Cloning, Sequencing, and Expression of Equinatoxin II

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Received February 1, 1996

Equinatoxin II (EqtII), a basic protein of 179 amino acids lacking cysteine residues, is the most abundant cytolysin isolated from the sea anemone Actinia equina. Its mode of action is still poorly understood. In order to initiate further structure–function studies by protein engineering, cDNA library was prepared from the whole animal and hybridized with a PCR-derived probe, deduced from the EqtII primary structure. The longest positive clone of 899 bp was shown to encode a 214 residue precursor of EqtII. The mature protein region was amplified by PCR, cloned into a T7 RNA polymerase-based expression vector and expressed in Escherichia coli. Recombinant toxin was isolated by a simple, two-step isolation procedure including separation on CM-cellulose and gel filtration using an FPLC system. Its biochemical properties and hemolytic activity were practically indistinguishable from those of native toxin.© 1996 Academic Press, Inc.

Sea anemones (order Actiniaria, class Anthozoa, phylum Cnidaria) are sedentary coelenterates. In their specialized stinging organelles, nematocysts, several toxic peptides and proteins can be found (1). One group of these toxins consists of short neurotoxic peptides and the other of larger, 10 to 20-kDa cytotoxic proteins. More than 20 such proteins have been isolated from different sea anemones (2). All of them are basic proteins, with pI values ranging from 8 to 12, which exhibit pore-forming activity (hence named actinoporins). From the body and tentacles of Actinia equina, three basic toxins of about 20 kDa have been isolated, designated equinotoxins I, II and III (EqtI, II and III) (3). The most abundant, EqtII, exhibits several pharmacological activities (4,5) including cytolytic and cytotoxic effects on different types of cells (summarized in 6). Its primary structure was recently determined (7) and compared to the sequences of tenebrosin-C (8) and cytolysin III (9), similar toxins isolated from the sea anemones Actinia tenebrosa and Stichodactyla helianthus, respectively. Interestingly, although isolated from two different species, the sequence of EqtII is practically identical to that of tenebrosin-C. During amino acid sequencing, microheterogeneity was observed in both protein samples, at position 81 (Pro or Asp) of EqtII and at position 177 (Ser or Thr) of tenebrosin-C. The significance of the existence of an identical protein in two distinct species has still to be established. A partial solution at least could be provided by analysis of their mRNAs and genes.

The action of EqtII on various types of cells and lipid vesicles has been thoroughly studied (6,10). However, the molecular mechanism of its action is not yet completely understood. It is believed that the toxin forms a cation-selective channel composed of 3 or 4 monomers (11) in the lipid membrane. The diameter of such a pore was estimated to be about 1.1 nm (10). This pore formation can be effectively inhibited with sphingomyelin. On the contrary, when sphingomyelin is present in the membrane, permeabilization is increased, which can be explained if sphingomyelin acts as a low-affinity acceptor (12). Similar observations have also been reported for cytolysin III (13). However, sphingomyelin is not essential for the pore-forming activity of actinoporins since EqtII can also act on model lipid membranes lacking sphingomyelin. At present, the structural basis of the membrane insertion and oligomerization of the toxin is still unknown. Heterologous ex-
pression of its mutants would help us to understand better which parts of the EqtII molecule are involved in the primary cytolytic and cytotoxic events. In order to initiate such studies, we report here the molecular cloning of EqtII and its production in *Escherichia coli*.

**MATERIALS AND METHODS**

*Materials.* Restriction and DNA-modifying enzymes were purchased from Boehringer-Mannheim or Pharmacia. All other chemicals of analytical grade were from Sigma and Serva, unless otherwise stated. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by a double precipitation with sodium acetate and ethanol. The bacterial strains *Escherichia coli* Y1090hsdR, *E. coli* DH5α and *E. coli* BLR(DE3) were obtained from Amersham, Gibco BRL and Novagen, respectively.

*Construction and screening of cDNA library.* Total RNA was isolated from the sea anemone *Actinia equina* L. by homogenization in guanidinium thiocyanate (14) and centrifugation in a solution of cesium trifluoracetate (15). Poly(A)*^+* RNA was purified on oligo(dT)-cellulose (16) and cDNA synthesized using an Amersham cDNA synthesis system. After fractionation by gel chromatography, size-enriched cDNA of more than 500 bp was used for construction of a cDNA library in Agt11, using a kit from the same manufacturer. The library was amplified once (17) using the *E. coli* Y1090hsdR strain.

