STUDIES ON THE BLUE DISCOLORATION IN THE MEAT OF CANNED CRAB

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CONTENTS

INTRODUCTION

Although fishing and canning of king crab (Paralithodes camtschatica) have been restricted to international environment such as continental shelf sovereignity proclamation in 1964 or protection of resources (Japan-U.S. king crab negotiation (1964), Japan-Soviet crab talks (1969)), Japanese packs are about 1,100,000 cases per year.[1] Approximate 30 per cents of the production are assorted to export. The production of canned king crab is about two-third among total canned products. The canned crab ~~have~~ has so high commercial values among canned sea foods that ~~they are~~ it is very important on economical viewpoint. ~~They are~~ It is mostly produced at floating canneries and a little ~~are~~ is at land. At the floating canneries, the materials are usually fresh, but at land they are not so good in freshness because they are transported from a distant waters. Therefore, the products of land canneries have associated frequently with the blue discoloration and browning. King crab, Paralithodes camtschatica, horse hair crab, Erimacrus isenbeckii, and "zuwaigani", Chionoecetes opilio, are used for the materials of canning. The blue discoloration reveals at every species, and more frequent in canned horse-hair crab meat. For the reason, it has been suggested that muscle fibers are ~~more~~ finer and thinner than other species. It is necessary to improve the quality to prevent grade down at inspection.

In a case of king crab canning, most proportion of the total product is packed on the floating canneries, and it

does not so often yield browning and blue discoloration; while that packed at land canneries, even the production is a little, but ~~they~~ it frequently causes browning and blue discoloration. A statistical data um on per cent occurrence of the browning and blueing in annual production of various kinds of canned crab from 1965 to 1969 is summarized at Table I-1.

It has already been passed about 70 years after first canning demonstration, but still not been made clear the blue discoloration as well as browning. The blue discoloration appears in the meat changing blue or bluish gray in colour after processing. The investigation concerned with the blue discoloration was first reported in 1926 by Fellers and Parks.[2] Then numerous papers have been reported on the blue discoloration for 44 years, but there is no general agreement of this problem.

Onuma and Sato[3] suggested that possible portions in which blue discoloration develops are body meat, meats near to joints, surface of leg meats, claw meat, and belly meat. It has also been found through practical experiences from commercial crab canning factories that deterioration of raw materials, insufficiency of bleeding or washing, imperfect boiling and exposure of crab meat to air for long time after boiling or picking procedures would be a cause of frequent blue discoloration. The degree of blue discoloration develops during storage of the can about one or longer weeks at room temperature, and sometimes at early stage after retorting.

Many investigators have provided presumptions on the cause and mechanism of the problem. First information reported by

Table I-1. The percentages of grade down at canned crab inspection for export by blue and browning discolorations.※

		1965	1966	1967	1968	1969
Blue discoloration	P. camtschatica	0.15	0.0	0.02	0.06	1.82
	E. isenbeckii	2.07	0.0	1.54	0.0	5.55
	P. brevipes	0.36	0.15	0.11	4.83	3.05
	C. opilio	0.70	0.0	0.35	7.63	3.94
Browning	P. camtschatica	1.01	0.90	1.17	1.35	0.76
	E. isenbeckii	1.36	0.0	1.90	0.0	0.0
	P. brevipes	0.0	2.76	2.86	1.49	6.57
	C. opilio	0.0	0.0	0.24	0.0	0.23

※ Quoted from the data of Japanese Canned Foods Inspection Association

Fellers and Parks in 1926[2] proposed that the blue discoloration would not be caused by iron or copper sulphides, but probably depends on a modified biuret reaction product which are produced from biuret(urea-NH_3) and copper, both are contained in the blood of Crustacea with ammonia. However, it is still doubtful, because even biuret reaction is completed at an alkaline side, the blue discoloration appears at an acidic pH range from 5.8 to 6.8. Biuret compounds are soluble in water, but this substance has not been found in the solid or liquid portions in canned crab as an objectionably coloured material. Since the binding of copper in haemocyanin is very strong,[4),5)] liberation of the copper seems to be difficult in canned crab. Therefore, the copper would not involve in biuret reaction because only liberated copper may react with urea.

Oshima, Koizumi and Takisawa,[6)] after an investigation on the causative substance of the blue discoloration, reported that iron sulphide, melanin, and biuret complex would not be the possible substance, but that copper sulphide had shown blue discoloration through the model experiments. They also suggested that the copper sulphide is produced by the reaction of the copper freed from haemocyanin with hydrogen sulphide generated from the crab meat during heat processing. Takayasu and Fukuhara,[7)] however, claimed that the presumption as described above was uncertain since copper salt solution which had been contained 0.1 mg copper in 100 ml water changed brown in colour after passing hydrogen sulphide. They further investigated iron or copper sulphides, indigo, amino acid – metal compounds, biuret complex with referring to enzymatic

and bacteriological standpoints, but could not obtain any positive result. Without any evidence, they suggested that a causative substance would be a sulphide derivative of haemocyanin or oxidized haemocyanin, with a consideration of the necessity of the presence of haemocyanin and hydrogen sulphide to develop blueing the crab meat. The proposed fomular were R-Cu-S-Cu-R or R-Cu-S, where R stands for a protein moiety of haemocyanin molecule. It appears that the presence of oxygen and slightly denatured haemocyanin would accelerated the reaction. However, they failed to isolate the causative substance.

Fellers and Harris[8] proposed that the discoloration was caused by the formation of blue copper-ammonia complexes and to some extent to sulphides indicating copper liberated from haemocyanin and ammonia and sulphide gernerated from crab meat during processing formed blue complex. The blue copper-ammonia complex including copper-ammine as $\left[Cu(NH_3)_4^{++}\right]$ is not only isolated from the blue meat, but also is soluble in water. For this reason, the complex is not confident of the causative matter.

Osakabe[9] succeeded in preventing the blue discoloration in canned crab meat removing the haemocyanin by the "low temperature and fractional heating" of the carcasses from which carapace had been removed. According to his experiments, the coagulating temperature of blood proteins of crabs is from 69°C to 70°C, and that of meat protein of crabs is from 59°C to 60°C. Thus, if the carcasses are heated at 59°~60°C

the meat coagulates, but the uncoagulated blood will run out. After removing the meat from the carapace in a half-heated condition, the blood will run out leaving the meat alone. When the meat from which the blood has been removed is boiled for a few minutes and packed in can as the usual manner, the blue meat did not appear in the finished product. He also reported that heating made haemocyanin oxidize which would develop blue colouring. This finding is completely different from those that blue discoloration may develop under the condition of reduction.

Gordievskaya[10] established that blue discoloration of crab meat is caused by a copper-protein complex formed from the decomposition products of protein(H_2S) and oxyhaemocyanin. According to the observation, these complexes give a blue colour in the presence of ammonia; an increase of the intensity of the blue colour of the crab meat is accompanying with an increase of copper content.

Groninger and Dassow[11] studied the dark discoloration called "blueing" of king crab blood coagulum after heat-processing, and reported that the colour and redox properties are similar to those shown by a copper protein or a copper-peptide complex(biuret). From this result, they concluded that the blued product is probably a type of biuret complex.

Little information is available with respect to isolation of the causative substance from the blue meat, because the difficulties concern extraction of the blue pigment from heat-denatured crab haemocyanin using organic solvents.

In consequence, the finding that haemocyanin involves in the blue discoloration as the main factor is agreeable to the results reported in past in this respect, but it still remains the problem to be solved. Chemical properties and mechanism of the formation of the causative substance should be clarified.

On the other hand, numerous preventions have been proposed. One method is to boil the crab meat completely at high temperature, make cool the carcase as thoroughly as possible after boiling, or immerse in diluted hydrochloric acid or hydrogen peroxide solution after boiling.[12] Takayasu and Fukuhara[13] recommended to use fresh crab meat as raw material, and to avoid the meat expose to air or to sunshine for a long time. The treatments with a 1~2% solution of lactic acid[10] or ascorbic acid[11] are reported to be effective. With respect to removal of blood from crab meat, it offered several methods as to bleed by removing the end appendages,[13),14] to wash thoroughly the meat with a fresh mixture of 1% sodium citrate – 5% sodium chloride solution,[15] to remove haemocyanin by the low temperature and fractional heating method,[9] to wash thoroughly the heat-coagulated blood by running tap water,[13] to stabilize the copper contained in the haemocyanin of crab blood and flesh with protective brine dip containing small amounts of aluminum and/or zinc salts(less than 400 ppm)[8] and to add salts of zinc(zinc oxide, zinc chloride or zinc sulphate) or sodium nitrite.[13]

These methods for prevention of the blue discoloration are effective partially. In general, however, the blood remains

among muscle fibres after bleeding, even washing of the picked meat is done as thoroughly as possible. The low temperature and fractional heating method is comparatively effective for the prevention of the blue discoloration, while it causes less flavor and loss weight of the canned product. Although ascorbic acid treatment is effective on the discoloration of boiled and frozen crab meat, this method is not sufficiently effective on canned crab meat. Adding of salts to absorb hydrogen sulphide gives inferior flavor of the product. The conclusion, after reviewing the papers reported on prevention of the blue meat, reaches that the methods are not completely satisfactory even they are partially effective. An attempt, therefore, was made to carry out studies in order to investigate the cause and the mechanism and prevention of the development of the blue discoloration in canned crab meat.

The purpose of the present study was at first to ascertain haemocyanin as a main factor, to detect of haemocyanin in the blue meat, to clarify the substance which react with haemocyanin, to isolate and to determine chemical compositions of the causative substance, and eventually to discuss the mechanism of the blue discoloration. Further, the present author tried to investigate the complete prevention of the blue discoloration according to the findings obtained.

Details of the investigations are described in following chapters.

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II. Chemical Analysis and Histological Observation of Blue Meat

Although the prevention of blue discoloration of the meat of canned crab has been established,[9] its causes and mechanisms are unknown, and there is no general agreement about its occurrence. Existing chemical studies of canned crab with special reference to blue discoloration concern the copper content of the meat, and it has been found that the degree of blue colouring depends on the copper content.[10] The present chapter describes chemical composition and histological characteristic of the blue meat.

Materials and Methods

King crab, Paralithodes camtschatica, stored at -20°C for 2 months, was divided into the meat portions of shoulder (protopodus), first leg(merus), second leg(carpus), third leg (propodus) and claw(dactylus)(Fig. II-1). The portions were thawed and packed in tin containers, vacuum sealed, and processed for 60 minutes at 115.2°C. The copper content of the meat was determined before and after canning. Blue meat and normal meat in the contents of each can were separated as thoroughly as possible by hand. They were ground separately and each was added to ten times its volume of deionized water, allowed to stand for 20 minutes and filtered, the filtrate being used for pH and volatile basic nitrogen measurements. Volatile basic nitrogen was determined by microdiffusion analysis and total nitrogen by the Kjeldahl method. Tomiyama's method using 10 g of ground meat was employed for the determination

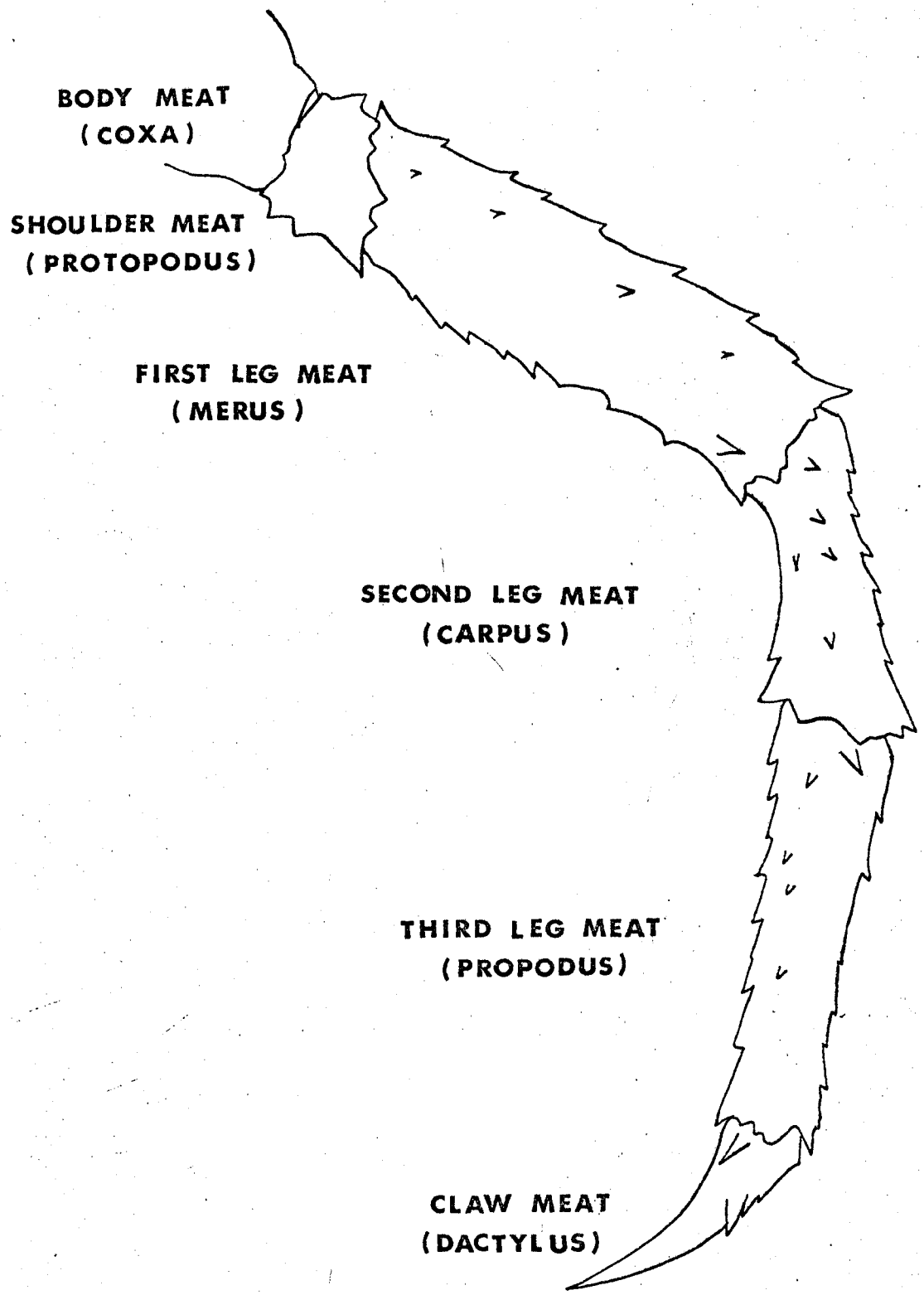


Fig. II-1. Nomenclature of the various parts of a king crab, Paralithodes camtschatica, leg.

of hydrogen sulphide.[16] The iron and copper contents were measured by the methods of o-phenanthroline[17] and A.O.A.C.,[18] respectively. Both blue and normal meats were fixed in Bouin's solution, embedded in paraffin, and sliced at 10 μ for microscopic observation. Raw king crab shoulder muscle containing blood was heated for 60 minutes at 115.2°C and treated as above.

Results

As seen Table II-1, a higher copper level was found in the blue meat, while volatile basic nitrogen, total nitrogen, iron, hydrogen sulphide and ash contents were almost the same in the two types of tissue. Copper contents in blue and normal meats were 2.80 and 0.49 mg%(wet weight) in average, respectively(Fig. II-2). It was found that shoulder, surface of first leg, claw and near joint meats contain comparatively large amounts of copper, and blue discoloration has been found frequently in these parts(Table II-2). Where the parts had been canned, the copper content maintained high level (Table II-3). Histological differences between the blue and the normal meat is shown in Figs. II-3 and II-4. Amorphous coagulum was found among muscle fibres from the blue meat (Fig. II-3), though little was found in the normal meat(Fig. II-4). The meat containing blood gave the appearance shown in Fig. II-3. The fact suggests that the coagulum found in the blue meat might be heat-coagulated blood, but this should be confirmed in a further study.

Discussion

The meat of king crab exhibiting blue discoloration contained

Table II-1. Chemical analysis of blue and normal meats in canned king crab.

	Blue meat	Normal meat
pH	6.95	6.89
Volatile basic nitrogen(mg%)	23.3	22.4
Total nitrogen(%)	4.36	4.23
Copper(mg%)	3.52	0.41
Iron(mg%)	0.74	0.81
Hydrogen sulphide(mg%)	2.01	1.80
Ash(%)	2.34	2.16

Table II-2. Copper content of the meat of different parts of king crab(mg%, wet weight of meat).

Parts	Copper content
Shoulder meat	2.15
First leg meat	1.25
First leg meat(cut)	2.10
First leg meat(surface)	3.16
Second leg meat	1.04
Second leg meat(broken)	1.30
Third leg meat	0.83
Third leg meat(cut)	2.05
Third leg meat(broken)	1.22
Claw meat	2.15
Claw meat(Immovable finger)	1.26

Table II-3. Copper content of the canned meat of different parts of king crab(mg%, wet weight of meat).

Parts	Copper content
Shoulder meat	1.64
First leg meat	1.14
First leg meat(surface)	1.19
Second leg meat(broken)	1.09
Third leg meat(cut)	1.61
Third leg meat(broken)	1.15
Claw meat	1.51

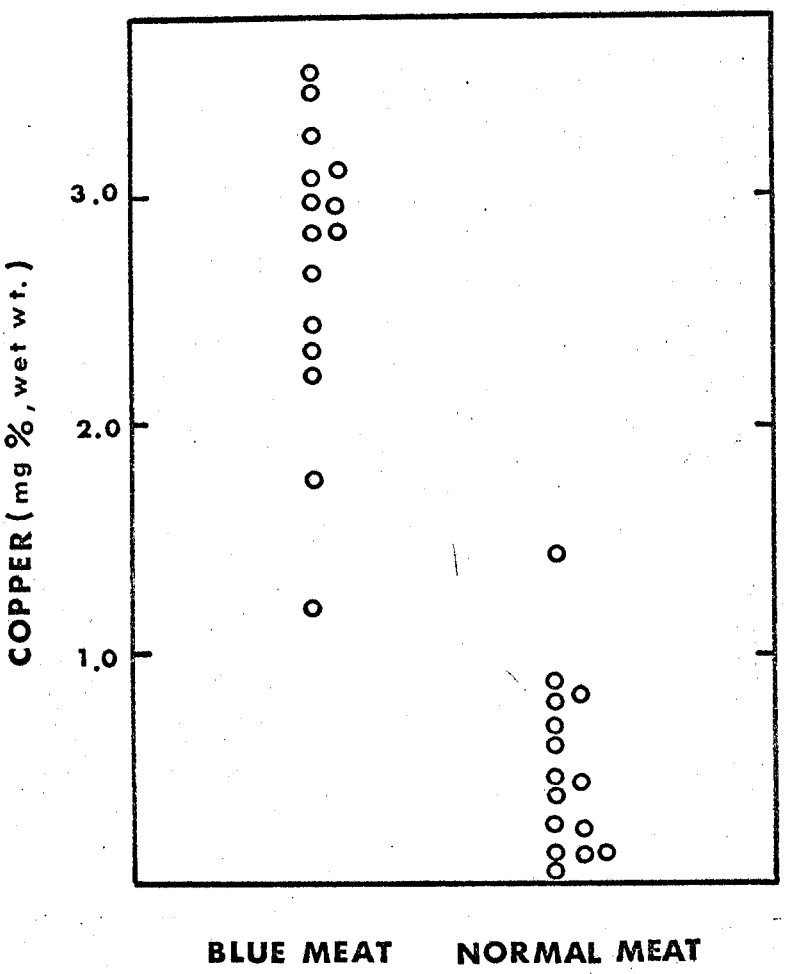


Fig. II-2. Copper content of blue and normal meats in canned king crab.

