Physicochemical Properties of a Lethal Hemolysin Isolated from the Sea Anemone *Anthopleura japonica*

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Hemolysins were purified from whole bodies of the sea anemone *Anthopleura japonica* by acetone precipitation, ion-exchange chromatography on CM-cellulose, gel filtration on Sephadex G-75 and chromatofocusing. Two hemolysins (AJH-1 and 2) were detected and only AJH-2 was obtained in pure form as judged by analytical disc electrophoresis. AJH-2 exhibited a lethal activity in mice (LD_{50} 78 μ g/kg, *i.v.* injection) as well as a hemolytic activity (106,500 HU/mg against sheep erythrocytes). It was stable over a wide range of pH values from 2 to 11 but rather unstable to heat treatment. The molecular weight was determined to be 17,800 by sedimentation equilibrium or 19,500 by SDS-polyacrylamide gel electrophoresis, indicating that AJH-2 has no subunit structure. AJH-2 was a slightly basic protein (pI 8.0) and its amino acid composition was characterized by the absence of half-cystine. It was notable that these properties of AJH-2 were closely similar to those of parasitoxin, the major lethal hemolysin isolated from the sea anemone *Parasicyonis actinostoloides*.

Sea anemones are important sources of hemolysins, some of which have been isolated in pure form and well characterized.¹⁻³⁾ In the course of our survey for hemolysins in sea anemones commonly inhabiting the coastal waters of Japan, we also detected powerful hemolysins in two species, *Parasicyonis actinostoloides* and *Anthopleura japonica*.³⁾ The major lethal hemolysin in the former species, which was tentatively named parasitoxin, has been isolated and its biological and physicochemical properties have been disclosed.³⁾ The present paper deals with the isolation and characterization of a lethal hemolysin in *A. japonica*.

Materials and Methods

Materials

Specimens of *A. japonica* were collected at Kominato, Chiba Prefecture, in May 1984. They were transported alive in sea water to our laboratory and, after removal of small stones and shells attached to their body walls, kept at -20°C until use.

CM-cellulose was purchased from Brown; Sephadex G-75, Polybuffer Exchanger PBE 94 and Polybuffer PB 96 from Pharmacia Fine Chemicals; a Zeineh dialyzer L-D-1 from Funakoshi Pharmaceutical; sheep blood from Nippon Bio-Supp.

Center; male mice (ddY strain) weighing about 20 g from Sankyo Labo Service. The reagents used were all of analytical grade.

Purification Procedure

Sheep erythrocytes, which were most sensitive to the *A. japonica* hemolysins,³⁾ were used to trace the hemolysins in the purification procedure.

Two hundred and fifty specimens (500 g) were extracted twice with an equal volume of cold distilled water. The crude extract was applied successively to acetone precipitation, ion-exchange chromatography on CM-cellulose and gel filtration on Sephadex G-75 essentially in the same manner as adopted for the purification of parasitoxin.³⁾ The partially purified preparation thus obtained was dissolved in 5 ml of 0.025 M ethanolamine-HCl buffer (pH 9.4) and put onto a Polybuffer Exchanger PBE 94 column (1 $\times$ 50 cm) equilibrated with the same buffer. Elution was achieved by Polybuffer PB 96 (pH 7.0, 0.0075 mM/pH/ml). Active fractions were collected and subjected to salting-out with ammonium sulfate of 90% saturation to remove Polybuffer.

Estimation of Hemolytic Activity

Hemolytic activity against sheep erythrocytes was estimated in the absence of lecithin as reported

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Fig. 1. CM-cellulose column chromatography of *A. japonica* hemolysins. To the crude extract was added 2.5 volumes of cold acetone (-20°C) and the precipitate was applied to a CM-cellulose column (2×59 cm) equilibrated with 0.01 M phosphate buffer (pH 5.6). A gradient of NaCl in the buffer was initiated at fraction 50. Fractions of 15 ml were collected at a flow rate of 75 ml/h.

previously.⁹⁾ The percentage of hemolysis was measured from the absorbance at 542 nm of the hemoglobin released. One hemolytic unit (HU) was defined as the amount of protein required to cause 50% hemolysis. Hemolytic activity in the presence of lecithin was also determined using the following assay system: 2.9 ml of sample solution made in buffered saline (0.15 M NaCl in 0.01 M phosphate buffer, pH 7.0), 0.1 ml of ethanolic lecithin solution (containing 200 μg lecithin) and 1 ml of 2% erythrocyte suspension made in buffered saline. The addition of the ethanolic solution to the assay system had no ill effect on the estimation of hemolytic activity.

