CHEMICAL AND PHARMACOLOGICAL STUDIES ON GLOBEFISH POISON.

II. PAPERCHROMATOGRAPHY OF THE CONSTITUENTS OF THE GLOBEFISH LIVERS AND OVARIES

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Purification of globefish poison has been reported by Tawara (1911[1]), Yokoo (1950[2]) and Tsuda and Kawamura (1952[3]). So far as their and other reports indicate the removal of fat in the crude poison is relatively easy, but sugars and proteins are difficult to remove. It seems likely that the impurities contained in the extract, such as sugars, free amino-acids and proteins may affect the toxicity of a crude poison, thereby causing varied potencies among different preparations. In earlier papers this point has not been considered. A careful investigation into the interference of impurities of the extract on the toxic potency is justified. The present study was therefore undertaken to clarify some of thsese points.

MATERIALS AND METHODS

Various poisonous extracts possessing different toxic potencies have been selected and used. The starting materials were those used in the previous report (1) and they consisted of nine "deadly", two "strong" and seven "weak" and three "none-poisonous" livers and 13 "deadly" ovaries.

1). Reducing sugar: About 0.02 cc of the sample was applied onto a Toyo Roshi No. 50 (3 × 40 cm). The development was achieved by one dimensional ascending method in a closed glass jars at 18–20°C for 15 hours. An acetic acid-Butanol-water solution (BuOH : AcOH : H_2O = 4 : 1 : 3) was used as a partioning solvent. The spots were made visible with aniline hydrogen phthalate (an equimolar mixture of aniline and phthalic acid in water saturated butanol), and with ammonia silver nitrate solution (mixture of equal volumes 0.5 per cent ammonia water and 0.1 N silver nitrate solution).

2). Free amino acid: About 0.02 cc of the sample was applied onto a Toyo Roshi No. 50 (40 × 40 cm). Two-dimensional ascending paperchromatography

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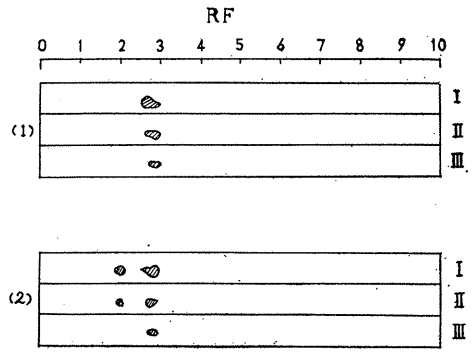
was run with (Bu OH : AcOH : H_2O=4 : 1 : 3) as the first solvent and with 80 per cent phenol (containing 0.1% NH_3) as the second, at 20–22°C for 15 hours. Ninhydrin (0.2% in watersaturated butanol) was used to spot the amino acids.

RESULTS

1). Reducing sugar: (a) When aniline hydrogen phthalate was used to determine the location of spots, one brown spot appeared with almost all liver samples and in a few ovaries and one pink spot in certain liver samples. The former was located at Rf 0.260–0.290 and the latter at 0.180–0.200 (Fig. 1). The color of the spot varied and may be graded into five intensities, (卅), (++), (+), (±), and (−), (Table 1, 3). (b) When ammonia silver nitrate solution was used, one brown spot was located at Rf 0.285–0.300 in almost all liver materials; no such spot was obtained with the ovary (Fig. 1). This spot may also be graded into 5 color intensities (Table 1, 3). Individual difference in the color of reducing sugar was marked in the each liver sample, and the appearance of the pink spot had no bearing on the color intensity of the brown spot (Table 2, 4). Brown spots in (a) and (b) were both assumed to be a hexose judging from their Rf values, and subsequently identified as glucose by comparing their Rf values with that of pure glucose. Pink spots were assumed to be a pentose, but no identification was made. When the relationship was studied between the toxicity and the color intensity of the spots obtained with livers and the aniline hydrogen phthalate procedure, poisonous samples generally

