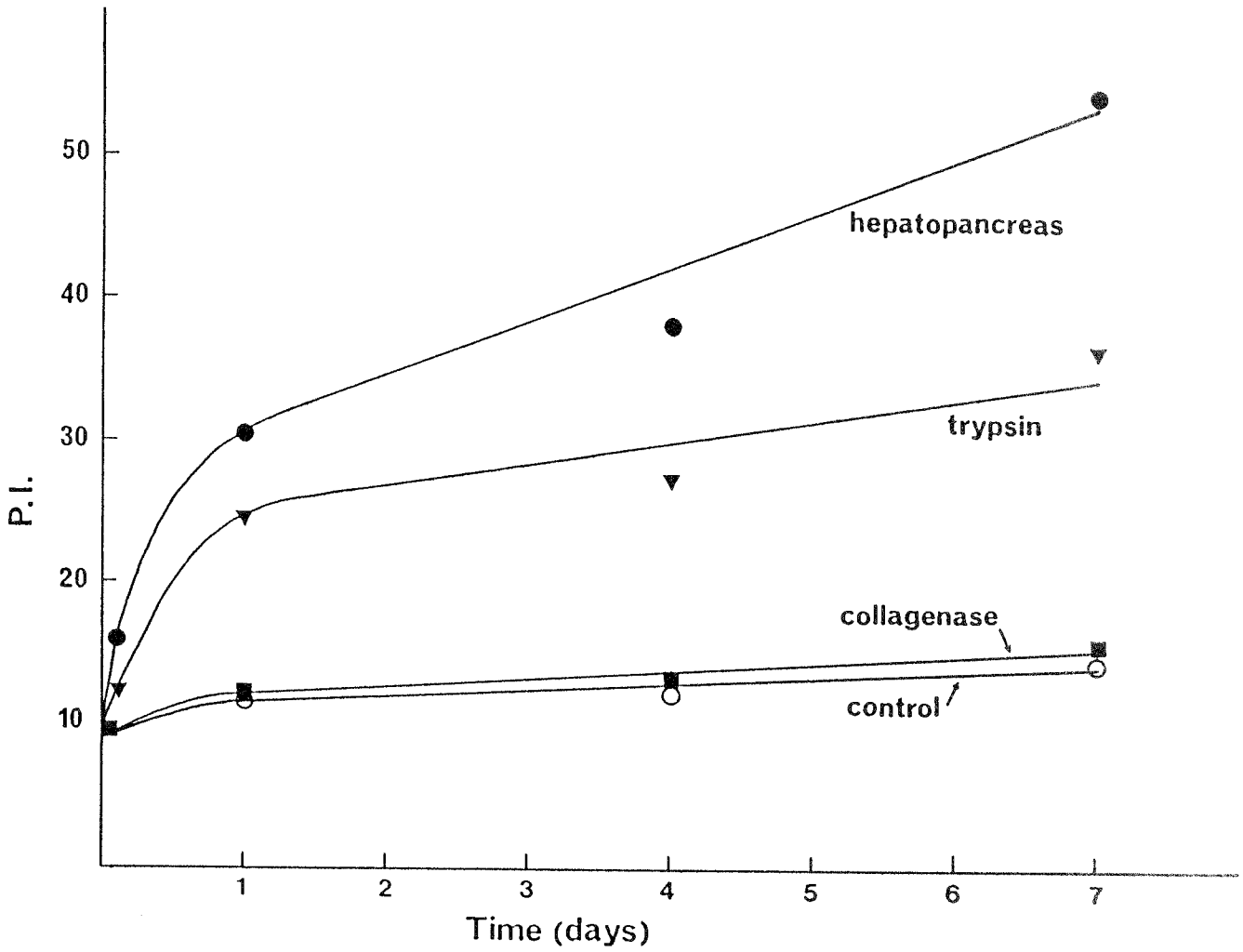


Figure 18. The effect of enzymes on P.I. of crab flesh during storage at 5°C

The results on crab muscle show that there are some endogenous proteases present, although at very low levels of activity at pH 7.5. The activation with calcium ions and inhibition with EDTA suggests that calcium-dependent protease(s) are at least partly involved. In contrast to muscle, hepatopancreas has highly active proteases, and hence contamination or infiltration of muscle with even minute amounts of hepatopancreas will greatly enhance the possibility of flesh degradation if correct handling procedures are not followed.

## Chromatofocusing of peak 6

Chromatofocusing of peak 6 from ion exchange chromatography was attempted using a pH gradient from 5.0 to 3.4. The results, shown in Figure 19, reveal that all proteins including the trypsin-like enzyme were still bound to the column at pH 3.4, and eluted only after washing with 1 M NaCl. A pH gradient with a lower end point (Pharmalyte pH 5.0-2.5) was also tried; however despite the elution of proteins at around pH 3.0 and in the 1 M NaCl wash, all tryptic activity was lost. Further studies showed that trypsin was inactivated at pH values below 3.5. Although inconclusive, the results indicate that the trypsin of peak 6 has a highly acidic character, with an isoelectric point below 3.4. This finding is supported by its tight binding to DEAE-Sepharose, where 0.65 M NaCl is necessary for its elution (Figures 15 and 16).

Trypsin-like enzymes with extremely low isoelectric points have been purified from American lobster, *Homarus americanus* (Brockerhoff *et al.*, 1970), and Antarctic krill, *Euphausia superba* (Kimoto *et al.*, 1983). The discovery of a third enzyme with these properties suggests that they may belong to a group of trypsins peculiar to crustacea. Unlike these enzymes, most trypsins of mammalian origin have isoelectric points around 7.