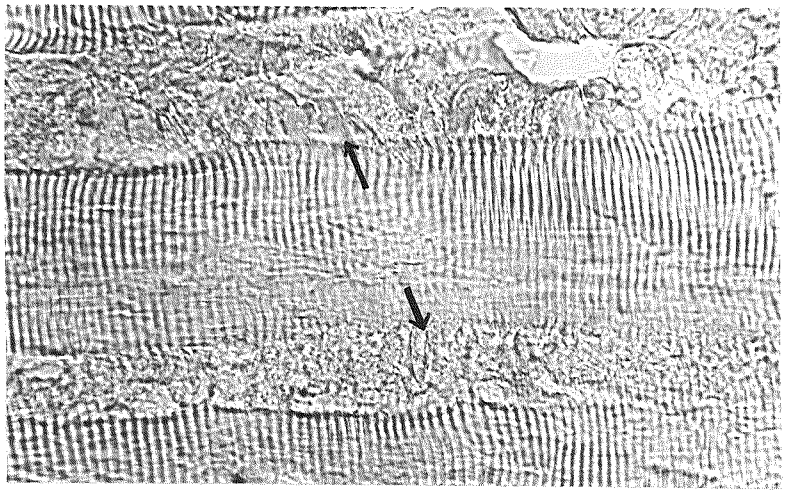


Fig. II-3. Blue meat of canned crab meat. (× ~~400~~ 656) Arrows indicate amorphous coagula.

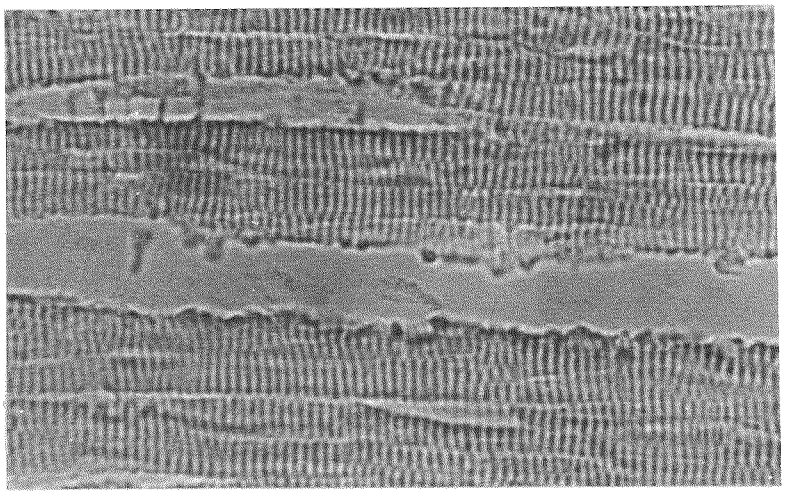


Fig. II-4. Normal meat of canned crab meat (× ~~400~~ 656).

an elevated level of copper. Oshima, Koizumi and Takisawa[6] have reported that the amounts of copper in blue and normal meats were 0.035 and 0.028%, respectively, while Sasa and Takeda[19] estimated the copper contents of blue and normal meats to be 6.964 and 3.417 mg%, respectively. The result from the present investigation is smaller than that of these papers, and is rather similar to that obtained by Fukuhara and Ohigashi[20] : 1.26 mg% in blue meat and 0.78 mg% in normal meat. The blue discoloration may occur whenever the copper content reaches more than 2 mg% in crab meat(Fig. II-2). Sasa and Takeda[19] suggested that the limit of copper content observed blue discoloration was 151~308 ppm(against dry matter) or 3.417~6.964 mg%(wet weight) in the meat. The result of the present study indicates that the limit of copper is less than their limit. Fukuhara and Ohigashi,[20] and Gordievskaya[10] suggested that the intensity of blue discoloration depended on the content of copper. In the present study, it was also found the same result. Though Gordievskaya[10] reported that lean meat and meat from the legs contain a large amount of copper and body and claw meats contain a small amount, in this study the claw meat contained a larger amount of copper than did the leg meats. This may reflect a difference in experimental conditions. The result of histological study suggests that the source of copper in blue meat is copper-protein(haemocyanin).

III. Detection of Haemocyanin in the Blue Meat

Many investigators[2),3),6),7),9),10)] have reported that haemocyanin or copper in haemocyanin might be a factor involved in the blue discoloration. The finding in a previous chapter also suggested that haemocyanin in haemolymph would be an amorphous coagulum found in a part of the blue meat.

The present chapter reports studies on haemocyanin in the blue meat following a modified method of a specific staining technique.[21)]

Materials and Methods

(1) First experiment

A sample of canned king crab was divided into blue and normal meat portions. Each 1 g of the portions was added to 5 ml of N/20 sodium hydroxide solution, and was left for 12 hours. After centrifuging at 1,500 × g for 20 minutes, the supernatant was removed and used as the sample. Crab haemocyanin solution was prepared as follows: The legs of freshly caught king crab, Paralithodes camtschatica, were separated from carapace, and any body fluid exuding from the cut end was collected. The body fluid was stirred for 10 minutes and filtered through a thin glass wool pad. The filtrate was stored -20°C. The frozen filtrate was thawed at 5°C, filtered through a glass wool pad and then dialyzed against deionized water for two days. The precipitate was centrifuged off at 2,000 × g for 20 minutes, and the supernatant was used for experiment. The haemocyanin thus obtained was heated at 100°C for 15 minutes, and was treated with the procedure as to the blue and normal meats.

Detection of haemocyanin was followed by a modified method of Manwell and Baker.[21] A reagent solution was prepared by mixing 10 ml of 1% dianisidine solution, 10 ml of 0.6M acetate buffer (adjusted pH 5.7), and 30 ml of ethyl alcohol. The mixture was made up to 100 ml of final volume with deionized water ("Diluted dianisidine solution"). For haemocyanin detection 1 ml of the test solution was added to 1 ml of the diluted dianisidine solution with few drops of 30% hydrogen peroxide. Haemocyanin was detected by a colour change.

(2) Second experiment

The blue and normal meats were collected from commercially packed king crab. Heat-treated crab haemocyanin was prepared as similar as that in the above experiment. The blue and normal meats and heat-treated crab haemocyanin were used for the sample. Each of the samples was washed 3 times with deionized water and dipped directly into the diluted dianisidine solution for 5 minutes. A few drops of hydrogen peroxide was then added. The haemocyanin was detected within 10 minutes after dipping the samples in the diluted dianisidine solution, because a brown colour develops during leaving for a longer period.

Results

The results are summarized in Table III-1. A haemocyanin-like reaction was found in sodium hydroxide extracts of the blue meat, while it was not detected in the normal meat extracts. If the normal meat extracts were left for several hours, the solution became brown in colour. This is caused through oxidation of dianisidine, so could be removed by adding

Table III-1. Haemocyanin-like reaction of blue meat, normal meat, coagulated haemocyanin by heating and sodium hydroxide extractives of them.

	Haemocyanin-like reaction
Blue meat	+
Sodium hydroxide extractive of blue meat	+
Normal meat	-
Sodium hydroxide extractive of normal meat	-
Coagulated haemocyanin by heating	+
Sodium hydroxide extractive of coagulated haemocyanin by heating	+

+; Haemocyanin-like reaction is positive.

-; Haemocyanin-like reaction is negative.

sodium hydrosulphite. In the second experiment, it was also found that the blue meat and heat-coagulated haemocyanin gave a positive result of haemocyanin reaction, while the normal meat gave negative. As seen in Figs. III-1 and III-2, haemocyanin was found in the joint portions of raw and boiled crab meats. This fact obviously indicates that there would be a large amount of haemocyanin in the joint portion. This fact also agrees with the finding reported in a previous chapter in which a high copper content was measured in joint portion.

Discussion

Osakabe[9] reported that the blue discoloration could be prevented by using a low temperature and fractional heating. According to his experiments, the coagulating temperature of blood protein of crabs is from 69 to 70°C, and that of meat protein of crabs is from 59 to 60°C. If the carcasses are heated at 59 to 60°C the meat coagulates, but the uncoagulated blood will run out. This method is based on the idea that haemocyanin is the causative substance in the blueing reaction. The copper of haemocyanin also correlates to blueing in canned crab meat.[6),7)] Earlier investigations on the cause of blue discoloration have reported that the removal of haemocyanin was less [9)] the frequencies of discoloration, and that a large amount of copper was detected in the portion of blue discoloration.[6),7),10),19)] However, these studies have not provided data confirming the presence of haemocyanin in the region of blue discoloration, but have only given indirect evidence. From the result in the present study, haemocyanin

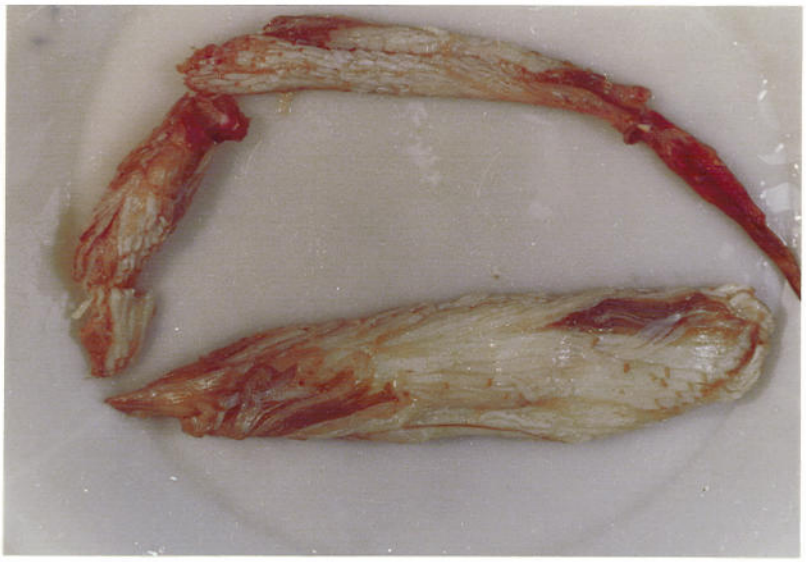


Fig. III-1. Haemocyanin detection in raw king crab leg meat by dianisidine – hydrogen peroxide.

Fig. III-2. Haemocyanin detection in boiled king crab leg meat by dianisidine – hydrogen peroxide.

was found in the portion of higher copper content in crab meat. Consequently, a causative substance of the blue discoloration is undoubtedly haemocyanin contained in the haemolymph.

IV. Influence of Hydrogen Sulphide upon Copper in Crab Haemocyanin with Reference to Blue Discoloration

A previous chapter in this series described the involvement of haemocyanin in the blue discoloration of canned crab meat. However, haemocyanin itself does not give any blue colour by heating. This fact suggests that a certain of chemical composition must involve in the colour development of crab haemocyanin.

As to the blue discoloration of fish products, it is known that a reactant produced from myoglobin, cysteine and trimethylamineoxide is a causative substance of green meat of canned tuna.[22] Thus, the present author has first examined the reaction of haemocyanin with trimethylamineoxide and cysteine in order to ascertain whether or not these compositions may involve in the blue discoloration of canned crab.

While, it has been reported that the blue discoloration of crab meat is caused by a Cu-protein complex formed from the decomposition products of protein(H_2S) and oxyhaemocyanin.[10] This indicates that hydrogen sulphide forms a blue pigment through the reaction with oxyhaemocyanin.

The present chapter describes influence of some chemical compositions upon copper in crab haemocyanin with special reference to the blue discoloration.

IV-1. Colouring of crab haemocyanin with sodium hydrosulphite, trimethylamineoxide, cysteine, ammonium sulphide, and hydrogen sulphide

A study was made to observe which compound among sodium hydrosulphite, trimethylamineoxide, cysteine, ammonium sulphide and hydrogen sulphide may develop blue colour with crab haemocyanin by heating.

Materials and Procedures

Haemocyanin solution was prepared from king crab, Paralithodes camtschatica, as described in a previous chapter (III). Two ml of the haemocyanin solution was poured into each of 5 test tubes. In the first tube, hydrogen sulphide was passed through until the solution became brown in colour. To the second was added 1 ml of 0.1 M ammonium sulphide solution. To the third and the fourth tube was added 1 ml of 2.16 mM trimethylamineoxide hydrochloride and 3.24 mM cysteine hydrochloride, respectively. And to fifth tube was added 1 ml of 2.16 mM trimethylamineoxide hydrochloride and 3.24 mM cysteine hydrochloride. They were heated for 15 minutes at 100°C, and were used as the sample for organoleptic colour assessment. In order to examine the effect of the compound contained in crab meat on the colour development, raw king crab was homogenized in a mortar, 1 g was removed, and was added to an equal volume of deionized water. The suspension was treated with hydrogen sulphide and was boiled for 15 minutes. Sodium hydrosulphite was the reducing agent in this reaction. Haemocyanin was detected by the method described in a previous chapter.

Results

The results are shown in Table IV-1. The colour of the haemocyanin solution changed brown after treating with hydrogen

Table IV-1. Colour of coagulum of crab haemocyanin when it was heated with hydrogen sulphide or with other reagents.

Reaction mixture*	Blue discoloration by heating**
Hcy	-
Hcy + H_2S	+
Hcy + H_2S + SHS	+
Hcy + $(NH_4)_2S$	+
Hcy + TMAO	-
Hcy + Cys	-
Hcy + TMAO + Cys	-
Crab leg meat + H_2S	-

*, Hcy; Haemocyanin

SHS; Sodium hydrosulphite

TMAO; Trimethylamineoxide

Cys; Cysteine

**, Heated at 100°C for 15 minutes.

+; Blue green coagulum was observed on standing after the heating.

-; White coagulum remained on standing after the heating.

sulphide, and produced a white coagulum on heating. Characteristic absorption bands at 338 mμ and 570 mμ of oxyhaemocyanin disappeared after adding hydrogen sulphide(Fig. IV-1). The white coagulum obtained by heating haemocyanin changed light brown in colour at first, then gradually changed to blue-green within 10 minutes(Fig. IV-2). When ammonium sulphide was added to the haemocyanin solution the colour changed to brown. This solution was not coagulated by heating, but coagulated and its colour changed to blue-green when neutralized with hydrochloric acid. This coagulum was similar in colour to that prepared by hydrogen sulphide. When the haemocyanin solution was heated without any sulphides, it only produced a whitish coagulum. Similarly whitish coloured coagula were obtained by heating the haemocyanin solution with trimethylamineoxide hydrochloride and/or with cysteine hydrochloride. In the case of dipping heat-coagulated haemocyanin into hydrogen sulphide solution, the colour changed bluish green on reheating for 15 minutes (Fig. IV-2). This fact suggests that heat coagulated haemocyanin may change to blue colouring compound in the presence of hydrogen sulphide. After leaving for 20 hours at 37°C, the colour of the haemocyanin changed from blue to brown. However, even if the haemocyanin deteriorated, its colour changed to blue-green after heating with hydrogen sulphide. Haemocyanin with or without oxygen became blue in colour after heating with hydrogen sulphide. There could not be observed any change in colour of ground meat of crab leg by heating with hydrogen sulphide. This fact suggests that the compound

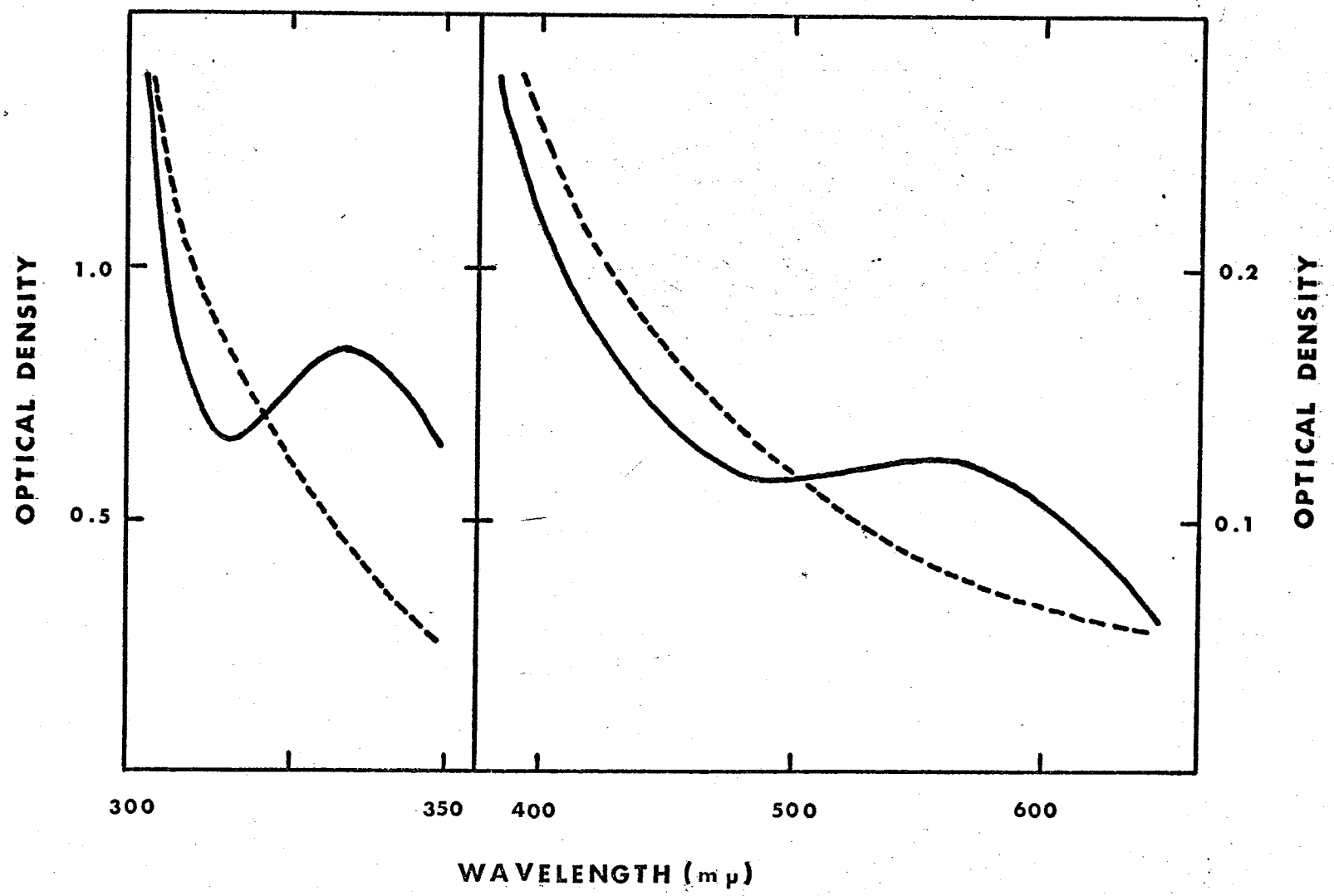


Fig. IV-1. Absorption spectra of oxyhaemocyanin and haemocyanin treated with hydrogen sulphide. Full line ——— , oxyhaemocyanin; dashed line ----- , haemocyanin treated with hydrogen sulphide.

Fig. IV-2. Coloured coagulum by heating (100°C, 15 minutes) with hydrogen sulphide.

in crab muscle apart from haemocyanin seldom affect development of the blue colour. In haemocyanin heated with hydrogen sulphide, the haemocyanin-like reaction was also detected by dianisidine – hydrogen peroxide.

From the results as above described, a cause and mechanism of the blue discoloration of canned crab meat is thought to be completely different from that of the green meat of canned tuna.

IV-2. Some properties of copper in haemocyanin

In the previous article, it was found that the blue discoloration is caused by the reaction between haemocyanin and sulphide. However, little is known which factor involves to the blue discoloration among copper in haemocyanin molecule, haemocyanin molecule including copper, or copper free protein molecule(apohaemocyanin). In the present article, a study was made in this respect.

Materials and Methods

Haemocyanin of king crab, Paralithodes camtschatica, was prepared as described in a previous chapter(III). Removal of copper from haemocyanin molecule and detection of copper were done according to the following procedure: The pH value of haemocyanin(2 ml) was adjusted to 2.3 by adding N/100 hydrochloric acid. With this treatment, the blue in colour of oxyhaemocyanin was faded away. This solution was dialyzed against 200 ml of deionized water for 16 hours at 5°C. This procedure was repeated 3 times. The total volume of the deionized water after dialysis was about 600 ml, and was concentrated to 5-6 ml by means of vacuum evaporator at 37°C. An aliquot of the

solution was used for polarographic detection of cupric ion in a mixture of N-ammonium chloride and N-ammonia as a supporting electrolyte solution. Copper content of the concentrated deionized water was estimated by the method of A.O.A.C.[18] The dialysate was precipitated by N/100 sodium hydroxide adjusting pH value to isoelectric point, collected by centrifugation at 1,500 × g for 20 minutes, and washed three times with deionized water(apohaemocyanin).

