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PART II

ISOLATION OF CHOLAGOGUE SUBSTANCES FROM *SETA-SHIJIMI* (*CORBICULA SANDAI* REINHARDT)

BY

Hiromi YAMADA

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Introduction

In 1933, Kayaba[2] reported that the cholagogue activity in crude extracts of *Shijimi* (*Corbicula* sp.) could be precipitated by trichloroacetic acid or as high as the saturated concentration of NaCl. These results suggest that the active component(s) have protein nature. More recently, Hori[5] made the partial purification of active component(s) and showed that the purified fraction has a basic property and gives several amino acids by hydrolysis. From these results, the active component(s) were supposed to be basic peptide(s).

Further purification of the active component(s) were carried out by the author. Two fluorescent substances which stimulate bile pigment excretion in rabbit were isolated from *Seta-Shijimi* (*Corbicula sandai* Reinhardt). These substances thus isolated are acidic and quite difference was demonstrated from the results formerly obtained by Kayaba and Hori.

The present paper deals with processes of purification and some properties of the isolated cholagogue substances.

Experimental

Biological Test

Determination of the cholagogue activity was made as described in the previous paper.

Charcoal Treatment of the Shijimi extract

The shell-fish was boiled in water (0.5 liter per kg. of the shell-fish) for 30 minutes, and the turbid extract obtained was clarified by filtration through a layer of celite. To the filtrate active charcoal (1 g. per kg. of the shell-fish) was added and the suspension was kept for 30 minutes. The charcoal was collected by filtration, washed with water, and eluted twice with 50% aqueous ethanol (100 ml. per kg.). The combined eluate was evaporated to dryness and the residue was redissolved in a small amount of water.

Ion Exchange Resins

Dowex 50×8 (200 mesh) was used in its Na form. It was prepared by treating with 10 volumes of 2 M NaCl.

Dowex 1×8 (200 mesh) was used in Cl or acetate form. The chloride form was obtained by treating with 10 volumes of 2 M NaCl, and the acetate form was prepared with 10 volumes of 0.25 M sodium acetate and 10 volumes of 8 N acetic acid.

Amberlite IR-120 (30 mesh) was used in H form. Treatment with 10 volumes of 2 N HCl converted the resin into its H form. The acetate form of Amberlite IR-4B (30 mesh) was prepared as follows. The resin was soaked in 10 volumes of 1 N NH_4OH for 12 hours with occasional stirring. After washing with water, it was treated with 10 volumes of 1 N acetic acid.

Filter Paper for Chromatography

Toyo Roshi No. 50 (40 cm.×40 cm.) was used throughout the experiments.

Cellulose Powder

Cellulose powder used was Shleicher and Schull No. 123.

Instrument for Detection of Chromatogram by Ultraviolet Light

A mercury lamp with an ultraviolet film filter was used. The filter was kindly supplied by Iwase Laboratory of the Institute of Physical and Chemical Research, Tokyo.

Spectrophotometric Measurements

All the spectrophotometric determinations in ultraviolet and visible ranges were made by a Beckman DU spectrophotometer.

Results

Ion Exchange Resin Fractionation

The charcoal eluate (12 ml.) was prepared from 6 kg. of *Shijimi* as described previously. The solution was passed through a column of Dowex 50 Na form (0.8 $cm.^2$× 10.5 cm.) at a flow rate of 1 ml. per minute, and the column was washed with 200 ml. of water. The brown effluent and the washing were combined and concentrated to 12 ml. on a boiling water bath. The concentrate was then passed through a column of Dowex 1 Cl form (0.8 $cm.^2$× 10.5 cm.) at the same flow rate as above and the column was washed with 110 ml. of water. The effluent and the washing were combined and concentrated to 9 ml. (neutral fraction). On standing of this fraction a considerable amount of white crystalline precipitate was formed. It was separated from mother liqor by filtration and washed with water (white precipitate). Basic components were eluted from the column of Dowex 50 with 160 ml. of 2 M NaCl solution. To the eluate 2 g. of active charcoal was added and allowed to stand for 15 minutes with occasional

