Isolation and Characterization of Equinatoxins from the Sea Anemone *Actinia equina* L.

Setsuko KOMATSU,[a] Kazuo FURUKAWA,[b] Keisuke ABE,[b] Hisashi HIRANO[a] and Masakazu UEDA*,[b]

Department of Molecular Biology, National Institute of Agrobiological Resources,[a] Kannondai 2-1-2, Tsukuba, Ibaraki 305, Japan and Department of Surgery, School of Medicine, Keio University,[b] Shinanomachi 35, Shinjuku-ku, Tokyo 160, Japan. *Received May 6, 1992*

Equinatoxins were purified from the tentacles and bodies of the sea anemone *Actinia equina* by the use of acetone precipitation, as well as column chromatographies on Sephadex G-50 and CM-cellulose according to the modified method of Macek and Lebez (1988). The equinatoxins obtained, equinatoxin 1 and 2, were hemolytic glycoproteins with a relative molecular mass of 20000. Equinatoxin 2 was found to be rich in glycine, alanine and valine. The amino acid sequences of equinatoxins 1 and 2 were partially determined. A portion of the N-terminal amino acid sequence of equinatoxin 2 was similar to those of pyruvate kinase and sialidase.

Keywords sea anemone; equinatoxin; amino acid sequence; hemolytic activity

Introduction

The specific targeting of monoclonal anti epidermal growth factor receptor antibody, after conjugation to gelonin and antitumour agents, is well documented.[1-3] However, the efficacy of such conjugates for causing cell destruction is low, because many antigenic determinants on the target cell surface fail to internalize, despite the successful binding and targeting of the cell by the monoclonal antibody. The work presented here describes the isolation, purification and characterization of an equinatoxin which causes hemolysis of cells without the need for internalization.[4]

Equinatoxins[4] have been isolated from the sea anemone *Actinia equina* L. One of these equinatoxins, equinatoxin II, is a lethal protein with a molecular mass of 19000 and a pI of 10.5.[5] It has been shown to be a hemolytic[6] and cytotoxic protein for different mammalian cells.[7,8] The hemolytic and lethal activities of equinatoxin II are inhibited by sphingomyelin and serum lipoproteins.[9]

In many respects, equinatoxin II is closely related to cytolysins from the sea anemone *Stichactis* (*Stichodactyla*) *helianthus*.[10-17] However, neither the structure nor the detailed mechanism of action of the equinatoxin has been well understood. The work presented here aims to characterize the equinatoxins from *Actinia equina* in order to determine the nature of the hemolytic active site and it is one of many studies in which the equinatoxins, conjugated to specific monoclonal antibodies, have been successfully used to target and destroy carcinoma cells.

Materials and Methods

Materials Specimens of sea anemone (*Actinia equina* L.) were collected from the rocky coast on the side of the Japan Sea. The live animals were transported to the laboratory and frozen at −20 °C.

Isolation of Toxins The equinatoxins were purified from the tentacles and the bodies of the sea anemone according to the modified method of Macek and Lebez.[5] A liquid including tentacles and bodies was cooled at 0 °C. The material was minced and homogenized with 30 strokes of a homogenizer, and filtered through a nylon sieve to discard the tentacles. Following one cycle of freezing and thawing the fairly clear filtrate was centrifuged (3000 *g*, 15 min) and the supernatant used for fractional precipitation. The supernatant was sequentially precipitated using 20%, 50% and 80% final concentrations of acetone. The fractions between 50% and 80% acetone were dissolved in 1 mM acetic acid and were subjected to Sephadex G-50 (1.5 × 37 cm, Pharmacia, Uppsala, Sweden) column chromatography. The eluted fractions were monitored spectro-photometrically at 280 nm for protein content and their hemolytic activities were assessed. Fractions having hemolytic activity were pooled, diluted with 0.5 M ammonium acetate, pH 6.8, to a final concentration of 0.05 M ammonium acetate, and applied directly to a CM-cellulose column (1.5 × 11.6 cm, Sigma, St Louis, U.S.A.) previously equilibrated with 0.05 M ammonium acetate, pH 6.8. The elution was carried out with a linear gradient of 0—1.0 M NaCl in 0.05 M Tris–HCl buffer, pH 8.5. Protein contents and hemolytic activities of the column fractions were monitored and the purity confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[18]

Haemolytic Tests The hemolytic activities of the eluted fractions and isolated equinatoxins were assessed by a turbidimetric method.[5] Two μl of the sample were tested. Sheep red blood cells were prepared, and suspended in a solution of 0.13 M NaCl, 10 mM Tris–HCl (pH 7.4), and 1 mM $CaCl_2$ to give an apparent absorbance of 0.5 at a wavelength of 660 nm.