After treatment by hydrogen sulphide, the concentrated deionized water and apohaemocyanin was heated for 15 minutes at 100°C.

Haemocyanin added hydrogen sulphide(16 mg%) was precipitated by N/100 hydrochloric acid, then was separated by centrifugation at 1,500 × g for 20 minutes. The precipitate and supernatant were heated for 15 minutes at 100°C, and were observed organoleptically change in colour.

Results

Metal freed from haemocyanin by dialysis against deionized water was cupric ion as shown in Fig. IV-3. As seen in Table IV-2, throughout the dialysis, 72.4~74.0% of copper in native haemocyanin was liberated to the deionized water.

Apohaemocyanin being suspended in a solution containing 16 mg% of hydrogen sulphide, and heated for 15 minutes at 100°C, did not change in colour. On the contrary, the concentrated deionized water after dialysis changed greenish blue in colour by boiling after treating with hydrogen sulphide(Table IV-3). When the haemocyanin precipitated with hydrochloric acid was

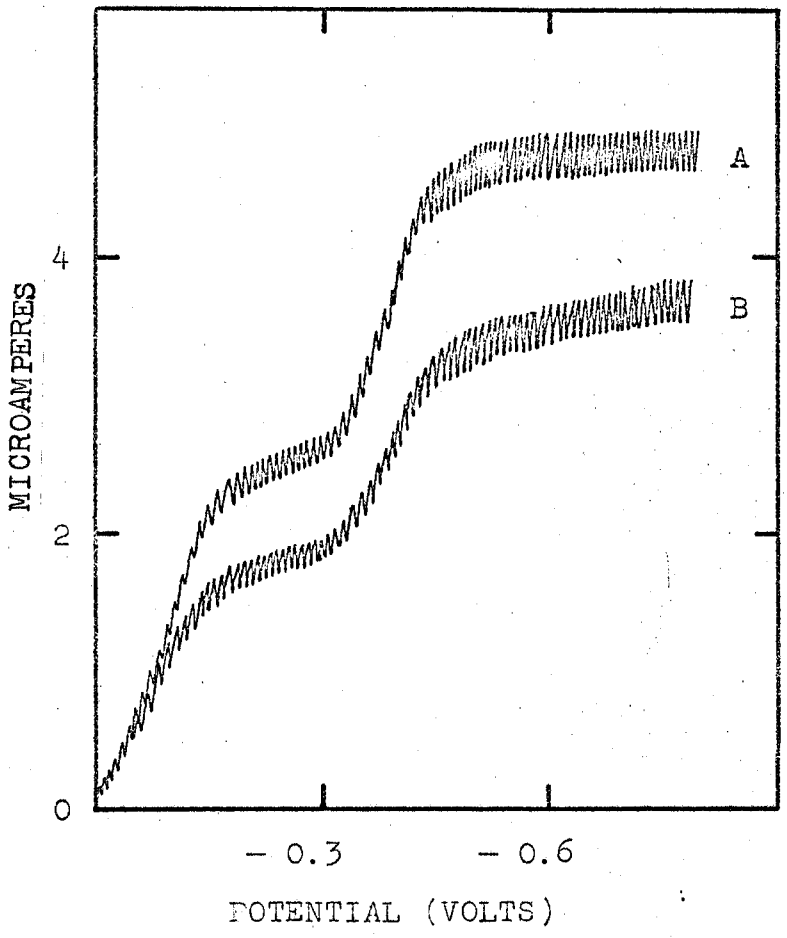


Fig. IV-3. Polarograms of Cu(II)(A) and metal ion of haemocyanin(B) in aqueous solution.

Table IV-2. Copper liberated by dialysis of haemocyanin at pH 3.2.

No.	Total copper (μg)	Copper liberated by dialysis (μg)	Percentage of copper liberated by dialysis
1	31.6	23.4	74.0
2	32.5	23.6	72.4

Table IV-3. The effect of the components of haemocyanin in colour change by hydrogen sulphide and heating.

	Hydrogen sulphide treatment	Heating for 15 minutes at 100°C
Haemocyanin	Brown	Greenish blue
Apohaemocyanin	White	White
Concentrated de-ionized water after dialysis	Brown	Greenish blue

Table IV-4. The effect of heating between cuprous or cupric ion and hydrogen sulphide.

	Hydrogen sulphide treatment	Heating for 2 minutes at 100°C	After heating for 15 minutes at 100°C
Cuprous ion	Brown	Dark green	Greenish blue precipitate
Cupric ion	Brown	Dark green	Greenish blue precipitate

added with hydrogen sulphide the colour changed to brown, while the supernatant was colourless. The brown coloured precipitate changed to greenish blue by heating for 10 minutes at 100°C. From the result, it appears that copper in crab haemocyanin acts an important rôle involving in the blueing with hydrogen sulphide. Protein moiety of the haemocyanin molecule seems to be not concerned with in this respect.

IV-3. Reaction between copper ion and hydrogen sulphide

In above article, confirmed that the copper ion from crab haemocyanin reacts with hydrogen sulphide to develop bluish colour by heating. The fact that a reactant between haemocyanin and hydrogen sulphide develops brownish colour, has been observed by Takayasu and Fukuhara.[7] They, however, made a denial of the copper-sulphide complex which is a causative compound of the blue discoloration because it was different in colour from the blue meat colour. In this view, the following experiment was undertaken in order to learn whether cupric or cuprous ion may react with hydrogen sulphide to change blue-green in colour by heating.

Materials and Methods

Cupric sulfate and cupric chloride were used as cupric ion solutions(0.72 mM). Cuprous ion solution was prepared from the cupric ion solutions to add 4% hydroxylamine hydrochloride.[24]

Hydrogen sulphide was passed through untill the solution became brown in colour. The solution treated with hydrogen sulphide was heated for 15 minutes in a boiling water bath. Absorption spectrum was measured with a Hitachi Perkin-Elmer

UV-VIS Spectrophotometer 139.

Results

When hydrogen sulphide was passed through the solution of cuprous or cupric ions, the colour of them changed brown immediately after the treatment. However, the colour of the solutions changed dark green after heating for 2-3 minutes. Continuous heating the solution for 15 minutes formed precipitate of green bluish in colour(Table IV-4). Without heating, the solution only changed greenish or dark-green after leaving for two days at room temperature, then formed precipitate of green-bluish colour after leaving for three days.

Fig. IV-4 shows the change in colour intensity measured by absorption spectra. A minimum absorption was observed at 580~585 mμ after heating for 3 minutes. This result indicates that brown colour in the hydrogen sulphide treated solution change dark green or blue-green, then stable greenish blue by heating or stand for a long time.

Discussion

Takayasu and Fukuhara reported that blue colouration in canned crab meat is caused by sulphide derivative of haemocyanin.[13] However, little evidence was provided on the blueing based on a reaction between haemocyanin and hydrogen sulphide. From the findings in the present study, it is now clear that the colour of haemocyanin after heating with sulphides becomes bluish even it is heat-coagulated material. Although an earlier experiment[10] has shown that the blue colour was given in the presence of ammonia, the present work proves that ammonia

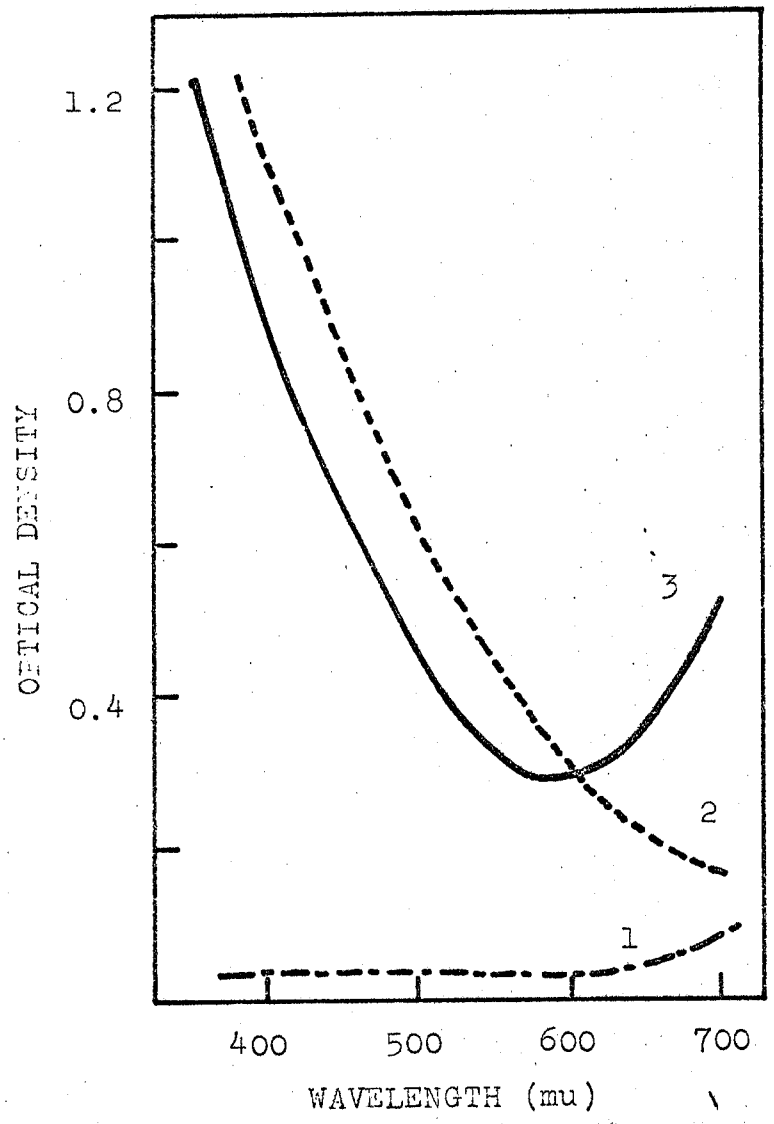


Fig. IV-4. Absorption spectra of Cu(II) solution, Cu(II) treated with hydrogen sulphide and its heat treated solution.
1, - - - - Cu(II); 2, ----- Cu(II) treated with hydrogen sulphide; 3, ——— heat treated solution of 2.

is not an essential compound to reveal the colour. The reason of the fact will be reported in a further chapter.

In the crab canning procedure, crab meat which is packed into tin container is the cooked meat. Therefore, crab haemocyanin must be heated twice times through cooking and processing. This means that cooked crab meat may cause blue discoloration if it is packed in a can and is heat processed. Thus, haemocyanin being coagulated is still active and can react with hydrogen sulphide to result in blueing.

Table IV-1 indicates that trimethylamineoxide and/or cysteine may not involve in the blue discoloration of canned crab meat. This fact suggests that the mechanism of the blue discoloration of canned crab meat differs completely from that of cooked tuna in which a causative compound of the green meat depends on forming of disulphide bond between cysteine and the sulfhydryl group on the denatured myoblobin.[22),23)] The results in Tables IV-3 and IV-4 indicate obviously that the copper in haemocyanin molecule reacts with hydrogen sulphide to cause colour change by heating. This fact suggests that the copper in haemocyanin molecule is the factor to cause the blue discoloration as well as hydrogen sulphide evolved to crab meat by heating.

V. Spectrophotometric Study of Blue Meat

In the inspection of canned crab meat, the colour has up to now only been assessed organoleptically, whereas any systematic study of discoloration, in this case the development of a blue colour, requires an objective assessment.

It has already been shown that crab haemocyanin can yield a dark greenish colour when it is heated with hydrogen sulphide in the previous chapter IV, and it seemed possible to us that there might be a similarity in reflectance spectrum between the compound so formed and the blue meat of unacceptable canned crab.

The present chapter describes spectrophotometric observations on blue and normal meats of canned crab and the reaction between crab haemocyanin and hydrogen sulphide.

Materials and Methods

A batch of raw king crab, Paralithodes camtschatica, caught in the Bristol Water in the summer fishing season was divided into two groups in a floating cannery. A group consisting of 5 crabs was packed in a parchment lined 1/2 lb flat can lacquered with zinc oxide for the inside(C-enameled) in order to prepare 25 of cans following the usual method. A second group consisting of 5 crabs was used for preparing haemocyanin solution as described in a previous chapter III. Blue and normal meats were separated from the canned crabs as described in a previous chapter II. A haemocyanin-sulphide complex was prepared as follows: the haemocyanin solution(2 ml) in a test tube was heated with 2 ml of solution containing 44 mg% of

hydrogen sulphide for 15 minutes at 100°C, cooled and the coagulated material was ground in a mortar. As a control sample, the haemocyanin solution without hydrogen sulphide was heated for 15 minutes at 100°C.

Red pigment in the epidermis was picked out by hand from the meat of the canned crab. Meat samples were then placed on filter paper, and any excess of water was removed. Three g were then inserted in a glass cell (diameter 2.0 cm), and the reflectance was measured within the range of wavelength 400 to 700 mμ. The blue meat was mixed with the normal meat in various proportions ranging 0 ~ 100% to make 3 g in total weight. The haemocyanin-sulphide complex was mixed with the heat-coagulated haemocyanin prepared as described above. The percentage reflectances of the mixtures were measured at 550 mμ. The colour intensity was calculated from the following equation.

Colour intensity = Percentage reflectance of normal meat at 550 mμ minus percentage reflactance of blue meat at 550 mμ

or

Colour intensity = Percentage reflectance of heat-coagulated haemocyanin at 550 mμ minus percentage reflectance of haemocyanin-sulphide complex at 550 mμ

A Hitachi Perkin-Elmer 139 Spectrophotometer fitted with diffuse reflectance apparatus was employed.

A stock copper solution contained copper 1.2 μg/ml was

prepared and used for the experiments. Each 5 ml of the solution was passed hydrogen sulphide. The solution in 50 ml volume test tube with cotton, was added 10 ml of egg white and mixed thoroughly. A final concentration of the mixture was heated for 15 minutes at 100°C. After cooling, reflectances were measured at 550 mμ and estimated the colour intensity.

Results

Reflectance spectra of blue and normal meats are illustrated in Fig. V-1. A maximum reflectance was observed at 550~570 mμ in the sample of blue meat, but not in the sample of normal meat within the range studied. If the colour intensity of a blue meat sample was slight, the maximum reflectance at 550 mμ was not obvious, but the percentage reflectance decreased as the meat became more deeply coloured. A maximum reflectance was also found at 530~550 mμ in the sample of haemocyanin-sulphide complex, but not in the heat-coagulated haemocyanin(Fig. V-2). Red pigment from the epidermis showed a minimum reflectance at 480~490 mμ(Fig. V-3). The results in Figs. V-1 and V-2 indicated that the blue meat and the haemocyanin-sulphide complex yielded slight differences in the wavelengths of maximum reflectance. However, where the red epidermal pigment was added to the haemocyanin-sulphide complex, minimum and maximum reflectances were found at 480 mμ and 550~570 mμ respectively, closely resembling those of the blue meat(Fig. V-4). Since the blue meat contained considerable amounts of the red pigment, the wavelength of the maximum reflectance of the blue meat seems to be longer than that of the haemocyanin-sulphide complex

alone. As shown in Fig. V-1, the percentage reflectance of blue meat is decreased by an increase in the intensity of pigmentation. A linear relationship was found between the logarithm of blue meat content in normal meat and the colour intensity(Fig. V-5). In the case of haemocyanin-sulphide complex, a linear relationship was also found between the logarithm of haemocyanin-sulphide complex content in heat-coagulated haemocyanin and the colour intensity(Fig. V-6). The relationship in Fig. V-5 can be represented by the equation

$$y = 2.0e^{0.138x}$$

where y is the blue meat content of the sample and x the colour intensity. The relationship for Fig. V-6 is

$$y = 2.1e^{0.138x}$$

where y is the content of haemocyanin-sulphide complex in the heat-coagulated haemocyanin and x again the colour intensity.

The colour intensities were increased by increasing of copper concentration(Table V-1). When the copper concentration was 0.5 mg%, light greenish colour was found visible. At 1.0 mg% of copper concentration, greenish colour was found clearly.

Discussion

Fukuhara and Ohigashi[13] and Gordievskaya[10] suggested that the intensity of blue discoloration depended on the copper content. These are also evident from the present results. There is a similarity between both equations(Figs. V-5 and V-6) which suggests that the colour intensity is related to the copper content combined with sulphide, and the analysis of percentage reflectance of the blue meat offers good possi-

bilities for colour assessment of the blue discoloration. For meat to quality as desirable, the colour intensity must be less than 10%, at which level it cannot be distinguished from normal. The copper content was 0.87~1.4 mg% at this level. Table V-1 shows that the copper concentration required for visible change is 0.5~1.0 mg% in egg white. These suggests that desirable copper content is less than 1.0 mg%.

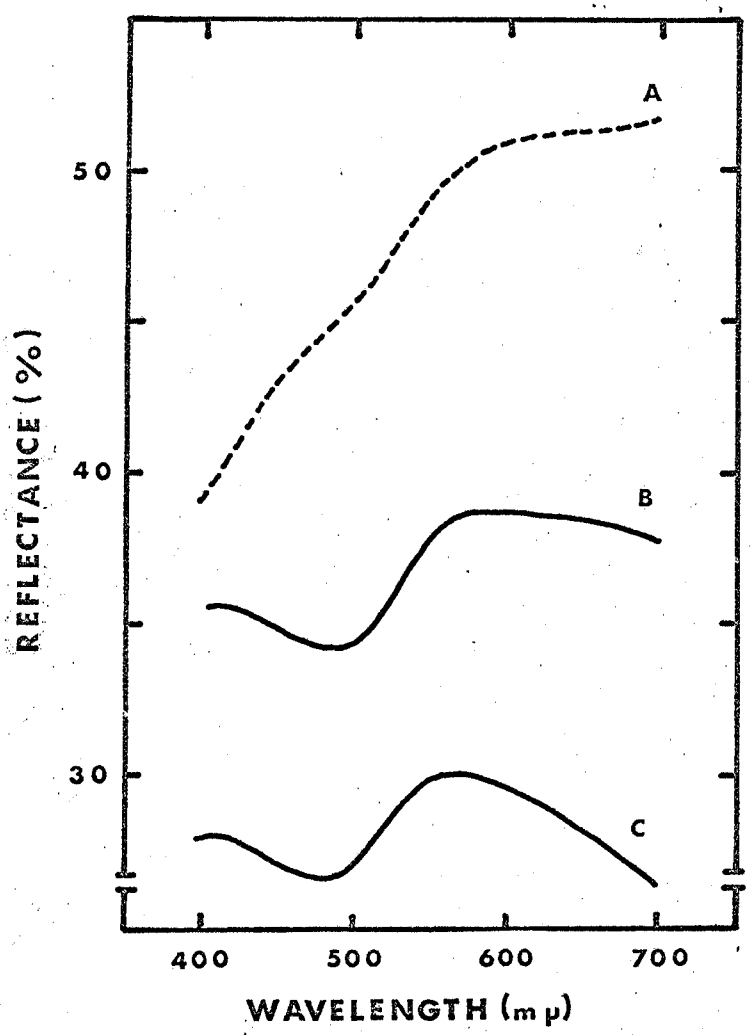


Fig. V-1. Reflectance spectra of the normal meat(A), slightly coloured blue meat(B) and strongly coloured blue meat(C) in canned king crab.