stirring. The charcoal was collected by filtration and washed with water until NaCl was completely removed. The washed charcoal was eluted with 200 ml., 100 ml., and 100 ml. each of 50% aqueous ethanol successively. The charcoal eluates were combined and dried. The resulting brown residue was redissolved in 9 ml. of water (basic fraction). Acidic components were also eluted from the column of Dowex 1 and desalted in the same way as above. The almost colorless residue thus obtained was dissolved again in 9 ml. of water (acidic fraction). Cholagogue activity was determined with all these fractions and the results obtained are shown in *Table* 1. It was clearly shown that only the acidic fraction is effective. Thus active component(s) in the *Shijimi* extract are proved to be acidic substance(s).

Table 1 Fractionation of Charcoal Eluate with Acidic and Basic Resins
Experimental conditions are as described in the text.

Animal No.	Fraction	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
36	Acidic	0.5	2.5
	"	1.2	20
	Neutral	0.5	0
	"	1.0	0
	"	2.4	0
	White precipitate	3.5	0
	Basic	0.5	ca. 0
	"	1.3	ca. 0
42	Acidic	8	5
	Neutral plus white precipitate	8	0
	Basic	8	0

* Dose was expressed in terms of the weight of the original shell-fish.

To simplify the resin fractionation procedure, the following experiments were conducted. Ten ml. of charcoal eluate (equivalent to 1 kg. of *Shijimi*)was directly flowed onto a column of Dowex 1 acetate form (0.8 cm.^{2}×2.5 cm.) and the column (column A) was washed with 10 ml. of water. The effluent and the washing were combined and concentrated to 10 ml. In this case, basic and neutral components were not distinguished. The washed column was eluted with 100 ml. of 2% aqueous acetic acid. The eluate was evaporated and redissolved in 10 ml. of water. Two more such columns (column B and C) were prepared and eluted with 10 ml. of 0.25%, and 10 ml. of 1% sodium acetate solution successively. These fractions were tested for their biological activities. As shown in *Table* 2, the results revealed that the active component(s) were adsorbed on Dowex 1 acetate form and were able to be eluted with 0.25 to 1% sodium acetate or 2% aqueous acetic acid.

Table 2 Adsorption and Elution of Active Component (s) with Dowex 1
Experimental conditions are as described in the text.

Animal No.	Fraction	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
57	Column A {effluent plus washing	0.2	0
	Column A {2% AcOH eluate	0.2	4
53	Column B {effluent plus washing	0.4	0
	Column B {0.25% NaOAc eluate	0.4	11
56	Column C {effluent plus washing	0.4	0
	Column C {0.25% NaOAc eluate	0.4	0
	Column C {1% NaOAc eluate	0.4	2.5

* See foot-note in Table 1.

Ion Exchange Chromatography

Charcoal eluate (800 ml., equivalent to 80 kg. of *Shijimi*) was applied to a column of Dowex 1 acetate form (20 cm.^{2}×5 cm.) at a flow rate of 10 ml. per minute. The column was washed with 3.2 liters of water and eluted with 5 liters of 2%, then 5 liters of 5% aqueous acetic acid successively. The effluent, the washing, and the eluates were collected in 1 liter fractions. The absorbancy at 260 mμ of each fraction was determined. From each fraction 2.5 ml. aliquot was withdrawn and evaporated to dryness on a boiling water bath. Residual material was dissolved in 2 ml. of water and subjected to the biological test. The results obtained are summarized in *Fig.* 1. It is shown

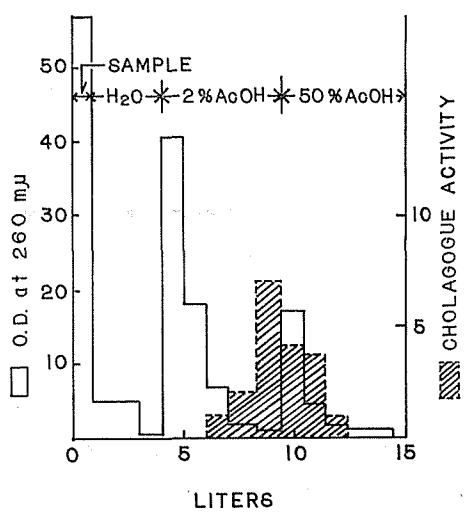

Fig. 1. Column chromatography of charcoal eluate on Dowex 1. Experimental conditions are as described in the text.

that most of ultraviolet-absorbing materials were separated from active component(s) by this procedure.