Protein Content The protein content of each column fraction was determined by a modified method of Stanffer.[19] The absorbances at 280 nm of equinatoxins dissolved in 0.05 M ammonium acetate, pH 6.8, were measured with a spectrophotometer (Beckman, DU-65, CA, U.S.A.).

Internal Amino Acid Sequence Analysis Each protein was dissolved in 20 μl of SDS sample buffer (pH 6.8).[20] Twenty μl of a solution containing 10 μl of *Staphylococcus aureus* V8 protease (*S. aureus* V8, Piece, Rockford) (0.1 μg/μl) in deionized water and 10 μl of SDS sample buffer (pH 6.8) containing Bromophenol blue were overlaid. Electrophoresis was performed until the sample was stacked in the upper gel and then interrupted for 30 min to allow the digestion of the protein.[21] SDS-PAGE was then continued and the separated digests were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Problott, Applied Biosystems, Foster City).[22] Portions (10—100 μg) of the protein were also digested with lysyl endopeptodase and chymotrypsin, analyzed by SDS-PAGE and electroblotted onto PVDF membrane.[22]

On the other hand, the proteins were cleaved under nitrogen, with a tenfold excess by weight of cyanogen bromide (CNBr) over protein, in 70% formic acid for 48 h at room temperature. Peptide fragments were dried, re-dessolved in 20 μl of SDS sample buffer (pH 6.8), analyzed by SDS-PAGE and electroblotted onto PVDF membrane.[22]

Amino Acid Sequence Analysis and Homology Search of Amino Acid Sequences The membrane was applied to the upper glass block of the reaction chamber in a gas-phase protein sequencer (477A, Applied Biosystems). Edman degradation was performed according to the standard programme Applied Biosystems. The released phenylthiohydantoin amino acid derivatives were identified by the on-line system of high performance liquid chromatography (120A, Applied Biosystems).

The amino acid sequences obtained were compared with sequences of over 16000 proteins contained in the amino acid sequence data base SEQDB (Protein Research Foundation, Osaka).

Amino Acid Composition Analysis The protein samples were hydrolyzed with 0.5 ml of 6 M HCl containing 0.1% phenol at 110 °C for 24 h. Amino acid composition of the hydrolysate was determined on an amino acid analyzer (A-5550, IRICA, Kyoto).

Detection of Glycoprotein with an *N*-Linked Oligosaccharide Chain The protein samples were separated by SDS-PAGE, electroblotted onto a PVDF membrane and reacted with peroxidase-coupled concanavalin A (Honen, Tokyo) according to the procedure described by Kijimoto-Ochiai *et al.*[23]

Results and Discussion

Hemolytic toxins from the tentacles and bodies of the sea anemone *Actinia equina* L. were purified essentially by the method of Macek and Lebez,[5] with additional purifications by acetone precipitation and Sephadex G-50 column chromatography. About 30 mg of equinatoxin was obtained from sea anemones weighing 65 g. Regardless of the biological origin (tentacles or bodies) of the protein preparation, three major protein peaks were obtained by gel permeation chromatography, of which only the second peak (Fig. 1, fractions 30—50) showed hemolytic activity. By CM-cellulose chromatography of the second hemolytically active peak from Fig. 1, the proteins were separated into two major fractions (Fig. 2). Fractions 50 to 60 (Fig. 2) were pooled and the proteins contained therein named equinatoxin 2 fraction, while the proteins eluted in fractions 30 to 40 were termed equinatoxin 1.