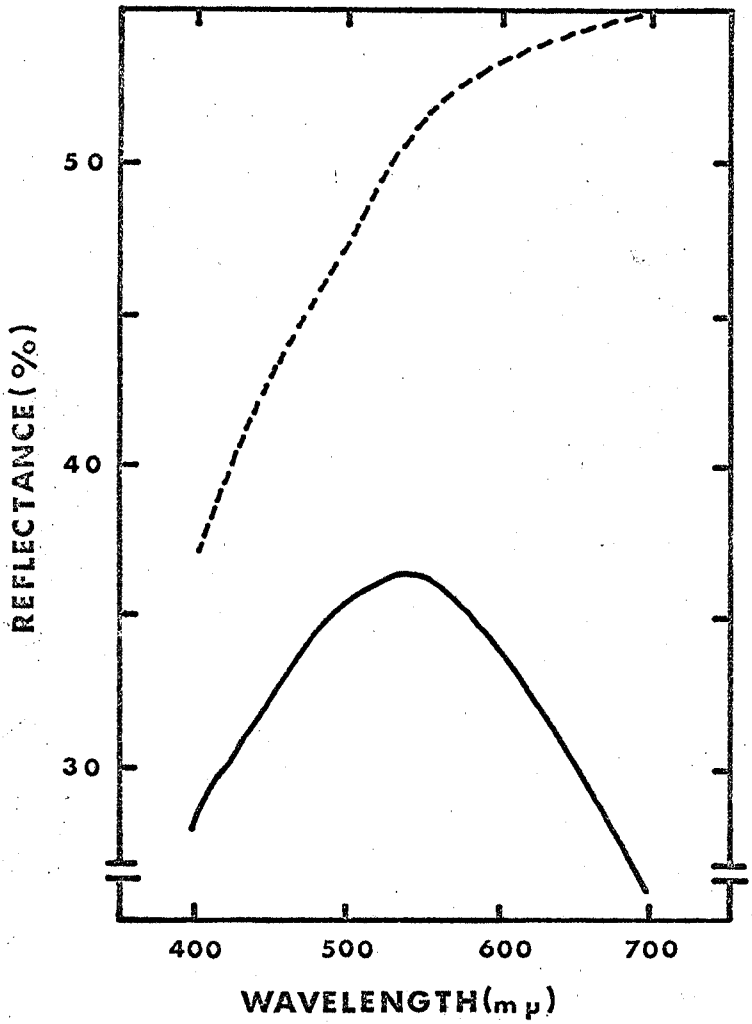


Fig. V-2. Reflectance spectra of the heat-coagulated haemocyanin and haemocyanin-sulphide complex. Dashes line ---- heat-coagulated haemocyanin; full line —— haemocyanin-sulphide complex.

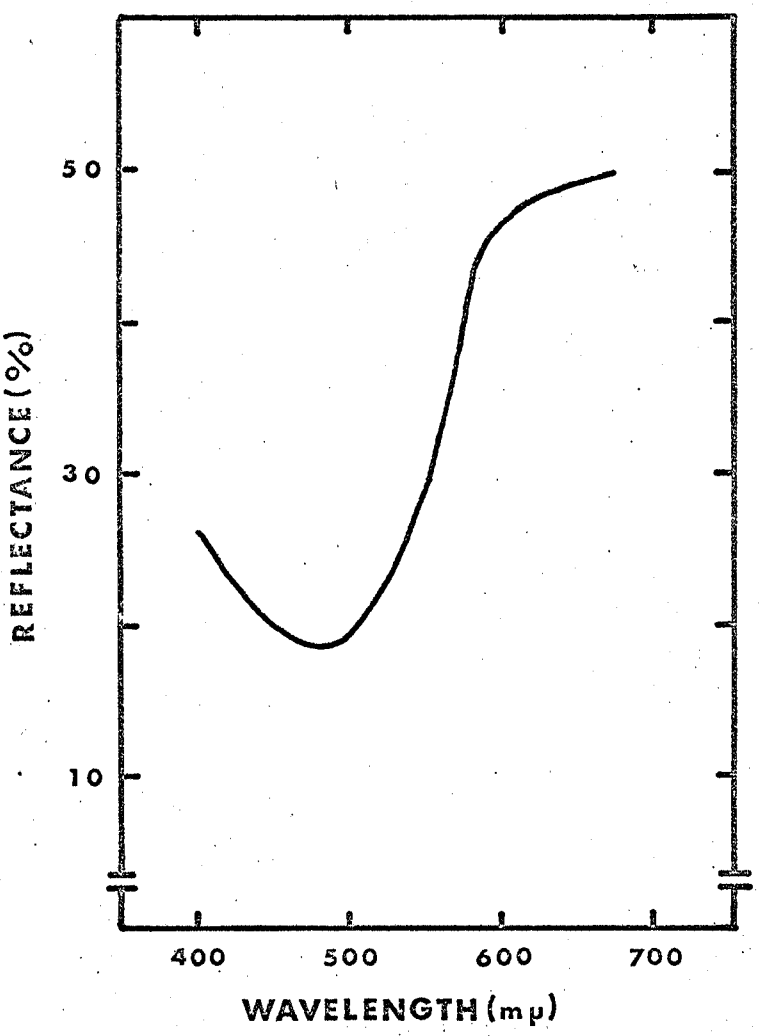


Fig. V-3. Reflectance spectrum of the red pigment of epidermis.

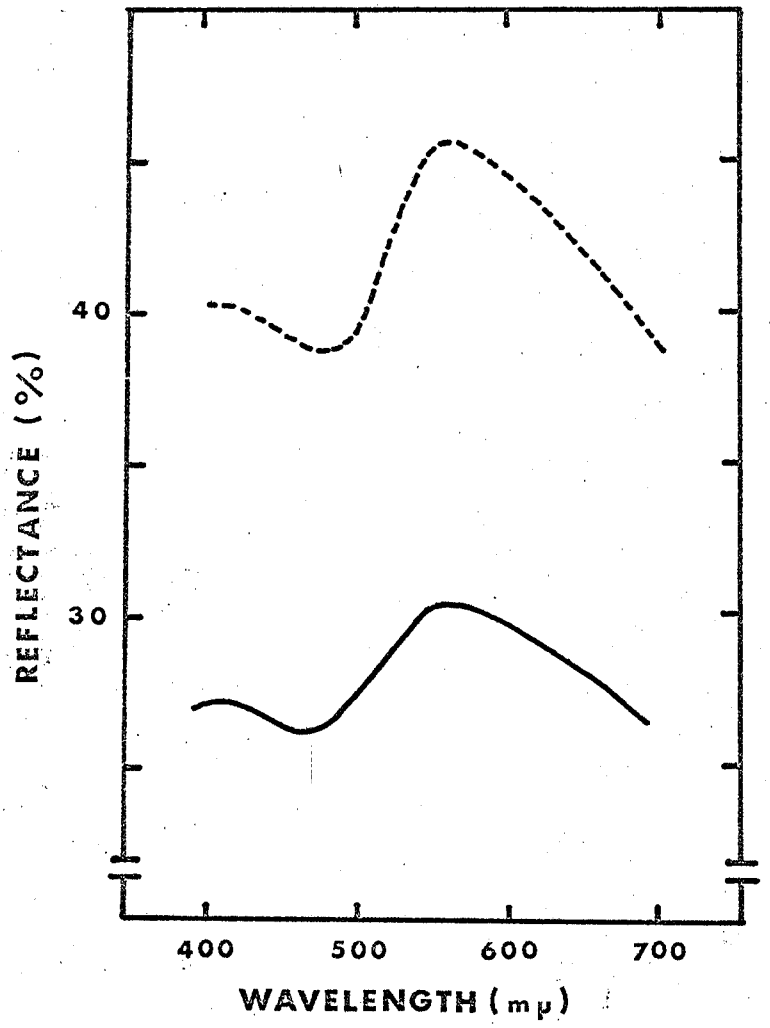


Fig. V-4. Reflectance spectra of blue meat and haemocyanin-sulphide complex with red pigment of epidermis. Dashes line ---- haemocyanin-sulphide complex with red pigment in epidermis; full line —— blue meat.

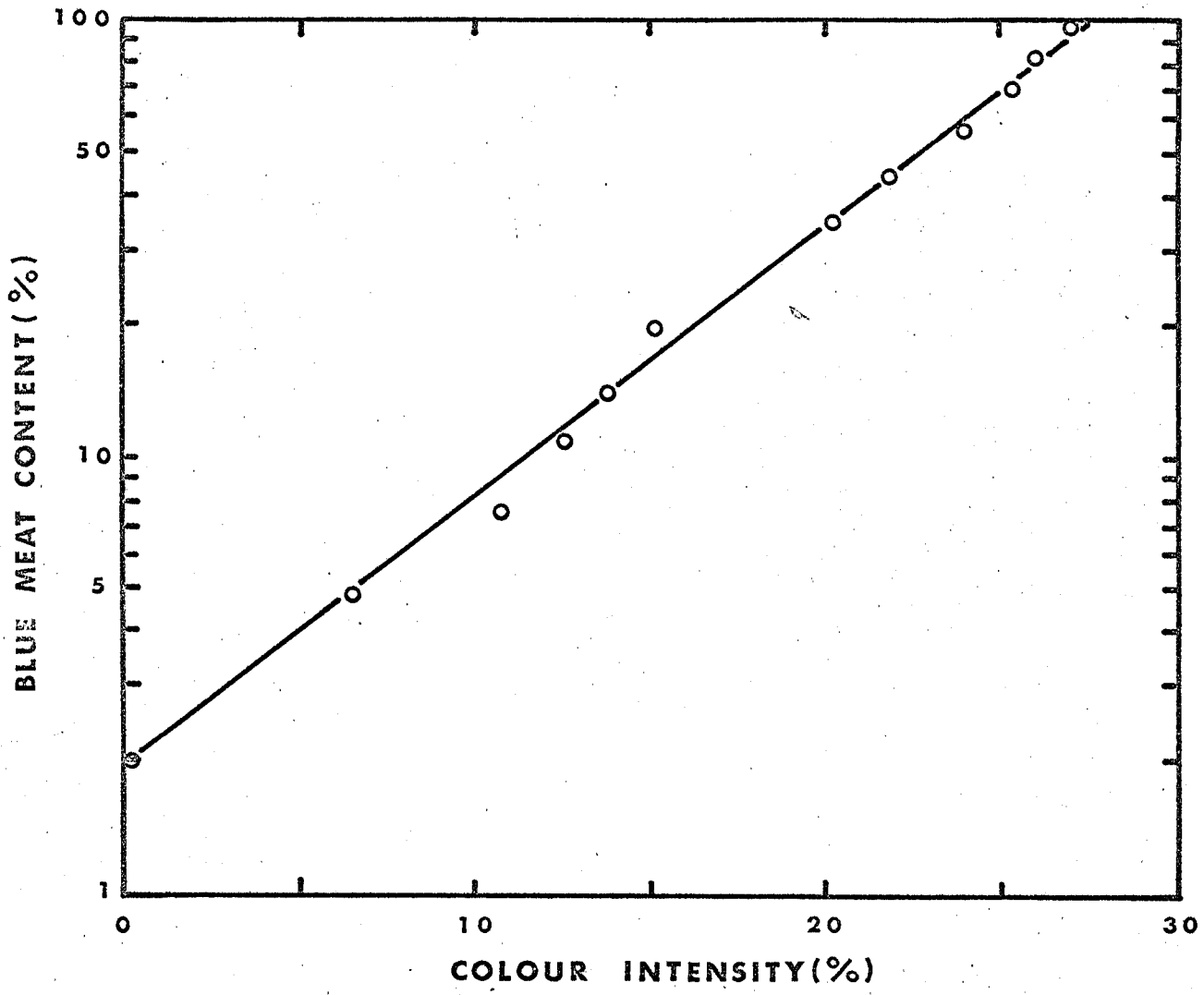


Fig. V-5. Relationship between the blue meat content in normal meat and the colour intensity.

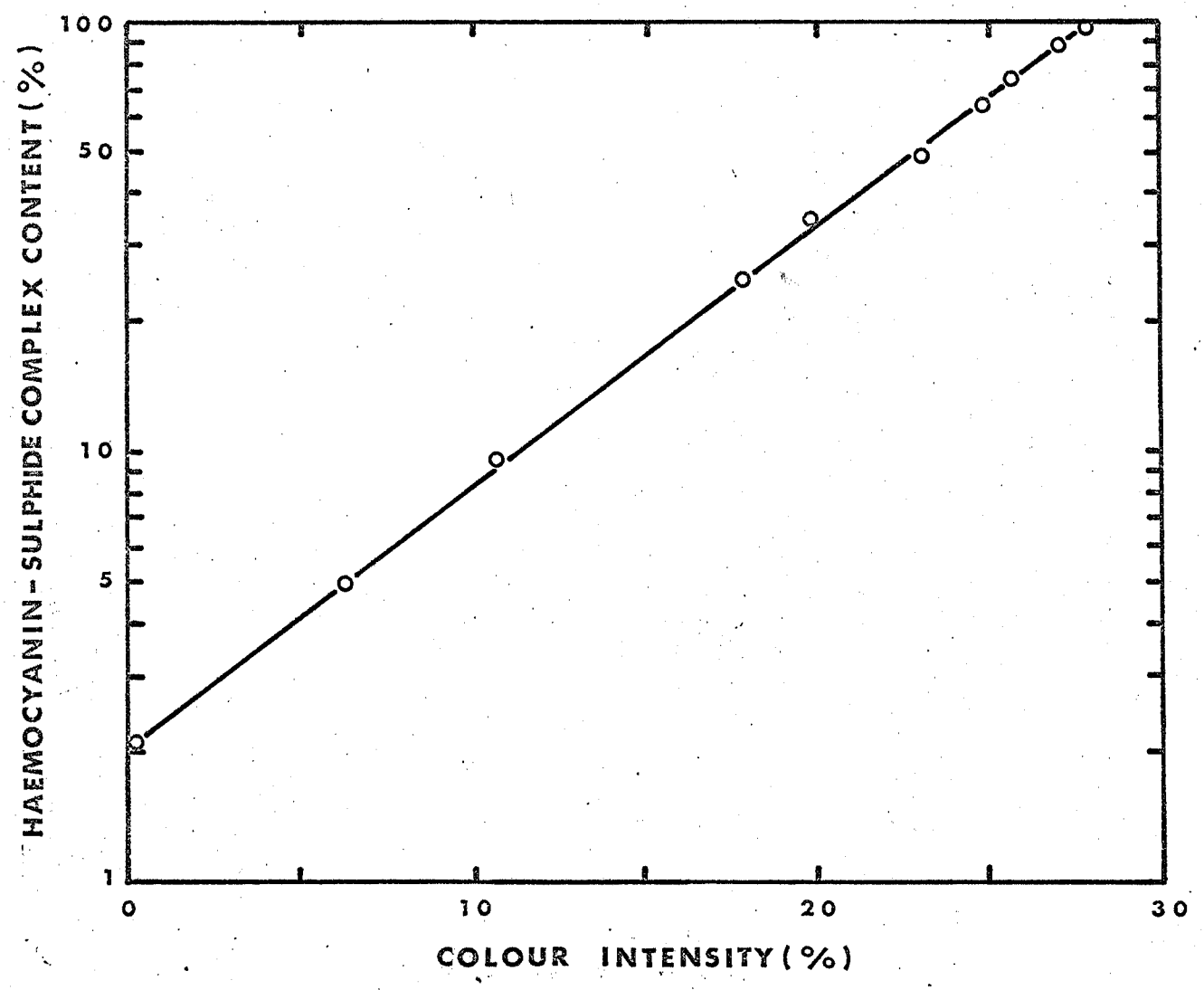


Fig. V-6. Relationship between the haemocyanin-sulphide complex content in heat-coagulated haemocyanin and the colour intensity.

Table V-1. Copper limits of visible blue colouration

Copper concentration (mg%)	Colour intensity	Colour development※
0	-	-
0.5	7.4	±
1.0	10.4	+
2.0	12.0	+
3.0	14.0	+

※, -; No colour change was found.

±; Colour change was found slightly.

+; Colour change was found clearly.

VI. Isolation of Causative Substance of Blue Meat

Although haemocyanin and hydrogen sulphide are involved in the blue discoloration of canned crab meat, processing studies in this laboratory have created a need for more detailed knowledge of the causative substance.

This work was undertaken to isolate it and study the chemical binding of copper in the blue substance.

Materials and Methods

Blue and normal meats in commercially canned king crab were separated as thoroughly as possible. Forty grams of blue meat was extracted by 200 ml of 1N sodium hydroxide, and fractionated as shown in Fig. VI-1. As the control sample, normal meat was treated as blue meat as well. After the fractionation, the fraction B was purified by a following procedure in order to obtain blue substance: The solution of fraction B was centrifuged at 32,600G for 30 minutes. The supernatant thus obtained was adjust to pH 6.2 and centrifuged at 32,600G for 1 hour. Blue-green pellet was finally obtained, and was dissolved in N/10 sodium hydroxide for the use of chemical analysis. Protein and copper were determined by biuret method[54] and the method of A.O.A.C.,[18] respectively. In order to study chemical characteristic of copper in haemocyanin, the effect of diazobenzenesulphonic acid(Table VI-3) was applied following the procedure of Wood and Bannister.[55] The amount of free copper was measured as cuprous by the modified method of Felsenfeld.[28] Haemocyanin-like reaction was detected by the method described in the chapter III.

Paper electrophoresis was carried out by veronal buffer(pH 8.6, μ = 0.05) at 5 mA for 4 hours.

Results

The fraction B extracted from blue meat contained high level of copper and showed remarkable haemocyanin-like reaction (Table VI-1), while fractions A and C showed slight haemocyanin-like reaction. Paper electrophoretic patterns were shown in Fig. VI-2. As seen in Fig. VI-2, the fraction B contained electrophoretically less fragments than the other fractions. While, the fractions extracted from normal meat contained no copper and indicated negative haemocyanin-like reaction. Protein, copper contents and haemocyanin-like reaction of blue substance purified from fraction B of blue meat were shown in Table VI-2, and the pattern of paper electrophoresis of the blue substance was shown in Fig. VI-3. As seen in Table VI-2, the blue substance contained larger amount of copper and less amount of free copper than that in the fraction B. This fact might be based on the purification of the fraction B. It appears that the blue substance contains less fragments than the fraction B in comparison with the Figs. VI-2 and VI-3. The copper of blue substance and king crab haemocyanin dialysed in the presence of EDTA was possible to remove after treating with 10 μmoles diazobenzenesulphonic acid per mg protein(Table VI-3).

Discussion

Wood and Bannister[55] reported that it was possible to remove over 90% of the copper of haemocyanin by treating it

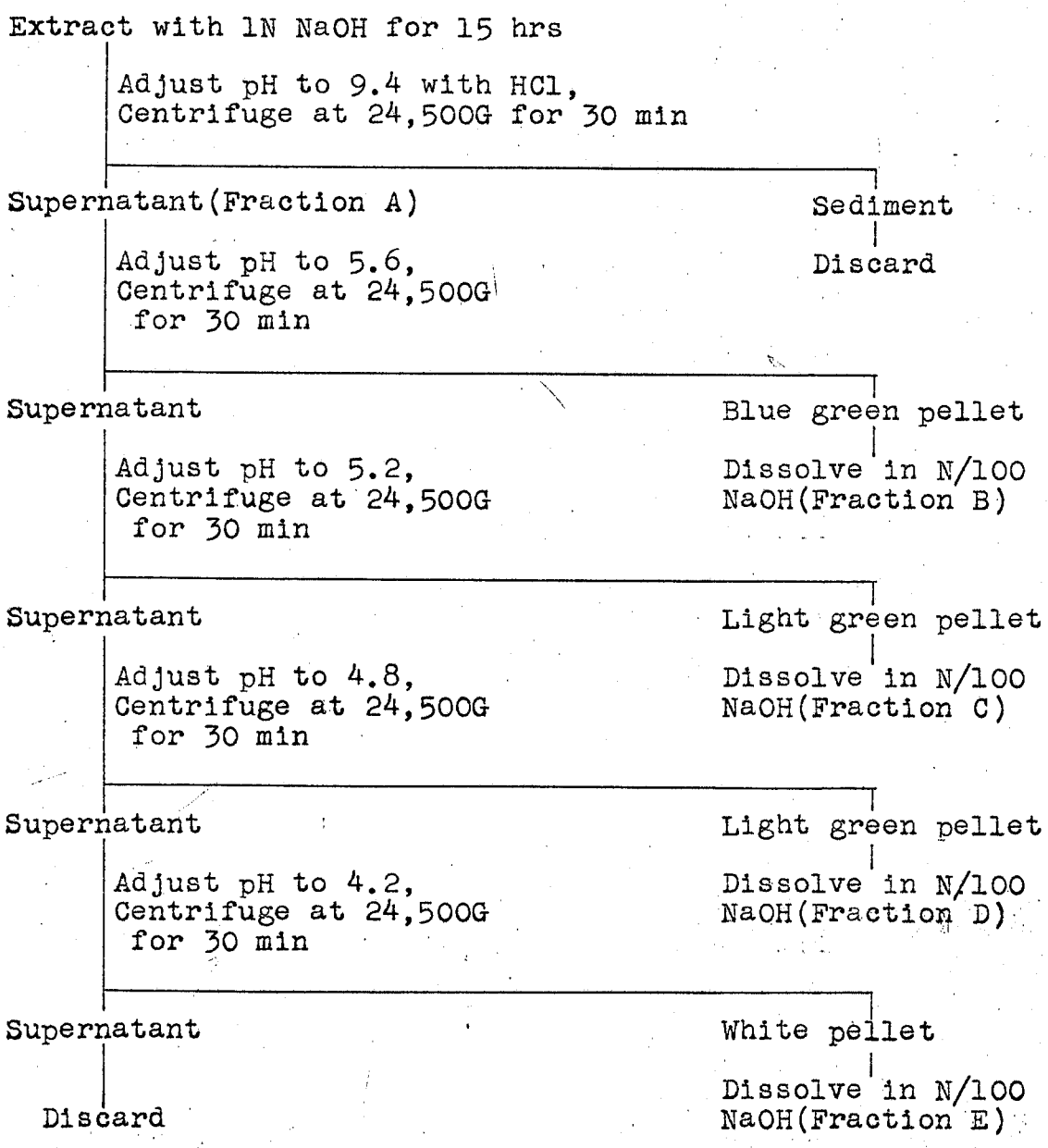


Fig. VI-1. Fraction preparation flow diagram from the blue meat

Table VI-1. Protein, copper contents and haemocyanin-like reaction of fractions extracted by 1N sodium hydroxide from the blue and normal meat

	Fraction	Protein (mg)	Copper (μg/mg)	Haemocyanin-like reaction※
Blue meat	A	2303	0.21	+
	B	49.5	7.03	++
	C	385.0	0.30	+
	D	800.0	0.0	-
	E	255.0	0.0	-
Normal meat	B	26.3	0.0	-
	C	182.0	0.0	-
	D	1055	0.0	-
	E	397.5	0.0	-

※ ++; Haemocyanin-like reaction is very clear.