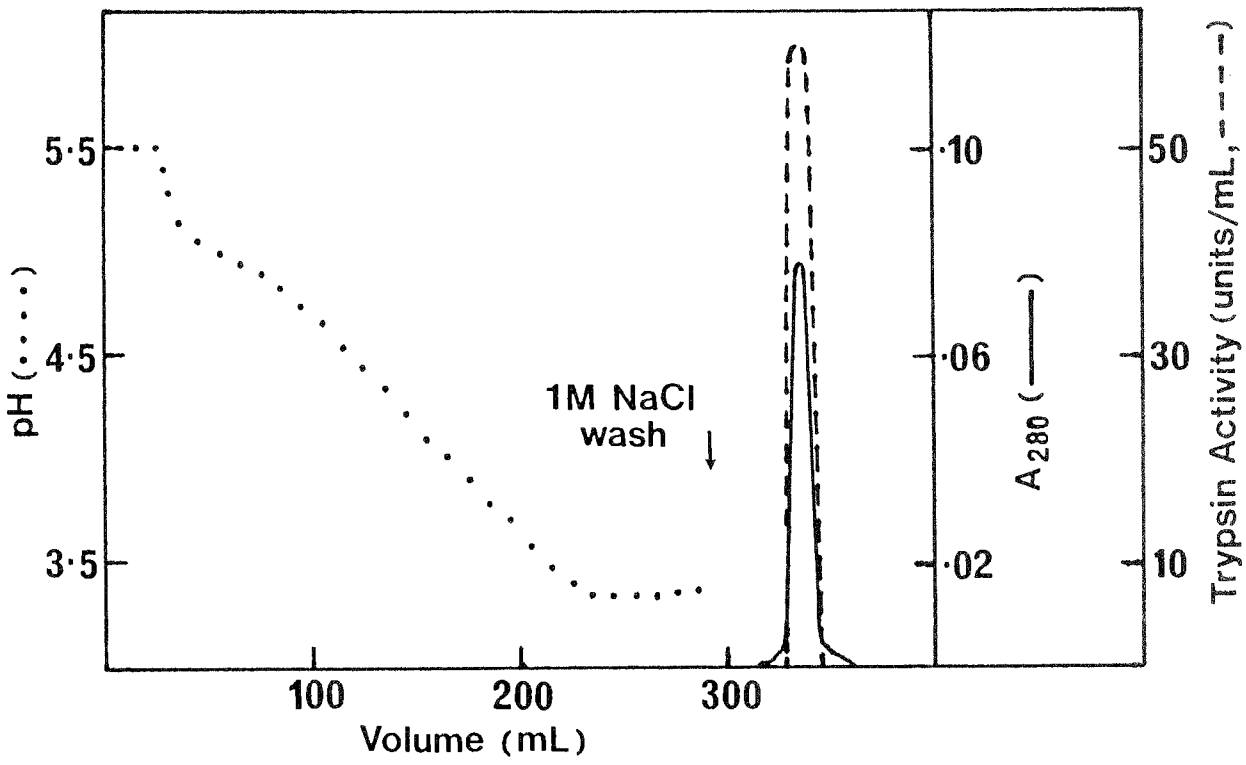


Figure 19. Chromatofocusing of peak 6, DEAE-Sepharose chromatography, on PBE 94 ion exchanger, pH 5.5-3.5

# PART 4

# GENERAL DISCUSSION

**Technological aspects**

**Biochemical aspects**

## TECHNOLOGICAL ASPECTS

The quality of seafood is dependent on the procedures used during handling of the catch, and the storage conditions under which the catch is held until the product is sold to the consumer. During the first stage of this project, the documentation of the incidence of mushiness, it was found that the handling and storage techniques used by industry personnel were numerous and varied. No standard guidelines were consistently adhered to, with the result that many of the practices were the result of 'father to son' passing-on of information. Many of the suggested causes of mushiness put forward by fishermen were based on trial and error experimentation, but almost as many were anecdotal. In the technological part of the project, studies were undertaken to determine which of the numerous handling and storage techniques had a deliterious effect on the quality of the flesh. Physiological characteristics were also examined.

Perhaps the most popular reason given for the cause of mushiness was overcooking. The findings reveal that in fact the opposite is true. Overcooking will not result in textural defects, but rather enhance the sensory characteristics, so that crabs cooked for 15 minutes were more acceptable than those cooked for 5 or 10 minutes. Undercooking on the other hand may lead to problems if the product is subsequently stored for too long. These findings form the basis for recommendations on cooking times (see Part 5). The improvement in textural and chemical quality of the flesh with increased cooking time is paralleled by a decrease in proteolytic activity in the hepatopancreas; the inference from this is that enzymes from the digestive organ are involved in mushiness.

The practice of spiking is commonly used by commercial crabbers to prevent claw loss during cooking and thus present a more appealing product. In experienced hands the technique may result only in damage to the nervous system, but when used by novices other internal organs may be ruptured. In nearly all instances during trials on spiking the yellow fluid characteristic of hepatopancreatic juices (see cover photograph) was observed to leak from the mouthparts. It could reasonably be expected that these digestive fluids also penetrate the flesh, the degree of infiltration dependent on the severity of damage to the hepatopancreas and the method of storage, i.e. mouthparts facing downwards, or otherwise. The results of spiking trials clearly indicate that the practice effects greater deterioration in quality of ice-stored, uncooked crabs. Again, hepatopancreatic proteases are suggested as having an involvement. While spiking can be used to inactivate lively crabs or to prevent claw loss, the crabs must be cooked immediately afterwards.

Backing of crabs has been suggested as a means of improving the keeping quality until the crabs can be cooked. The results show that the method can be used but total removal of hepatopancreas must be ensured or the problem will only be exacerbated. Precautions against desiccation of the meat should also be taken.

Trawler fishermen have often been nominated, without valid reason, for producing the majority of mushy crabs. The findings of comparative studies involving pot-caught and trawl-caught crabs reveal that if the catch is cooked and stored on ice, no differences in quality will result. Uncooked crabs, if trawl-caught, do have poorer quality. This is possibly related to damage during, or stress of capture, and the hepatopancreas is

one possible source of the increased enzyme activity in the flesh. However the activation of endogenous muscle enzyme systems may also be responsible. Whatever the cause of the problem, it can be overcome by correct cooking.

Storage trials revealed that cooked crabs may be stored on ice for up to 5 days and still retain acceptable texture. Uncooked crabs lasted only 3 days, while any uncooked crabs which were held in brine could not be transferred to ice without greatly accelerating flesh degradation. Brine storage alone of uncooked crabs produced good quality flesh. The temperature of brine tanks on board trawlers may be even lower than that used in these trials (0.2° to 1.8°C), and give even better quality flesh. Bremner (1985) presents a formula which relates the deterioration rate in seafood to the square of the storage temperature. These results notwithstanding, the practice of storing uncooked crabs in brine should be discouraged because of the likelihood of some cold storage after removal from brine and before cooking. No such problems could be envisaged for the storage of properly cooked crabs in brine.

The studies on frozen storage of crabs have produced some clear-cut findings. As with many other trials, problems occur with uncooked crabs, and the results suggest that it is the freezing and/or thawing process itself, and not the length of storage time which is critical, since even after one week at -28°C the flesh quality was unacceptable. It would be expected that storage for even one day would be enough to produce mushiness, and it is therefore recommended that uncooked crabs should not be frozen.

A popularly held belief is that crabs with soft shells will also have soft flesh. This has been shown to be incorrect for crabs which have been cooked prior to storage, while problems may arise with storage of uncooked post-moult crabs. The conclusion from these results is that it is not the presence of lower amounts of flesh which bring on the mushiness, but the conditions under which those crabs are held. Bremner (1985) found no defects in the texture of moulting scampi, *Metanephrops andamanicus*.