The molecular weight of the isolated hemolytic protein, equinatoxin 2, as determined by SDS-PAGE, was 20000 (Fig. 3A-2), and differed from that previously reported (19000) by Macek and Lebez.[5] Protein c in the equinatoxin 1 fraction, had a relative molecular mass of 19000 which corresponds with that of the equinatoxin 2 purified by Macek and Lebez.[5] However, N-terminal amino acid sequencing demonstrated homology between protein b of the equinatoxin 1 fraction and equinatoxin 2 (Table I). The sequencing results showed that whereas equinatoxin 1 was a mixture of four proteins, equinatoxin 2 consisted of only one protein. We regarded that the two proteins, a and d, in Table I were impurities. Equinatoxin 2 was selected for further study.

Electroblotting and concanavalin A peroxidase methods[23] demonstrated that equinatoxin 2 was a glycoprotein with an N-linked oligosaccharide chain (Fig. 3B). The amino acid composition of equinatoxin 2 is shown in Table II. Equinatoxin 2 was rich in glycine (about 38.0 residues), alanine (about 25.9 residues) and valine (about 18.7 residues), with 13.7%, 11.1% and 10.5%, respectively, but was found to be poor in histidine, and aspartic acid or asparagine.

In order to determine the amino acid sequence, equinatoxin 2 was degested with *S. aureus* V8, CNBr, lysyl endopeptidase and chymotrypsin, and electroblotted from the gel onto a PVDF membrane. Four major fragments of equinatoxin 2 were obtained, one of which had an N-terminal amino acid sequence and the others internal amino acid sequences. In total, the sequence of 49 amino acid residues were determined (Table III). The results of a structural homology search of N-terminal amino acid sequence are shown in Fig. 4. The amino acid sequence of equinatoxin 2 was homologous with those of pyruvate kinase[24] and sialidase,[25] which had both been deduced from cDNA sequences. It is of interest that the hemolytic

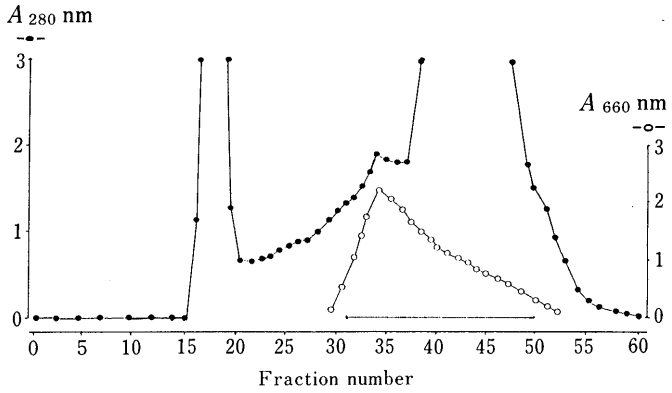

Fig. 1. Sephadex G-50 Column Chromatography of Acetone Precipitated Proteins from the Tentacles and Bodies of *Actinia equina*

●—●, A_{280} nm; ○—○, hemolytic activity, $1/t_{0.5}$ (2 μl were tested as described in Materials and Methods). Fractions of 4.0 ml were collected at a flow rate of 11.6 ml/h.

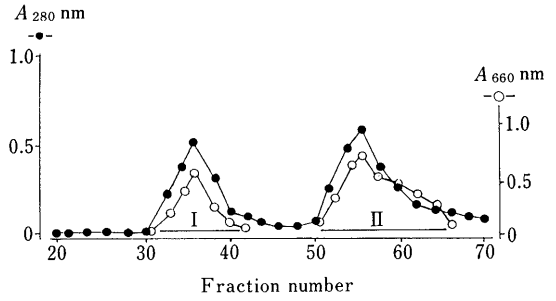

Fig. 2. CM-Cellulose Column Chromatography of Fractions, Identified as Ones Having Hemolytic Activity, Obtained from the Sephadex G-50 Column Shown in Fig. 1

●—●, A_{280} nm; ○—○, hemolytic activity, $1/t_{0.5}$ (2 μl were tested as described in Materials and Methods). Fractions of 3.5 ml were collected at a flow rate of 11.8 ml/h.