Fig. 1 Reducing sugars



Note: One dimensional paperchromatogram of globefish liver and ovary extract.
Filter paper: Toyo Roshi No. 50, 2×40 cm
Solvent: BuOH:AcOH:H_2O=4:1:3
Ascending system at 20~22°C for 15 hours
Spraying reagent: (1). 0.1 N ammonia silver nitrate solution
(2). Aniline hydrogen phthalate

Table. 1 Toxicity and reducing sugar isolated from individual globefish liver (1)

Sample No.	Sex	Toxic potency (Mouse unit)	A. H. P		A. S. N	
			Brown	Pink	Brown	Pink
1	♂	+ (36~1,600)	+	+	+	−
2	♂	+++ (960~2,160)	+	−	+	−
3	♀	+++ (1,920~3,600)	−	−	−	−
4	♂	+++ (3,400~5,700)	+	−	±	−
5	♂	+++ (1,140~2,160)	+	−	+	−
6	♀	+ (36~ 840)	++	+	++	−
7	♀	− (<36)	+	−	±	−
8	♀	+ (38~ 720)	++	−	++	−
9	♀	− (<36)	+	−	++	−
10	♀	+ (36~ 340)	++	+	+	−
11	♀	++ (400~ 680)	+++	−	++	−
12	♀	+++ (3,060~4,080)	+	+	+	−
13	♀	+ (34~ 340)	++	−	+	−
14	♀	− (<36)	+	−	+	−
15	♀	+ (38~ 340)	+++	−	++	−
16	♂	+++ (3,600~6,300)	+	+	++	−
17	♂	+++ (4,320~6,080)	+++	−	+++	−
18	♀	+ (38~ 80)	+++	+	+++	−
19	♀	++ (144~ 228)	+	−	+	−
20	♂	+++ (9,520~11,520)	++	−	++	−
21	♂	+++ (3,800~5,400)	+	−	+	−

Note: A. H. P; Spots tested by aniline hydrogen phthalate
A. S. N; Spots tested by ammonia silver nitrate
(1). Spots were graded grossly into the following five degrees of the intensity of colour and size (+++), (++), (+), (±), (−).
(2). Degree of toxicity: deadly=+++, strong=++, weak=+, none=−.

Table. 2 Toxicity and reducing sugar isolated from individual globfish liver (2)

Aniline hydrogen phthalate.

Tox. \ Col.	+++	++	+	±	−	Samples
+++	1	1	6	0	1	9
++	1	0	1	0	0	2
+	2	4	1	0	0	7
−	0	0	3	0	0	3
Samples	4	5	11	0	1	21

Ammonia silver nitrate.

Tox. \ Col.	+++	++	+	±	−	Samples
+++	1	2	4	1	1	9
++	0	1	1	0	0	2
+	1	3	3	0	0	7
−	0	0	3	0	0	3
Samples	2	6	11	1	1	21

Note: (1). Spots were graded grossly into the following five degrees of the intensity of colour and size (+++), (++), (+), (±), (−).
(2). Degree of toxicity: deadly=+++, strong=++, weak=+, none=−.
(3). Tox.: Toxicity, Col.: Colour.

Table. 3 Toxicity and reducing sugar isolated from individual globefish ovary (1)

Sample No.	Toxic potency (Mouse unit)		A. H. P		A. S. N	
			Brown	Pink	Brown	Pink
1	⫲	(1,440~1,700)	−	−	−	−
2	⫲	(5,400~7,200)	+	−	−	−
3	⫲	(3,400~4,560)	±	−	−	−
4	⫲	(3,400~4,800)	−	−	−	−
5	⫲	(3,400~4,160)	−	−	−	−
6	⫲	(1,360~2,560)	−	−	−	−
7	⫲	(3,400~4,480)	−	−	−	−
8	⫲	(1,920~3,200)	±	−	−	−
9	⫲	(2,880~3,840)	±	−	−	−
10	⫲	(3,200~5,320)	−	−	−	−
11	⫲	(1,900~2,880)	−	−	−	−
12	⫲	(9,180~9,600)	−	−	−	−
13	⫲	(1,700~3,200)	±	−	−	−

Note: A. H. P; Spots tested by aniline hydrogen phthalate
A. S. N; Spots tested by ammonia silver nitrate
(1). Spots were graded grossly into the following five degrees of the intensity of colour and size (⫲), (⫲), (+), (±), (−).
(2). Degree of toxicity: deadly=⫲, strong=⫲, weak=+, none=−.

