ON THE PREVENTION OF THE BLACK SPOT OF SHRIMP. *

I. EFFECT OF SOME CHEMICAL REAGENTS ON THE BLACK SPOT OF SHRIMP.

II. THE ISOLATION AND PURIFICATION OF THE ENZYME RELATED TO THE FORMATION OF BLACK SPOT.

(Received in 29/4/1966)

YASUZO ITÔ **

SYNOPSIS

The causes of the black discoloration of three species of shrimp commonly caught off the coast of the State of São Paulo, Brazil, are studied and measures for its prevention are advocated.

Landings of shrimp in Santos in 1964 equaled 13% of the total landing, by weight, while economically it is the principal fishery.

I — Blackening was effectively prevented by dipping the shrimp in cooled chemical solution for 30 minutes and then storing them at low temperatures. L-ascorbic acid gave the best results, delaying discoloration up to 9 days at 0° to —2°C and for a longer period at colder temperatures. The effect of Sodium-thiosulfate and EDTA at different temperatures is also discussed.

II — Tyrosinase was isolated and assayed by measuring the formation of Dopa-chrome from DL-Dopa at pH 6.8 an 37°C. Tyrosinase was obtained from the shrimp liver and was purified by approximately 25 times its activity from the initial extract by absorption chromatography through Celite 545, as judged by the rate of dopachrome formation.

INTRODUCTION

Since 1923, several authors (HARRISON & HOOD 1923; TANNER 1944; GREEN 1949; ALMEIDA 1955; DECKWITZ 1954; BAILEY 1958; KAKIMOTO 1956; FIEGER 1956) reported on the black discoloration of shrimp and suggested various methods to prevent it.

Earlier procedures may be summarized as follows:

1 — The fresh shrimp was boiled and then stored at low temperature (— 10°C ~ — 15°C);

2 — The fresh shrimp was rapidly freezed and stored at low temperature (about — 10°C ~ — 15°C);

3 — The fresh shrimp's head was taken off and the headless shrimp was stored at low temperature (about — 10°C);

4 — The fresh shrimp was covered with ice with added chemicals or dipped in cooled chemical solutions; after that stored at low temperature (— 5°C ~ — 10°C);

5 — The fresh shrimp was stored with ice with added antibiotic;

6 — The fresh shrimp was freeze dried and stored at low temperature.

In Brazil, people usually prefer to consume fresh or dried shrimp. Therefore, it seems that we must study according to procedures outlined under points 4, 5 and 6.

The shrimp landed in Santos is mainly 'camarão-sete-barbas', and 'camarão-legítimo'. The amount of shrimp landed in Santos from 1962 to 1964 is shown in Table I.

As shown in Table I, in 1964 the total landings of shrimp reached about 13% of the total landings of fish in Santos. Parallel to the increase of the catch of shrimp, the problem of the black spot of shrimp has become increasingly important also in Brazil.

In this connection, for a prevention of the black spot, fishermen have already used chemicals, called

* This work was accomplished while under contract as Visiting Researcher at the Instituto Oceanográfico da Universidade de São Paulo, on a Ford Foundation Grant.

** Present address: Hokkaido Kyoiku University, Hokkaido, Japan.

TABLE I — Landings in Santos from 1962 to 1964

Species \ Year	1962 Quantity (kg)		1963 Quantity (kg)		1964 Quantity (kg)	
'Camarão-sete-barbas'	922,152	6.8%	1,194,639	6.3%	1,247,378	7.2%
'Camarão-rosa'	604,997	4.5%	817,751	4.3%	891,022	5.1%
'Camarão-legitimo'	45,706	0.3%	34,133	0.2%	67,321	0.4%
Total	1,572,955	11.6%	2,046,523	10.8%	2,205,721	12.7%

% ratio of total landings

'pó-de-camarão', at Santos, they are sodium sulfite for 'camarão-rosa' and at São Sebastião, boric acid for 'camarão-sete-barbas'.

Other chemicals used or tested in other countries were sodium thiosulfate, sodium bisulfite and a mixture of L-ascorbic and citric acids.

The fishermen of U.S.A. boats fishing along the Guyana coast, dip shrimp in dilute sodium bisulfite solution at low temperature for a few minutes. After that, they cut off the heads and stored the tails at low temperature. Moreover, since 1940 many researchers have studied the value of antibiotics to keep the quality of fish. However, from the view point of food hygiene, the Ministry of Health and Welfare in the U.S.A., Canada and Japan has permitted fishermen to use this procedure only on a few fishes (salmon, sea trout etc.).

On shrimp, Deckwitz (1954), Fieger (1956), Green (1949), Southcott *et al.* (1965) and Mucciolo & Schneider (1965) studied the effect of antibiotic to keep quality and to prevent black discoloration. Except for the result of Mucciolo & Schneider (1965) they reported that antibiotics kept the quality of shrimp but had no value in preventing black spot formation which is caused by tyrosinase.

From these results it was observed that chemicals, such as sodium thiosulfate, sodium bisulfite and others were significant in preventing the black spot but did not contribute towards keeping the quality of shrimp. Therefore, shrimp treated with chemicals should be stored at low temperature.

It seems that the black spot of shrimp is caused by the action of the contained enzyme (tyrosinase) and of contaminating bacteria. Therefore, for a study of this problem, the author separated it into two parts: a study of the contained enzyme and a study of the contaminating bacteria.

