ISOLATION AND CHARACTERISATION OF THREE POLYPEPTIDES WITH NEUROTOXIC ACTIVITY FROM *ANEMONIA SULCATA*

László BÉRESS, Rosemarie BÉRESS
Institut für Meereskunde an der Universität Kiel, 23 Kiel, Düsternbrooker Weg 20, West-Germany

and

Gert WUNDERER
Institut für Klinische Chemie und Klinische Biochemie der Universität München, 8 München 2, Nußbaumstr. 20, West-Germany

Received 4 December 1974

1. Introduction

The sea anemone *Anemonia sulcata* captures and kills small fishes and crustaceans by means of its tentacles. Contact with nematocysts in the tentacles causes injection of toxic substances into the prey, paralysing it. We were interested in the biochemical and physiological mechanisms leading to this paralysis. As the most active neurotoxic components of *Anemonia sulcata* have been shown to be polypeptides [1], we began with the purification and characterization of the neurotoxic polypeptides.

In this work three toxic polypeptides (toxins I, II and III) were isolated in analytically pure form using alcoholic extracts of whole animals, batchwise adsorption on cation exchangers, gel filtration and ion-exchange chromatography. The neurotoxic activity was tested on the shore crab *Carcinus maenas*. LD_{100} for both toxins I and II is less than 2 μg/kg *Carcinus*, whereas for toxin III it is less than 50 μg/kg *Carcinus*. The three toxins are heat-stable basic polypeptides.

2. Experimental

2.1. *Purification of the toxins*

The sea anemones (*Anemonia sulcata*) were collected in the Bay of Naples. Whole wet animals (5 kg; dry weight 13% of wet weight) were homogenized in ethanol, heated to 60°C, centrifuged and dialysed as described previously [1]. Most of the basic polypeptides were removed from this material by batchwise adsorption on CM-cellulose (batch I: pH 6.5 and 4 mS × cm^{-1}, batch II: pH 5 and 2 mS × cm^{-1}). Toxins I, III and most of toxin II were adsorbed on SP-Sephadex at pH 3 and 2 mS × cm^{-1} conductivity (batch III). After elution with 1 M NaCl, 0.05 M Tris–HCl pH 8, the toxins were concentrated by ultrafiltration (Amicon UM 05) followed by gel filtration on Sephadex G-50 (fig.1). Toxic fractions were desalted and lyophilised. Toxins I and II were separated by chromatography of fraction 3 on SP-Sephadex. Toxin II was rechromatographed on SP-Sephadex. Toxin I was submitted to QAE-Sephadex, Sephadex G-25 and SP-Sephadex chromatography. Fraction 6 from Sephadex G-50 chromatography contained toxin III which was purified by Sephadex G-25, G-10 and Biogel P-2 gel filtration. Toxicity measurements were performed on Carcinus maenas as described previously [1].

2.2. *Electrophoresis*

The purity of toxic samples was checked using polyacrylamide gel electrophoresis at pH 8.6, following the method of Zwisler and Biel [2]. The mol. wt of toxin II was determined by SDS polyacrylamide

