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Expression of fully active ammodytoxin A, a potent presynaptically neurotoxic phospholipase A_2, in *Escherichia coli*

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A cDNA encoding the most presynaptically neurotoxic phospholipase A_2, ammodytoxin A, from the venom of the long-nosed viper (*Vipera ammodytes ammodytes*) has been expressed in *Escherichia coli*. Ammodytoxin A was produced as a fusion protein with the 81 N-terminal residues of adenylate kinase followed by the tetrapeptide recognition site for factor Xa (IEGR) just preceding the first amino acid residue of the toxin. The fusion protein was expressed under the control of *tac* promoter without IPTG induction in the form of insoluble inclusion bodies. It was dissolved in guanidine hydrochloride, S-sulfonated and refolded in a reoxidation mixture including a reduced/oxidized glutathione redox couple. Ammodytoxin A was fully activated by limited hydrolysis with trypsin that preferentially cleaves the fusion protein at the factor Xa recognition site and purified by cation-exchange chromatography. The correct N-terminus was confirmed by protein sequencing. Recombinant ammodytoxin A has been proved to be indistinguishable from the native toxin in its enzymatic activity and toxicity.

Phospholipase A_2; Neurotoxin; Refolding; Expression; Snake; *Vipera ammodytes*

1. INTRODUCTION

Phospholipases A_2 (PLA_2s) are a diverse family of mostly calcium-dependent lipolytic enzymes that catalyze the hydrolysis of the 2-acyl ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides [1]. Snake PLA_2s represent the top of evolutional diversity of 14 kDa class of PLA_2s. Structurally similar snake PLA_2s differ considerably in their pharmacological activities including presynaptic and postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, convulsive, hypotensive, hemolytic, hemorrhagic and edema-inducing effects [2]. Presynaptically neurotoxic snake PLA_2s are very potent toxins that block release of acetylcholine at the neuromuscular junction in a multi-step process [3]. Their mode of action at the molecular level is still not completely understood. During the first, presumably non-enzymatic step in this blockade, neurotoxin binds to a receptor in the presynaptic membrane, apparently one of potassium channels [4]. Experiments with different PLA_2 neurotoxins also indicate that probably several types of such receptors exist [3]. In the following steps, which include PLA_2 enzymatic activity, toxin produces a complete neuromuscular blockade [5]. So far, it has not been possible to locate all the amino acid residues responsible for high toxicity of these PLA_2s neither those responsible for the initial binding to the receptor. This problem could be best approached by protein engineering which requires, however, expression of a fully active toxin to be achieved. Thus far this had eluded all attempts.

Three single-chain presynaptically neurotoxic PLA_2 isotoxins, ammodytoxins A, B and C, have been isolated from the venom of the long-nosed viper (*Vipera ammodytes ammodytes*) and their primary structures determined [6–8]. They show more than 97% identity in their amino acid sequences, but differ in toxicities by more than a factor of ten. Recently, all the three ammodytoxins have been cloned [9]. In this paper, we present the expression of the most neurotoxic, ammodytoxin A, as a fusion protein in *E. coli*, its refolding and final activation to produce recombinant toxin which is in its enzymatic and neurotoxic properties indistinguishable from the native neurotoxin. This is the first report of the expression of a presynaptically neurotoxic snake PLA_2.

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Abbreviations: cDNA, complementary DNA; DTT, 1,4-dithiothreitol; EDTA, disodium ethylenediaminotetraacetate; GdnHCl, guanidine hydrochloride; IPTG, isopropyl-β-D-thiogalactoside; kDa, kilodalton; NTSB, disodium 2-nitro-5-thiosulfobenzoate; PAGE, polyacrylamide gel electrophoresis; PLA_2, phospholipase A_2; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction and DNA modifying enzymes were purchased from Boehringer-Mannheim (Germany). Other chemicals of analytical grade were from Sigma (USA) or Serva (Germany) unless otherwise stated. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer (USA) and purified by polyacrylamide gel electrophoresis. Trypsin from bovine pancreas (type III) was obtained

from Sigma. [α-^{35}S]dATP used for nucleotide sequencing was from Amersham (UK). The cDNA encoding ammodytoxin A used in this study was isolated from a *V. ammodytes ammodytes* venom gland cDNA library as described previously [9]. The expression plasmid pMAX, a derivative of pKK223–3 and pUC18 (both from Pharmacia, Sweden) encoding the N-terminal part of adenylate kinase of 81 amino acid residues under the control of *tac* promoter, was a generous gift of Prof. Tatsuya Samejima of Aoyama Gakuin University, Tokyo, Japan. The bacterial strain used for cloning *E. coli* DH5α was from Gibco BRL (USA) and the one for expression *E. coli* JM109 from Promega (USA).