The effect of chemicals in preventing discoloration by tyrosinase in shrimp was studied. It was observed again that reducing reagents were useful in preventing it. Furthermore, it was observed that a chelate reagent, such as EDTA, was similarly valuable as a reducing agent.

The tyrosinase of shrimp was also studied. Bodine & Allen (1941) reported on the presence of protyrosinase in blood of cray-fish and Kakimoto (1956) on the tyrosinase in blood, liver and other organs of Japanese spiny lobster. However, no purified enzyme has hitherto been obtained from marine animals.

The author has undertaken the isolation and purification of the tyrosinase of 'camarão-sete-barbas' for a study of its properties and obtained the enzyme purified approximately 25-times specific activity. In the present paper, the experimental results on the effect of chemicals in preventing black spot caused by a tyrosinase and a method of the isolation and purification of enzyme from the 'camarão-sete-barbas' liver are described.

EXPERIMENTAL PROCEDURE

I. *On the effect of some chemical reagents on the black spot of shrimp*

Material — 'Camarão-sete-barbas' (*Xyphopenaeus kroyeri*) samples were taken at the offing of Santos, from March to November, 1965. 'Camarão-rosa' (*Penaeus aztecus*) was bought at the central market of Santos.

Chemicals — Sodium sulfate, L-ascorbic acid, Sodium thiosulfate, Ethylendiamin tetra acetic (EDTA), Acetic acid, n-Buthanol, Phenol, Acetone and Ninhydrine were used. All reagents were Merck A.G.

Organoleptic tests were done in two methods: A — Chemicals were added directly on shrimp and the treated shrimp were stored at low temperature. That is, at first, shrimp were separated into three masses (I, II and III). Next, each mass was separated into three or five groups containing 10 or 20 shrimps. Then, each chemical (one of tenth of weight of shrimp) was added to each mass. That is, sodium sulfate was added to mass I, L-ascorbic acid to mass II and EDTA to mass III, respectively. Then, shrimp treated were stored in the electric refrigerator at 0°C ~ −2°C. B — Shrimp were dipped in each cooled chemical solution for 30 minutes at low temperature and then stored at low temperature. That is, shrimp were separated on the same way as described above, and then, each mass was dipped in each cooled chemical solution pre-

pared at 0°C ~ −2°C, for 30 minutes. After that, shrimp were taken out from each solution and stored in the electric refrigerator at 0°C ~ −2°C and −5°C ~ 10°C. The concentration of various chemicals solution in shown in Table II.

TABLE II — Concentration of chemical solutions

Chemicals	Concentration (%)
Na_2SO_4	1
L-Ascorbic acid	1
$Na_2S_2O_3$	1
EDTA	0.5
EDTA	1
EDTA	1.25
EDTA	2.5
EDTA	5

After the procedure described above, daily observations were made to see whether the shrimp in each group became black or not.

CHEMICAL TEST — a) The change of the free amino acids content in muscle of shrimp during storage at 0°C ~ −2°C was observed by means of paper chromatography.

Preparation of sample for the paper chromatography — One gram of muscle was homogeneized for a few minutes with 10 volumes of water. After 10 minutes, 5 ml of 20% trichloracetic acid were added and the precipitate was filtered off. The filtrate obtained was dried in a vacuum dessiccator, then, 0.1 ml of warm water (60°C) was added and the precipitate was completely dissolved. At first, one third volume of the solution was applied to one dimension paper chromatography. Two third volumes of the remaining solution were applied to two dimension paper chromatography at room temperature (about 25°C).

Solvent system — n-Buthanol — acetic acid — water (4:1:5 sup.) and phenol-water (4:1) were used.

Development of color — 0.25% ninhydrin acetone solution and special reagent for a few amino acids (isatin for proline, Pauly test for histidine and tyrosinase and Sakaguchi test for arginine) were used.

b) pH was measured with Metrohm A G Herisou Type E 148 C.

II. *On the isolation and purification of tyrosinase from shrimp liver*

MATERIAL — Shrimp ('camarão-sete-barbas', *Xyphopenaeus kroyeri*) was captured at the offing of Santos, from March to November, 1965. All reagents used were analytical grade, unless otherwise indicated. Celite 545 was washed several times by suspending it in distilled water and decanting. The slurry was poured into the column and equilibrated with 0.005 M sodium phosphate buffer, at pH 7.2 by running several hold-up volumes of the buffer through each column. Sephadex G-50 was prepared in the similar way as the Celite 545 except that 0.01 M sodium phosphate buffer was used for washing and equilibration.

Enzyme assay — Tyrosinase was assayed by measuring the formation of 2-carboxy-2,3-dihydroindol-5,6-quinone (Dopa-chrome) from 3,4-dihydroxy-DL-phenylalanine (DL-dopa) at pH 6.8 and at 37°C. The assay system consisted of 0.2 ml of enzyme extract preparation, 1.8 ml of 0.1 M sodium phosphate buffer at pH 6.8, and 0.1 mg of DL-dopa dissolved in 5 ml of the same buffer. The formation of dopa-chrome at 37°C was measured colorimetrically using a Bausch & Lomb Spectronic 20 at 480 mμ.

Tyrosinase unit — According to the method of HAROWITZ (1960) and FLING (1963), an amount of enzyme which produces an absorbancy increase of 0.1 at 480 mμ in the first 5 minutes, was adopted as a unit for the tyrosinase.