Table. 4 Toxicity and reducing sugar isolated from individual globfish ovary (2)

Aniline hydrogen phthalate.

Tox. \ Col.	⫲	⫲	+	±	−	Samples
⫲	0	0	1	4	8	13
⫲	0	0	0	0	0	0
+	0	0	0	0	0	0
−	0	0	0	0	0	0
Samples	0	0	1	4	8	13

Ammonia silver nitrate.

Tox. \ Col.	⫲	⫲	+	±	−	Samples
⫲	0	0	0	0	13	13
⫲	0	0	0	0	0	0
+	0	0	0	0	0	0
−	0	0	0	0	0	0
Samples	0	0	0	0	13	13

Note: (1). Spots were graded grossly into the following five degrees of the intensity of colour and size (⫲), (⫲), (+), (±), (−).
(2). Degree of toxicity: deadly=⫲, strong=⫲, weak=+ none=−.
(3). Tox.: Toxicity, Col.: Colour.

had stronger color than none-poisonous samples. The (+) spots appeared most frequently in both "deadly" and "none-poisonous" samples. The (⫲), (⫲), or (+) spots were frequently found in "deadly" and "weak" poisonous samples. The results in ammonia silver nitrate tests were similar (Table 2). In contrast, only four spots (±) out of thirteen ovaries were obtained with aniline hydrogen phthalate, while all were (−) with silver nitrate solution (Table 4).

2). Free amino acid: The ninhydrin pattern of all the amino acids of the liver samples separated with paperchromatography are shown in Fig. 2 and that of the ovary samples in Fig.3. Six to ten spots were detected from the liver samples and

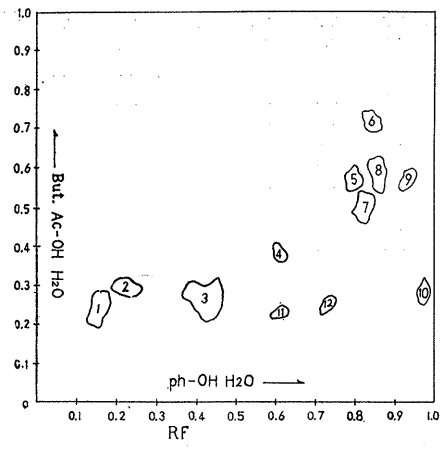


Fig. 2 Free amino acids

Note: Two dimensional paper chromatogram of globefish liver extract.
Filter paper: Toyo Roshi No. 50, 40×40 cm
Solvent: (1). BuOH : AcOH : H_2O=4:1:3
(2). Phenol (0,1% NH_3)
Ascending system: at 20~22°c for 15 hours
Spraying reagent: 0.2% Ninhydrin butanol solution.

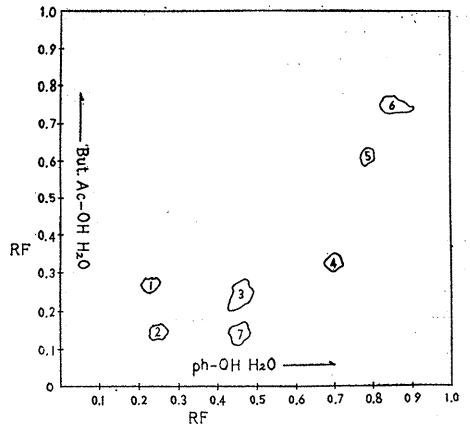


Fig. 3 Free amino acids

Note: Two dimensional paper chromatogram of globefish ovary extract.
Filter Paper: Toyo Roshi No. 50, 40×40 cm
Solvent: (1). BuOH : AcOH : H_2O=4:1:3
(2). Phenol (0.1% NH_3)
Ascending system: at 20~22°c for 15 hours
Spraying reagent: 0.2% Ninhydrin butanol

Table. 5 Toxicity and free amino acids isolated from liver extract of individual globefish.