Further Purification by Paper Chromatography

The fractions 4 to 8 of acetic acid eluate were combined and evaporated to dryness under reduced pressure at about 50°. The residue was dissolved in 10 ml. of water and paper chromatography was conducted with butanol-acetic acid-water (4 : 1 : 1) as solvent. Spots on the chromatogram were detected under the ultraviolet light as shown in *Fig.* 2. The paper was cut in pieces as indicated in the figure and extracted with water. The extracts were concentrated to 30 ml. and the cholagogue activity in each fraction was tested. As shown in *Table* 3, the experimental result indicated that the activity was

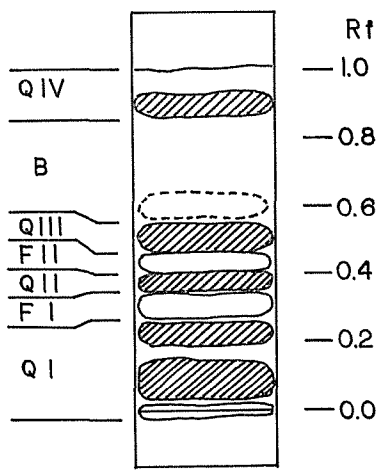

Fig. 2. Paper chromatography of Dowex eluate. Experimental conditions are as described in the text.

Table 3 Fractionation by Paper Chromatography

Experimental conditions are as described in the text.

Animal No.	Fraction	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
72	Q-I	0.6	0
	Q-II	0.6	0
	F-II	0.6	10
73	F-I	0.6	15
	Q-III	0.6	0
	B	0.6	0
	Q-IV	0.6	0

* See foot-note in Table 1.

located only in two fractions, which are in accord with two fluorescent spots on the chromatogram. Rf values of these spots were 0.32 (F-I) and 0.41 (F-II) respectively. Each active fraction was rechromatographed on paper with the same solvent as above and the fluorescent spot was extracted with water. The extracts were then chromatographed with acetone-water (2 : 1) as solvent. The Rf value of spot F-I was 0.73 and that of spot F-II was 0.79 with this solvent system. Spots FI- and F-II were then cut out, extracted with water, and concentrated to 5 ml. Upon testing the biological activities of these fractions, both the purified fractions F-I and F-II were found to have cholagogue activities to rabbit as shown in *Table* 4. The two fractions were evaporated to dryness and yellow residues remained were less than 1 mg. each.

Table 4 Cholagogue Activity of Purified F-I and F-II Fractions

Experimental conditions are as described in the text.

Animal No.	Fraction	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
74	F-I	0.6	7
	F-II	0.6	5
75	F-I	0.3	5
76	F-II	0.06	2.5

* See foot-note in Table 1.

Adsorption of Active Components on Amberlite IR-4B

Since the charcoal adsorption is not convenient for large scale preparation, an anion exchange resin was used for the concentration of the active components from the crude extract. A weak anion exchanger, Amberlite IR-4B (30 mesh), was selected for its large exchange capacity and its facility in passing of rather slime extract. A pilot column of the acetate form of the resin (0.8 $cm.^2 \times 6$ cm.) was prepared and 8 liters of the crude extract from 5 kg. of the shell-fish was passed through at a flow rate of 20 ml.

Table 5 Adsorption of Active Components on Amberlite IR-4B

Experimental conditions are as described in the text.

Anmal No.	Fraction	Dose (kg. equivalent)*	Increase in biliverdin condentration (units)
76	Effluent 1	0.07	0
	" 2	0.07	0
	" 3	0.07	0
	" 4	0.075	0
	" 5 plus 6	0.08	0
	Crude extract	0.08	12

* See foot-note in Table 1.

per minute. One liter fractions were collected and 100 ml. aliquot from each fraction was concentrated to 3 ml. Upon centrifugation at 10,000 X *g* for 15 minutes, the supernatant was subjected to the biological test. The results are shown in *Table* 5. Under the present condition, 5 ml. of Amberlite IR-4B was found to adsorb the active components contained in at least 6 liters of the crude extract, which corresponded to 3.8 kg. of the shell-fish.