Protein determination — During the course of the isolation, protein concentration was measured colorimetrically by the biuret method using a Bausch & Lomb Spectronic 20 at 560 mμ or 750 mμ.

RESULTS

I. *On the effect of some chemical reagents on the black spot of shrimp*

The results of organoleptic methods are shown in Tables III-IV. The value indicated in the Tables are shown as a ratio of blackening against total shrimp. Table III shows the results of method A.

From the results of Table III, L-arcorbic acid was indicated as having a better effect than others, but in this case, the surface of a few shrimp were

TABLE III — Discoloration during storage (powder) at 0°C ~ −2°C

Storage period days	Reference %	Chemicals		
		Na_2SO_4 %	L-Ascorbic acid %	EDTA %
0	0	0	0	0
1	15	0	0	0
2	28	20	0	30
3	70	30	0	40
4		30	0	40
5		/	/	/
6		60	20*	50
7			80*	

* to become yellow

covered with a yellow color (that is yellow discoloration) instead of blackening. The results of method B are shown in Tables IV, V and VI. Tables IV and V show the results for 'camarão-sete-barbas' when storage temperature was 0°C ~ — 2°C (Tab. IV) and — 5°C ~ — 10°C (Tab. V), respectively. EDTA solution was indicated as having a effect than others. Table VI show the results for 'camarão-rosa'.

From the results of Table VI, it is evident that L-ascorbic acid strongly delayed black spot formation. Sodium thiosulfate and EDTA delayed black spot formation too, but their effects were not as strong as L-ascorbic acid, while, sodium sulfate had hardly any value in preventing black spot formation. At the 9th day, pH value of shrimp, which showed no black spot up to that day, was measured and the results obtained are shown in Table VII. The author was able to indicate slight differences between three chemicals, except sodium sulfate.

TABLE IV — Discoloration during storage of 'camarão-sete-barbas' (solution) at 0°C ~ — 2°C

Storage period days	Reference %	Chemicals				
		L-Ascorbic acid %	EDTA %			
			0.5%	1.25%	2.5%	5.0%
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	15	0	0	10	0	10
3	40	10	0	20	10	20
4	55	40	20	20	60	40
5	100	/	/	/	/	/
6		50	30	40	60	50

TABLE V — Discoloration during storage of 'camarão-sete-barbas' (solution) at — 5°C ~ — 10°C

Storage period days	Reference %	Chemicals		
		Na_2SO_4 %	L-Ascorbic acid %	EDTA %
0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	10	0	0	0
8	20	0	0	0
9	20	10	0	0
10	20	10	0	0
11	/	/	/	/
12	/	/	/	/
13	50	20	10	10

TABLE VI — Discoloration during storage of 'camarão-rosa' (solution) at — 5°C

Storage period days	Reference %	Chemicals			
		$Na_2S_2O_3$ %	Na_2SO_4 %	L-Ascorbic acid %	EDTA %
0	0	0	0	0	0
1	0	0	10	0	0
2	20	10	20	0	10
3	60	10	50	0	10
4		10	50	0	10
5		10	50	0	10
6		10	50	0	10
7		20	60	0	20
8		20	70	0	20
9		40	80	0	40

From the results of Tables IV and V, it was observed that the formation of black spot was strongly delayed at low temperature. In this case, shrimp didn't become black for about 6 days without chemicals, but after that, the black spot appeared rapidly on the shrimps. The author was unable to indicate organoleptic difference between these chemicals up to 8 days, but he could indicate the differences between three chemicals from pH value measured at 8th days. The results of the pH value were shown in Table VII. At high temperature (0°C ~ — 2°C), diluted

The results of the chemical test are shown in Figures 1-5.

TABLE VII — pH value of each fraction during storage

	Chemicals	'Camarão-sete-barbas'				'Camarão-rosa'
		pH value				
		— 5°C ~ — 10°C		0°C ~ — 2°C		— 5°C ~ — 10°C
		8th days	After blackening	6th days	After blackening	9th days
Solution	L-Ascorbic acid	6.80	7.70	7.40	7.70	7.11
	0.5 % EDTA	—	—	7.20	7.80	—
	1.25 % EDTA	7.20	7.80	7.50	7.50	7.38 (1)
	2.5 % EDTA	—	—	—	7.80	—
	5.0 % EDTA	—	—	—	8.00	—
	Na_2SO_4	7.50	8.00	8.00	8.00	7.59
	$Na_2S_2O_3$	—	—	—	—	7.39
Powder	Na_2SO_4	—	—	7.50	8.20	—
	L-Ascorbic acid	—	—	6.80	5.00 (2)	—
	EDTA	—	—	6.20	6.70	—

1) Concentration of EDTA is 1%

2) Yellow color

Figure 1 shows the results of one dimension paper chromatography.