A similar conclusion may be drawn from the studies on "hermaphroditic crabs" infected with *Sacculina granifera*. As noted by Phillips and Cannon (1978), some yellow discolouration of the flesh was often found, but the textural and chemical quality of the flesh was not affected. Any mushiness found in parasitised crabs must arise from incorrect handling and/or storage procedures, and not as a result of the parasite's presence. A myxosporidian parasite similar to that which causes proteolysis in kingfish (Lester, 1982a) has also been found in the sand crab population of Moreton Bay (Potter *et al.*, 1987). While it is present at only low levels (1%) within the crab population, its occurrence could explain the infrequent appearance of individual highly mushy crabs in treatments where the rest of the batch was of good quality. Another parasite identified as *Polyprocephalus* sp. was found in two crabs intended for histological examination.

In all trials where mushiness was found to occur, it was evident in uncooked batches only. Hence, in studies on handling (spiking, pot *vs* trawl methods), storage (brine/ice and frozen storage) and physiological traits (post-moult) the rate of deterioration in quality was significantly greater in uncooked crabs than in cooked crabs. These findings are

supported by the cooking trial data (Figure 4); an increased time of cooking resulted in an improvement in the textural and chemical properties of the flesh. The biochemical processes which effect the undesirable change in quality are stopped with adequate cooking. Therefore whatever the mechanism by which these changes are initiated, correct cooking procedures can effectively stop its progression. The message is clear and simple : **if crabs are cooked as soon as possible after capture, and cooked well, the problem of mushiness will virtually disappear.**

Several findings point to the involvement of the hepatopancreas and its proteolytic enzymes in the processes of flesh degradation. The results of the cooking trial (Figure 4) suggest that enzyme inactivation accompanies improvement in texture, and while it may be guilt-by-association, hepatopancreatic enzyme activity is almost fully lost at the same time (10 min) as there is a significant decrease in the P.I. of the muscle. Spiking was observed to rupture the hepatopancreas and release the orange-coloured juices characteristic of this organ. This procedure also led to decreases in quality greater than in undamaged crabs under identical conditions, with the severity of the execution affecting the rate of deterioration. It is likely that digestive juices can infiltrate the flesh more quickly once the organ's membranes have been ruptured. The brine data of Table 5 show the differences in protease activity and P.I. of the flesh after storage for 1 or 2 days. While 1-day storage yields low P.I. values and activities approximating normal levels in muscle (see Part 3), 2-day storage gives high P.I. values and protease concentrations up to 8 times the normal level. Either endogenous enzyme systems are greatly activated by some means, or there is an influx of protease into the flesh. The likely source of exogenous proteases is the hepatopancreas. It is also possible to explain the results of freezing trials in terms of damage to hepatopancreas membranes and subsequent proteolysis by the released enzymes. These interpretations, backed by literature reports of hepatopancreas involvement in tissue degradation in species including krill (Kawamura *et al.*, 1981), lobster (Wessels and Olley, 1973), prawn (Rowland *et al.*, 1982) and capelin (Hjelmeland and Raa, 1980) point strongly to this organ and its proteases as being responsible for mushiness in the flesh of the sand crab.

## BIOCHEMICAL ASPECTS

The proteolytic enzymes of the digestive system have been found to be responsible for textural defects in the flesh of species such as prawns, krill, lobster, mud crab, cod and capelin. In all of these studies the conditions of storage and/or handling led to enzyme release from the digestive organs and infiltration of the surrounding flesh, resulting in autolytic digestion. The results presented in this report strongly implicate the enzymes from the hepatopancreas as the cause of mushiness in the flesh of the sand crab, *Portunus pelagicus*. The characterisation of proteases of the hepatopancreas has been undertaken to augment the technological studies, with an aim to understanding the biochemical processes involved in the degradation.

The post-mortem pH of crab meat is slightly alkaline and it could therefore be expected that proteases with optimum activity around pH 7 or above would be largely responsible for muscle protein hydrolysis. Most of the enzymes from the hepatopancreas fall into this category; the extract has an optimum activity around pH 7.5, with a much smaller activity peak in the acid range (pH 3 to 4). While recognising that acid proteases endogenous to muscle have been reported (Makinodan *et al.*, 1982; Ting *et al.*, 1968; Konagaya, 1985b), their activity would be restricted to intracellular metabolism of living animals, when a microenvironment of low pH could be maintained. Upon death these favourable conditions would rapidly disappear as a pH around neutrality was established throughout the tissue.

A wide variety of protease types was found in the hepatopancreas : acid proteases, serine endopeptidases (chymotrypsin and trypsin), aminopeptidases (aminoacyl arylamidase and leucine aminopeptidase), carboxypeptidases (A and B) and connective tissue proteases (collagenase and elastase). This is to be expected of course, in view of the organ's role in metabolism. After death however, the tight regulation of these enzymes is lost, and catabolic processes predominate. It has been shown that some of these enzymes are very active in the degradation of crab flesh. One enzyme activity in particular, peak 6 from DEAE-Sepharose chromatography, which has high levels of trypsin activity, has attracted attention for several reasons; firstly, the peak has significant activity towards crab flesh, even at 5°C; secondly, commercial trypsin preparations have been shown to produce extensive proteolysis in crab flesh; and thirdly, the enzyme(s) of peak 6 have significant thermal stability. These factors combined point to trypsin as being one of the causative agents in mushiness. This peak most likely is a homogeneous solution of trypsin, a conclusion supported by the high specific activity of the fraction towards TAME. The enzyme is also distinguished by its very low isoelectric point (<3.4), an unusual property for an enzyme with trypsin activity. In fact, it is unusual for any protein to be so acidic in character.

The importance of proteases which specifically attack connective tissues *viz.* collagenase and elastase, should not be overlooked in this discussion. While the amount of connective tissue in crustacea may be very low (stroma protein comprises only 3.1% of total meat protein in *Portunus pelagicus* [Badawi, 1971]), it serves as a binding agent around the bundles of muscle fibres. When this coating is removed by enzymes

such as collagenase, the myofibril is vulnerable to attack by other specific proteases. Hence, while connective tissue proteases cannot, because of their specificity, produce extensive proteolysis, they may be the initiators of the entire process. Nip *et al.* (1985a) found a collagenase to be responsible for textural changes in prawns during ice-chilled storage. Our results showed that collagenase and elastase were present in the hepatopancreas (peak 2, DEAE-Sepharose chromatography). Their action would have enhanced the hydrolytic capability of the putative non-specific protease of this fraction, with the result that peak 2 is very active in the degradation of crab flesh.