2.2. *Construction of the expression plasmid*

The original cDNA clone encoding ammodytoxin A in pUC9 contains two internal *Pst*I sites, the first one in the middle of the coding region and the second just after the stop codon. Additionally, an *Eco*RI site is present at the codons encoding Glu-4 and Phe-5. The ammodytoxin A cDNA sequence was cleaved with *Eco*RI/*Pst*I and inserted between the *Nco*I and *Pst*I sites of the expression plasmid pMAX using an *Nco*I/*Eco*RI synthetic cassette. The cassette was constructed from the oligonucleotides 5′-C-ATG-GTG-ATC-GAG-GGC-CGC-AGC-CTG-CTC-G-3′ and 5′-AA-TTC-GAG-CAG-GCT-GCG-GCC-CTC-GAT-CAC-3′. It encodes the recognition sequence IEGR for cleavage with factor Xa just preceding the first amino acid residue, serine, in the sequence of mature ammodytoxin A. Finally, the missing *Pst*I-*Pst*I fragment coding for the second half of ammodytoxin A was ligated into the *Pst*I site of the expression plasmid. Its correct orientation and the entire sequence of the construct were verified by nucleotide sequencing using ^{T7}Sequencing kit of Pharmacia. The final expression plasmid named pMAXA thus encodes ammodytoxin A as a fusion protein with the N-terminal sequence of adenylate kinase which can be cleaved off by factor Xa or trypsin.

2.3. *Expression of the fusion protein*

Routinely, a single fresh bacterial colony was picked from a M9 plate supplemented with 50 μg/ml ampicillin, inoculated into 2.5 ml of M9 medium with ampicillin and grown overnight at 37°C. The overnight culture was used to inoculate 250 ml of LB medium containing 100 μg/ml ampicillin in a 2 l Erlemeyer flask. Bacterial culture was grown by shaking at 250 rpm and 37°C for 8–10 h to yield at the end about 2.2 g wet weight bacteria per liter of culture medium. If IPTG was used for induction, it was added at the final concentration of 0.4 mM when the cell density had reached $A_{600} = 0.6$. Cells were harvested by centrifugation at $4000 \times g$ for 10 min. Inclusion bodies were isolated according to the procedure of Marston et al. [10].

2.4. *Refolding, activation and purification of the recombinant protein*

Inclusion body pellet recovered from 2 l of bacterial culture was thoroughly washed with a solution containing 2 M urea, 1% Triton X-100 and 5 mM DTT, and sulfonated with NTSB in 6 M GdnHCl, 0.3 M Na_2SO_3, pH 8.5 [11]. The fusion protein was precipitated by 1% acetic acid and dissolved in 100 ml of 6 M GdnHCl, 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 3 mM EDTA and 2 mM reduced glutathione. Refolding was performed by dilution 1:10 into a solution of 0.3 M GdnHCl, 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 3 mM EDTA, 2 mM reduced and 1 mM oxidized glutathione. After standing for about 48 h at room temperature, the reoxidation mixture was concentrated to 100 ml by ultracentrifugation through a YM-10 membrane (Amicon, USA) and trypsin was added to the final concentration of 4% by weight according to the fusion protein. Production of active ammodytoxin A was followed by measurement of PLA_2 activity. After 2.5 h of stirring at room temperature, the pH was adjusted to 5.0, supernatant concentrated by ultrafiltration and dialysed against 50 mM sodium acetate, pH 5.0, 1 mM EDTA. This solution was loaded onto a Mono-S HR 5/5 FPLC column (Pharmacia) and purified with a linear gradient to 50 mM sodium acetate, pH 5.0, 1 mM EDTA and 2 M NaCl. Recombinant, more than 95% pure ammodytoxin A eluted as a sharp peak at about 0.6 M NaCl. The protein solution was desalted using a Centricon-10 microconcentrator (Amicon).