Two oligonucleotide primers, sense EQT-1 (5’-GACGAATTCCGNGTNGGTAAAYGA-3’) and antisense EQT-2 (5’-GACGATCGCGTCGGCTGCTCAGAT-3’), with degeneracy of 1024 and 128, respectively, were synthesized. PCR was performed with the two primers using 75 ng of the synthesized cDNA within 30 cycles of the following conditions: denaturation, 94°C for 1 min; annealing, 33°C for 1 min; extension, 72°C for 1 min. The PCR product was labeled by [35S]dATP (Amersham) using Random Primed DNA Labeling Kit of Boehringer. A portion of the cDNA library was spread on LBA plates and, after blotting to Hybond-N membranes (Amersham), plaques were hybridized by the standard method (17). Positive plaques were subjected to two rounds of purification.

*Cloning and sequencing.* Positive clones were excised from ADNA with BamHI and cloned into pUC19. The largest cDNA clone was sequenced in both directions using a T7 sequencing kit (Pharmacia) and [35S]dATP (Amersham). The nucleotide and amino acid sequences were analyzed by the DNASIS (Pharmacia) program.

*Expression of EqtII.* The coding region of EqtII of the cDNA clone was amplified by PCR with E2(+) (5’-GGAAATTCTATGGTCGCAGGCTGCT-3’) and E2(−) (5’-CGAATTCCGATCATCTATCAAGCTCTTGCTACG-3’) primers. 30 amplification cycles were performed with denaturation at 94°C for 1 min; annealing at 48°C for 1 min and extension at 72°C for 1 min. The product was digested with *Nde*I and *Eco*RI and inserted into the pT7-7 expression vector (18). Finally, the entire coding sequence was verified by nucleotide sequencing. The constructed expression vector was named pAG2.1. It was used to transform the *E. coli* strain BLR(DE3). Bacterial cells were grown in an enriched M9-LBA medium. Expression of heterologous protein was induced in middle log phase by addition of IPTG to a final concentration of 0.4 mM. The cells were grown until they reached the stationary phase.

*Purification and determination of activity of recombinant EqtII.* After expression, cells were spun down and resuspended in 50 mM ammonium acetate buffer, pH 6.8, containing 25% glycerol. Bacteria were pelleted, frozen and thawed again. Osmotic shock was achieved by addition of 3 volumes of the same buffer without sucrose. After 1 h of vigorous shaking, the suspension was sonicated on ice 3 × 60 s at 80 W (Ultrasonics W185 sonicator cell disrupter). After centrifugation at 6000 g for 10 min, the supernatant was applied on a 1 × 10 cm CM-cellulose column. Recombinant toxin was eluted with the same buffer containing 0.5 M NaCl. Active fractions were pooled and concentrated on an Amicon YM-10 membrane. Final purification was performed by FPLC on a Superdex HR75 column (Pharmacia) in 50 mM phosphate buffer, pH 6.5, containing 0.1 M NaCl. SDS-PAGE was performed using a Phast System (Pharmacia) or according to Laemmli (19). Purified recombinant EqtII was analyzed by HPLC on an RP18 column (Aquapore). The N-terminal sequence was determined by Edman degradation using an Applied Biosystems 470A protein sequencer. Hemolytic activity of the isolated protein was measured on bovine red blood cells as described (3).

**RESULTS AND DISCUSSION**

A cDNA library in Agt11 was prepared from a single specimen of *Actinia equina*. The probe for screening the library was prepared by PCR using EQT-1 and EQT-2 mixed oligonucleotide primers. The two oligonucleotides were deduced from the amino acid residues Ala34-Glu40 and Met132-Asn139 of EqtII (7), respectively. The PCR product was of the expected size, about 335 bp. Its deduced protein sequence perfectly matched that of the corresponding region of EqtII. The library was hybridized with the probe and two positive clones, E7 and E9, were isolated and subcloned into pUC19 where their sequences were determined. The nucleotide sequences of both cDNAs were identical. The E7 clone, however, lacked a part of the 5’-end encoding the first 24 residues, so the larger, E9 clone was chosen for further analysis.

The E9 cDNA, including a polyA tail, consists of 899 bp. Its nucleotide and derived amino acid
sequences are shown in Figure 1. An open reading frame of 642 bp was identified coding for a precursor protein of 214 amino acids, in which the mature EqII of 179 residues is preceded by a 35 residue peptide. Two potential translation start sites were found at positions 94–96 and 130–132, respectively. Both possess highly conserved A residue at positions −3 and +4, and some other elements recognized as a consensus in invertebrates (20). The first site is more likely to represent an actual start codon, since it provides translation of a more characteristic eukaryotic signal peptide (21). Within the last third of the 35 residue peptide, two potential proteolytic cleavage sites consisting of a pair of basic residues were recognized at positions 23–24 and 34–35, respectively, the last one just preceding the first residue of the mature EqII (see Figure 1). Consequently, the first 35 residues of the protein deduced from the cDNA were recognized as a prepropeptide.