Estimation of Lethal Activity

The lethal activity of the purified hemolysin was examined by intravenous injection into ddY male mice. For the determination of LD_{50} value by the method of Litchfield and Wilcoxon,⁹⁾ groups of six mice were injected with four different doses (99.0, 84.4, 73.0 and 59.0 $\mu\text{g}/\text{kg}$).

Stability Tests

The purified hemolysin was used for stability tests. Sample solutions were buffered at various pH values (pH 2 and 3, glycine-HCl buffer; 4 and

5, acetate buffer; 6 and 7, phosphate buffer; 8 and 9, Tris-HCl buffer; 10, 11 and 12, glycine-NaOH buffer) and assayed for hemolytic activity after being kept at 4°C for 24 h. For heat stability sample solutions were heated at 40, 60, 80 and 100°C for 10 min and the residual hemolytic activity in each solution was examined.

Amino Acid Analysis

An aliquot of the purified hemolysin was submitted to hydrolysis by 5.7 N HCl in an evacuated tube at 110°C for 24 h and then analyzed on a Hitachi 835 amino acid analyzer. Tryptophan was estimated optically according to the method of Goodwin and Morton.¹⁰⁾

Other Analytical Methods

Protein determination, analytical disc electrophoresis, sodium dodecylsulfate (SDS) disc electrophoresis and sedimentation equilibrium were carried out as reported previously.⁹⁾

Results and Discussion

Purification of Hemolysin

The *A. japonica* hemolysins were precipitated with acetone from the crude extract, with a yield of 80%. As illustrated in Fig. 1, the hemolysins

Fig. 2. Gel filtration on Sephadex G-75 of the active fraction obtained in CM-cellulose column chromatography.

The active fraction obtained in CM-cellulose column chromatography was applied to a Sephadex G-75 column (2.5 x 90 cm) equilibrated with buffered saline which was used as an eluting solvent. Fractions of 15 ml were collected at a flow rate of 45 ml/h.

were adsorbed by CM-cellulose and eluted by a linear gradient of 0–0.5 M NaCl in 0.01 M phosphate buffer (pH 5.6). In gel filtration on Sephadex G-75 (Fig. 2) only one and symmetrical hemolytic peak was observed. However, when the active fraction, after re-chromatography on Sephadex G-75, was applied to chromatofocusing (Fig. 3), the existence of two hemolysins was established. One hemolysin (AJH-1) passed through a Poly-buffer Exchanger PBE 94 column without interaction, suggesting that its isoelectric point is higher than 9.0. This hemolysin was not further examined due to scarcity of the sample available. Another hemolysin (AJH-2) was eluted between fractions 51 and 58 as a symmetrical peak as to both hemolytic activity and protein and its homogeneity was evidenced by analytical disc electrophoresis (Fig. 4). Although the yield in activity was very low (2.5%), 2.3 mg of pure AJH-2 was obtained from 500 g of wet animals (250 speci-

mens).

Properties of the Purified Hemolysin (AJH-2)

The specific hemolytic activity of AJH-2 was determined to be 106,500 HU/mg. The activity was not enhanced in the presence of lecithin, suggesting that AJH-2 itself directly attacks and destroys the erythrocyte membrane. In addition to hemolytic activity AJH-2 also exhibited a lethal activity in mice; the LD_{50} was 78 μ g/kg (95% confidence limit, 65–94 μ g/kg). The mice given lethal doses showed agitation immediately after injection followed by paralysis and convulsion, and died of respiration arrest. The death time was within 10 min at $2 \times LD_{50}$ and about 24 h at nearly LD_{50} but doses of more than $4 \times LD_{50}$ killed mice instantly. Sublethal doses induced no ill signs in mice.

The activity of AJH-2 was unchanged during storage at 4°C for 1 week or at –20°C for 6

Fig. 3. Chromatofocusing of the active fraction obtained in gel filtration on Sephadex G-75.