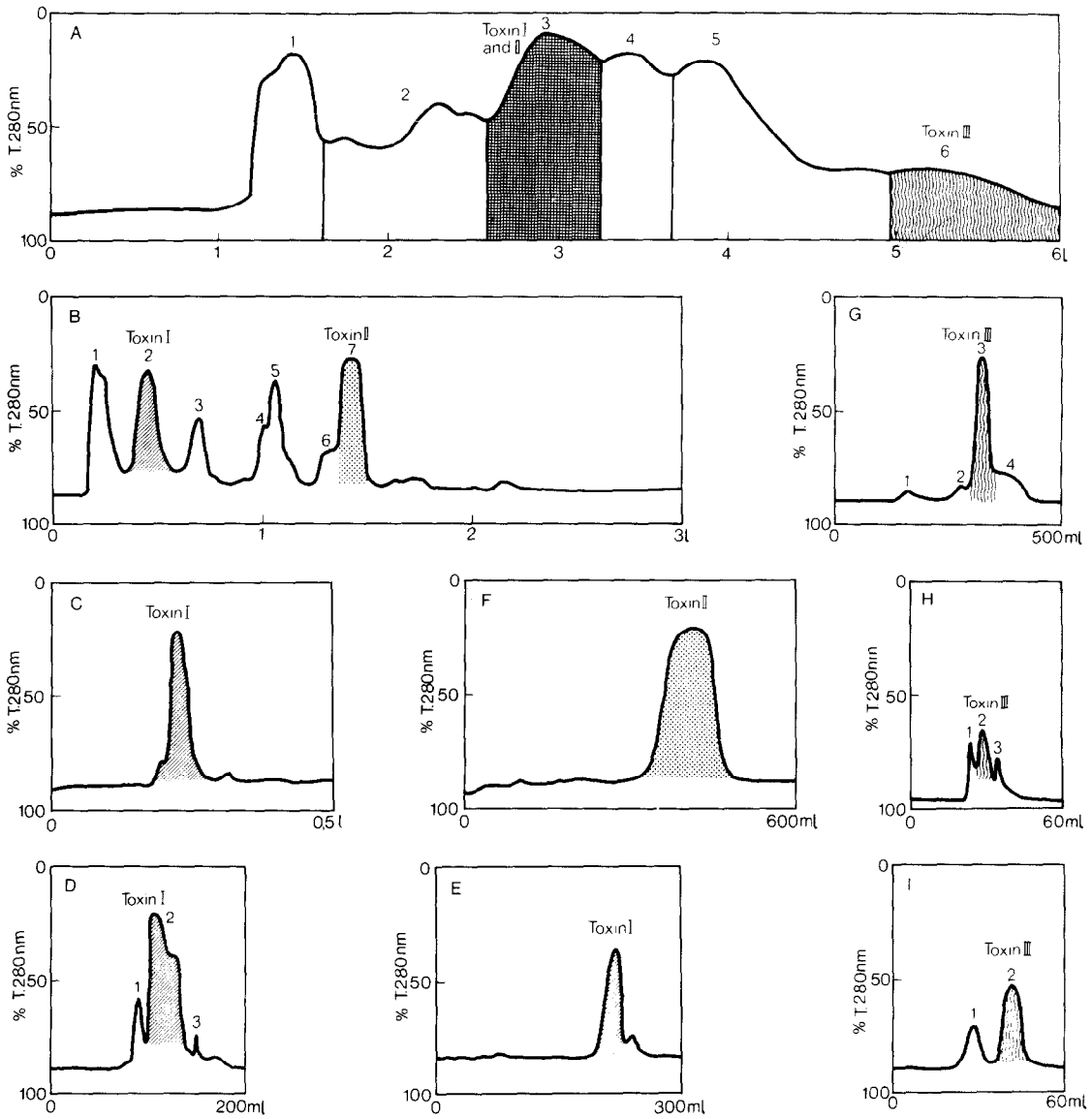

Fig. 1. Purification of neurotoxins from *Anemonia sulcata*. TI = toxin I, TII = toxin II, TIII = toxin III. A) Sephadex G-50 gel filtration of batch III proteins of 5 kg of sea anemones. Column: 7 × 140 cm; eluting solvent was 1 M NaCl, 0.05 M Tris/HCl buffer pH 8; flow rate, 5 ml/min. Indicated fractions 3 and 6 were desalted and lyophilized. B) SP-Sephadex C-25 chromatography of fraction A3. Column: 3 × 100 cm; eluting solvent was 2.4 litre phosphate buffer (40 mM), pH 6; linear gradient of NaCl (80 – 380 mM Na^+); flow rate, 3 ml/min. Fraction 2 is TI, fraction 7 is TII, both were desalted and lyophilized. C) QAE-Sephadex A-25 chromatography of fraction B2. Column: 2 × 100 cm; eluting solvent was 1 litre Tris/HCl buffer (50 mM), pH 8; linear gradient of NaCl (50 – 350 mM Cl^-); flow rate, 1 ml/min. The toxic fraction was desalted and lyophilized. D) Sephadex G-25 superfine gel filtration of toxic fraction from C. Column: 2 × 60 cm; eluting solvent was distilled water; flow rate, 0.3 ml/min. Fraction 2 was lyophilized. E) SP-Sephadex C-25 rechromatography of D2. Column: 1 × 100 cm; eluting solvent was 300 ml phosphate buffer (20 mM), pH 5; linear gradient of NaCl (40–100 mM Na^+); flow rate, 0.5 ml/min. The toxic fraction was desalted and lyophilized and shown to be pure toxin I, F) SP-Sephadex C-25 equilibrium rechromatography of toxin II, fraction B7. Column: 2 × 100 cm; eluting solvent was phosphate buffer (50 mM) pH6 containing 250 mM Na^+; flow rate, 1 ml/min. The main toxic fraction was desalted and lyophilized and shown to be pure toxin II. G) Sephadex G-25 superfine gel filtration of A6 = TIII. Column: 3.5 × 50 cm; eluting solvent was distilled water; flow rate, 6 ml/min. The toxic fraction G3 was lyophilized. H) Sephadex G-10 gel filtration of G3. Column: 1 × 50 cm; eluting solvent was distilled water; flow rate, 0.4 ml/min. Toxic fraction 2 was lyophilized. I) Biogel P-2 minus 400 mesh gel filtration of 1.3 mg of fraction H2. Column: 0.6 × 150 cm; eluting solvent was 0.1 M NH_4HCO_3 buffer pH 8.6; flow rate, 1.2 ml/hr. The toxic fraction 2 was shown to be pure toxin III.