2.5. *SDS-PAGE*

Electrophoresis was performed on 8 25% gradient gels using the PhastSystem of Pharmacia. The gels were stained by Coomassie blue R 350 according to the instructions of the manufacturer (Pharmacia).

2.6. *Amino-terminal protein sequence analysis*

Prior to sequencing, recombinant ammodytoxin A was desalted by HPLC (Milton Roy, USA) on a Chrompack reversed-phase C8 column (100 mm × 3 mm) equilibrated with 0.1% TFA in water and eluted by a linear gradient of 80% acetonitrile containing 0.1% TFA (solution B). The flow rate was 1 ml/min. Absorbance was monitored at 215 nm. Fraction A was lyophilized and subjected to sequencing. For the N-terminal sequence determination of the recombinant protein, an Applied Biosystems liquid-pulsed protein sequencer 475A, on-line connected to a phenylthiohydantoin-amino acid analyzer 120A of the same manufacturer was used.

2.7. *Phospholipase A_2 activity*

PLA_2 activity was measured titrimetrically on mixed micelles using a simple egg yolk assay [12]. The reaction mixture consisted of egg yolk phosphatidylcholine as a substrate in the presence of 1% Triton X-100 and 10 mM $CaCl_2$. All the measurements were done at 40°C and pH 8.0. One enzyme unit is defined as consumption of 1 mmol of NaOH per minute. Concentrations of ammodytoxin A were calculated from the absorbance at 280 nm using an $A_{1\%}$ of 11.6 cm^{-1} [13].

2.8. *Toxicity testing*

Lethality of recombinant ammodytoxin A was determined by dissolving different quantities of the toxin in 0.5 ml of 0.9% NaCl and intravenous injection to NMRI albino mice. The LD_{50} was calculated using the method of Reed and Muench [14].

3. RESULTS

The ammodytoxin A cDNA sequence was fused with a sequence coding for the first 81 amino acid residues of adenylate kinase by a linker encoding factor Xa recognition site (shown in Fig. 1). As expected, expression of ammodytoxin A in *E. coli* led to formation of inclusion bodies in the cytoplasm. The fusion protein accumulated to a level of about 10% of the total bacterial protein (lane 3 in Fig. 2). We have also tested two other bacterial expression systems, a short-fusion protein expression (MIEGR preceding the first amino acid residue of ammodytoxin) using the same *tac* promoter and periplasmic expression under the control of T7 promoter (data not shown). However, the expression levels were much lower.

In order to obtain a good expression from the plasmid pMAXA in *E. coli* JM109, it was crucial to start inoculation with a colony from a fresh transformation plate. Our attempts to express the fusion protein in a 10 litre fermentor were not successful, as we found that the expression was relatively low in comparison to the one using shaking flasks. Additionally, it has been observed that in this system *tac* promoter is not tightly regulated. The recombinant protein could be produced to the same expression level with or without IPTG induction. Although expression of the fusion protein was not fatal for