+; Haemocyanin-like reaction is slight.

-; Haemocyanin-like reaction is negative.

Table VI-2. Protein, copper contents and haemocyanin-like reaction of fraction B and blue substance

	Protein (mg)	Copper (μg/mg)	Free copper (μg/mg)	Haemocyanin-like reaction※
Fraction B	49.5	7.03	1.67	++
Blue substance	19.5	7.52	0.12	++

※ See Table VI-1

Table VI-3. Effect of diazobenzenesulphonic acid on the blue substance and king crab haemocyanin

EDTA※	Percentage copper remaining	
	Blue substance	King crab haemocyanin
with	18.8	23.3
without	75.8	82.2

※ Dialysed against 0.1M phosphate buffer(pH 7.0) with or without the addition of 0.01M EDTA. Amount of diazobenzenesulphonic acid, 10 μmoles/mg protein; Duration of experiment, 10 minutes at 24°C.

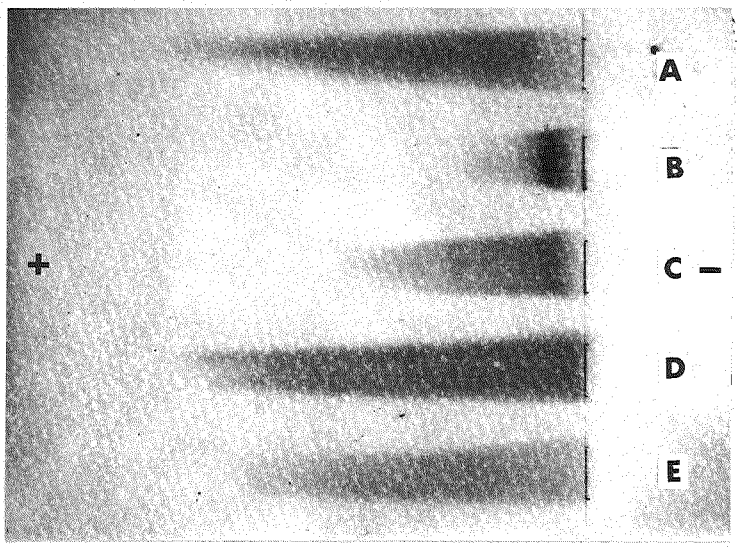


Fig. VI-2. Paper electrophoresis of fractions extracted by 1N sodium hydroxide from the blue meat
Veronal buffer(pH 8.6, μ = 0.05), Current at 5 mA for 4 hours, Stained with Amido black 10B

with 5~10 μmoles diazobenzenesulphonic acid per mg protein, provided that the product was dialysed in the presence of EDTA. They suggested that imidazole groups probably participate in the binding of copper in haemocyanin. In the present study, the copper of blue substance and king crab haemocyanin could be removed in the presence of EDTA. This would seem to indicate that the copper of blue substance was involved in haemocyanin derivative. The high level of copper content in blue substance indicates that the structure of haemocyanin might be destroyed during extraction procedure of blue meat with 1N sodium hydroxide. From the results of this chapter and previous chapters, it is suggested that the causative substance of the blue discoloration is haemocyanin derivative.

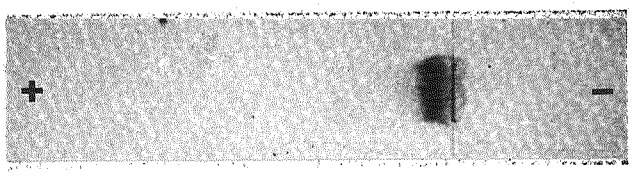


Fig. VI-3. Paper electrophoresis of blue substance Veronal buffer(pH 8.6, μ = 0.05), at 5 mA for 4 hours, stained with Amido black 10B

VII. A Mechanism of the Reaction between Copper and Sulphide

Fukuhara and Ohigashi[13] proposed that the blue discoloration seen in, for example, canned crab meat is caused by sulphide derivative of haemocyanin with a formula R-Cu-S-Cu-R or R-Cu-S, where R stands for a protein molecule. Gordievskaya[10] suggested that the blue discoloration of crab meat is caused by a copper-protein complex. In the study of this series also, it has been reported that the blueing was caused by a haemocyanin-sulphide complex. Little information exists with respect to the role played by copper in the haemocyanin-sulphide reaction. This chapter reports that state of copper in heat-coagulated haemocyanin and in haemocyanin-sulphide complex.

Materials and Methods

Heat-coagulated haemocyanin was prepared from haemocyanin of king crab, Paralithodes camtschatica, by heating at 100°C for 20 minutes, dehydrating with 95% ethanol, and drying under vacuum. A haemocyanin-sulphide complex was prepared as follows: heat-coagulated haemocyanin was dipped in 42 mg% hydrogen sulphide solution for 30 minutes, then heated, dehydrated and vacuum-dried. Blue and normal meats were prepared as described in a previous chapter VI, dehydrated and vacuum-dried. Infrared spectrum was measured by an infrared spectrophotometer, Japan Spectroscopic Co., Ltd, Model DS-301. X-ray diffusion spectrum was measured by X-ray spectrometer, Rigaku-Denki Co., Ltd, "Geigerflex".

Cuprous copper concentration determined by the method of

Felsenfeld.[28] A suspension was prepared by mixing 25 mg of the sample with 2 ml of distilled water, adding 1 ml of 4% hydroxylamine hydrochloride or 1 ml of 0.1M ethylenediaminetetraacetic acid(EDTA), or p-chloromercuribenzoate(PCMB) and 2 ml of a 0.05%(weight per volume) solution of 2,2'-biquinoline in acetic acid. The mixture was stirred well, diluted to 6 ml, and centrifuged at 10,000 × g for 20 minutes. The optical density of the supernatant was then estimated at 540 mμ. Total copper or cupric copper contents were determined by the A.O.A.C. method.[18] For the determination of sulphide content, the sample was subjected to reduction with divalent tin-phosphoric acid by the method of Ohashi.[29] The reduced sample was used for the determination of sulphide content by the method of Fogo and Popowsky.[30] Strong phosphoric acid and divalent tin-strong phosphoric acid were prepared as follows: 300 g of commercial orthophosphoric acid was concentrated by heating up to 300°C. Twenty g of tinnous chloride dihydrate was dissolved in 200 g of this phosphoric acid and the mixture was heated at 300°C in a carbon dioxide stream. The tinnous salt-strong phosphoric acid thus prepared was kept in a 100 ml buret fitted with a guard tube of anhydrous calcium chloride. The whole apparatus is shown in Fig. VII-1. Four to five mg of sample weighed accurately were placed at the bottom of the reaction vessel and 7 ml of tinnous salt-strong phosphoric acid added from the stock buret. Twenty ml of 1% zinc acetate solution was placed in each absorption flask. Air in the reaction and absorption vessels was displaced by a rapid flow

of carbon dioxide, which was reduced after 5 minutes to a rate of 120 bubbles per minute in the absorption flask. The reaction medium was then heated electrically for 20 minutes at 250~300°C. The hydrogen sulphide liberated from the medium was swept into the absorbent solution by the carbon dioxide, where it formed a white precipitate of zinc sulphide. After completion, 1 ml of 12% sodium hydroxide was added to the absorbing flask. The flask was stoppered and 5 ml of 0.5% N,N'-dimethylphenylenediamine hydrochloride and 1 ml of 0.023M ferric chloride were added. After leaving for 1 hour, the total volume was diluted to 50 ml and the absorbance was determined at 670 mμ.

Results

Fig. VII-2 illustrates the results of infrared spectra. From the result in Fig. VII-2, in no case, however, was any relation found which would help to explain the difference among blue and normal meats and heat-coagulated haemocyanin. X-ray diffraction patterns are shown in Fig. VII-3. As seen in Fig. VII-3, it also indicates little variation in the diffraction in patterns of the heat-coagulated haemocyanin and haemocyanin-sulphide complex.

As shown in Table VII-1, under reducing condition with hydroxylamine, most of copper in the heat-coagulated haemocyanin was detected as cuprous copper. No cuprous copper was detected with EDTA. When the heat-coagulated haemocyanin was not mixed with reducing or chelating reagents, one-half of the copper was

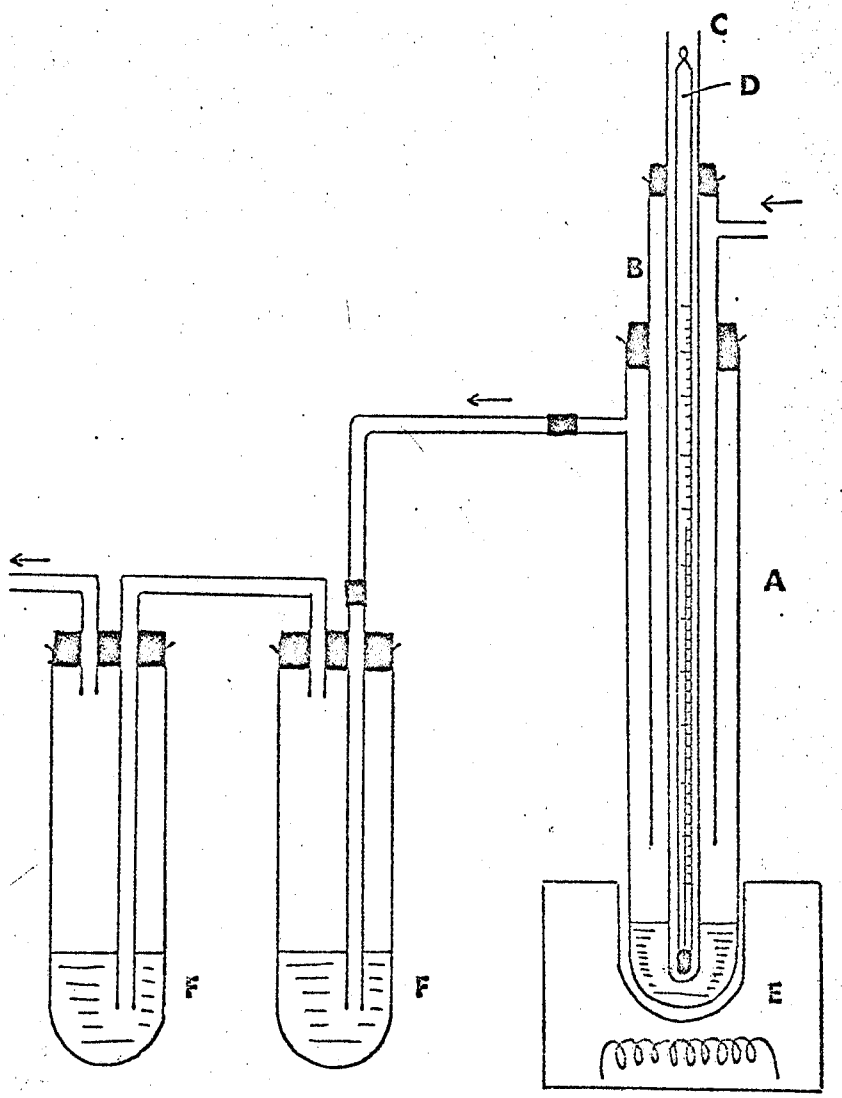


Fig. VII-1. Apparatus for estimation of hydrogen sulphide. A; reaction vessel, B; gas introducing tube, C; thermometer-protecting tube, D; thermometer, E; electric heater F; absorbing flasks.

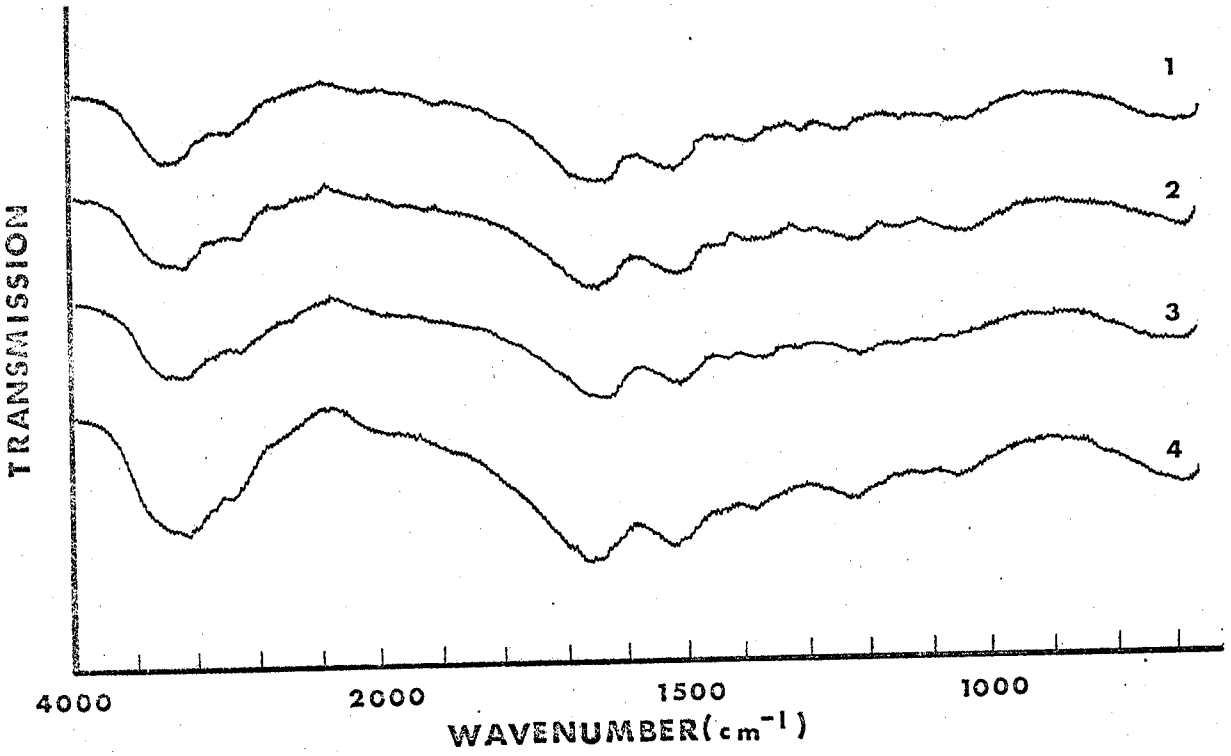


Fig. VII-2. Infrared absorption spectra of the normal(1) and blue(2) meats, heat-coagulated haemocyanin(3) and haemocyanin-sulphide complex(4) in KBr.

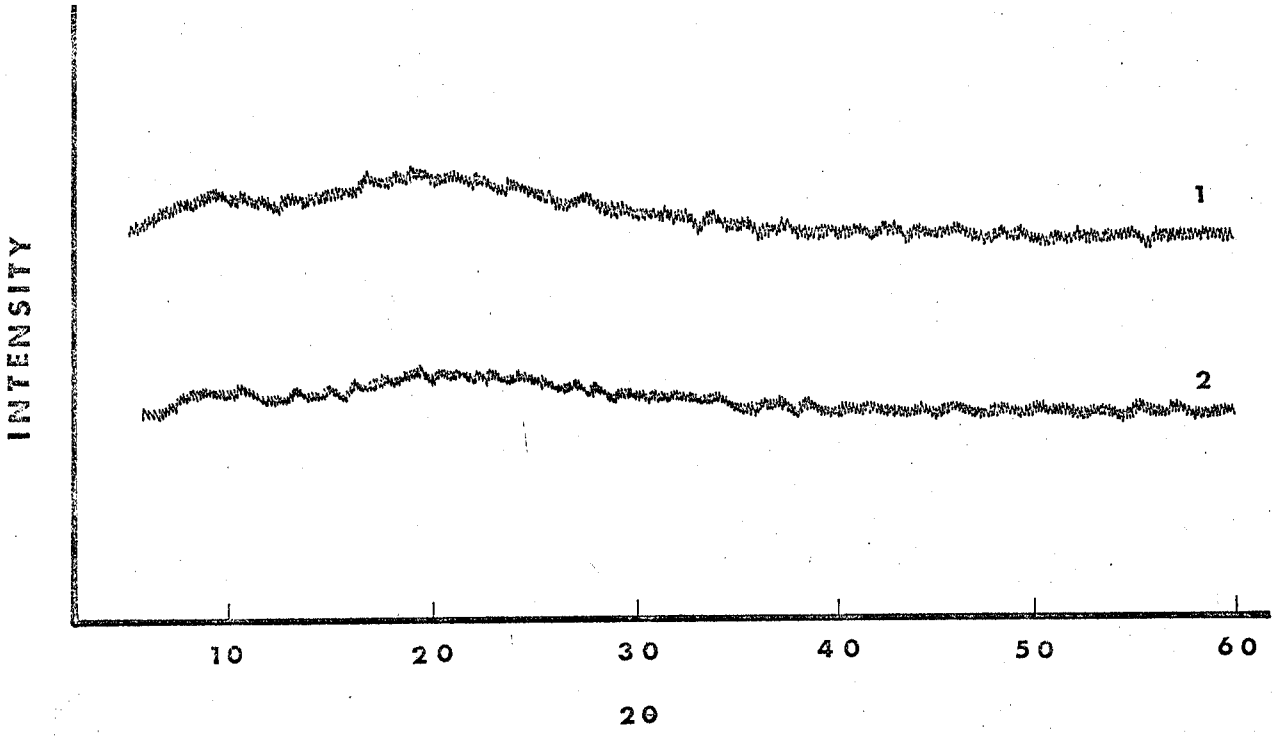


Fig. VII-3. X-ray diffraction patterns of the heat-coagulated haemocyanin(1) and haemocyanin-sulphide complex(2). Condition: Cu(K) radiation, Voltage; 35 kV, Current; 10 mA, Time const; 2, Divergence slit; 1°, Scattering slit; 1°, Receiving slit; 0.2 mm, Scanning speed; 2°/min.

Table VII-1. Copper state in the heat-coagulated haemocyanin at various conditions.

	Cuprous copper (μg/mg)	Cupric copper (μg/mg)
No addition	0.49	
Hydroxylamine	0.97	
EDTA	0.00	
Residue after cuprous copper estimation		0.17
Total copper		1.17

Table VII-2. The effect of p-chloromercuribenzoate(PCMB) concentration upon cuprous-ion concentration measured by biquinoline-glacial acetic acid reagent.