gel electrophoresis [3] on the reduced form of the toxin.

2.3. *Amino acid analysis*

Samples of native or oxidised toxins were hydrolysed in 5.7 N HCl (3 times distilled) for 22 hr at 110°C in sealed evacuated tubes. The hydrolysates were analysed on a Beckman Multichrom and/or a nanomol analyser [4]. Tryptophan was determined spectrophotometrically [5,6].

2.4. *End group determination*

End group determination was done by manual or automated Edman degradation [7] and/or by the dansylation technique [8].

3. Results

Table 1 summarizes the data obtained in the purification of the three neurotoxins from *Anemonia sulcata*. For the toxin preparation only the eluate of batch III (71% of the total toxicity) was used. The eluates of batch I and batch II were used for the isolation of the polyvalent proteinase inhibitors [9,10].

The final yield in toxicity was 62.5% of that of the crude homogenate and 92.7% of that of the material adsorbed on batch III. Fig. 1 shows the elution patterns corresponding to the different chromatographic steps. Toxin III was purified by gel filtration. Toxin II was purified by equilibrium chromatography, whereas toxin I had to be chromatographed more extensively to remove small amounts of accompanying proteins.

The amino acid compositions of the three toxins are given in table 2 together with the mol. wts, the N-terminal residues and the molecular adsorption coefficients. The iso-electric point of toxin I was 8.6; toxin II was still more basic, as estimated from

Table 2
Amino acid compositions of *Anemonia sulcata* toxins

Amino acid	Toxin I	Toxin II	Toxin III
Asx	5.10 (5)	4.00 (4)	1.00 (1)
Thr	1.91 (2)	1.89 (2)	--
Ser	3.70 (4)	3.58 (4)	1.69 (2)
Glx	1.96 (2)	1.14 (1)	1.99 (2)
Pro	2.29 (2)	3.80 (4)	4.21 (4)
Gly	7.97 (8)	7.20 (7)	4.46 (4)
Ala	3.07 (3)	1.05 (1)	--
Cys	5.87 (6)[F]	5.37 (6)[F]	4.18 (4)[F]
Val	0.96 (1)	1.56	0.95 (1)
Met	0.51 (1)	-- } (4)[T]	--
Ile	1.79 (2)	2.79	--
Leu	0.99 (1)	2.95 (3)	--
Tyr	1.14 (1)	--	1.93 (2)
Phe	1.05 (1)	--	--
Trp	2.30 (2)	2.05 (2)	1.93 (2)
His	--	1.90 (2)	--
Lys	1.96 (2)	3.02 (3)	1.03 (1)
Arg	1.95 (2)	0.95 (1)	0.92 (1)
Total	45	44	24
Mol. wt	4702	4197	2678
N-terminus	Gly	Gly	Arg
$A^{1\%}_{1\ cm}$ at 280 nm	24.4	27.9	

[F] Determined as cysteic acid after performic acid oxidation.
[T] Microheterogeneity Ile/Val in position 2 of the sequence [11].