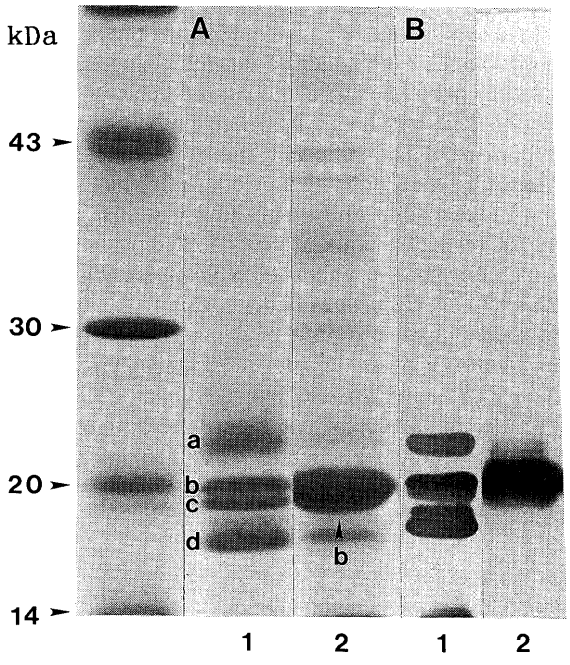

Fig. 3. SDS-PAGE Pattern of Equinatoxin 1 and 2

1, equinatoxin 1; 2, equinatoxin 2. A, Coomassie stain; B, concanavalin A stain. a, b, c and d identify the component of the equinatoxin.

TABLE I. N-Terminal Amino Acid Sequences of Equinatoxin

Toxins	Type	Amino acid sequence
Toxin 1	a	TSYNQAEVQE ISK
	b	SADVAGAVID GASLXFDXLX
	c	SVAVAGAVIE GATLTFNVLQ
	d	WPSISTMLSN YPLYEXYXXN
Toxin 2	b	SADVAGAVID GASLSFDIL

TABLE II. Amino Acid Composition of Equinatoxin 2

Amino acid	Amino acid residue	%
Asx	2.7	1.8
Thr	9.8	5.6
Ser	13.8	7.0
Glx	8.8	6.3
Gly	38.0	13.7
Ala	25.9	11.1
Cys	n.d.	n.d.
Val	18.7	10.5
Met	3.0	2.1
Ile	7.2	4.5
Leu	9.3	5.9
Tyr	8.2	7.1
Phe	4.0	3.2
Lys	12.4	8.7
His	3.6	0.8
Arg	8.7	7.3
Pro	7.8	4.3
Trp	n.d.	n.d.
Total	181.9	99.9

Amino acid compositions were determined as described in Materials and Methods. The number of each amino acid is an average number calculated from three amino acid analyses. n.d.: not determined.

TABLE III. N-Terminal and Internal Amino Acid Sequences of Equinatoxin 2

Treatment	Amino acid sequences
N-Terminal	SADVAGAVID GASLSFDIL
S. aureus V8-A	SADVAGAVID GAXLXXD
S. aureus V8-B	LYYNLSPFRG DNG
CNBr-A	SADVAGAVID GASLXXD
CNBr-B	YEE LYYNLSPFRG DNG
Chymotrypsin	SADVAGAVXD GAXLXF
Lysyl endopeptidase-A	SADVAGAVXD GA
Lysyl endopeptidase-B	ALLYNGQKDRGPVA

equinatoxin shows partial homology with these proteins, since pyruvate kinase is involved in the glycolytic pathway and sialidase functions during blood coagulation. However, we could not show the relationship between these proteins and hemolytic equinatoxin. The complete amino acid sequence has been determined for tenebrosin-C,[26] from the Australian sea anemone *Actinia tenebrosa*. The amino acid sequence of equinatoxin 2 determined in this paper was highly homologous with that of tenebrosin C.