All peaks from ion exchange chromatography were capable of breaking down crab flesh, yet in some peaks specific enzymes responsible for the proteolysis were not identified. In peak 2 only acid protease, collagenase and elastase were identified, and these enzymes would not be expected to effect the extensive proteolysis that this peak exhibits against azocasein and crab flesh. Similarly, no specific proteases were found in peak 5 which was active against both the abovementioned substrates, albeit weakly against crab flesh. The conclusion to be drawn from these findings is that one or more non-specific enzymes with neutral protease activity are present in these peaks. In fact most of the peaks may contain non-specific proteases, whose role *in vivo* may be one of metabolic protein turnover.

The enzymes studied in this report were obtained from a homogenate of hepatopancreas tissue, and would therefore contain all the enzymes present in the organ. The studies on tissue proteolysis also used this same collection of enzymes, and it could be argued that the digestive fluids often seen leaking from the crab on storage may have a different composition of proteins and enzymes. However, chromatographic analysis of the fluid passively leaked from the crab on storage revealed no differences in protein composition compared to homogenate. Moreover, all enzyme peaks were present, and the relative levels of activity were also identical to homogenate activity peaks. The argument therefore holds that whatever the origin of the digestive fluid entering the flesh, its effect on the flesh will be the same.

The studies on temperature effects and activity show that proteolytic enzymes are still active at cold storage temperatures, ie. $0^{o}$ to $5^{o}C$. If contamination of flesh with hepatopancreas has occurred, and enzyme activity has not been totally removed by cooking, then degradation will continue unabated under these conditions. Even at temperatures below $0^{o}C$, proteolysis may still proceed. Lovern and Olley (1962), in studies on lipid hydrolysis in fish flesh, showed that the enzymatic reactions continued below $0^{o}C$, with a maximum around $-4^{o}C$. These findings further emphasise the need to fully inactivate the proteolytic enzymes prior to cold storage at <u>any</u> temperature.

Neutral proteases endogenous to muscle tissue of fish and crustacea have been reported in the literature, and it is possible that limited proteolysis could result from their action; the decrease in textural quality of raw separated crab meat over time could be caused by such enzymes. However, their level of activity is very low compared to hepatopancreas enzymes (approx. 0.05%). Makinodan *et al.* (1979) commented that the lack of investigation into the neutral proteinases of muscular tissue is possibly due to difficulties in the detection of enzyme

activity. It is clear that only slight contamination with, or diffusion of hepatopancreas enzymes into the crab flesh would greatly increase the proteolytic activity in the flesh. The studies reported here strongly suggest that it is the enzymes of the hepatopancreas that are responsible for the onset of mushiness, as the dramatic textural changes observed in the numerous trials could not have been effected by the very low levels of endogenous muscle proteases.

In summary, it has been shown that several of the fractions separated by ion exchange chromatography are important in the degradation of crab muscle. These include peak 2, which contains collagenase and elastase, peak 6 which contains trypsin, and peak 3, with a mixture of protease types. The significance of peak 6 increases in undercooked crabs. This can be seen in Table 15, where the activities of peaks towards crab flesh following heat treatment of peaks (30 min at 60°C) have been calculated, using data from Tables 11 and 13.

**TABLE 15**

**Peak activities against crab flesh following heat treatment (60°C/30 min)**

| Peak | Activity *vs* crab flesh (units) | % of total remaining activity |
|---|---|---|
| 1 | 78 | 1.4 |
| 2 | 1057 | 18.8 |
| 3 | 1520 | 27.0 |
| 4 | 224 | 4.0 |
| 5 | 223 | 4.0 |
| 6 | 1970 | 35.0 |
| 7 | 483 | 8.6 |

Following this heating regime, peak 6 accounts for 35% of the total activity remaining, compared with 18% in fully active, ie. unheated preparations of enzyme.

It is interesting to speculate that trypsin activity may be used as an indicator of the mushiness potential of crab flesh. There are several good reasons for this : the enzyme is highly active against crab flesh, its activity is easily measured, and as already stressed, the enzyme is relatively heat-stable. This means that in cases of undercooking, where mushiness potential is high, or indeed in any instance where hepatopancreas enzymes are present in the flesh, trypsin activity levels could be measured, and offer an indicator as to the likelihood of mushiness.

# PART 5

# RECOMMENDATIONS

The recommendations on correct storage and handling of sand crabs contained in this section are based on the research findings presented in Part 2 of this report. The aim of these recommendations is to provide the fisherman, wholesaler, retailer and consumer with information on procedures which will ensure a product of good quality.

**COOKING**

1. Unless crabs are required in an uncooked state for a specific purpose, they should be cooked as soon as possible after capture.

2. Correct cooking procedure is essential if mushiness is to be prevented.

The recommended cooking times are as follows:

small crabs - 10 to 12 minutes
large crabs - 12 to 14 minutes

(Cooking time starts when the water returns to the boil)

If crabs are cooked immediately on removal from cold storage (around 5°C), the cooking time should be increased by 3 to 5 minutes.

Undercooking of crabs may result in mushiness, for while the crab outwardly appears cooked, the digestive system responsible for the onset of mushy flesh may not be fully inactivated. Overcooking of crabs (20 minutes or longer) will not cause mushiness, but some sweetness of the flesh may be lost.

**HANDLING**

1. Immediately after capture, crabs should be put on ice or kept moist in a cool, shaded area eg. covered with a wet bag.

2. Crabs which have been spiked should be cooked immediately afterwards. Degradative processes can cause extensive damage within hours of spiking if the crabs are left uncooked.

3. Crabs which have had their carapaces removed ("backed") can be kept uncooked provided care is taken to remove all traces of internal organs. Failure to do so will accelerate the degradative processes. Precautions against desiccation of the flesh should also be taken.

If the carapace is damaged, backing of the crab is recommended if cooking cannot be done immediately.

**STORAGE**

Crabs should not be stored in an uncooked state. Mushiness only occurs in uncooked and undercooked crabs.

1. Cooked crabs may be stored on ice for up to 5 days and still retain acceptable texture.

2. Cooked crabs may be stored in refrigerated brine for several days. Uncooked crabs should not be stored in brine.

3. Cooked crabs may be stored frozen, preferably at $-20^{\circ}C$ or lower, for at least 4 months. Although still of acceptable quality after this time, the flesh has a stringy texture. To prevent dehydration, the crabs should be glazed in fresh water and stored in air-tight containers. Uncooked crabs should not be frozen.

# PART 6

# DISSEMINATION OF FINDINGS

Dissemination of the findings from this project has been approached with the aim of reaching as wide an audience as possible. A leaflet providing recommendations on correct handling procedures has been prepared for distribution. QDPI fisheries libraries are assisting in this distribution throughout Queensland. The Queensland Commercial Fishermen's Organisation is co-operating in the distribution of leaflets to all professional crabbers in Queensland. The leaflet is shown in Appendix 2.