The sample dissolved in 5 ml of 0.025 M ethanolamine-HCl buffer (pH 9.4) was subjected to a Polybuffer Exchanger PBE 94 column (1×50 cm) equilibrated with the same buffer. Polybuffer PB 96 (pH 7.0, 0.0075 mM/pH/ml) was used as an eluting solvent. Fractions of 4 ml were collected at a flow rate of 16 ml/h.

months. While about 80% of the original hemolytic activity was lost at pH 12, AJH-2 was stable over a wide range of pH values between 2 and 11. When heated at various temperatures, the activity was decreased linearly with increased temperature; heating at 40, 60 and 80°C caused loss of 15, 90 and 95% of the original activity, respectively, and heating at 100°C eventually destroyed all activity.

The isoelectric point of AJH-2 was assumed to be 8.0 from its behavior in chromatofocusing (Fig. 3). The molecular weight of AJH-2 was determined to be 17,800 by sedimentation equilibrium or 19,500 by SDS disc electrophoresis, indicating that AJH-2 has no subunit structure. In the latter method the homogeneity of AJH-2 was supported again since it afforded a single protein band. As listed in Table 1, the amino acid composition of AJH-2, which was characterized by the absence of half-cystine, was closely similar to that of parasitoxin isolated from *P. actinostoloides*.³⁾

Aside from a few exceptions such as metri-

diolysin, the *Metridium senile* hemolysin,³⁾ it is generally known that hemolysins in sea anemones are basic proteins of molecular weight 10,000–20,000 and that they have no subunit structure. AJH-2 well conforms to this general rule. Furthermore, AJH-1 is a basic protein with an isoelectric point of more than 9.0 (Fig. 3) and, judging from the behavior on Sephadex G-75 (Fig. 2), its molecular weight is comparable with that of AJH-2. Therefore, AJH-1 may also conform to the general rule although it is unknown whether it has no subunit structure. On the other hand, as pointed out by Bernheimer *et al.*,¹⁷⁾ the sea anemone hemolysins isolated in pure form show significant differences in amino acid composition. In this respect it is particularly noticeable that the amino acid compositions of AJH-2 and parasitoxin resemble overall each other (Table 1). As summarized in Table 2, both hemolysins also have similarities in specific hemolytic activity, LD₅₀ value, isoelectric point and molecular weight. In addition AJH-2 behaved similar to parasitoxin³⁾

Fig. 4. Disc electrophoretic pattern of the purified hemolysin (AJH-2). A 7% polyacrylamide gel and Tris-glycine buffer (pH 8.3) were used. Origin and cathode are at top. Protein was stained with Amido Black 10 B.

in column chromatographies on CM-cellulose, Sephadex G-75 and Polybuffer Exchanger PBE 94. These results may lead us to assume that AJH-2 is an analogue of parasitoxin.

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Table 1. Amino acid compositions of the purified hemolysin (AJH-2) and parasitoxin

Amino acid	Mole %	
	AJH-2* ¹	Parasitoxin* ²
Asp	10.4	10.6
Thr	4.9	8.3
Ser	9.2	8.3
Glu	5.6	4.6
Pro	2.9	1.6
Gly	12.1	11.2
Ala	10.0	8.0
Val	8.6	8.3
Cys (half)	0.0	0.0
Met	1.9	1.7
Ile	3.8	4.5
Leu	7.6	9.4
Tyr	6.0	7.6
Phe	3.0	3.0
Trp	2.7* ³	2.1
Lys	4.5	6.1
His	2.1	1.7
Arg	4.6	3.0

*¹ Mean value of two determinations.

*² Cited from the previous paper.⁸⁾

*³ Determined optically.

Table 2. Properties of the purified hemolysin (AJH-2) and parasitoxin

	AJH-2	Parasitoxin*
Molecular weight		
Sedimentation equilibrium	17,800	17,000
SDS disc electrophoresis	19,500	19,000
Isoelectric point	8.0	7.9
Specific hemolytic activity against sheep erythrocytes (HU/mg)	106,500	119,900
LD ₅₀ (μg/kg, i.v. injection into mice)	78	65

* Cited from the previous paper.⁸⁾