Table 1

Purification step	Reference to fig. 1	Toxicity in crab units (CU)	Yield of toxicity %	Spec. toxicity CU/μg
5 kg *Anemonia sulcata* alcohol. extr.	–	45×10^6	100	–
Batch III eluate	–	32×10^6	71	–
Sephadex G 50 gel filtration	A 3	30.4×10^6	67.5	16
	A 6	0.25×10^6	0.64	–
Toxin I	E 1	7.5×10^6	16.7	50
Toxin II	F	20.6×10^6	45.8	50
Toxin III	I 2	0.0148×10^6	0.03	2

electrophoresis data. In SDS-electrophoresis, reduced toxin II migrated between the basic pancreatic trypsin inhibitor (mol. wt 6500) and the B-chain of insulin (mol. wt 3000).

4. Discussion

The procedure described here has proved to be a simple method for the isolation of three neurotoxins from batch III of the *Anemonia sulcata* homogenate. The purification of polyvalent proteinase inhibitors quantitatively adsorbed on CM-cellulose (batches I and II) has previously been described [9,10]. Part of the toxin II activity (29% of total neurotoxic activity) was found in batches I and II; batch III did not however contain any inhibitory activity.

Toxin II is analytically pure after an equilibrium chromatography (fig.1, F). This material is used for the elucidation of the primary structure [11]. Toxin I required more extensive chromatography. Toxin III could be purified by retardation on gels with low exclusion limits, due to its relatively high content of aromatic amino acids. Toxins I and II could also be purified from isolated tentacles of *Anemonia sulcata* [15].

Toxin III can not be merely a degradation product of toxins I or II. It contains two more proline and one more tyrosine residues compared with toxin I, and two more tyrosine and one more glutamic acid compared with toxin II.

Toxin I and toxin II are very potent paralysing toxins, acting on crustacea, fishes and mammals after parenteral application [1,12–14]. On mammals a cardiotoxic effect of toxin I and especially of toxin II has been shown [14].

Toxin III was tested only on *Carcinus maenas* where it caused cramp reaction with following paralysis of the extremities, similar to that caused by toxins I and II. The toxic effect of toxins I and II on the neuromuscular transmission of the crayfish and other crustacea has been shown recently [12]. The mode of action of these toxins on crayfish nerve-muscle preparations and on isolated guinea pig heart is at present the subject of further investigations [12,14]. There is evidence that toxins I and II are acting on crayfish neuromuscular transmission as neurotoxins and on guinea pig heart as cardiotoxins.

Acknowledgements

We wish to thank Professor Dr H. Fritz, Institut für Klinische Chemie und Klinische Biochemie der Universität München, for his generous support; Professor Dr Th. Bücher, Dr W. Machleidt and Dr E. Wachter, Institut für Physiologische Chemie und Physikalische Biochemie der Universität München, for kindly providing facilities for chromatography and protein chemistry and M. Meier, E. Mempel and J. Zwick for technical assistence. This work is supported by Deutsche Forschungsgemeinschaft, Grant Be 554/3 (L.B.), and Sonderforschungsbereich 51, München (G.W.).

References

[1] Béress, L. and Béress, R. (1971) Kieler Meeresforschungen 27, 117–127.
[2] Zwisler, O. and Biel, H. (1966) Z. klin. Chem. 4, 58–62.
[3] Swank, R. T. and Munkres, K. D. (1971) Anal. Biochem. 39, 462–477.
[4] Krejci, K. and Machleidt, W. (1969) Z. physiol. Chem. 350, 981–993.
[5] Edelhoch, H. (1967) Biochemistry 6, 1948–1954.
[6] Bredderman, P. J. (1974) Anal. Biochem. 61, 298–301.
[7] Wachter, E., Machleidt, W., Hofner, H. and Otto, J. (1973) FEBS Lett. 35, 97–102.
[8] Gray, W. R. and Hartley, B. S. (1963) Biochem. J. 89, 59 p.
[9] Fritz, H., Brey, B. and Béress, L. (1972) Z. physiol. Chem. 353, 19–30.
[10] Wunderer, G., Kummer, K., Fritz, H., Béress, L. and Machleidt, W. (1974) in: Bayer Symposium V. Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L. J. and Truscheit, F., eds.), pp. 277–281, Springer Verlag, Berlin, Heidelberg, New York, in press.
[11] Wunderer, G., Fritz, H., Machleidt, W., Wachter, E. and Béress, L. in preparation.
[12] Rathmayer, W. and Béress, L. to be published.
[13] Möller, H. and Béress, L. to be published.
[14] Alsen, C. in preparation.
[15] Béress, L., Béress, R. and Wunderer, G. in preparation.