Preliminary findings were published in the January 1987 edition of *The Queensland Fisherman*, and it is anticipated that a full article will appear in an upcoming edition of the same journal. Arrangements have been made for an article to appear in the *Menu* newsletter produced by the Restaurant and Caterers Association of Queensland.

A radio-talk discussing the major findings has been produced through the QDPI Information Branch and copies of the presentation have been sent to metropolitan and country radio stations. A transcript of this radio-talk is attached in Appendix 2. An earlier radio-talk publicising the research and releasing some early findings was prepared in mid-1986. A number of metropolitan radio stations have also broadcast short interviews on the subject eg. the ABC's 'Statewide' programme and FM 104.

Publicising the results to the scientific community commenced in May 1987 when major findings were presented at two conferences. The technological aspects were displayed in poster format at the annual conference of the Australian Marine Science Association in Townsville, while the enzymological data were presented to the Australian Biochemical Society conference in Perth. These posters, in reduced form, are shown in Appendix 3. It is anticipated that two full papers on the technological and biochemical aspects will be submitted to appropriate scientific journals in the near future. A full article for publication in *Australian Fisheries* is also in preparation.

# PART 7

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# PART 8

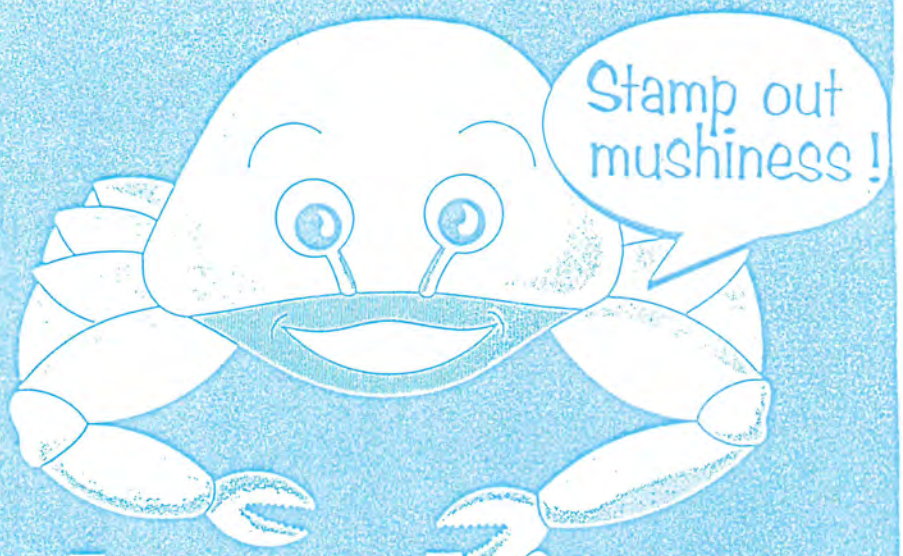
# APPENDICES

## APPENDIX 1

Publicity poster : "Have you found mushiness in sand crabs?"

Questionnaire : Mushiness in sand crabs

Press release




# Have you found mushiness in sand crabs ?

## What is mushiness ?

Before they are opened, mushy sand crabs look the same as crabs with firm flesh. However, their flesh is soft and, at worst, looks like toothpaste. Mushy crabs can cause consumer bias.

DPI Food Research Officers are seeking information on this problem, for example:

- numbers of mushy crabs caught;
- catching and handling methods; and
- suggestions on the cause.

**Contact :** Steve Slattery or David Dionysius,
QUEENSLAND FOOD RESEARCH LABORATORIES,
19 Hercules Street, Hamilton, Brisbane.
Phone: 268 2421

Queensland Department of Primary Industries

# QUESTIONNAIRE - MUSHINESS IN SAND CRABS

Department of Primary Industries Food Research Officers are investigating the problem of mushiness in sand crabs. They are seeking any information which might help in finding a solution to the problem. If you have any information could you please complete this questionnaire and return it to:

Mr. Steve Slattery,
Queensland Food Research Laboratories,
19 Hercules Street,
Hamilton, Q. 4007
Phone 268 2421

Name/Business:

Address:

Phone Number:

1. Occupation:

   FISHERMAN

   MARKETING

   RETAILER

   RESTAURATEUR

   CONSUMER

2. Have you encountered mushiness in sand crabs?

   YES

   NO

3. If yes, which of the following would you consider is/are the possible cause(s) of the mushiness?

| | Comments |
|---|---|
| PARTICULAR AREA | _____ |
| PARTICULAR BATCHES | _____ |
| SEASONAL | _____ |
| CATCHING METHOD | _____ |
| HANDLING METHOD | _____ |
| COOKING METHOD | _____ |
| MOULT STAGE | _____ |
| OTHER (Please explain) | _____ |

4. Can you give any other information which you think is relevant?

Your co-operation is much appreciated.

QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES

# NEWS ITEMS

For pictorial and further information, contact:
Information and Extension Training Branch,
DPI, 41 George St, Brisbane, Qld 4000.
Phone (07) 224 4265

1 July 1985
RELEASE DATE.....

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IN THIS ISSUE

# MUSHY SANDCRABS POSE PROBLEM

Scientists at the Queensland Department of Primary Industries are asking people to help them collect information on the increasingly common problem of "mushiness" in sandcrabs.

Dr Hilton Deeth, supervising chemist at the DPI's Food Research Laboratory, said that a "mushy" sandcrab was one that had soft and crumbly flesh, but appeared normal from the outside.

"Restaurateurs and other retailers are disappointing some of their customers by supplying them with mushy crabs," Dr Deeth said, "but, unfortunately, they have no way of identifying the undesirable crabs before sale.

"Consumers are now demanding assurance that the sandcrabs they buy will be firm.

"If that assurance could be given, demand for sandcrabs would increase."

Dr Deeth said that DPI research officers had begun to investigate possible causes of mushiness in sand crabs, and, as a first step, they needed more detailed information on the problem's frequency of occurence.

"Some restaurant owners claim that, at times, an entire lot of purchased crabs can be mushy, while sandcrab fishermen and consumers have reported both good quality and mushy crabs in the same batch," he said.

"We need to define the extent of the problem, so we are asking anyone with relevant knowledge to contact staff at the DPI Food Research Laboratory in Hamilton."

Dr Deeth said that, at present, there were only theories to explain the cause of the mushiness. Possible causes included post-capture handling, incorrect cooking or cooling, and possibly even the crab's moulting habits.

He said that the research project would be partly funded by a grant from the Fishing Industry Research Trust Account.