Sample No.	Sex.	Degree of toxicity	Degree of ninhydrin colour reaction											
			Number of amino acids											
			1	2	3	4	5	6	7	8	9	10	11	12
1	♂	+	++	++	+++	+++	+++	++	++	++	+	++	−	−
2	♂	+++	−	+	+	+++	+++	+++	+++	−	−	−	−	−
3	♂	+++												
4	♂	+++	+	+	+++	+++	++	++	+++	−	+	++	−	−
5	♂	+++	++	++	++	+++	++	++	+++	+	+	++	−	−
6	♀	+	±	+	++	+++	+++	+++	+++	−	−	−	−	−
7	♀	−	+	+	+++	+++	+	+++	+++	−	−	−	±	±
8	♀	+	+++	++	++	+++	+++	+++	+++	−	−	−	+	+
9	♀	−	+	+	++	+++	+++	+++	+++	−	−	−	±	±
10	♀	+												
11	♀	++	+	+	++	+++	+++	+++	+++	−	−	−	+	+
12	♀	+++	++	++	+++	+++	+++	+++	+++	−	−	−	+	+

Note: (1). Spots were graded grossly into the following five degrees of the intensity of colour and size (+++), (++), (+), (±), (−).
(2). Degree of toxicity: deadly=+++, strong=++, weak=+, none=−.

Table. 6 Toxicity and free amino acids isolated from ovary extract of individual globefish

Sample No.	Degree of toxicity	Degree of ninhydrin colour reaction						
		Number of amino acid						
		1	2	3	4	5	6	7
6	+++	±	±	++	−	+	+	+
8	+++	±	±	++	±	±	±	−
9	+++	±	±	+++	++	−	−	−
13	+++	−	−	+++	−	−	−	−
14	+++	−	−	+++	−	−	−	−

Note: (1). Spots were graded grossly into the following five degrees of the intensity of colour and size (+++), (++), (+), (±), (−).
(2). Degree of toxicity: deadly=+++, strong=++, weak=+, none=−.

Table. 7 Names of free amino acids isolated from liver extract, assumed by mean Rf value.

Spot No.	Butanol	Phenol	Assumed name
1	0.27	0.16	Aspartic acid
2	0.33	0.23	Glutamic acid
3	0.23～0.34	0.37～0.48	Glycine
4	0.37	0.60	Alanine
5	0.60	0.99	Leucine
6	0.76	0.84	Methionine
7	0.53	0.79	Valine
8	0.57	0.85	—
9	0.59	0.92	Phenylalanine
10	0.31	0.95	Proline
11	0.26	0.61	Hydroxyproline
12	0.30	0.72	Histidine

Note: (1). Butanol: mean Rf values with the first solvent (BuOH : AcOH : H_2O=4:1:3)
(2). Phenol: mean Rf values with the second solvent (80% phenol included NH_3).

Table. 8 Names of free amino acids isolated from extract, assumed by mean Rf value.

Spot No.	Butanol	Phenol	Assumed name
1	0. 31	0. 22	Glutamic acid
2	0. 19	0. 24	—
3	0. 29	0. 43	Glycine
4	0. 38	0. 66	Alanine
5	0. 60	0. 81	Valine
6	0. 73	0. 87	Leucine
7	0. 16	0. 45	—

Note: (1). Butanol: mean Rf values with the first solvent (BuOH : ACOH : H_2O=4 : 1 : 3)
(2). Phenol: mean Rf values with the second solvent (80% phenol included)

one to six spots from the ovary samples (Table 5, 6). The mean Rf values of free amino acids of the liver samples and of ovary samples together with their presumable identification are presented in Tables 7 and 8.