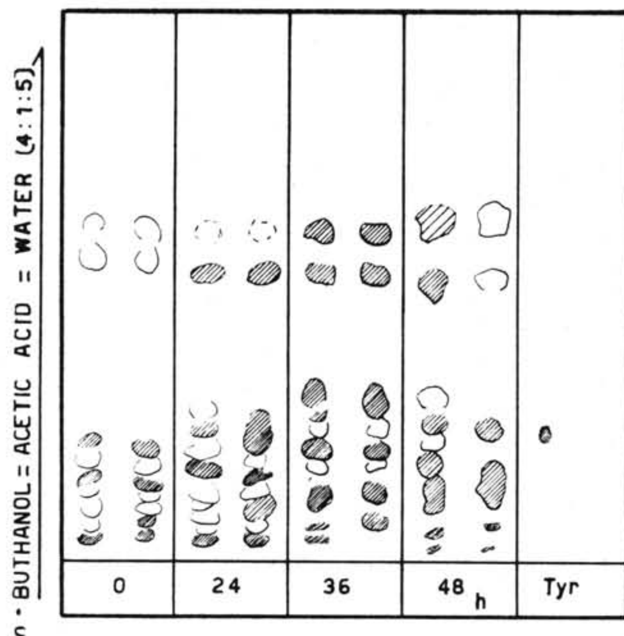

Fig. 1 — One dimension paper chromatogram of tyrosinase and shrimp muscle after 0, 24, 36 and 48 hours storage time at 0°C to −2°C.

Figue 2 shows the results of fresh material. Figure 3 shows the results after 24 hours of storage. Figure 4 shows the results after 36 hours. Figure 5 shows the results after 48 hours. After 48 hours,

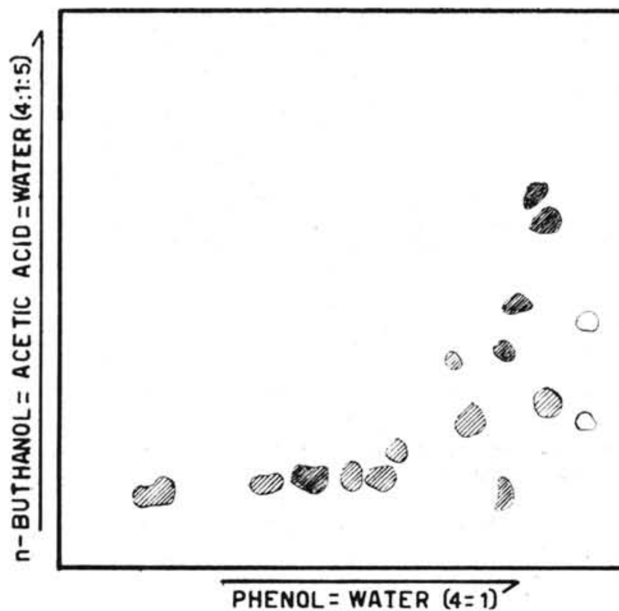

Fig. 2 — Two dimension paper chromatogram of fresh shrimp muscle.

the surface of shrimp's neck became slightly black. From Figures 2, 3, 4 and 5, the author was able to indicate that some amino acids (pro. arg. tyr. α — amino butyric acid etc) has disappeared and an unknown substance was produced during storage at 0°C ~ — 2°C. The results of the change of free amino acids in the muscle of shrimps are shown in Table VIII.

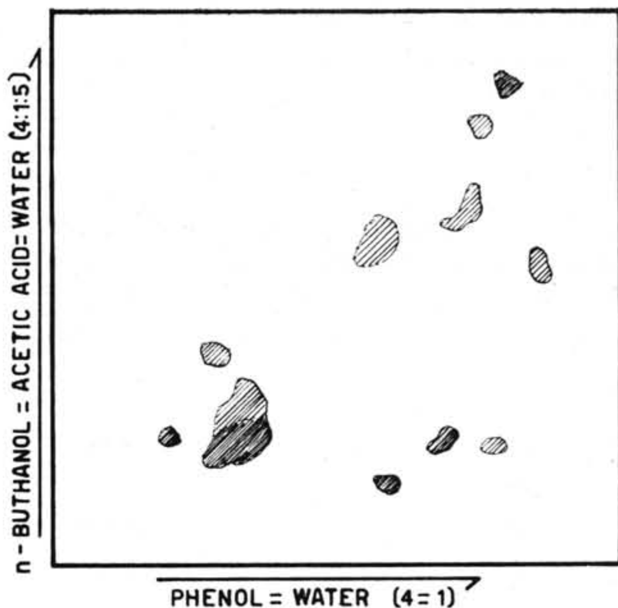

Fig. 3 — Two dimension paper chromatogram of shrimp muscle after 24 hours of storage time at 0°C to −2°C.

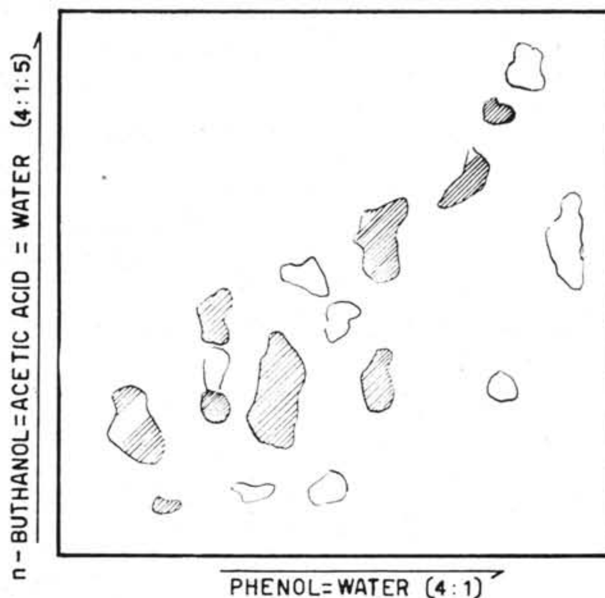

Fig. 5 — Two dimension paper chromatogram of shrimp muscle after 48 hours storage time at 0°C to −2°C.