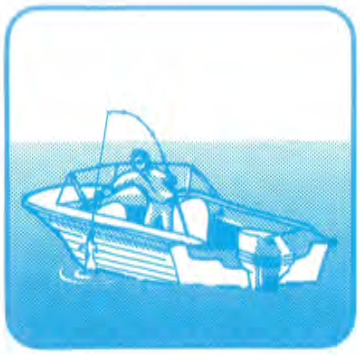
MEDIA CONTACT: Dr Hilton Deeth (07) 268 2421
Queensland Department of Primary Industries

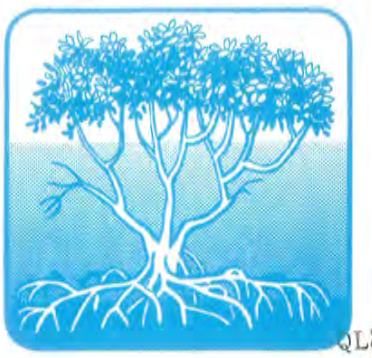
1 July 1985

# APPENDIX 2

QDPI leaflet : "Procedures for handling sand crabs"

Radio-talk transcript





Queensland Department of Primary Industries Leaflet

QL87017
replaces QL86008

# PROCEDURES FOR HANDLING SAND CRABS

Officers of Food Research & Technology Branch

These notes are based on the findings of research work carried out at the Queensland Food Research Laboratories, Hamilton, Brisbane. The two year project "Investigation of Mushiness in Sand Crabs" was funded by the Fishing Industry Research Trust Account. The recommendations are aimed at providing the consumer with a top quality product.

**Handling**

- store on ice immediately after capture
- **do not store uncooked crabs in brine**
- cooked crabs may be stored in refrigerated brine for several days with no apparent defects in meat quality
- physical damage e.g. spiking the brain, or crushing, may lead to mushiness in the flesh if the crab is not cooked within several hours
- **do not freeze uncooked crabs**
- cooked crabs can be stored frozen, preferably at -20°C or lower, in an air-tight container; glazing will help prevent dehydration under these conditions

**Cooking**

- cook the crabs **as soon as possible** after capture
- recommended cooking times:

  small crabs - 10 to 12 minutes (after the water returns to the boil)
  large crabs - 12 to 14 minutes

  If the crabs have just been removed from cold storage, increase the cooking time by 50%

- **Undercooking may lead to mushiness**
- **Overcooking will not cause mushy flesh**

  The flesh becomes slightly tougher with prolonged cooking (20 minutes or longer)

For further information contact:

Dr David Dionysius
Queensland Food Research Laboratories
19 Hercules Street
Hamilton Q 4007 (07) 2682421

Queensland Department of Primary Industries
Brisbane

**TRANSCRIPT OF RADIO-TALK**

Introduction to interview

For many years people who eat seafood have been baffled and annoyed over the mushiness in the flesh of some cooked sand crabs. The blame was usually levelled at dad the fisherman, for overcooking the crab, or the fish shop. But a research program conducted by the Queensland Department of Primary Industries has let these people off the hook. DPI chemist, Dr David Dionysius has released the results of two years research into mushiness in sand crabs.

Interviewer: David, we've spoken previously about mushiness in sand crabs and that the D.P.I. is investigating the problem. What has transpired since we last spoke?

Dr Dionysius: Firstly John, if I could describe what mushiness is. It's a condition where the flesh becomes soft, and loses its integrity, and in the worst cases, feels just like tooth paste. It occurs only in the body meat, and not in the claws.

We've done a lot of research on a number of handling and storage techniques commonly used by crabbers, eg. storage of cooked and uncooked crabs in refrigerated brine, frozen storage, storage of uncooked crabs on ice, and also various cooking conditions.

Interviewer: With what results?

Dr Dionysius: In all of these cases there was some detrimental effect on flesh quality.

Interviewer: We've spoken before about overcooking and mushiness. What are your findings on overcooking firstly?

Dr Dionysius: We've shown that overcooking will not cause mushiness in the flesh. Even if you cook the crab for 20 minutes it won't go mushy. In fact the flesh is perhaps a little tougher than usual. But it does possibly lose a little of its flavour if cooked for this long.

Interviewer: Is there a simple remedy which can stop the mushiness problem?

Dr Dionysius: Yes there is. If you cook the crabs as soon as you possibly can, and cook them well, you shouldn't have any problems. By proper cooking, I mean at least 10 minutes after the water returns to the boil. Undercooking them may lead to problems.

Interviewer: The fisherman's out in the boat; he catches a couple of nice crabs. What can he do, what measures can he take to keep them fresher?

Dr Dionysius: Well he should put the crabs on ice as soon as they're caught, and keep them out of the sun.

Interviewer: Not everyone carries ice in the little tinnie. What can he do?

Dr Dionysius: If you don't have any ice, put them in the shade, covered with a wet bag or cloth. Once you get back to shore, cook them as soon as you can, and you should end up with good quality crabs.

Interviewer: Now what's actually causing the mushiness? This is the thing we want to get at.

Dr Dionysius: It's the crab's digestive enzymes which can degrade the meat if the internal organs are ruptured or damaged in any way. Correct cooking can inactivate these enzymes, but if they're kept uncooked, or not cooked properly, then some problems may arise.

Interviewer: You're talking about ruptured enzymes. Is this damage done by the fisherman, with a spike or that type of thing?

Dr Dionysius: Yes that's one way. Or perhaps even freezing or some sort of damage like that may end up in rupturing the membranes.

Interviewer: So you recommend not doing that?

Dr Dionysius: It can be done, but we recommend that if you do spike them to kill the crab then you should cook them very quickly after you spike them. Don't let them go for any length of time after spiking before you cook them.

## APPENDIX 3

**"Mushiness in the sand crab, *Portunus pelagicus*"**, a poster presented at the Australian Marine Science Association Annual Conference, Townsville, May 1987.

**"Proteases of the sand crab, *Portunus pelagicus*"**, a poster presented at the Australian Biochemical Society Annual Conference, Perth, May 1987.

# MUSHINESS IN THE SAND CRAB, Portunus pelagicus.

Steven L. Slattery, David A. Dionysius and Ross A.D. Smith

FOOD RESEARCH AND TECHNOLOGY BRANCH, Qld. D.P.I, Hamilton, 4007.

## A. INTRODUCTION

- MUSHINESS IN SANDCRABS (BLUE SWIMMER) CAUSES A LOSS OF PRODUCT IMAGE WITH CONSEQUENT BUYER RESISTANCE TO THE PRODUCT. THIS LEADS TO SUBSTANTIAL ECONOMIC LOSS IN THE FISHERY.
- MUSHINESS, WHICH RESULTS FROM TISSUE DEGRADATION BY THE CRAB'S HEPATOPANCREATIC PROTEOLYTIC ENZYMES, IS MOST COMMONLY CAUSED BY POOR HANDLING TECHNIQUES.
- THESE INVESTIGATIONS WERE UNDERTAKEN TO IDENTIFY HANDLING TECHNIQUES WHICH LEAD TO MUSHINESS.