(a) Free amino acids in livers (Table 5): The number of the detected amino acids were twelve: There were two samples which yielded ten spots, six samples with nine spots and one sample with seven and six spots each. The following are the analysis of these spots from Rf values and the color intensity was expressed with the same marks that were used in the reducing sugar spots. (1) The spots 4 was usually (卅) or (++) in color and presumed to be alanine. (2) The spots 6 and 7 were usually (卅) or (++) and presumed to be methionine and valine, respectively. (3) The Spots 3 and 5 were between (+) and (卅) and presumed to be leucine and glycine, respectively. (4) The spot 2 was (+) or (++) and presumed to be glutamic acid. (5) The appearance of the spot 1 varied from (卅) to (−), no spot appearing in some samples and thought to be aspartic acid. (6) The spot 10 varied from (++) to (−) and thought to be proline. The spot 8 varied from (++) to (−) and was not identified. (7) The spots 9, 11 and 12 were (+) or (−), and probably phenylalanine, hydroxyprorine and histidine, restecpively. All the spots obtained with liver samples of various potencies may be classified into these seven groups. However, no correlation was found between the toxicity of these poisons and the intensity of color of free amino acid spots.

(b) Free amino acids in ovaries (Table 6): There were seven separated spots. However, some individual variation was seen in the number and the kind of the spot among the examined samples. Two samples yielded six spots, one sample had four spots and one had two spots. (1) The spot 3 was usually (卅) or (++) in color intensity and presumed to be glycine. (2) The spot 4 was (+) or (−), presumably alanine. (3) The spot 5 and 6 were (+) of (−), presumably valine and leucine. The spot 7 was (+) or (−), unidentified. The spot 2 was (±) or (−), unidentified. The spot 1 was (±) or (−), presumably glutamic acid. The spots obtained from the "deadly" ovaries may be classified into these four groups,

but no demonstrable correlation was seen between the toxicity and the color, as was the case with the livers.

DISCUSSION

GIucose was detected in almost all liver and ovary samples, but its amount varied considerably with the samples, no glucose being found in some. Another spot which was thought to be a pentose was not as yet identified. The appearance of this spot was not consistent, and this was not found in ovary samples. The findings on glucose in the ovary were in accord with the results of Tsuda and Kawamura[3]. Since there has been no report on glucose in liver samples, no attempt will be made to discuss our data. Our results indicated that the toxicity of poison was not cor related with the amount of glucose or pentose in either organ. A conclusion may be warranted that the reducing sugar has no direct bearing on toxicity. Our results on free amino of ovaries are the same as those of Tsuda and Kawamura[3] with respect to glycine, alanine and valine. However, they did not find glutamic acid and leucine, while taurine and leucine-phenylalanine which they reported were not found in the present study. Furthermore, two more unknown spots were found in our study. Since Tsuda and Kawamura identified their amino acid fractions and the author simply assumed these amino acid without further identification, it is perhaps unwise to compare their results with ours. Nonetheless, they recognized five spots and

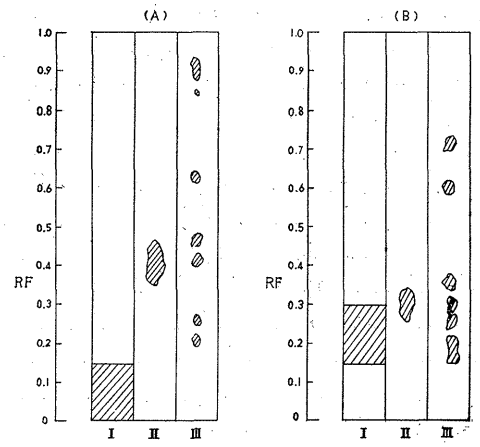


Fig. 4 One-dimensional chromatogram of the globefish ovary poison.