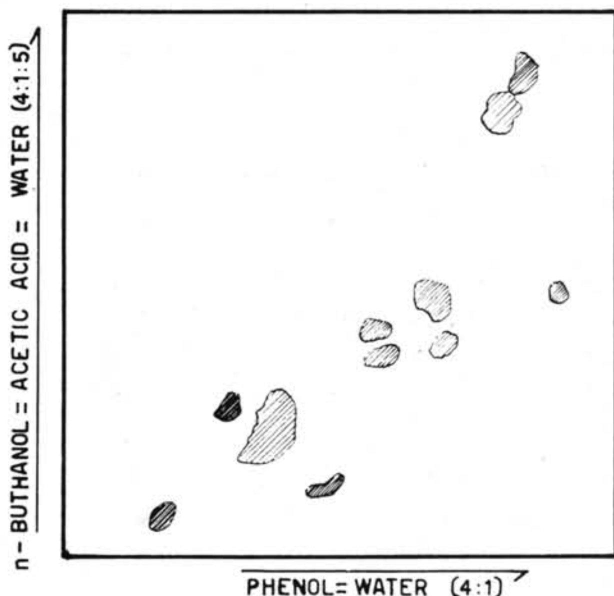

Fig. 4 — Two dimension paper chromatogram of shrimp muscle after 36 hours storage time at 0° to −2°C.

The author again measured the pH as an indicator of autolysis. These results are shown in Table IX.

II. *On the isolation and purification of the enzyme related to the formation of the black spot of shrimp*

A summary of the isolation procedure of tyrosinase from shrimp liver is shown in Figure 6 and Table X.

All the steps were carried out below 10°C. Unless otherwise indicated the buffer referred to below is 0.1 M sodium phosphate at pH 7.2.

Extration — Fresh liver of shrimp was mixed with two volumes of cooled buffer and allowed to stand for 24 hours; then the extract was filtered.

Preliminary purification — The filtrate was treated with 50 ml of 0.2 M Manganous sulphate per liter. The pH value of this solution has to be kept at 7.2. After standing for 24 hours, the precipitate was removed by centrifugation and washed once with chilled water.

Ammonium sulphate precipitation — To the mixture of the supernatant and the washing, solid ammonium sulphate was slowly added by stirring, to bring the concentration of ammonium sulphate to 60%, and then allowed to stand overnight. The precipitated protein, containing the tyrosinase, was

TABLE VIII — Change of free amino acid in the muscles of 'camarão-sete-barbas'

Amino acid	Phe	Leu	Val + Met	Try	Tyr	α - Amino - butyric acid	Pro	Ala	Arg	Thr	Ser	Gly	Glu	Asp
0	+	+	+	+	+	+	+	++	+++	+	+	++	+	+
24	+	+	+	±	+	+	±	++	++	+	+	+	+	+
36	+	+	+	±	±	?	—	+	±	+	+	+	+	+

TABLE IX — Change of pH value during storage

Storage period h.	0	12	24	36	48	Storage temperature
pH value	7.0	6.9	6.8	7.0	7.2	(—2°C)
	7.0	7.3	—	8.2	—	(—2°C)

TABLE X — Purification of tyrosinase

Fraction	Volume ml	Total units DOPA	Total protein mg	Specific activity DOPA unit/ mg	Purification
Crude extract	200	200.000	15	13.33	1
$MnSO_4$ supernatant	300	207.480	12.9	16.08	1.2
First ammonium sulfate precipitate	100	130.802	3.8	34.42	2.6
Extract of acetone precipitate	40	118.700	1.0	118.70	8.9
Solution passed on Sephadex column	20	104.160	0.64	162.75	12.2
Celite eluate	50	16.670	0.0536	329.88	24.9

FRESH LIVER
- add. 2 volumes of 0.1 M sodium phosphate buffer, pH 6.8 in the low temperature, after 24 hours, filtered.

FILTRATE
- add. 0.2 M Mn-SO_4 (50 ml/l) keep pH 7.2, after 24 hours, filtered.

FILTRATE
- add. solid $(NH_4)_2SO_4$, at 0°C, after 24 hours, the precipitate was collected by centrifugation at 2,000 r.p.m. for 30 minutes.

PRECIPITATE
- dissolved in 0.1 M sodium phosphate buffer pH 7.2, add. 2 volumes of acetone at −10°C the precipitate was collected by centrifugation.

PRECIPITATE
- extraction with small quantities of buffer, add. solid $(NH_4)_2SO_4$, the precipitate was collected by centrifugation.

PRECIPITATE
- dissolved in buffer, passed on Sephadex G-50 column, add. equal volume of chilled water.

CELITE CHROMATOGRAPHY

Fig. 6 — Isolation of enzyme from 'camarão-sete-barbas'.

collected by centrifugation at 2,000 r.p.m. for 30 minutes.

Acetone precipitation — The precipitate was dissolved in a minimal amount of buffer and cooled to — 10°C, then two volumes of acetone were added slowly by stirring. The suspension was centrifuged, the supernatant of which was discarded. The tyrosinase was taken out of the precipitate by extracting it several times with small amounts of buffer and was precipitated from the combined extracts by addition of solid ammonium sulphate.

Procedure on Sephadex G-50 — The ammonium sulphate precipitate from the preceding step was dissolved in buffer. The solution was made free from ammonium sulphate by passing it through Sephadex G-50 column (2.5 x 8 or 2.5 x 15 cm) which had been equilibrated with 0.01 M buffer.