## B. METHODS

IN SEPARATE TRIALS FRESHLY CAUGHT CRABS WERE

STORED 1. IN REFRIGERATED BRINE AND ON ICE (-3 TO 1°C)

2. FROZEN AT -28°C.

COOKED IN BOILING SEAWATER FOR 4 TO 12 MINUTES COOLED AND STORED FOR 2 DAYS AT 5°C.

SPIKED TO PREVENT CLAW LOSS BY INSERTING A STEEL SPIKE THROUGH THE ORAL CAVITY. THESE WERE STORED AT 5°C FOR UP TO 4 DAYS.

MEAT ASSESSMENT WAS PERFORMED ON MUSCLE FIBRES FROM THE THE POSTERIOR CEPHALOTHORACIC SEGMENT FOR ANALYSIS BOTH

PHYSICAL - SUBJECTIVE ASSESSMENTS WERE MADE BY A 3 MEMBER EXPERT PANEL TO GIVE A SENSORY GRADE ON A 1(MUSHY) TO 5(VERY FIRM) SCALE.

OBJECTIVE MEASUREMENTS WERE MADE USING A MODIFIED KRAMER SHEAR CELL ATTACHED TO AN INSTRON UNIVERSAL TESTING MACHINE.

CHEMICAL - PROTEOLYTIC ACTIVITY WAS DETERMINED ON HOMOGENISED TISSUE EXTRACTS USING THE SUBSTRATE AZOCASEIN.

A PROTEOLYSIS INDEX (P.I.) WAS CALCULATED FROM THE FORMULA-

$$\frac{100 \times \text{TCA SOLUBLE PROTEIN BREAKDOWN PRODUCTS (FOLIN)}}{\text{TOTAL PROTEIN (KJELDAHL)}}$$

## C. RESULTS

EACH POINT ON THE GRAPHS IS THE MEAN OF DATA FOR AT LEAST 10 CRABS

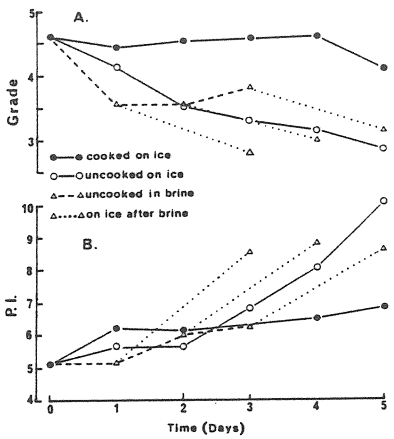



FIGURE 1 A&B STORAGE CHANGES IN GRADE(A) AND P.I.(B) OF COOKED CRABS ON ICE AND UNCOOKED CRABS ON ICE AND IN BRINE.

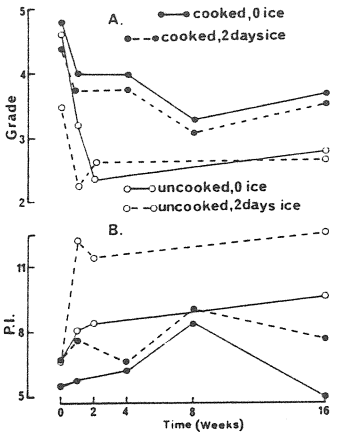



FIGURE 2 A&B STORAGE CHANGES IN GRADE(A) AND P.I.(B) OF COOKED AND UNCOOKED FROZEN CRABS.

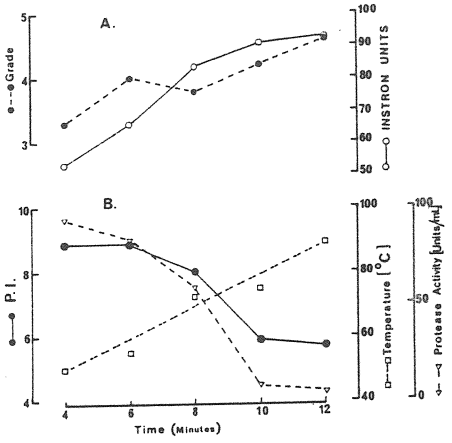



FIGURE 3 A&B CHANGES IN PHYSICAL(A) AND CHEMICAL(B) PARAMETERS WITH COOKING TIME.

## D. CONCLUSIONS

- UNCOOKED CRABS HAVE A LIMITED SHELF LIFE WHETHER STORED IN ICE OR REFRIGERATED BRINE. CRABS SHOULD BE COOKED AS SOON AS POSSIBLE UNLESS SPECIFICALLY REQUIRED UNCOOKED.
- UNCOOKED CRABS SHOULD NOT BE FROZEN. FROZEN COOKED CRABS RETAIN THEIR QUALITY FOR LONG PERIODS IF GLAZED TO PREVENT DRYING.
- INACTIVATION OF PROTEOLYTIC ENZYMES OCCURS AFTER 10 MINUTES BOILING. COOKING FOR A PERIOD OF 10 TO 12 MINUTES AFTER THE WATER HAS RETURNED TO THE BOIL IS RECOMMENDED. LARGE AND COLD STORED CRABS REQUIRE 12 TO 14 MINUTES.
- SPIKING OF CRABS TO PREVENT CLAW LOSS DURING COOKING CAUSES PHYSICAL DAMAGE TO INTERNAL ORGANS AND RELEASES PROTEOLYTIC ENZYMES INTO THE FLESH. THIS CAUSES PROTEIN DEGRADATION DURING SUBSEQUENT STORAGE UNLESS CRABS ARE COOKED IMMEDIATELY.

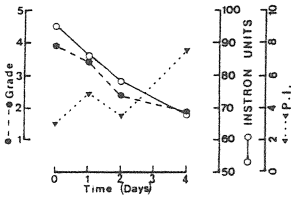



FIGURE 4 EFFECT OF STORAGE ON THE QUALITY OF SPIKED CRABS.

THIS WORK WAS SUPPORTED BY A GRANT FROM THE FISHING INDUSTRY RESEARCH TRUST ACCOUNT.

# PROTEASES OF THE SAND CRAB, PORTUNUS PELAGICUS

D.A.Dionysius and S.L.Slattery FOOD RESEARCH AND TECHNOLOGY BRANCH, Qld D.P.I., Hamilton, 4007.