Concentration of PCMB (moles PCMB/mole of total Cu)	Cuprous copper (μg/mg)
5	0.44
10	0.41
15	0.42
20	0.42

Table VII-3. Copper in the haemocyanin-sulphide complex.

	Cuprous copper (μg/mg)	Cupric copper (μg/mg)
Free cuprous copper with hydroxylamine	0.11	
Total copper		1.23

Table VII-4. Sulphide content in the heat-coagulated haemocyanin and haemocyanin-sulphide complex.

	Sulphide (μg/mg)		Number of determinations
	Average	Range	
Heat-coagulated haemocyanin	3.33	2.90-3.44	4
Haemocyanin-sulphide complex	3.61	3.29-3.90	4

detected in the cuprous state(Table VII-1). The small amount of residual copper after estimating cuprous copper is assumed to be associated with inactivated sites during preparation of heat-coagulated haemocyanin. A small change in the amount of cuprous ion was detected by increasing the p-chloromercuribenzoate concentration(Table VII-2). The cuprous copper of heat-coagulated haemocyanin with p-chloromercuribenzoate is found to be 36% of the total copper. As for the haemocyanin-sulphide complex, very little free cuprous copper was detected with hydroxylamine(Table VII-3). This suggests that the copper of heat-coagulated haemocyanin was combined, presumably with sulphide. The content of sulphide in the haemocyanin-sulphide complex was 0.28 μg/mg more than in the heat-coagulated haemocyanin(Table VII-4). The copper combined with sulphide in haemocyanin-sulphide complex was 1.12 μg/mg of dry matter. The copper and the sulphide combined with copper were 17.6×10^{-3} and 8.7×10^{-3} μM/mg respectively.

Discussion

The blued material and the white coagulum from crab blood have been compared by infrared spectroscopy by Groninger and Dassow.[11] They, however, reported great similarities between the infrared spectra. In the present study also, it is not advisable to distinguish the blue and normal meats by infrared or X-ray analysises.

The result in Table VII-1 suggests that the copper in heat-coagulated haemocyanin makes a chelated compound with EDTA, and that the activity of copper of haemocyanin or heat-

coagulated haemocyanin probably be controled by the chelating reagents. Felsenfeld[28] found that the serum of horseshoe crab, Limulus polyphemus, contained material capable of reducing the copper. Needham[31] also reported that cupric copper was reduced by proteins. In the present investigation, one-half of the copper in heat-coagulated haemocyanin was detectable as the cuprous copper, the remainder being cupric(Table VII-1). There are numerous investigations on physical and biochemical properties of haemocyanin.[32),33)] These investigations have presented the data that the copper binding sites are grouped in pairs and each pair acts a function of oxygen transportation.[5)] It has been known that haemocyanin contains cuprous and cupric copper, even the ratio of cuprous and cupric coppers is not confirmed yet. A proposition concerns to the binding of copper in haemocyanin which attributes to thio groups or imidazole groups. In the present study even in heat-coagulated haemocyanin, inaccordance with the finding in the previous works,[34)] one-half of copper is cuprous.

VIII. Some Observation of Clotting of Crab Haemolymph

It is now obvious that haemocyanin-sulphide complex is a causative substance of the blue discoloration in canned crab, but the reason why the haemocyanin remains in the gaps of muscle fibres throughout the course of canning procedure where a large amount of water is used for washing of the meat. It might be due to physical properties of the haemolymph. The haemolymph of Crustacea has been studied dealing with clotting phenomenon by many investigators.[37] It is generally agreed that the clotting process of crustacean blood may be divided into two distinct phases: (1) agglutination or clumping of cells, and (2) coagulation of plasma.[37] While, plasma alone is not capable of coagulation, unless haemocytes are present.[38] Dumont, Anderson and Winner[38] investigated some characteristics of the haemocytes of horseshoe crab, Limulus polyphemus, by means of electron microscope. The components of the granule were dispersed in the plasma where they presumably contributed to the formation of the gelatinous clot. It has been reported that haemolymphs of crabs, Cancer irroratus, C. borealis and Hyas coarctatus, were not possible to find any distinct difference that could be attributed to the disappearance of fibrinogen from the plasma by electrophoretic patterns.[39] However, little information is available on clotting of haemolymphs of horse hair crab or king crab. It may be thus wondered whether the phenomenon is much contributive to the blue discoloration of canned crab. The present chapter reported the coagulation of crab haemolymph.

Materials and Methods

Nine horse hair crabs, Erimacrus isenbeckii, consisting all male, caught 7/17/70 at off Mori, Hokkaido, and the length of carapace of them was 8~10 cm. Fresh haemolymph of crab was separated from the carapace which allowed to flow by removing of end appendages. In comparison with horse hair crab, the blood of a squid, Todarodes pacificus, was obtained from the heart by syringe. The clotting process was observed by microscope. The haemolymph of the horse hair crab was dropped into an aliquot of the acid and alkali solutions of definite pH value, stirred, observed whether or not it makes clot and measured pH value. For the estimation of the preventive effect to coagulation of the haemocytes, each 1 ml of 0.5% sodium citrate, 0.5% sodium oxalate, and 0.5% sodium hexametaphosphate was poured in a test tube, was added with fresh haemolymph drop by drop until the final volume became 1.5 ml, then the formation of coagulum was observed.

Results

The haemocytes of haemolymph were agglutinated within 5~10 seconds by stirring in various salts solutions after withdrawal, while the haemocytes dropped directly into deionized water were immediately agglutinated. Therefore, the agglutination of haemocytes in various salts was slower than that in deionized water. The agglutination of haemocytes in various pH value is shown in Table VIII-1. In acidic side, an acid coagulum was produced in the solution at pH 4.0~5.2. The acid coagulum is thought to be the plasma containing haemocyanin, because plasma coagulates at pH 3.8~5.0. While, it

was recognized an agglutination of haemocytes in pH 1.4 where haemocyanin dessociates. In alkali side, agglutination of haemocytes was found in a pH range from 7.0 to 10.0. There is no noticeable effect to control agglutination of the haemocytes by adding chelating reagents such as sodium citrate, sodium oxalate and sodium hexametaphosphate.

The micrographs presented in Fig. VIII-1 depict the changes in haemolymph of the horse hair crab. Fig. 1-A shows haemocytes immediately after withdrawal. Fig. 1-B shows the agglutination of haemocytes which have been existed separately in haemolymph. There occurs a change in the state of inner haemocyte structure. As seen in Fig. 1-C, many granules appeared in the cytoplasm of haemocyte, and the cell membrane ruptured during clotting. After being leave for 4 hours from death of the horse hair crab, the haemolymph contained the haemocytes separated from the crab after leaving for 4 hours agglutinated easily(Fig. 1-E). It was found gelatinous substance around agglutination of haemocytes after 15~20 minutes from the start of clotting(Figs. 1-F and 1-G). The quantity of the gelatinous substance was 2.62%(wet weight) in the haemolymph. The gelatinous substance made a network structure, and haemocyanin was contained in the network. The gelatinous substance changed blue-green after treating with hydrogen sulphide for 15 minutes at 100°C, but no change was observed by similar treatment after removing haemocyanin by washing with water. Fig. VIII-2 shows the gelatinous substance remained in the muscle tissue of horse hair crab after a day.

Fig. VIII-3 shows the clotting of squid blood as the comparison with horse hair crab, agglutination of haemocytes was only observed.

Discussion

The clot formation process in the haemolymph of horse hair crab is completed through two steps. The agglutination of cells initiates the process and coagulation of the plasma follows subsequently. This type of clot formation is similar as the second one of clotting classified by Tait.[37] This fact suggests that the haemocytes agglutinate easily in the gaps of muscle fibres during leaving the carapace for a long time after catching, and lead in successive coagulation of plasma in the crab tissue. A difficulty concerns to remove the gelatinous substance(Fig. 1-G) being coagulated in the muscle tissue by washing with water(Fig. VIII-2). The haemocyanin is contained in haemolymph, and it does not coagulate itself by leaving the haemolymph. However, if the network structure of clotted gelatinous substance holds haemolymph in it, or if it is heated, the haemolymph might not be removed completely by washing with water. As seen in Fig. VIII-2, this phenomenon is observed apparently showing remaining the coagula in the tissue. Consequently, haemocyanin in the haemolymph being held in clotted gelatinous clot is thought to be related to blue discoloration.

As to the clot formation process in the haemolymph of squid was different from that of horse hair crab. As shown in Fig. VIII-3, any gelation of plasma was not observed. Therefore, there is distinct coagulation pattern between horse hair crab and squid haemolymphs. This fact suggests that there might

not cause blue discoloration in canned squid meat because squid haemolymph containing haemocyanin is thought to be removed off easily from muscle tissue.

Table VIII-1. Effect of pH value on the agglutination of haemocytes.

pH	Agglutination
1.2	-
1.4	+
5.6	+
6.8	+
8.6	+
9.2	+
10.0	+
11.0	-

+; Haemocytes were agglutinated.

-; Haemocytes were not agglutinated.

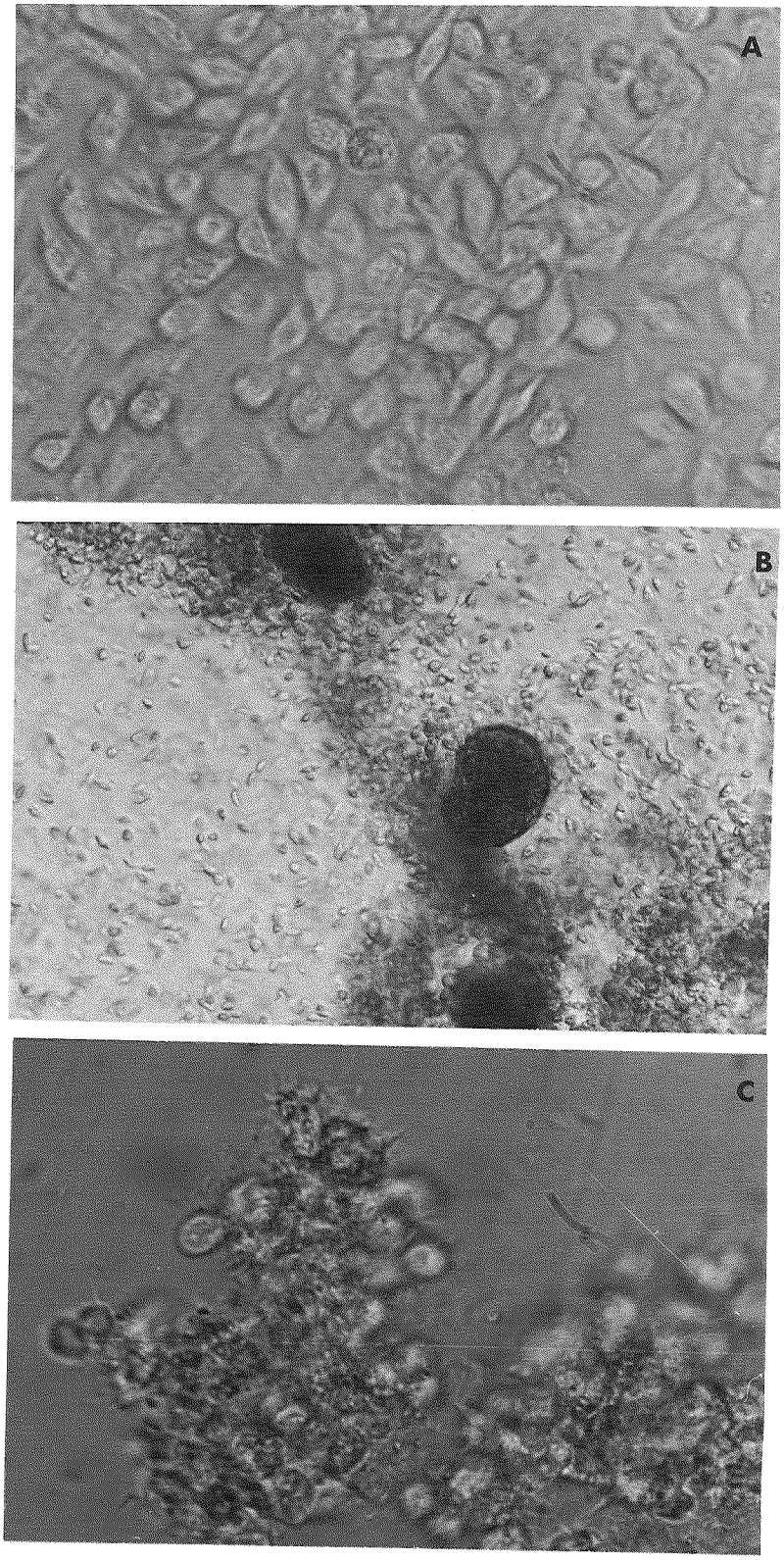
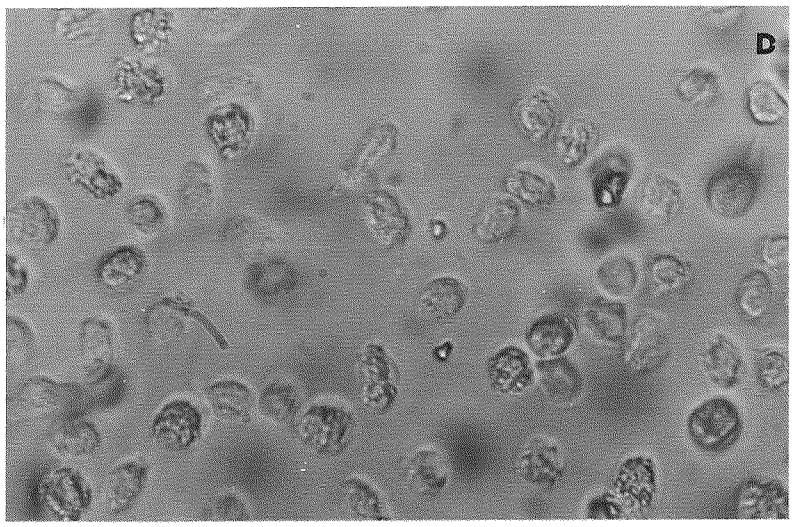


Fig. VIII-1-1. Change of haemocytes and plasma at various stages. A, fresh haemocytes; B, appearance of haemocytes after 5~10 seconds; C, the agglutination of haemocytes. (A, C; ×656, B, ×164)



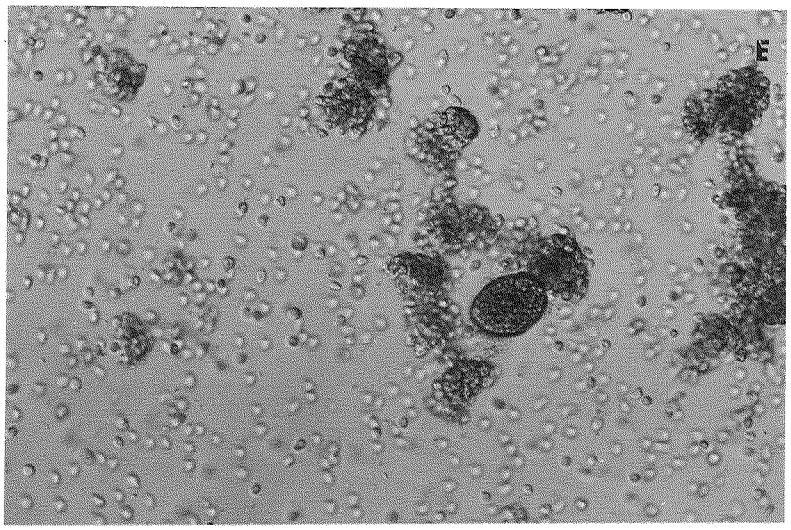
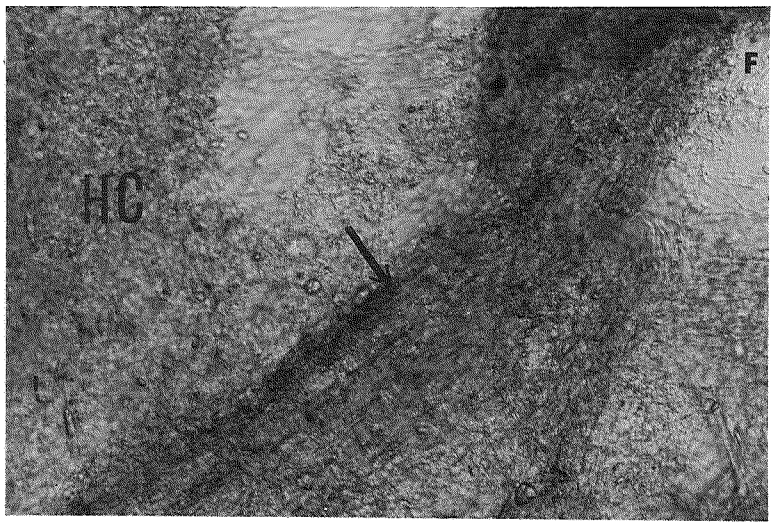


Fig. VIII-1-2. Change of haemocytes and plasma at various stages. D, the appearance of haemocytes after leaving for 4 hours from death of crab; E, the agglutination of haemocytes of above haemolymph. (D, ×656; E, ×164)



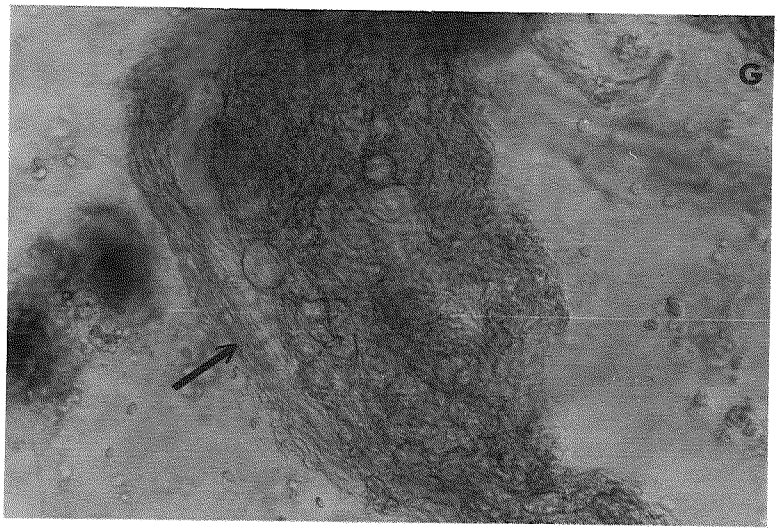


Fig. VIII-1-3. Change of haemocytes and plasma at various stages. F, the gelatinous substance(arrow) of plasma and ruptured haemocytes(HC); G, the gelatinous substance(arrow) of plasma.
(F, G; x 164)

Fig. VIII-2. The gelatinous substance (arrows) in muscle fibres. (×164)

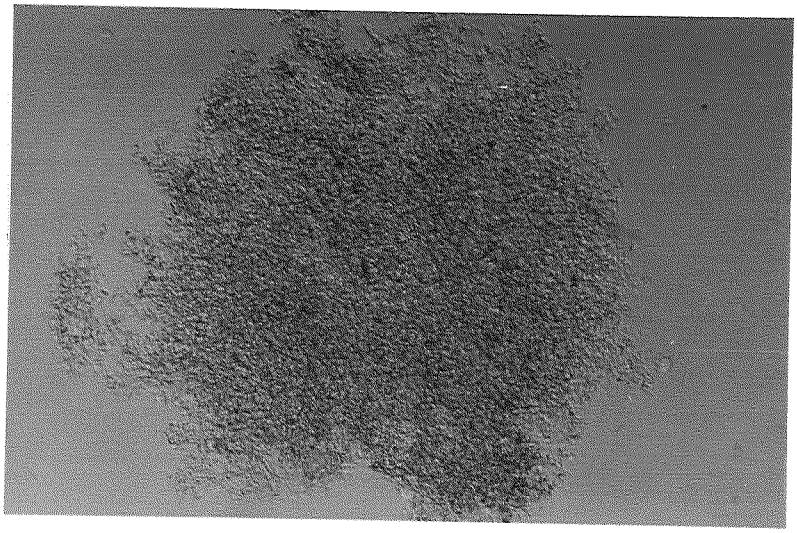


Fig. VIII-3. The agglutination of haemocytes in squid blood. (×164)

IX. Influence of pH, heating time and temperature on reactability of native and heat-coagulated haemocyanins

In commercial crab canning, it has been known that the use of unfresh crab may often be a cause of the outbreak of the blue discoloration after processing. One of the reason for it, as described in a previous chapter VIII, is apparently based on the agglutination of haemocytes and subsequent clotting of plasma which sticks on muscle fibre of crab. If raw crab is left for several hours, then the freshness falls gradually, and accordingly the haemolymph in the muscle tissue of crab changes to stick on muscle fibre holding haemocyanin in its network structure. Few evidence, however, has been provided on the relation between degree of freshness of crab meat and blue colouring.