Celite chromatography — The ammonium-free eluate from the preceding was diluted with two volumes of water and placed on a column (2.5 x 8 or 2.5 x 15 cm) of Celite 545 which had been equilibrated with 0.005 M buffer. Stepwise elution of the column was employed for the chromatographic separation of the enzyme. The effluent volume of each tube was controlled for 5 ml in 30 minutes.

About 70% of the activity originally placed on the column was recovered in the 0.02 M buffer. These results are shown in Figure 7.

DISCUSSION

The author studied the effect of chemicals against shrimp blackening with organoleptic and chemical methods.

Fishermen except for those operating from São Sebastião did not use chemicals for the prevention of black spot formation in 'camarão-sete-barbas'. Therefore is valuable to use 'camarão-sete-barbas' for studies in the laboratory on the effect of chemicals on the prevention of the black spot formation.

From the results of Table III and V, the author observed that L-ascorbic acid had very strong value in preventing black spot formation.

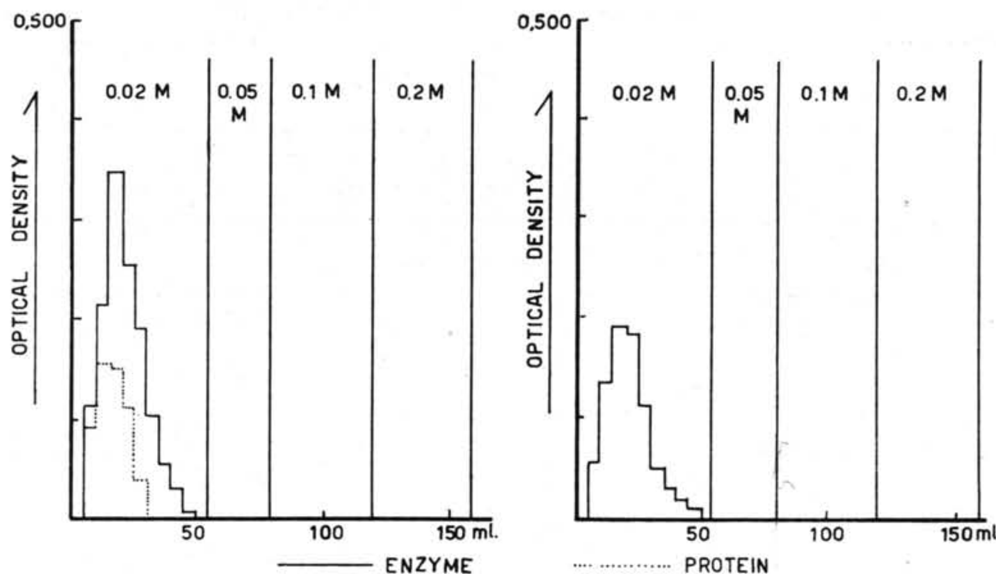

Fig. 7 — Elution of tyrosinase from Celite 545 column.

If the phenomenon of blackening is caused by the action of tyrosinase in shrimp, it should be inhibited when copper ion is removed from the shrimp muscles, because it is subject to activation by tyrosinase. It is known that copper ions are contained in the muscles of shrimp, therefore a chelate reagent (EDTA) was used to remove it. And it was observed that the effect of EDTA was much the same as that of L-ascorbic acid when dilute solution of EDTA was used.

'Camarão-rosa' treated with chemicals (sodium sulfate) were used. From Table VI, the author observed that L-ascorbic acid, sodium thiosulfate and EDTA were valuable in preventing black spot formation caused by tyrosinase. L-ascorbic acid and sodium thiosulfate were reducing reagents and strongly delayed black spot formation. The fact agreed well with the results of other authors.

Sodium thiosulfate, sodium sulfate and sodium bisulfite have various uses, are widely used and their price is reduced. But, when we use these reagents, we must keep within the limits allowed by legislation on the hygenie of food. While L-ascorbic acid and EDTA are harmless their price is high. The author thinks that this is the difficulty to the widespread adoption of these chemicals as preservatives.

In this experiment, the author observed that reducing reagents and chelate reagents are valuable in preventing black spot formation in shrimp. From these results, the author is also able to conclude that blackening occurs by the action of tyrosinase in shrimp.

The author recommends the following method for the prevention of the black spot formation of shrimp as follows:

Procedure — The fresh shrimp are dipped in the cooled chemical (reducing reagent or EDTA) solution for a few minutes. After that, treated shrimp are put in a box and stored at low temperature for the prevention of the evaporation of moisture from shrimp and to inhibit the action of contaminated bacteria.

Moreover, the author studied the change of the free amino acids in shrimp muscles when stored at about $-2°C$. At this temperature, black spot appeared on the surface of the shrimp within 48 hours. The fresh shrimp have many free amino acids in the muscle as shown in Figure 2. This fact agreed well with the results of ALMEIDA (1954) and CAMIER *et al.* (1951).

On paper chromatogram, the author did not identify some substances that would have occurred during storage. As shown in Table IX it was observed that autolysis occurred in shrimp. The author was unable to clear the relation of blackening and free amino acids in muscle, but he thinks that part of amino acids will be used as substrate of tyrosinase, because tyrosinase and tryptophane disappeared at once.

Moreover, the author studied the isolation and purification of the enzyme related to the discoloration of shrimp.