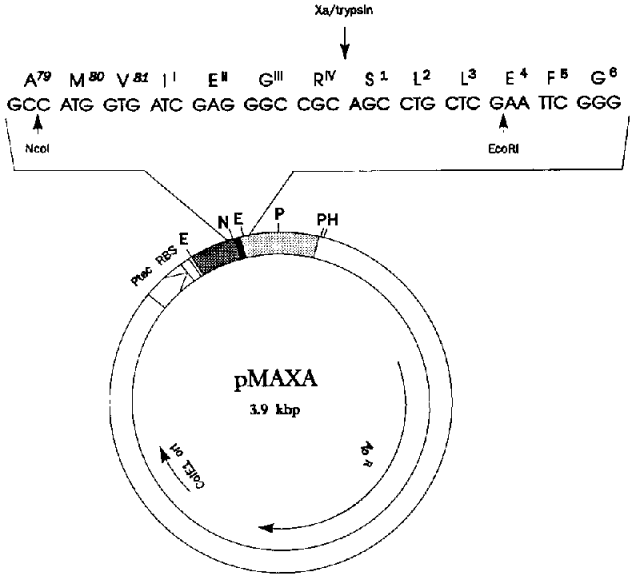

Fig. 1. Schematic presentation of the expression plasmid pMAXA. Restriction enzyme sites: E, *Eco*RI; N, *Nco*I; P, *Pst*I; H, *Hin*dIII. Other abbreviations used: Ptac, *tac* promoter; RBS, ribosome binding site; ColE1 ori, ColE1 origin of replication; Ap^R, β-lactamase gene for ampicillin resistance. The N-terminal fusion part of adenylate kinase is indicated by a heavy shaded box, recognition site IEGR for factor Xa by a solid box and the coding sequence of ammodytoxin A by a light shaded box. The linker region is additionally presented enlarged. The amino acid residues of adenylate kinase are indicated by numbers in italics, residues of the IEGR site by Roman numerals and those of ammodytoxin A by upright numbers. Cleavage sites for *Nco*I, *Eco*RI and trypsin or factor Xa are denoted by arrows.

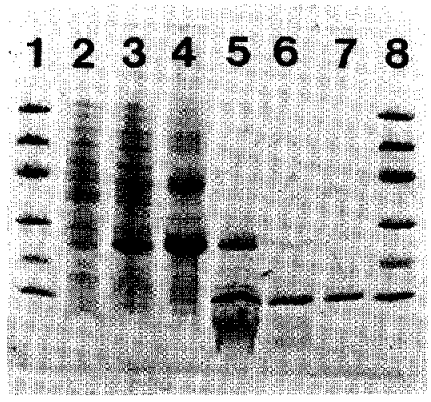

Fig. 2. SDS-PAGE analysis of expression and purification of ammodytoxin A. Lanes 1 and 8, molecular weight standards (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa); lane 2, total protein from *E. coli* JM109 without the expression plasmid; lane 3, total protein from the same strain with the expression plasmid pMAXA at the end of expression; lane 4, washed inclusion body pellet; lane 5, fusion protein after cleavage with trypsin; lane 6, recombinant ammodytoxin A purified by FPLC; lane 7, native ammodytoxin A. Before loading, samples were boiled in loading buffer for 5 min in the presence of 0.2 M DTT.

the host bacteria, about 50% retardation in growth was observed by measuring the final cell density.

After careful washing of the inclusion body pellet, the recombinant protein was more than 70% pure (see lane 4 in Fig. 2). Before the final purification, the fusion protein was refolded and activated. The fusion protein was solubilized in 6 M GdnHCl, *S*-sulfonated and refolded at a concentration of about 50 μg/ml in a redox buffer including 0.9 M GdnHCl. After two days of reoxidation and subsequent concentration, activation of ammodytoxin A was performed with trypsin. In the first experiments, we used factor Xa for a specific cleavage. However, it was less efficient than trypsin, which was also able to degrade most of the incorrectly folded fusion protein and is considerably cheaper. Using limiting conditions for tryptic activation, such as room temperature, short time of incubation and presence of 0.9 M GdnHCl, we were able to cleave about 70% of the available fusion protein (lane 5 in Fig. 2). The final purification by FPLC on a cation-exchange resin yielded about 0.5 mg of active ammodytoxin A per liter of bacterial culture. Recombinant ammodytoxin A appeared to be more than 95% pure (lane 6 in Fig. 2). Before application to a protein sequencer, it was analysed by HPLC to show its homogeneity (Fig. 3). The N-terminal sequence up to the 10th residue completely corresponded to the sequence of native toxin. Specific enzymatic activity of recombinant ammodytoxin A was about 300 U/mg in the egg yolk assay and its intraperitoneal LD_{50} about 0.02 mg/kg of white mice. Almost the same values have been reported for ammodytoxin A isolated from the venom [15].

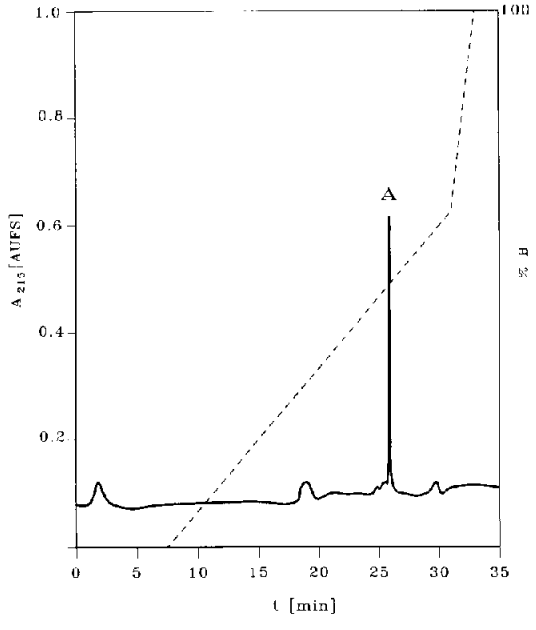

Fig. 3. HPLC separation of recombinant ammodytoxin A (A) on a reversed-phase C8 column before N-terminal protein sequencing. For details see section 2.