The present chapter reports on some factors involved in reactability of native and heat-denatured haemocyanins with hydrogen sulphide.

Materials and Methods

Material Haemocyanin solution of king crab, P. camtschatica, was prepared as described in a previous chapter III.

Effect of heating time Each of haemocyanin solutions(5 ml) was added 1.5 ml of a solution containing hydrogen sulphide (13.5 mg%) and the mixture was allowed to stand for 5 minutes, then heated at 100°C in a water bath for 1, 2, 3, 6, 8, 10 and 15 minutes. After cooling, they were measured reflectance at 550 mμ.

Effect of heating temperature Each of haemocyanin solutions (5 ml) was added with 0~1.5 ml of a solution containing hydrogen sulphide(12.0 mg%) and the mixture was allowed to stand for 5 minutes, and heated at 80°, 90° and 100°C in a water bath for 15 minutes. After cooling, they were measured reflectance at 550 mμ.

Effect of pH of haemocyanin solution pH of each of haemocyanin solution was adjusted at 4.6, 7.0, 8.2 and 8.8. Five ml each of solutions was added with 0~1.5 ml of a solution containing hydrogen sulphide(21 mg%) and left for 5 minutes. They were heated at 100°C for 15 minutes. After cooling, they were used for reflectance measurement.

Effect of deterioration of fresh and heat-coagulated haemocyanin on blue discoloration Haemocyanin solution(5 ml) was allowed to stand for 20 hours at 5°, 20°, 24°, and 37°C, into which a solution containing 24 mg% of hydrogen sulphide was added to make 0, 24, 48, 72 and 96 μg/ml of haemocyanin solution, and was then left for 5 minutes. They were heated for 15 minutes at 100°C, cooled with tap water then measured reflectance at 550 mμ.

A portion each of haemocyanin heated for 15 minutes at 100°C was allowed to stand for 24 hours at 5°, 20°, 30° and 35°C. Hydrogen sulphide solution was added to heat-coagulated haemocyanin to make 15, 30, 45 and 60 μg/ml of haemocyanin solution. They were allowed to stand for 5 minutes and heated 15 minutes at 100°C. After cooling, they were used to measure reflectance.

Results

Effect of heating time The results are shown in Table IX-1 and Fig. IX-1. The colour of haemocyanin solution changed brown after treatment with hydrogen sulphide. The haemocyanin was coagulated by heating within a minute and the colour of the coagulum was light brown. The brownish colour disappeared after heating the haemocyanin for two minutes, while light greenish blue colour appeared after three minutes. The colour gradually developed through continuous heating and the coagulum contracted strongly. The change in the percentage reflectance of the haemocyanin was marked at earlier stage of heating period, but was not significant at the stage of heating period from 6 to 15 minutes.

Effect of heating temperature and pH of haemocyanin solution

Colour intensity of heated haemocyanin progressed accompanying with increasing of heating temperature and hydrogen sulphide concentration at a definite temperature(Table IX-2). Haemocyanin did not coagulate completely at lower temperature than 80°C so that it was unable to measure the reflectance. This fact suggests that high temperature and high concentration of hydrogen sulphide may aid haemocyanin in reacting with hydrogen sulphide. Colour intensity of heated haemocyanin was weakest at pH 4.8(Table IX-3).

Effect of deterioration of fresh and heat-coagulated haemocyanins

The results are shown in Tables IX-4 and IX-5. The colour intensity increased with increase of concentration of hydrogen

sulphide. This indicates that blue discoloration develops in increasing with hydrogen sulphide which react with copper of haemocyanin. Visible change of colour intensity was observed when 48 μg of hydrogen sulphide was charged to 1 ml of haemocyanin.

As for the heat-coagulated haemocyanin, a similarity was found as the finging in fresh haemocyanin. In this case, a visible colour change was observed when 30 μg of hydrogen sulphide was added to 1 ml heat-coagulated haemocyanin.

Discussion

Takayasu and Fukuhara[7] reported that the colour of haemocyanin treated with hydrogen sulphide changed brown, but did not change greenish blue. In the present study, however, the colour of haemocyanin from crab changed from brown to greenish blue after both treatments with hydrogen sulphide and heating. This result suggests that the haemocyanin reacted with hydrogen sulphide is stabilyzed by heating. Haemocyanin-sulphide complex became stable at high temperature(Table IX-2). Therefore, it can be concluded that heat treatment is required to the change of the colour of the complex. pH value of the haemocyanin does not affect to the change of colour(Table IX-3). In the present study, there could not found any significant difference in colour change between fresh haemocyanin and heat-coagulated haemocyanin.

Osakabe[9] has reported that the blueing develops in heat-coagulum of crab haemolymph during leaving it at room temperature after insufficient boiling. This phenomenon was also found

by Motohashi,[12] and it was recognized as oxidation of coagulum because the blueing was not observed in the absence of oxygen. Based on the finding, they presumed that the blue discoloration was oxidation of haemocyanin. However, any explanation of the reason was not described in their papers. This presumption is completely different from the result of the present study. The blue discoloration appears in the absence of oxygen(chapter IV), and is seen in the canned crab meat which has been processed by the usual method("high temperature boiling method"). Although the blueing has been observed in coagulum of insufficiently heated haemolymph during leaving it in the air, the insufficiently heated haemolymph was not found in the processed can. The coagulum in processed can must be a completely heated haemolymph even if insufficiently heated haemolymph has been packed.

A possible consideration on blueing based on oxidation of haemocyanin may concerns in oxyhaemocyanin. If insufficiently heated haemolymph would be left for some period in the air, haemocyanin in haemolymph is undoutfully converted to oxyhaemocyanin which develops blue colour. However, this process is only limited in the case of before packing the meat into can. Through the consideration, a conclusion reaches that the blue discoloration in canned crab may not be caused by oxidation of haemocyanin, but hydrogen sulphide must involve in the discoloration.

As for the effect of freshness of haemocyanin on the blue discoloration, any significant difference was not found between

fresh and unfresh haemocyanin. Therefore, if fresh raw material is used for canning, there always be existing a possibility to cause the blue discoloration in the final product.

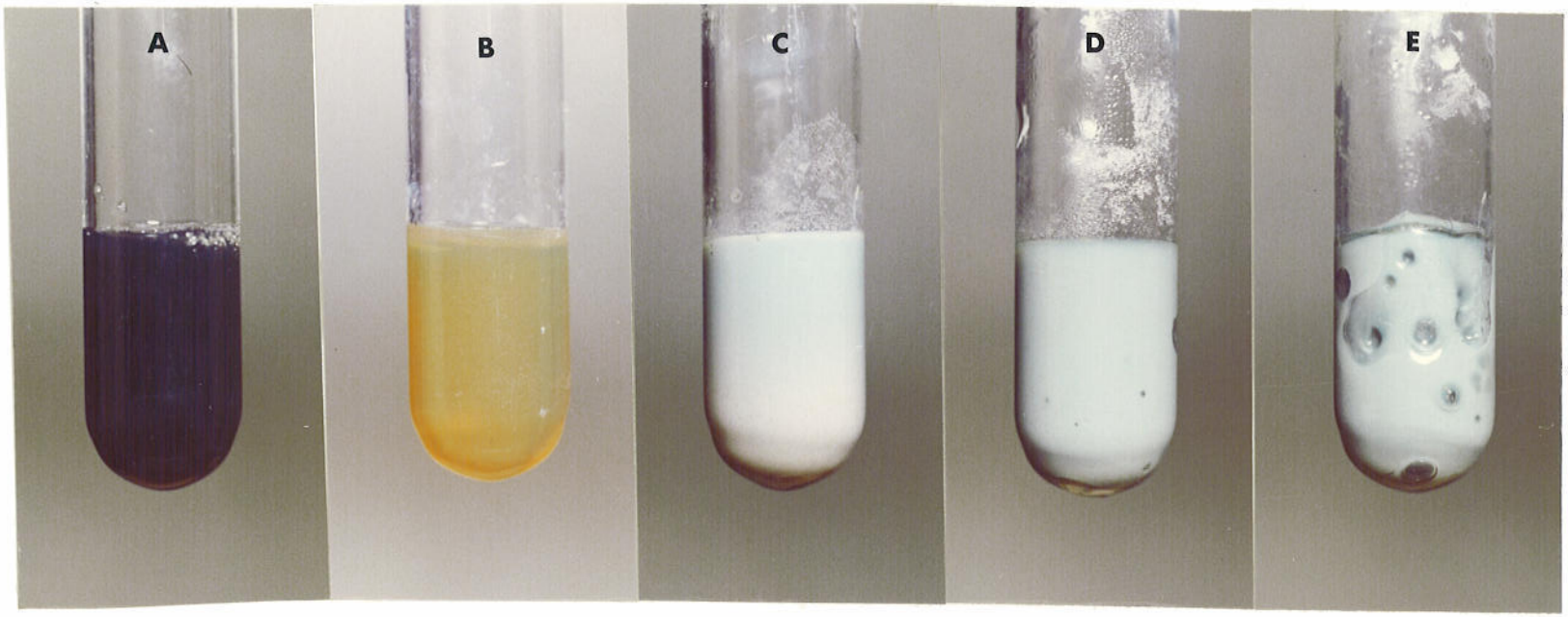


Fig. IX-1. The colour changes of haemocyanin after treatments with hydrogen sulphide and boiling. A, native haemocyanin; B, after treatment with hydrogen sulphide; C, after boiling for 3 minutes with hydrogen sulphide; D, after boiling for 8 minutes; E, after boiling for 15 minutes.

Table IX-1. Effect of heating time on haemocyanin-sulphide complex.

Heating time (min)	Colour intensity
1	–
2	–
3	–
6	16.2
8	16.6
10	16.8
15	16.8

Copper content was 30 µg/ml of haemocyanin.

Heating temperature was 100°C.

Hydrogen sulphide was added 41.7 µg/ml of haemocyanin.

Table IX-2. Effect of heating temperature on haemocyanin-sulphide complex.

Heating temperature (°C)	Hydrogen sulphide (µg/ml of haemocyanin)	Colour intensity
100	0	-
	12	7.7
	24	13.6
	36	13.9
90	0	-
	12	1.8
	24	8.7
	36	10.0
80	0	-
	12	0.2
	24	7.4
	36	10.0

Copper content was 30 µg/ml of haemocyanin.
Heating time was 15 minutes.

Table IX-3. Effect of pH on haemocyanin-sulphide complex.

pH	Hydrogen sulphide (µg/ml of haemocyanin)	Colour intensity
4.8	0	-
	21	12.0
	42	12.0
	63	13.0
	84	13.3
7.0	0	-
	21	12.8
	42	14.2
	63	15.0
	84	15.4
8.2	0	-
	21	14.2
	42	15.0
	63	15.6
	84	16.0
8.8	0	-
	21	13.4
	42	15.8
	63	16.2
	84	15.9

Table IX-4. Effect of allowing temperature on haemocyanin-sulphide complex by fresh haemocyanin.

Allowing temperature (°C)	pH	Hydrogen sulphide (µg/ml of haemocyanin)	Colour intensity
5	6.4	24	6.9
		48	15.7
		72	16.5
		96	17.2
20	6.2	24	8.3
		48	16.3
		72	17.6
		96	17.6
24	6.2	24	11.7
		48	15.9
		72	15.1
		96	15.4
37	6.0	24	9.1
		48	13.9
		72	15.3
		96	18.1

Copper content was 32.9 µg/ml of haemocyanin.
Haemocyanin was allowed to stand for 20 hours at definite temperatures.

Table IX-5. Effect of allowing temperature on haemocyanin-sulphide complex by heat-coagulated haemocyanin.

Allowing temperature (°C)	Hydrogen sulphide (μg/ml of haemocyanin)	Colour intensity
5	15	8.9
	30	15.1
	45	15.1
	60	16.3
20	15	6.1
	30	16.3
	45	17.9
	60	17.9
30	15	11.1
	30	14.3
	45	15.3
	60	15.3
35	15	9.9
	30	14.1
	45	14.7
	60	14.7

Copper content was 32.9 μg/ml of haemocyanin.
Heat-coagulated haemocyanin was allowed to stand for 24 hours.

X. Hydrogen Sulphide evolved from Raw and Canned Crab Meats

Hydrogen sulphide in canned crab is thought to be evolved from crab meat by decomposition during retorting. Because of an importance in connection with the blue discoloration, evolution of hydrogen sulphide in canned crab must be studied. In the present chapter, therefore, hydrogen sulphide from crab meat is described in relation to temperature of boiling and pH and deterioration of crab meat.

Materials and Methods

Raw meat of king crab, Paralithodes camtschatica, was used for sample. Hydrogen sulphide was estimated by a modified method of Almy.[27] Twenty-five grams of ground meat were taken in a 250 ml volumetric flask and 50 ml of deionized water was added. Aeration was continued for 15 minutes. Into a receiver which previously contained 5 ml of 5% zinc acetate, hydrogen sulphide was led from the sample by aeration. The solution absorbed hydrogen sulphide was added 5 ml of diamine reagent and 1 ml of 0.02M ferric chloride solution. After leaving for 1 hour, optical density was measured at 610 mμ by Hitachi Perkin-Elmer UV-VIS Spectrophotometer.

Effect of heating temperature The second leg meat was used as the sample for the determination of the amount of hydrogen sulphide produced at the temperatures from 60°C to 100°C. A portion of the meat was weighed and subjected to the procedure as above described. Aeration was continued for 15 minutes at 60°, 70°, 80°, 90° and 100°C, respectively. The pH value and

the amount of volatile basic nitrogen(VB-N) of the sample were 6.2 and 12.5 mg%, respectively.

In the case of quantitative determination of hydrogen sulphide evolved at the temperature of retorting of canned crab, it was estimated by means of an apparatus shown in Fig. X-1 as it needed a steam pressure. The samples used for the experiment were first leg, second leg, third leg, belly and claw meats. Each of 20 g of the meats was mixed with 50 ml of deionized water. After reaching 5 lbs. pressure, the retort was kept for 15 minutes as it was.

Effect of pH In the first experiment, claw meat only was used as the sample. The meat was mixed with 50 ml of deionized water, and the pH was adjusted to 5.5~8.5 with N/10 hydrochloric acid or N/10 sodium hydroxide solutions. Aeration to the samples was continued for 15 minutes at 100°C. The initial pH and VB-N were 6.4 and 10.9 mg%, respectively.

In the second experiment, the canned meat was used as the sample. Commercially packed belly, shoulder and third leg meats of king crab were used for estimation of hydrogen sulphide content. Twenty-five grams of the canned meats were taken and treated as similar way as raw meat.

Effect of deterioration of raw meat The first and second leg meats were used for sample. They were allowed to stand for 0, 18, 24, 42, 48 and 72 hours at room temperature(19°C), and were used for estimation of hydrogen sulphide content.

Results

Effect of temperature The results are shown in Table X-1.

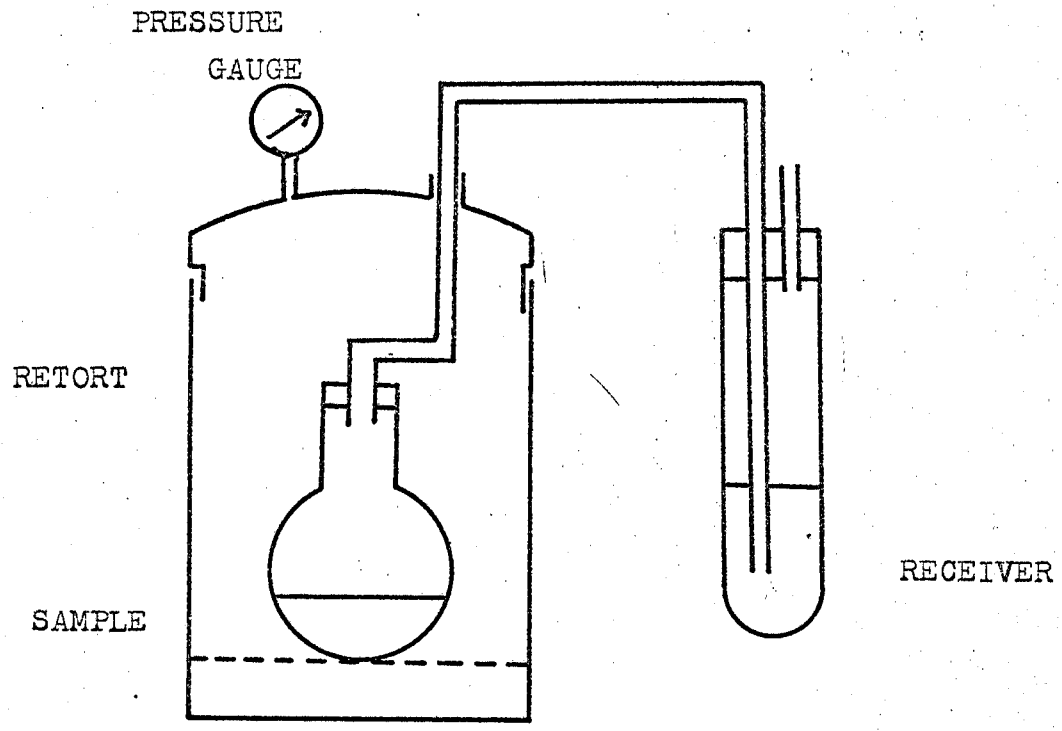


Fig. X-1. Apparatus used for measurement of hydrogen sulphide at high pressure.

The estimation of hydrogen sulphide content was difficult at the temperature below 70°C as the aeration of sample had formed bubbles which prevented evolution of hydrogen sulphide. The amount of hydrogen sulphide from crab meat became higher by increase of the heat temperature in the range 70°~100°C. At a higher temperature than 100°C, a large amount of hydrogen sulphide was produced from the raw meat(Table X-2). Comparing the result in Tables X-1 and X-2, it is notable that the pressure cooked second leg meat was about 8 folds higher level of hydrogen sulphide than that cooked at 100°C.

Effect of pH The effect of pH value on the evolution of hydrogen sulphide was shown in Table X-3. In alkaline region, a larger concentration of hydrogen sulphide was produced than in acidic region. The amount of hydrogen sulphide liberated from canned crab meats was shown in Table X-4. In comparison with the amount of hydrogen sulphide in portions of crab meat, the largest amount was found in belly meat. At similar pH value, the belly meat was about 28 and 8 folds higher level of hydrogen sulphide than third leg and shoulder meats, respectively.

Effect of deterioration of raw meat Table X-5 shows the amount of hydrogen sulphide generated from the deteriorated meat. Hydrogen sulphide content, VB-N, and pH value increased with standing time of crab meat. There is little variation in the amount of hydrogen sulphide, VB-N, and pH values between first leg and second leg meats at a definite leaving time.

Table X-1. Hydrogen sulphide from leg meat at various heating temperature.

Heating temperature (°C)	Hydrogen sulphide (μg/100 g of meat)
60	-
70	21.6
80	23.6
90	24.4
100	27.0

The pH value was 6.2 and volatile basic nitrogen was 12.5 mg% before heating.

Table X-2. Hydrogen sulphide liberated from crab meat at high temperature(5 lbs., 15 mins.).

	Hydrogen sulphide (μg/100 g)
First leg meat	154.8
Second leg meat	202.3
Third leg meat	262.0
Claw meat	202.0

Table X-3. Hydrogen sulphide from claw meat at various pH value.

pH	Hydrogen sulphide (μg/100 g)
5.5	16.4
6.0	26.6
6.5	53.2
7.0	58.4
8.0	88.0
8.5	72.2

Table X-4. Hydrogen sulphide liberated from the meat of canned king crab at various pH.