In this paper, equinatoxins were purified from the tentacles and bodies of the sea anemone *Actinia equina* L. using acetone precipitation and column chromatographies on Sephadex G-50 and CM-cellulose. The purified equinatoxin, equinatoxin 2, was a hemolytic polypeptide and was determined to be a glycoprotein, rich in glycine, alanine and valine, and it had a molecular mass of 20000. The N-terminal and internal amino acid sequences of

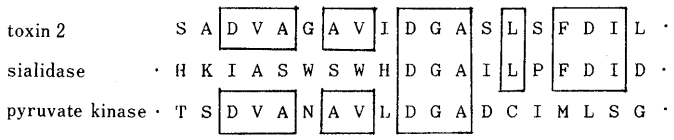

Fig. 4. Amino Acid Sequence Homology among Equinatoxin 2, Sialidase (Dale *et al.*, 1987) and Pyruvate Kinase (Cognet *et al.*, 1987)

equinatoxin 2 were homologous with those of pyruvate kinase and sialidase. In this study, we could not determine the hemolytic active site. However, Furukawa *et al.* have demonstrated that when monoclonal antibodies are conjugated to equinatoxin 2, carcinoma cells expressing the antigenic determinant are killed (paper in preparation). Thus, the equinatoxins described here, when combined with monoclonal antibodies, can be developed as cell-specific, targeted killing agents.

Acknowledgment We thank Dr. R. N. Waterhouse for her reading of the manuscript.

References

1) N. Hirota, M. Ueda, S. Ozawa, O. Abe and N. Shimizu, *Cancer Res.*, **49**, 7106 (1989).
2) S. Ozawa, M. Ueda, N. Ando, O. Abe, S. Minoshima and N. Simizu, *Int. J. Cancer*, **43**, 152 (1989).
3) M. Osaku, M. Ueda, N. Ando, Y. Shinozawa, N. Hirota, N. Shimizu and O. Abe, *Anticancer Res.*, **11**, 1951 (1991).
4) I. Ferlan and D. Lebez, *Toxicon*, **12**, 57 (1974).
5) P. Macek and D. Lebez, *Toxicon*, **26**, 441 (1988).
6) P. Macek and D. Lebez, *Toxicon*, **19**, 233 (1981).
7) T. Giraldi, I. Ferlan and D. Romed, *Chem. Biol. Interact.*, **13**, 199 (1976).
8) U. Batista, K. Jezernik, P. Macek and B. Sedmak, *Period. Biol.*, **89**, 347 (1987).
9) T. Turk and P. Macek, *Period. Biol.*, **88**, 216 (1986).
10) J. P. Devlin, *J. Pharm. Sci.*, **63**, 1478 (1974).
11) A. W. Bernheimer and L. S. Avigad, *Toxicon*, **19**, 529 (1981).
12) K. M. Blumenthal and W. R. Kem, *J. Biol. Chem.*, **258**, 55 (1983).
13) P. Macek, L. Sencic and D. Lebez, *Toxicon*, **20**, 181 (1982).
14) D. Mebs, M. Liebrich, A. Reul and Y. Samejima, *Toxicon*, **21**, 257 (1983).
15) A. W. Bernheimer, L. A. Avigad, G. Branch, E. Dowdle and C. Y. Lai, *Toxicon*, **22**, 183 (1984).
16) K. Shiomi, E. Tanaka, H. Yamanaka and T. Kikuchi, *Toxicon*, **24**, 8865 (1985).
17) R. S. Norton, P. Macek, G. E. Reid and R. J. Simpson, *Toxicon*, **30**, 13 (1992).
18) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
19) C. E. Stanffer, *Anal. Biochem.*, **69**, 646 (1975).
20) P. H. O'Farrell, *J. Biol. Chem.*, **250**, 4007 (1975).
21) D. W. Cleveland, S. G. Fischer, M. W. Kirschner, and U. K. Laemmli, *J. Biol. Chem.*, **252**, 1102 (1977).
22) H. Hirano and T. Watanabe, *Electrophoresis*, **11**, 573 (1990).
23) S. Kijimoto-Ochiai, Y. U. Katagiri and H. Ochiai, *Anal. Biochem.*, **147**, 222 (1985).
24) M. Cognet, Y. K. Lone, S. Vaulont, A. Kahn and J. Marie, *J. Mol. Biol.*, **196**, 11 (1987).
25) B. Dale, R. Brown, J. Miller, R. T. White, G. M. Air and B. Cordell, *Virology*, **115**, 460 (1986).
26) R. J. Simpson, G. E. Reid, R. L. Moritz, C. Morton and R. S. Norton, *Eur. J. Biochem.*, **190**, 319 (1990).