Note: Filter paper: Toyo Roshi No.50, 3 × 40 cm
Solvent: (A). 90% phenol
(B). BuOH : AcOH : H_2O = 4 : 1 : 3
Ascending System: at 20~22°C for 15 hours
I. Poison map
II. Reducing sugar map
III. Amino acid map

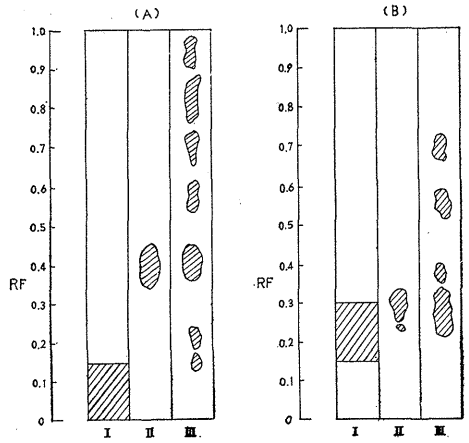


Fig. 5 One dimensional chromatogram of the globefish liver poison.

Note: Filter paper: Toyo Roshi No. 50, 3 × 40 cm
Solvent: (A). 90% phenol
(B). BuOH : AcOH : H_2O=4:1:3
Ascending System: at 20~22°C for 15 hours
I. Poison map
II. Reducing sugar map
III. Amino acid map

our study revealed seven spots. The author investigated with individual fresh materials as they became available and Tsuda and Kawamura[3] perhaps carried out their experiments afrer prolonged collection of materials for their purpose. Therefore, such difference could have brought about a discrepancy. In fresh materials the free amino acid obtained was usually glycine only. The relative location of amino acids, reducing sugar and the poison in livers and ovaries of globefish (Spheroides rubripes) is illustrated in Fig. 4 and Fig. 5. Nuki and Kawakami (1960[4]) reported that the ovary poison was stronger than the liver poison in respiratory dipressant action, and conversely the latter is stronger than the former in the effect of lowering blood pressure. A study is underway to study the respiratory depressant action and hypotensive action of these sugars and amino acids shown in Fig. 4 and 5 by means of reextraction in order to determine whether such impurities have any effect on the toxicity of the poison. A mention may be made that the report of the pure crystalline tetrodotoxin from globefish ovaries being a polyhydroxyacyl compound (Tsuda and Kawamura. 1953[5]) suggested to the author that globefish poison might be ralated to a reducing sugar in its biosynthesis and decomposition in organs. The presence of −(NH)− and CO-N= groups in crystalline tetrodotxin as studied with absorption spectra was reported by Tsuda and Kawamura (1953[5]).

The presence of −(NH)− and −CO−N= groups may suggest a correlation between the poison and free amino acid. Therefore further studies on reducing sugars

and amino acids seem to be necessary. In the past, Tawara et al. reported some chemical substance beside the toxin in ovary. Their study was conducted along purification of the globefish poison and had a different purpose. Our objective was to study the rerationship between variation of potency of the toxin and reducing sugars and amino acids and was somewhat different from theirs. Further investigation into the relationship between the toxicity and reducing sugars or amino acids by different methods is being contemplated.

SUMMARY

1). Paperchromatographry of acidified aqueous extracts of livers and ovaies of globefish (Spheroides rubripes), which have different toxic potencies, was carried out to find reducing sugars with aniline hydrogen phthalate and ammonia silver nitrate, and free amino acids with ninhydrin.
2). Two reducing sugar spots were obtained. One spot which was assumed to be a hexose, was identified to be glucose, but the other spot, presumably a pentose, was not identified.
3). With liver extracts, six to ten spots of amino acid were obtained, and with ovaries, one to six spots. The spot of presumably alanine gave the strongest color in the former material, and the spot of presumably glycine was the strongest in color in the latter material.
4). No correlation between reducing sugars or amino acids and the potency of the globefish poison could be recognized.

ACKNOWLEDGEMENT

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