In our preparatory experiment, it was studied wheter tyrosinase present or not in various organs of 'camarão-sete-barbas' and on some solvents for the extraction of tyrosinase from the 'camarão-sete-barbas' liver. The results obtained were shown in Table XI.

From the results of Table XI, tyrosinase was extracted from 'camarão-sete-barbas' liver with phosphate buffer, at pH $6.8 \sim 7.2$. Also the activity of tyrosinase of three sorts of shrimp ('camarão-sete-barbas', 'camarão-rosa' and 'camarão-legítimo') was compared. The results obtained were shown in Figures 8 and 9.

From the results shown on Figures 8 *a*, *b*, and 9, it may be seen that the activity of tyrosinase from these shrimps indicated the same activity, after 24 hours.

TABLE XI — The enzymatic activity of the various organs from 'camarão-sete-barbas'

Hour / Sample			0.5	1	1.5	2	3	5	8	15	24
A	R	tyr	Y	DB	+	+				++	+++
		H_2O	—	—	—					+	+
	Q	tyr	+	+	+	±	++	++		+++	+++
		H_2O	—	—	±	±	+	+		+	++
B	R	tyr	Y	DR	DV	+				+	+
		H_2O	—	—	—	—				±	±
	Q	tyr	+	+	+	+	+	+		+	+
		H_2O	—	—	—	—	—	—		±	±
C	R	tyr	RV	DR	DR	+				+++	+++
		H_2O	—	—	—	—				+	+
	Q	tyr	+	+	+	+	+			+++	+++
		H_2O	—	—	—	—	—	—		—	+
D	R	tyr	±	±	±	±	±	±	±	+	+
		H_2O	—	—	—	—	—	—	—	—	±
	Q	tyr	±	±	±	±	±	±	±	+	+
		H_2O	—	—	—	—	—	—	—	—	—
E	R	tyr	—	—	—	—	—	—	—	—	—
		H_2O	—	—	—	—	—	—	—	—	—
	Q	tyr	—	—	—	—	—	—	—	—	—
		H_2O	—	—	—	—	—	—	—	—	—
F	R	tyr	—	—	—	—	—	—	—	—	—
		H_2O	—	—	—	—	—	—	—	—	—
	Q	H_2O	—	—	—	—	—	—	—	—	—
		tyr	—	—	—	—	—	—	—	—	—

Sample A — extract solution by physiological salt.

B — extract solution by phosphate buffer, pH 6.8.

C — extract solution by 30% acetone.

D — liver; E — muscle and F — intestine. D, E and F were extract solution by phosphate buffer, pH 6.8.

R = raw material; Q = heated material; Y = yellow; DB = dark brown; DR = dark red; DV = dark violet; RV = redish violet; +, black (darkeing), —, no black.

As shown in Table X, the tyrosinase was purified, by approximately 25 times its activity from the initial extract, by means of absorption chromatography through Celite 545, as judged by the rate of the formation of dopachrome.

Recently, the purification of tyrosinase from various sources such as mushroom, hamster and Neurospora was published (Harowitz 1960; Fling 1963; Bouchilloux *et al.* 1963; Pomerantz 1963). The author was unable to compare his results with the present ones, because of the lack of more detailed data on the physical and chemical properties of this enzyme, which will be reported elsewhere.

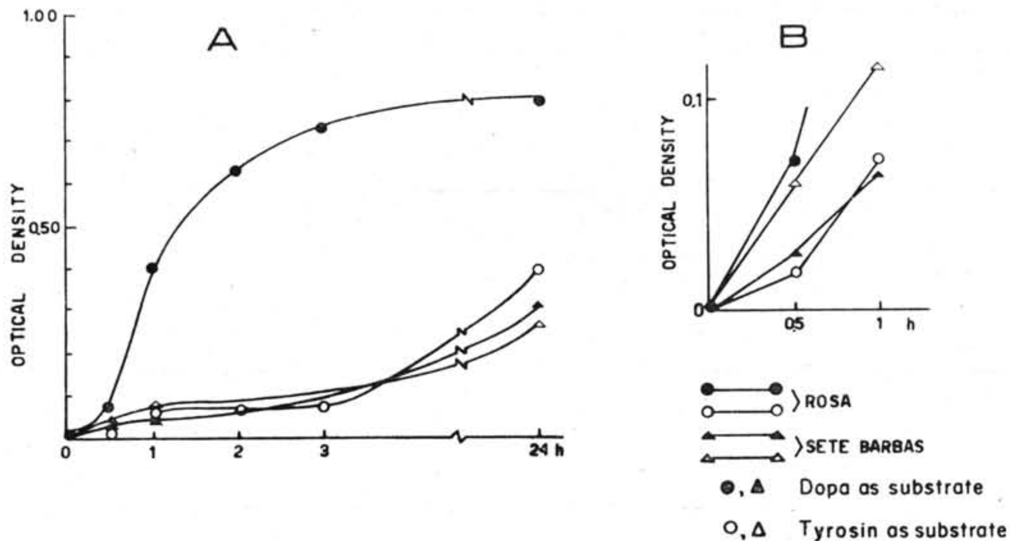

Fig. 8 — Enzyme activity of 'camarão-sete-barbas' and 'camarão-rosa'.