Elution of Active Components from Amberlite IR-4B

Several columns of Amberlite IR-4B (5 ml. each) were used for the adsorption of the active components. They were eluted with the following solutions respectively; 1 N, 0.5 N, and 0.1 N HCl: 1 N AcOH; 1 M $(NH_4)_2SO_4$; 1 N NH_4OH; and 0.01 N NaOH. The eluates were collected in 10 ml. fractions and evaporated to dryness. Alkaline eluates were treated with H form of Amberlite IR-120 to remove cations prior to the evaporation step. After the decationization the cation resin was washed with water (4 to 10 times of the resin bed) to recover the active components retained. Using purified samples of F-I and F-II, it was comfirmed that the loss of the active components during this decationization step was negligible. The dried eluates were chromatographed on filter paper with butanol-acetic acid-water (4 : 1 : 1) and examined under the ultraviolet light. The experimental results showed that 1 N HCl is sufficient to elute all the substances giving visible spots under the ultraviolet light within 150 ml. More than 300 ml. of other eluting reagents were required for the complete elution of these substances. In case of 0.01 N NaOH, 1.5 liter was necessary. The eluates with 1 N HCl obtained in different experiments were dried and redissolved in small amounts of water. Upon neutralization with 1 N NaOH, they were injected to animals and their cholagogue activities were confirmed as shown in *Table* 6.

Table 6 Cholagogue Activity of Amberlite IR-4B Eluate

Experimental conditions are as described in the text.

Animal No.	Dose (kg. equivalent)*	Increase in biliverdin concentration (units)
101	0.013	1
	0.04	6
102	0.16	0
	0.75	23
104	0.2	2
	0.5	10

* See foot-note in Table 1.

Large Scale Preparation

One hundred kg. of *Shijimi* was placed in a 180 liters can and 50 liters of water

was added. The content was heated and kept in boiling state for 1 hour. The extract was taken out through a cock at the bottom of the can. The residual shell was extracted with 50 liters of water two more times in the same way. The extracts were combined and allowed to cool down to room temperature. The cooled extract was passed through a column of IR-4B acetate form (20 cm^2. × 5 cm.) at a flow rate of 10 liters per hour, and the effluent was discarded. The resin was well washed by suspending it in water, and stored in the cold. In this way 1,200 kg. of the shell-fish was treated and 0.9 liter of the resin adsorbed most of the active components was soaked in 1.7 N HCl overnight. A red orange HCl layer was reserved and the resin was transfered to a glass tube to elute with 30 liters of 1.7 N HCl.

The eluates were combined and concentrated to 400 ml. Precipitated succinic acid was removed by filtration and the supernatant solution was saved.

Although butanol fractionation of the concentrated eluate was tried, any effective purification was not achieved. Column partition chromatography using cellulose powder or potate starch with butanol-acetic acid-water system was also carried out, and was proved to be ineffective to purification of the active components.

All the fractions obtained by the above treatments were recombined and chromatographed on 70 sheets of filter papers with butanol-acetic acid-water (4 : 1 : 1) as solvent. Zones on papers corresponding to F-I and F-II were cut out and extracted three times with about 1 liter each of distilled water. The extracts were combined, concentrated under reduced pressure and rechromatographed on filter papers with acetone-water (3 : 1). The fluorescent substances F-I and F-II were further rechromatographed with butanol-acetic acid-water and acetone-water as solvents successively. The finally purified fractions were concentrated to 1 ml. each in a boiling water bath and yellow solutions showing strong green fluorescence were obtained. Four volumes of acetone were added to the fluorescent solution and the resulted yellow precipitate was collected by centrifugation, washed with a small amount of 80% aqueous acetone,

Table 7 Cholagogue Activity of Crystals F-I and F-II

Experimental conditions are as described in the text.