Portion	pH	Hydrogen sulphide (μg/100 g)
Belly meat	6.72	1140.0
	6.32	1128.0
	5.89	268.0
	5.78	188.0
	5.56	39.6
	5.41	25.4
Third leg meat(cut)	7.70	450.2
	7.10	170.0
	6.80	40.3
	6.50	37.2
	6.28	21.1
	6.00	16.4
Shoulder meat	6.83	149.2
	6.40	45.7
	6.00	38.6
	5.72	35.2
	5.60	27.7
	5.44	11.4

Table X-5. Effect of deterioration concerned with release of hydrogen sulphide from crab meat

Portion	Allowing time (hrs)	VBN (mg%)	pH	Hydrogen sulphide (µg/100 g)
First leg meat	0	12.4	6.4	10.3
	18	13.8	6.6	20.8
	24	15.2	6.6	29.8
	42	76.3	6.8	33.3
	48	95.2	6.8	34.5
	72	125.4	7.0	129.0
Second leg meat	0	12.9	6.2	14.4
	18	14.7	6.4	21.4
	24	20.0	6.4	30.2
	42	81.4	6.6	52.6
	48	100.8	6.6	58.3
	72	111.3	6.8	74.2

Discussion

Since hydrogen sulphide is an important factor involving the blue discoloration as well as copper in haemocyanin, to control the evolution must be taken in consideration at practical crab canning. The present findings on the evolution would contribute in this respect. It has been reported that hydrogen sulphide evolves a large amount at higher temperature in Mollusca.[40] Further, it has also been known from the papers reported by Oya and Kawaguchi[41] and Shimada[42] that evolution of hydrogen sulphide is much more in alkaline region than in acidic region. However, a detailed knowledge on the evolution concerns obviously the variation of the produced amount of hydrogen sulphide in different portion of crab meat. In accordance with the finding reported previously, as for crab meat, fresh meat evolves only small amount of hydrogen sulphide,[40] while a deteriorated meat produces fairly large amount. As described in the previous chapter IX, deterioration of crab haemocyanin might not be relative in developing of the blue discoloration, but that of crab meat itself may cause increase of hydrogen sulphide content in tin container which would be an indirect factor involving the blue colour development. Though it has been reported that cystine, glutathione and methionine are the source of hydrogen sulphide during heat treatment,[43), 44] the precursor in crab meat is not clear yet.

XI. Prevention of Blue Discoloration in Canned Crab Meat

Osakabe[9] has succeeded in preventing the appearance of the blue meat of the canned crab by "low temperature and fractional heating". However, the method brought forward following problems; (1) browning, (2) black spots, (3) decrease of the yield of canned crab meat, (4) inferior taste, and (5) occurrence of crystals, etc. The low temperature and fractional heating, thus, is fewly employed in practical crab canning at present time because of the disadvantages of the method. In general, the prevention of the blue discoloration is classified into two methods. One is based on removal of haemocyanin thoroughly from the meat, or inactivation of copper in haemocyanin contained in the meat. The second method is repression of evolution of hydrogen sulphide from the meat during heat processing. However, completely effective method has not been developed yet.

In the present chapter, effect of some additives on inactivation of copper in haemocyanin is reported.

Materials and Methods

Heat-coagulated haemocyanin is prepared as described in a previous chapter V. Additives used in the experiment were ethylenediaminetetraacetic acid(EDTA), dipicolinic acid(DPA), sodium dipicolinate, phytic acid, sodium phytate and thiourea. The concentrations of EDTA, DPA and thiourea were 0.1M, 0.01M and 0.001M, respectively. While, the concentrations of phytic acid used in the experiment were 0.5%, 0.25% and 0.1%. Other

additives to be studied were 1% zinc acetate, 1% sodium citrate, 1% sodium thiosulphate, 0.01M ascorbic acid, 0.01M 2,2'-biquinoline, 1.5% lactic acid, 0.005% and 0.01% ammonia solution and 0.04% bathocuproine sulphonate.

Heat-coagulated haemocyanin(0.5 g, wet weight) was added to the test tube containing 5 ml of the solution of the additive for 1 hour. The test tube was plugged with cotton, allowed to stand for 30 minutes, added 6 ml of hydrogen sulphide solution (76 mg%), and heated for 15 minutes in a boiling water bath. After cooling, the reflectance of the heat-coagulated haemocyanin was measured at 550 mμ. In order to study the effect of phytic acid in practically packed crab meat, shoulder, third leg and belly meats were packed in a parchment lined 1/2 lb flat can lacquered with zinc oxide for the inside(C-enameled) and added phytic acid to make 0~0.5% for the content to each can. The packed can was heat processed for 90 minutes at 5.5 lbs, cooled in water, and stored for 3 months at room temperature. The stored can was opened and solid and liquid portions were separated. Each portion was used for the sample of pH value and VB-N determinations. The blue meat was observed organoleptically.

Results

The results on the effect of various additives to the blue discoloration are shown in Tables XI-1 and XI-2. Zinc acetate reacts with hydrogen sulphide, but not reacts with copper of haemocyanin. Various chelating reagents used for the experiment were effective to control the blue discoloration, and the

effectiveness was as following order: Na-DPA > EDTA > Phytic acid > Thiourea > DPA. By adding 0.1M DPA solution, the heat-coagulated haemocyanin swelled during reheating. The solution and the coagulum changed dark blue in colour in sodium phytate. Adding 0.1M and 0.01M Na-DPA solution into haemocyanin was effective to prevent blue colour development, but a disadvantage was colour change to brown during or after heating. Similar browning discoloration was observed in the haemocyanin solution containing dipicolinic acid also. Sodium citrate, ascorbic acid, 2,2'-biquinoline, bathocuproine sulphonate and lactic acid were not effective for prevention of blue discoloration(Table XI-2). While, ammonium hydroxide did not promoted colour intensity of the blue discoloration. The colour of haemocyanin coagulum was changed to reddish violet and orange with 2,2'-biquinoline and bathocuproine sulphonate, and further changed to greenish blue by heating with hydrogen sulphide(Table XI-3). As seen in Table XI-4, the blue discoloration of canned crab meat was not found by adding with more than 0.2% level of phytic acid to the content. However, above 0.3% level, the content assumed sour taste.

Discussion

Numerous methods for prevention of the blue discoloration are effective partially. Since the blue discoloration is caused by a reaction of haemocyanin with sulphide, to keep both compounds apart is required to prevent the blue discoloration; namely, removal of haemocyanin, repression of evolution of hydrogen sulphide, and chelating of copper in haemocyanin

Table XI-1. Prevention of haemocyanin-sulphide complex.

	Colour intensity	pH after heating
Heat coagulated haemocyanin	-	4.2
Haemocyanin-sulphide complex	29.6	4.2
1% Zinc acetate	0	4.4
M/10 EDTA	5.7	4.0
M/100 EDTA	8.7	4.0
M/1000 EDTA	12.0	4.2
M/10 Na-DPA	4.2	5.0
M/100 Na-DPA	7.3	4.2
M/1000 Na-DPA	17.2	4.2
M/10 DPA	22.3	2.0
M/100 DPA	17.4	2.6
M/1000 DPA	21.0	4.2
0.5% Phytate	5.1	1.6
0.25% Phytate	10.3	2.2
0.10% Phytate	21.5	2.6
0.25% Sodium phytate	-	-
M/10 Thiourea	14.4	4.2
M/100 Thiourea	20.4	4.2
M/1000 Thiourea	23.7	4.2

EDTA; Ethylenediaminetetra acetic acid

DPA; Dipicolinic acid

Table XI-2. Colour intensity of haemocyanin-sulphide complex by reagents.

	Colour intensity	pH after heating
1.5% Lactic acid	29.9	2.4
0.01M 2,2'-Biquinoline	27.0	2.6
0.01M Ascorbic acid	30.3	4.0
0.04% Bathocuproine sulphonic acid	28.3	5.2
0.01% Ammonium hydroxide not treated with H_2S	0.1	5.2
0.01% Ammonium hydroxide	27.3	4.2
0.005% Ammonium hydroxide	30.0	4.0
1% Sodium citrate	23.1	5.4
1% Sodium thiosulphonate	24.2	6.2

Table XI-3. The effect of heating with hydrogen sulphide on colour change.

	Colour produced under conditions shown	
	Heat for 15 min at 100°C	Heat for 15 min at 100°C with H_2S
0.04% Bathocuproine sulphonate	Orange	Light green
0.01M 2,2'-Biquinoline	Reddish violet	Greenish blue

Table XI-4. Effect of phytic acid for prevention of blue meat in canned king crab.

Parts	Amount added of phytate (%)	Liquid pH	Liquid VBN (mg%)	Solid pH	Solid VBN (mg%)	Blue meat	Taste
Shoulder meat	0	6.90	20.0	7.02	20.3	+	Normal
	0.1	6.52	20.8	6.64	17.3	+	Normal
	0.2	6.28	19.3	6.39	16.9	+	Normal
	0.3	6.08	21.0	6.08	18.3	-	Sour
	0.4	5.70	20.4	5.80	17.7	±	Sour
	0.5	5.68	20.6	5.69	18.2	±	Sour
Third leg meat	0	7.52	26.9	7.70	21.7	+	Normal
	0.1	6.88	23.1	7.10	18.6	+	Normal
	0.2	6.60	21.8	6.80	19.2	+	Normal
	0.3	6.38	21.1	6.50	19.9	±	Sour
	0.4	6.12	22.0	6.24	23.8	-	Sour
	0.5	5.90	21.3	6.00	20.8	-	Sour
Belly meat	0	7.00	24.8	6.90	22.3	+	Normal
	0.1	6.28	23.7	6.40	18.6	±	Normal
	0.2	6.10	21.5	6.14	16.2	+ -	Normal
	0.3	5.78	22.0	5.90	19.4	-	Sour
	0.4	5.60	22.8	5.72	19.6	-	Sour
	0.5	5.44	24.4	5.66	18.0	-	Sour

would be possible ways for this purpose. In the present study, 2,2'-biquinoline and bathocuproine sulphonate could react with cuprous ion, but the reactants could further react with hydrogen sulphide. This fact indicates sulphide is very stable and chemical binding is very tight(Table XI-3). Though Fellers and Parks[2] and Gordievskaya[10] have reported that ammonia would promote the blue colour development, the result in the present study indicates that there is few relation between ammonia and the blue colour development. Therefore, it appears that ammonia would not be related to the blue discoloration in canned crab meat. In consequence, as seen in Tables XI-1 and XI-4, to add 0.01M EDTA and 0.2% phytic acid appears to be comparatively effective for the prevention of the blue discoloration. However, care should be taken in consideration as the chelating complexes with copper and EDTA, phytic acid and DPA give green or greenish blue in colour.

XII. General Discussion and Conclusion

Many investigators[6),10),19),20)] have presented informations on the blue discoloration of canned crab meat. Their findings concerns mostly the amount of copper in the blue meat and have regard to the fact that there is certainly a relation between the amount of copper and the colour intensity of the blue meat. The finding of the present study also supports principally those of the previous works(chapter II). The reason why a larger amount of copper is contained in the blue meat would probably be due to a larger amount of haemolymph. Crustacea and Mollusca contain haemocyanin in haemolymph as an oxygen carrier and haemocyanin contains a large amount of copper in its molecule.[45),46),47),48)] The copper in crustacean haemocyanin is 0.17~0.18%, and that in molluscan haemocyanin is 0.24~0.26%. The muscle tissue of crab is filled with haemolymph being provided with an open blood-vascular system, and 85~95% of haemolymph is occupied with haemocyanin.[39),47),49)] Therefore, provided the haemolymph remains in crab muscle tissue, it must contain a larger amount of copper. In fact, there are plenty lot of amorphous coagula of haemolymph in the blue meat and little in the normal meat(chapter II). The crab haemolymph is so changeable in the physical property that it becomes easily sticky even immediately after separation from living crab(chapter VIII). Heating process may change the sticky haemolymph to a coagulum in the crab meat. Depending on the fact, it appears that complete leaching of haemolymph from crab meat would be of difficult.

Haemocyanin has been assumed a causative substance up to now, but has not been found in the blue meat yet. The reason for the difficulty of certification of the haemocyanin in the canned crab seems to be depending on detecting method to heat-denatured haemocyanin by boiling or retorting during canning. However, a staining method by Manwell and Baker[21] is available for the detection of heat-denatured haemocyanin(chapter III) and haemocyanin-sulphide complex(chapter IV).

The blue discoloration has been presumed to be owing to haemocyanin from following views; (1) a high copper content in blue meat,[6),10),19),20] (2) decrease of blue colour intensity after thoroughly bleeding.[13),14),15] It has been known that copper in haemocyanin involves in not only blue discoloration, but also blackening of canned meat.[50] However, the cause and the mechanism of blue discoloration has not been clarified yet. The causes of the discoloration have been thought to be associated with copper in haemocyanin and ammonia,[8] oxygen,[9),20] or sulphide,[8),20] and a modified biuret compound.[2),11] Therefore, an effort to be paid in the present study was to investigate a causative substance. Both fresh and heat-denatured haemocyanins react with hydrogen sulphide to change in colour to greenish blue after heating(chapter IV). Though Fukuhara and Ohigashi[20] have presumed that the blue discoloration develops through a reaction between haemocyanin and hydrogen sulphide without any evidence for it. Ammonia or oxygen is not essential factor

for formation of haemocyanin-sulphide complex(chapters IV and XI), but haemocyanin reacts directly with hydrogen sulphide to change in colour to greenish blue after heating(chapters IV and IX).

Apohaemocyanin and copper freed from haemocyanin were examined with respect to colour development. Apohaemocyanin did neither change in colour with hydrogen sulphide nor heat treatment. On the contrary, copper freed from haemocyanin reacted with hydrogen sulphide and changed greenish blue in colour by heating. This change in colour was also observed with inorganic copper ion(chapter IV). From these facts, copper in haemocyanin is thought to be the very factor involved in the blue discoloration. Takayasu and Fukuhara[7] claimed that copper sulphide would not be a causative compound in the blue discoloration since its colour is not similar as that of the blue meat, but is only brown. This claim seems to be not true. Their conclusion reached as above is the result of observation on colour change in unheated copper sulphide, but not in heated copper sulphide. If copper sulphide is heated, its colour changes to greenish blue from brown. Therefore, heat processing is required to develop the blue discoloration in canned crab.

Copper-protein linkage in haemocyanin is so highly stable[4),5)] that a difficulty concerns to release copper by heating.[49)] If the haemocyanin remains in the crab meat tissue(chapter VIII), it can be coagulated by heat processing and can react with hydrogen sulphide evolved from crab meat during retorting.

The causative substance of the blue discoloration have

never been isolated from the blue meat. However, a method is available for isolation applying fractionation by pH to the blue meat(chapter VI). In general, for the isolation of causative substance from the blue meat, extraction method by solvents has been applied, but the method in the present study which is including removal of impurities from the blue meat is effective to isolate the causative substance. Thus, the causative substance isolated by the present method indicates the chemical characteristic that it is undoubtly by copper combines amino acids in haemocyanin[35),52),55)] (chapter VI).

There is little difference in infrared spectra and X-ray diffusion patterns between the causative substance and blue meat from normal meat(chapter VII). However, to measure percentage reflectances seems to be effective to distinguish the blue meat. The percentage reflectance of blue meat decreases according to development of the intensity of blue discoloration. There is a correlation between the colour intensity and the content of copper combined with sulphide. The copper content should be less than 1.4 mg% for desirable appearance of crab meat. The blue coloured crab meat contains more than 2.0 mg% of copper(chapter II). The significant change of colour is found 0.5~1.0 mg% from the results of model experiment(chapter VII). Therefore, in order to obtain desirable appearance of the crab meat, copper-sulphide complex level should be less than 1.0 mg%.

Hydrogen sulphide evolves from crab meat by decomposition

during retorting, which reacts easily with copper(chapter X). If the amount of hydrogen sulphide is sufficiently enough, the copper content responds to the blue discoloration, as reported by Fukuhara and Ohigashi.[20]

The possible method for prevention of the blue discoloration is removal haemolymph away before clotting them in muscle tissue. From this point of view, the "low temperature and fractional heating" method of Osakabe is thought to be effective, but if one depends on the method there occurs many problems such as taste-fall, decreasing of the yields, etc.[51] While, the present author applied chelating additives to the copper of haemocyanin remained in crab muscle tissue. Some of the additives used in the present study are actually effective, but remains are not as they change green or blue in colour even they are able to make chelate complex. Further, the chelate complex yielded with copper and chelating additives may react with hydrogen sulphide. Therefore, in consequence, chelating additives should be stronger in reactivity than hydrogen sulphide to copper and in this respect phytic acid appears to be most suitable.

From the results of the present study, the uncertainty of earlier investigations became quite clear owing to the evidence on a cause and mechanism of the blue discoloration of canned crab meat. The present author believes that further studies on removal of copper complex will contribute to a possible complete method for prevention of the blue discoloration.

Summary

In order to clarify the cause, the mechanism and the prevention of the blue discoloration in the canned crab meat, this study was carried out. Results obtained through the course of the investigation may be summarized as follows:

(1) Copper contents in blue and normal meats of king crab were 2.80 and 0.49 mg%(wet weight) in average, respectively. Higher copper content was found in shoulder surface of first leg, and meats nearer a joint and claw meats. The result of histological study suggested that haemocyanin might be a causative material for blueing. King crab shoulder muscle containing haemocyanin heated for 60 minutes at 115.2°C gave the appearance as the blue meat by microscopical observation.

(2) The blue meat from canned crab, heat coagulated haemocyanin and haemocyanin-sulphide complex gave positive results for haemocyanin. Haemocyanin was not found in the normal leg meat, but was found markedly in the "joint" portion of the boiled material.

(3) The haemocyanin contained in crab haemolymph can react with hydrogen sulphide to produce a blue coloured pigment by heating for 15 minutes at 100°C. Heat coagulated haemocyanin also react with hydrogen sulphide to give a blue colour by heating.

(4) The reflectance spectra of blue meat and haemocyanin-sulphide complex showed maxima at 550~570 mμ and 530~550 mμ, respectively. A decrease of the percentage reflectance is related to an increase of blue discoloration.

A linear relationship was found between the colour intensity and the content of copper combined with sulphide.

(5) A blue substance could be isolated from alkaline extracted solution of the blue meat applying fractionation by adjusting pH value. The chemical characteristic of copper in the substance was similar as that of haemocyanin. The copper in the blue substance could be freed in the presence of EDTA. The blue substance contained high level of copper, and showed positive haemocyanin-like reaction. From the present finding, it was suggested that in fact the causative substance of the blue discoloration of canned crab meat was haemocyanin derivative.

(6) The copper in heat coagulated haemocyanin was detected to be cuprous ion under reducing conditions, and its activity was masked with chelating agents such as EDTA. In the haemocyanin-sulphide complex, the copper was combined with sulphide. The copper and sulphide contents in the haemocyanin-sulphide complex were found to be 17.6×10^{-3} and 8.7×10^{-3} μM/mg, respectively.

(7) The haemocytes of haemolymph agglutinated during leaving it. It appeared many granules in cytoplasm, then ruptured the cell membrane during agglutination. Gelatinous clot of plasma around agglutinated materials of haemocytes were found after 15~20 minutes following onset of clotting. The gelatinous clot was found in the crab muscle tissue. Only agglutination of haemocytes was found in the squid haemolymph containing haemocyanin, and subsequent gelation of plasma was not found.

(8) Haemocyanin-sulphide complex was formed by heating within

5~6 minutes at 100°C. The formation of haemocyanin-sulphide complex was not influenced by the degree of freshness of haemocyanin or heat-coagulated haemocyanin. The colour intensity increased accompaning with increasing of heating temperaure and hydrogen sulphide concentration. Oxygen and ammonia were not essential factors for formation of haemocyanin-suphide complex.

(9) A large amount of hydrogen sulphide was evolved from crab meat at higher temperature. Heating at alkaline region and use of deteriorated crab meat could promote the evolution of hydrogen sulphide. Hydrogen sulphide reacted thoroughly with copper in the meat of can.

(10) Chelating additives at higher concentration were effective for the prevention of the blue discoloration. The copper combined with the additives could not further react with hydrogen sulphide. More than 0.2% level of phytic acid in the content was most effective for the prevention of blue discoloration.

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