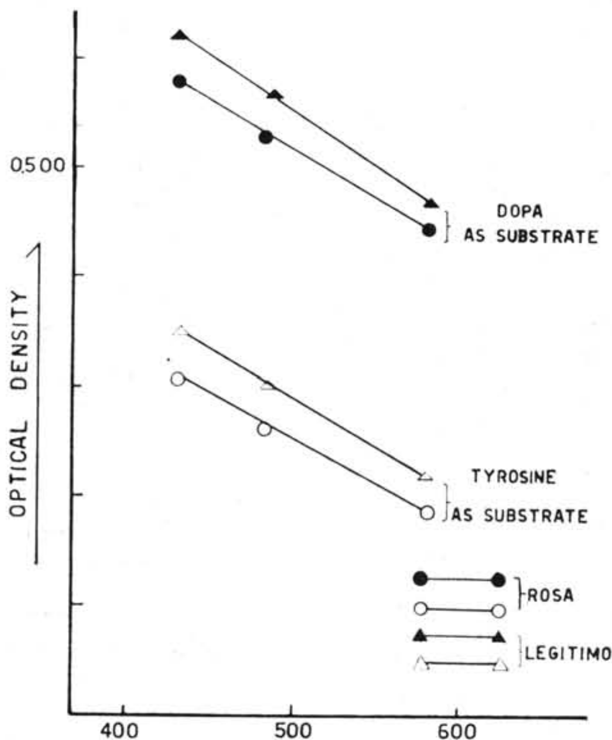

Fig. 9 — Enzyme activity of 'camarão-rosa' and 'camarão-legítimo'.

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Dra. M. Vannucci for her support and also wishes to thank Miss Dulcinda Rodrigues da Silva and Mr. Luiz Sanches for their helpful assistance.

RESUMO

Foram estudadas, no presente trabalho, as causas do enegrecimento do camarão, tendo-se verificado que o mesmo é causado, fundamentalmente, pela ação da tirosinase, produzida no hepato pâncreas do crustáceo. O isolamento e a purificação da enzima foram realizados. A purificação foi feita, até se obter uma atividade 25 vêzes maior do que o extrato inicial, por meio de cromatografia de absorção, através de Celite 545 e avaliada através da velocidade de formação de dopacromo.

O uso de diferentes drogas também foi tratado experimentalmente, tendo-se verificado que o melhor inibidor do processo de enegrecimento é o ácido L-arcórbico que evita a formação da mancha preta durante 9 dias à temperatura de 0 a —2°C e durante tempo mais longo em temperaturas mais baixas. Tiosulfato de sódio e EDTA também têm boa ação inibidora. As técnicas empregadas são descritas detalhadamente e é apresentada discussão com resultados obtidos por outros autores. Ao que se sabe, esta é aprimeira vez que tirosinase é isolada de um invertebrado.

REFERENCES

Almeida, M. E. W. de
1955. Aminoácidos livres em camarões — variações decorridas durante a decomposição. Revta Inst. Adolfo Lutz, vol. 15, n.º único, p. 158-167.

Bailey, M. E.
1958. Biochemical of melanin formation in shrimp. Ph. D. Thesis. Louisiana State Univ., Baton Rouge, Louisiana.

Bodine, J. H. & Allen, T. H.
1941. *In*: The proteins, ed. by Hans Neurath. New York, Academic Press, vol. 2, part A, p. 149.

Bouchilloux, S. *et al.*
1963. The multiple forms of mushroom tyrosinase. J. biol. Chem., vol. 238, p. 1699-1707.

Camier, *et al.*
1951. Nonprotein amino acids in muscle and blood of marine and fresh water crustacea. J. biol. Chem., vol 193, p. 881-885.

Degkwitz, E. *et al.*
1954. Untersuchungen uber die Ursachen der Schnillen Verderblichkeit des Krabben Fleisches. Arch. Fischereiwiss., vol. 5, p. 35-42.

FIEGER, E. A. *et al.*
1956. Objective tests applicable to quality studies of ice stored shrimp. Fd Res., vol. 21, p. 611-620.

FLING, M. *et al.*
1963. The isolation and properties of crystalline tyrosinase from Neurospora. J. biol. Chem., vol. 238, p. 2045-2053.

GREEN, M.
1949. Bacteriology of shrimp. I. Introduction and development of experimental procedures. II. Quantitative studies on freshly caught and iced shrimp. Fd Res., vol. 14, p. 365-371, p. 372-383.

HAROWITZ, N. H. *et al.*
1960. Genetic determination and enzymatic induction of tyrosinase in Neurospora. J. molec. Biol., vol. 2, p. 96-104.

HARRISON, E. G. *et al.*
1923. The red discoloration of cured cod fish. Trans. R. Soc. Can., vol. 3, n.º 16, p. 101-152.

KAKIMOTO, D.
1956. Studies on the black discoloration of lobster. Bull. Jap. Soc. scient. Fish., vol. 22, p. 471-479.

MUCCIOLO, P. & SCHNEIDER, I. S.
1965. Eficiência da clortetraciclina na conservação do pescado. Revta nac. Pesca S. Paulo, vol. 6. n.º 46, p. 14-15.

POMERANTZ, S. H.
1963. Separation, purification and properties of two tyrosinase from Hamster melanoma. J. biol. Chem., vol. 238, p. 2351-2357.

SOUTHCOTT, B. A. *et al.*
1965. Tetracycline antibiotics in shrimp preservation. J. Fish. Res. Bd Can., vol. 22, p. 117-129.

TANNER, F. W.
1944. The microbiology of foods. 2nd ed. Champaign, Ill., Garrard Press.