4. DISCUSSION

Until now, PLA_2s from various organisms have been produced in different expression systems including bacterial [16–25], yeast [26–28] and mammalian hosts [29–34]. Expression of an active PLA_2 possessing 5–7 disulfide bonds in a reductive environment of the bacterial cytoplasm is practically impossible. The only possibility to express a correctly folded PLA_2 using a bacterial system is expression into the periplasmic space which has been reported for expression of the M8L mutant of notechis 11'2 PLA_2 from the elapid snake *Notechis scutatus scutatus* [25]; the expression yield was, however, low. In most cases, bacterial expression of PLA_2s is accompanied by an in vitro refolding procedure. The early experiments on refolding of native PLA_2s either from group I [35] or group II [36] showed almost a quantitative recovery of their structures and activities. These encouraging results have been followed by renaturation of porcine pancreatic [16], bovine pancreatic [17,37], human non-pancreatic [19 21], snake non-neurotoxic [22,23] and bee venom PLA_2s [24] expressed in *E. coli*. However, no expression of a snake neurotoxic PLA_2, neither from family Elapidae (group I) nor from family Viperidae (group II), has been reported so far. One of the reasons might be that such a PLA_2 can only refold under the conditions which do not resemble those used for renaturation of acidic, non-neurotoxic snake PLA_2s from *Naja naja naja* [22] and *Agkistrodon piscivorus piscivorus* [23].

We expressed a potent presynaptically neurotoxic snake PLA_2 from *V. ammodytes* (Viperidae) in *E. coli* as a non-toxic fusion protein. The fusion protein can be renatured under the conditions similar to the ones used for reoxidation of human non-pancreatic PLA_2 [20]. Both PLA_2s, ammodytoxin A and human non-pancreatic PLA_2, are very basic proteins that belong to group II PLA_2s. Following reoxidation, recombinant ammodytoxin A is released from the fusion protein by limited trypsinolysis. The N-terminal fusion of adenylate kinase simulates the propeptide which is normally present in pancreatic type I PLA_2s and prevents enzymatic activity and toxicity of the expressed fusion protein. The use of trypsin instead of factor Xa was feasible as properly folded recombinant ammodytoxin A was shown to be resistant against limited tryptic digestion. In a separate experiment, this finding was confirmed by incubation of native toxin with trypsin using the same conditions. Subsequent sequencing revealed no additional N-termini that might result from trypsin treatment. Use of the enzymatic activation instead of frequently used chemical cleavage with cyanogen bromide does not require any preliminary mutation of internal methionine residues and enables expression of the protein with a native sequence. Additionally, possible chemical modifications of the amino acid side groups are excluded. Tryptic degradation of incorrectly folded protein molecules also contributes to more efficient subsequent purification of the correctly folded, active enzyme.

The overall recovery of about 4% of pure recombinant ammodytoxin A with respect to the amount expressed in a bacterial culture is somewhat lower than expected. There are at least two possible reasons for that. The most important one seems to be low efficiency of refolding including reoxidation of all 7 disulfide bonds which might be interfered by the presence of a single cysteine residue in the N-terminal part of adenylate kinase. Exchange of this residue could result in a better recovery, although it might be necessary to readjust the conditions for optimal refolding. The other major problem in refolding is extremely high affinity of this protein for surfaces. A considerable amount of recombinant ammodytoxin A was lost due to its irreversible non-specific binding during refolding where the concentration of the toxin has to be as low as 50 μg/ml.

In conclusion, the amount of fully-active, recombinant ammodytoxin A we could get from the fusion protein expressed in *E. coli* followed by its refolding, enzymatic activation and easy purification has enabled us to start with production of the first mutants to approach the yet unknown molecular mechanism of its presynaptic neurotoxicity by protein engineering.

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