Animal No.	Sample	Dose (γ)	Increase in biliverdin concentration (units)
98	F-II	ca. 10	5
99	F-I	2	0
	"	20	0
	F-II	2	10
101	F-I	100	4
106	F-I	60	12
117	F-II	15	2.7

and dried over H_2SO_4 under reduced pressure. About 10 mg. of yellow crystal was obtained from F-I fraction, and also about 2 mg. of brownish yellow crystalline material from F-II fraction. Each crystal showed single fluorescent spots on chromatograms using butanol-acetic acid-water and acetone-water as solvent systems. The crystals were dissolved in water and subjected to the biological test. As shown in *Table* 7, the experimental results revealed that both samples have distinct cholagogue activity to rabbit, and that F-II is much more active than F-I.

Properties of Active Substances

F-I and F-II are both sparing soluble in cold water and soluble in hot water or 50% aqueous acetic acid. They are insoluble in ether, chloroform, and acetone. Aqueous solution of F-I shows a pale blue fluorescence under the ultraviolet light, and that of F-II shows a greenish yellow fluorescence. Both compounds did not melt or decompose below 250°C. Ultraviolet spectrum of aqueous solution of F-I (*Fig.* 3) shows maxima at 230, 260, 290, and 370 mμ (ε_0=17,300, 10,900, 5,930, and 5,430,), and that of F-II (*Fig.* 4) at 258, 290, and 360 mμ (ε_0=8,100 5,800, and 2,600). These compounds are found to be rather unstable under irradiation of the visible and ultraviolet lights in neutral solutions. Paper chromatographic analysis revealed that F-I is tend to convert slowly to other fluorescent or ultraviolet-absorbing compounds. F-II was also proved to decompose into F-I and these unidentified compounds under the above conditions.

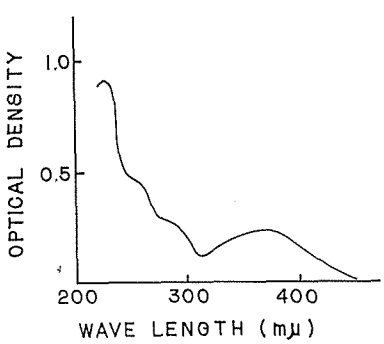

Fig. 3. Absorption spectrum of F-I.

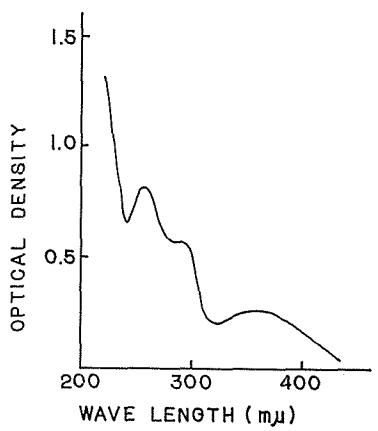

Fig. 4. Absorption spectrum of F-II.

Discussion

The active substances isolated by the author is rather low molecular compounds having acidic properties. This result dose not agree with that formerly obtained by Kayaba[2], in which a protein-like character of the active component was suggested.

The solution of this discrepancy may be brought by the following two interpretations.
(A) The effect caused by Kayaba's active substance was reported to be the increase in the volume of excreted bile, while that by the author's was the increase in biliverdin concentration. Therefore, it seems likely that the cholagogue substance which Kayaba examined is different from that of the author.
(B) The author's cholagogue substance may be coprecipitated with protein-like materials in Kayaba's experiment.

Another discrepancy between the author's result and Hori's is as follows. This is very incomprehensible one. Biological effect of *Shijimi* extract observed by these two investigators was the same, that is the increase in biliverdin concentration. By ion exchange resin fractionation method, however, Hori revealed the basic property of the active substance. While, the results of the author showed the acidic property by the similar method. This is as yet unsolved problem.

Hori[5] showed that the cholagogue activity of *Shijimi* extract considerably varied from season to season, and the shell-fish sampled in spring gave the most active extract. The author found that the amount of fluorescent components in the *Shijimi* extract changed remakably by season. Besides F-I and F-II, some other active substances seems to be present. Seasonal variations of active components in quality as well as in quantity should make the results of the different investigators more intricate.

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