## A. INTRODUCTION

- THE SAND CRAB (OR BLUE SWIMMER) FISHERY IN QUEENSLAND HAS SUFFERED SUBSTANTIAL ECONOMIC LOSSES DUE TO THE OCCURRENCE OF MUSHINESS IN THE FLESH OF THE CRAB.
- STUDIES IN OUR LABORATORY HAVE SHOWN THAT THE PROBLEM IS CAUSED BY PROTEOLYTIC ENZYMES RELEASED FROM THE CRAB'S DIGESTIVE ORGAN, THE HEPATOPANCREAS. SIMILAR FINDINGS HAVE BEEN OBTAINED BY WORKERS STUDYING TISSUE DEGRADATION IN OTHER CRUSTACEA (1,2).
- THIS WORK WAS UNDERTAKEN TO IDENTIFY THE ENZYMES RESPONSIBLE FOR THE ONSET OF MUSHINESS IN CRAB FLESH.

## B. METHODS

- ION EXCHANGE CHROMATOGRAPHY OF A CLARIFIED HOMOGENATE OF CRAB HEPATOPANCREAS WAS PERFORMED ON DEAE-SEPHAROSE CL-6B AT PH 7.0, USING A LINEAR NACL GRADIENT (0-0.8 M).
- GENERAL PROTEOLYTIC ACTIVITY WAS MEASURED USING 0.2% AZOCASEIN AS SUBSTRATE AT PH 7.5. ONE UNIT OF ACTIVITY PRODUCES AN INCREASE IN $A_{366}$ OF 0.01 PER HOUR AT 37°C.
- ONE UNIT OF ACTIVITY TOWARDS HOMOGENISED CRAB MEAT RELEASES ONE µG OF TYROSINE EQUIVALENTS PER G OF MEAT IN 48 HOURS AT 5°C. ONE UNIT OF ACID PROTEASE ACTIVITY (PH 3.4) PRODUCES AN INCREASE IN $A_{280}$ OF 0.01 PER HOUR AT 37°C. FOR ALL OTHER ACTIVITIES, ONE UNIT PRODUCES A CHANGE IN ABSORBANCE OF 0.01 PER MINUTE AT THE APPROPRIATE WAVELENGTH (PH 7.5, 37°C).
- CHROMATOFOCUSING WAS PERFORMED USING PBE 94 ION EXCHANGER AND A PH GRADIENT (5.5-3.5) WITH POLYBUFFER 74, PH 3.5, AS ELUENT.

## C. RESULTS

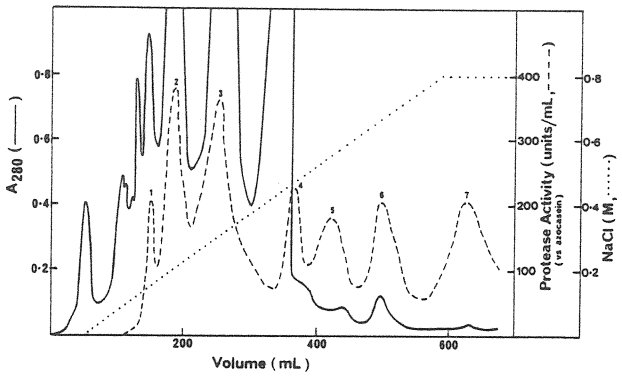



FIGURE 1. DEAE-SEPHAROSE CL-6B ION EXCHANGE CHROMOTAGRAPHY OF SAND CRAB HEPATOPANCREATIC HOMOGENATE AT PH 7.0.

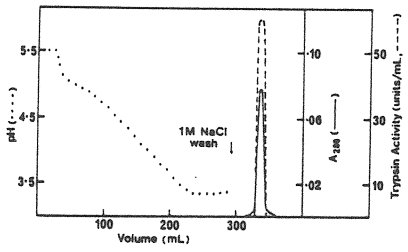



FIGURE 2. CHROMATOFOCUSING OF FRACTION 6, DEAE-SEPHAROSE CHROMATOGRAPHY, ON PBE 94 ION EXCHANGER, PH 5.5-3.5.

TABLE 1. PROTEOLYTIC ENZYME ACTIVITIES OF POOLED FRACTIONS FROM DEAE - SEPHAROSE CHROMATOGRAPHY OF CRAB HEPATOPANCREATIC HOMOGENATE.

| Column fraction | Total activity vs azocasein (units) | Total activity vs crab meat (units) | Specific Enzymes: Enzyme | Substrate | Activ (unit |
|---|---|---|---|---|---|
| 1 | 4120 | 510 | acid protease | Hb | 55 |
| | | | amino acid arylamidase | alanine-NA | 144 |
| 2 | 10760 | 4230 | acid protease | Hb | 90 |
| | | | elastase | N-succinyl-$(ala)_3$-NA | 310 |
| 3 | 17200 | 4110 | acid protease | Hb | 68 |
| | | | chymotrypsin | BTEE | 110 |
| | | | carboxypeptidase A | FA-phe-phe | 166 |
| | | | carboxypeptidase N | FA-ala-lys | 1140 |
| | | | leucine aminopeptidase | leucine-NA | 14 |
| 4 | 7620 | 900 | trypsin | TAME | 184 |
| 5 | 5600 | 690 | (no specific enzyme activities detected) | | |
| 6 | 6520 | 2530 | trypsin | TAME | 2780 |
| 7 | 6240 | 1200 | (no specific enzyme activities detected) | | |

Abbreviations: Hb, haemoglobin; NA,4-nitroanilide;BTEE, N-benzoyl-tyrosine ethyl ester; FA, N-(2-furanacryloyl); TAME, tosyl-arginine methyl ester.

## D. CONCLUSIONS

- SEVEN MAJOR FRACTIONS WITH PROTEOLYTIC ACTIVITY HAVE BEEN SEPARATED FROM CRAB HEPATOPANCREAS BY ION EXCHANGE CHROMOTAGRAPHY.
- OF THESE, FRACTION 2 WITH ONLY ACID PROTEASE AND ELASTASE ACTIVITIES IDENTIFIED TO DATE, AND FRACTION 3, WHICH CONTAINS A NUMBER OF EXO- AND ENDOPEPTIDASES, ARE THE MOST ACTIVE IN THE DEGRADATION OF RAW CRAB MEAT.
- THE TRYPSIN-LIKE ENZYME OF FRACTION 6 ALSO HAS SIGNIFICANT ACTIVITY TOWARDS CRAB MEAT. THIS ENZYME HAS AN UNUSUALLY LOW ISOELECTRIC POINT (<3.5). THE TRYPSIN ISOLATED FROM THE AMERICAN LOBSTER, HOMARUS AMERICANUS, IS ALSO AN ACIDIC PROTEIN (3).

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THIS WORK WAS SUPPORTED BY THE FISHING INDUSTRY RESEARCH COUNCIL.