FIG. 1. The nucleotide and deduced amino acid sequences of EqII cDNA. The putative prepropeptide and the polyadenylation signal are underlined. The protein sequence is numbered starting from the presumed initiation methionine residue. Numbering of the mature toxin is in parentheses. The nucleotide sequence presented has been deposited in the GenBank Data Library under Accession No. U41661.
Synthesis of EqtII in the precursor form is likely to be connected with its sorting to the cnidocyst, a specialized organelle which occupies the majority of the nematocyte (22). The proteolytic processing of the precursor could take place later in the cnidocyst. Additionally, the propart might assist a proper folding of the toxin.

The deduced sequence of mature EqtII is identical to the published protein sequence (7). Two isoforms of EqtII were identified within the course of sequencing the protein sample, differing at position 81, where equimolar concentrations of Pro and Asp were found. Our cDNA encodes Pro at this position. This microheterogeneity could be explained by different allelic forms of EqtII. Analysis of codon usage shows a random distribution and it is not biased towards A or T in the third position as was reported for some other cnidarian genes (23). A putative polyadenylation signal (AATAAA) at position 862, represents a typical eukaryotic signal sequence. The cDNA was found to be completed at the 3' end with a polyA tail of 22 residues.

The coding region of mature EqtII was amplified by PCR and cloned within the NdeI and EcoRI sites of the expression vector, which enabled direct expression of the protein. E2(−) primer for PCR was designed to code for a threonine residue at position 177. The deduced amino acid sequence of the cloned EqtII thus corresponded to a Pro81 and Thr177 isoform (see above). The E. coli strain BLR(DE3) was transformed with the expression vector. The basal level of T7 RNA polymerase expression was, as expected, very low, because without addition of IPTG no expression of EqtII could be observed even after a prolonged incubation (Figure 2A, lane 2). Expression was induced in log phase by addition of IPTG (Figure 2A, lanes 3 and 4). Recombinant EqtII produced by the bacterial cells was of the expected size of about 20 kDa. It represented approximately 5% of the total bacterial proteins as estimated from an SDS-PAGE gel. After expression, bacterial cells were disrupted by osmotic shock and sonication. The expressed protein was found after centrifugation

**FIG. 2.** (A) SDS–PAGE analysis of EqtII expression. Lane 1, induced cells with pT7-7 alone; 2, uninduced cells with pAG2.1; 3, cells with pAG2.1 1 h after IPTG induction; 4, cells with pAG2.1 2 h after IPTG induction; M, molecular mass standards. The arrow denotes position of the expressed toxin. (B) SDS–PAGE analysis of EqtII purification. Lane 1, total cell extract; 2, supernatant; 3, EqtII after purification on CM-cellulose; 4, EqtII after additional gel filtration by FPLC; M, molecular mass standards.
in two fractions. As estimated from an SDS-PAGE gel, half the protein formed insoluble inclusion bodies and the other half was soluble in the supernatant. The optimal growth temperature was determined to be 37°C. Either an increase of the temperature for 5 degrees or a decrease to even 25°C reduced the amount of soluble protein whereas the overall expression rate remained the same (data not shown).

The soluble recombinant EqtII was purified from the bacterial cytoplasm by a simple, two-step isolation procedure. Separation on CM-cellulose, used because of the basic character of the toxin, was followed by FPLC on a Superdex column, which provided final purification. Homogeneity was verified by SDS-PAGE electrophoresis (Figure 2B) and analytical RP-HPLC where a single, symmetric peak was observed (data not shown). The N-terminal amino acid sequence was determined and found to be identical to the sequence of EqtII. Less than 5% of the recombinant protein still possessed Met at the N-terminus in front of the first (Ser) residue of the toxin. This observation is in consistence with the study of Hirel et al. (24) for directly expressed proteins with different penultimate residues in E. coli. In the case of smaller side-chain residues (such as Gly, Ala, Ser) in second position, bacterial excision of the preceding, initial Met residue by methionine-aminopeptidase is more efficient.

Using the isolation procedure presented above, we were able to isolate about 1 mg of active protein from a liter of fermentation broth. Hemolytic activity of the isolated protein, measured on bovine erythrocytes, was practically the same as that of native EqtII (Figure 3). This indicates that substitution of Ser177 by Thr is not essential for cytolytic activity of EqtII. EqtII can thus be produced efficiently in a bacterial expression system without need of renaturation. It is also evident that, at least in in vitro conditions, the propart is not important for correct folding of the toxin.

The successful cloning of EqtII represents, to our knowledge, the first molecular cloning of actinoporins from sea anemones. Additionally, the bacterial expression system and subsequent isolation procedure can be applied for production of EqtII mutants, which we shall use to study the structure-function relationships of these interesting toxins.

ACKNOWLEDGMENTS

The authors thank Adrijana Leonardi for her help in protein isolation and amino acid sequencing. We also thank Dr. Roger H. Pain for critical reading of the manuscript. This work was supported by a grant from the Ministry of Science and Technology of Slovenia.

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