cDNA cloning and expression of Contractin A, a phospholipase A_2-like protein from the globiferous pedicellariae of the venomous sea urchin *Toxopneustes pileolus*

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Running title: Phospholipase A_2-like venom protein from a sea urchin

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Abbreviations: CF: carboxyfluorescein; EDTA, ethylenediamine tetraacetate; PLA_2, phospholipase A_2; RACE, rapid amplification of cDNA ends; SUL-I, sea urchin (*Toxopneustes pileolus*) lectin-I; TBS, Tris-buffered saline

Abstract

Venomous sea urchins contain various biologically active proteins that are toxic to predators. Contractin A is one such protein contained within the globiferous pedicellariae of the venomous sea urchin *Toxopneustes pileolus*. This protein exhibits several biological activities, such as smooth muscle contraction and mitogenic activity. N-terminal amino acid residues of Contractin A have been determined up to 37 residues from the purified protein. In this study, we cloned cDNA for Contractin A by reverse transcription-PCR using degenerate primers designed on the basis of its N-terminal amino acid sequence. Analysis of the cDNA sequence indicated that Contractin A is composed of 166 amino acid residues including 31 residues of a putative signal sequence, and has homology to the sequence of phospholipase A_2 from various organisms. In this study, recombinant Contractin A was expressed in *Escherichia coli* cells, and the protein was subjected to an assay to determine lipid-degrading activity using carboxyfluorescein-containing liposomes. As a result, Contractin A was found to exhibit Ca^{2+}-dependent release of carboxyfluorescein from the liposomes, suggesting that Contractin A has phospholipase A_2 activity, which may be closely associated with its biological activities.

Keywords: sea urchin, *Toxopneustes pileolus*, cDNA cloning, phospholipase A_2, liposome, carboxyfluorescein

1. Introduction

Venom obtained from the globiferous pedicellariae of the sea urchin *Toxopneustes pileolus* contains several biologically active proteins, including lectins (Edo et al. 2012; Nakagawa et al. 1996; Nakagawa et al. 1991; Nakagawa et al. 1999; Sakai et al. 2013; Satoh et al. 2002; Takei and Nakagawa 2006; Takei et al. 1991). We have previously reported cDNA cloning and expression of a galactose-specific lectin, SUL-I, which was originally isolated from large globiferous pedicellariae.

The amino acid sequence of SUL-I shares homology with rhamnose-binding lectins, which are mostly distributed in fish eggs, and this protein indeed exhibits high rhamnose-binding specificity (Hatakeyama et al. 2015). In addition to SUL-I, several lectins and other proteins have been found in the venom of *T. pileolus*. Among them, Contractin A has some intriguing biological activities such as inducing the contraction of smooth muscles, which might be mediated by the activation of cellular phospholipase C (Nakagawa et al. 1991). N-terminal sequence analysis revealed that Contractin A shares structural similarity with phospholipase A_2 (PLA_2), which also suggests that Contractin A exerts its biological activities by affecting signal transduction through the target cell membrane. Interestingly, another venom protein, UT841, was isolated from the globiferous pedicellariae of *T. pileolus* and has a very similar N-terminal sequence to that of Contractin A; only the residues at position 21 (Contractin A: Asn, UT841: Tyr) differ within the 37 residues in the N-terminal of these proteins. UT841 has been reported to have inhibitory activity toward Ca^{2+} uptake of the synaptosome fraction from chicken brain (Zhang et al. 2001). Since membrane lipid metabolism is closely related to cellular signal transduction processes, some similarities between Contractin A and UT841 with PLA_2 are of interest.

In the present study, we cloned cDNA for Contractin A, and expressed it in *Escherichia coli* cells. The results confirm that there is a close structural relationship between Contractin A and PLA_2 from various organisms. Lipolytic activity of the recombinant Contractin A was also observed in an assay in which degradation of phospholipid vesicles (liposomes) was monitored by the release of a fluorescent dye, carboxyfluorescein (CF), which strongly suggests that Contractin A has Ca^{2+}-dependent PLA_2 activity.

2. Materials and Methods

2.1. Materials

Oligonucleotides, egg yolk phosphatidylcholine, and CF were purchased from Sigma-Aldrich.

Oligotex-dT30 mRNA Purification Kit was from Takara (Otsu, Japan). Plasmid vector pTAC-2 was from BioDynamics Laboratory (Tokyo, Japan). Plasmid vector pET-3a and *E. coli* BL21(DE3)pLysS were from Novagen. *E. coli* JM109 cells, SMARTer cDNA Cloning Kit, and In-Fusion HD Cloning Kit (Clontech) were from Clontech. All other chemicals were of analytical grade for biochemical use. Sea urchin *T. pileolus* specimens were collected along the coast of Tokushima Prefecture, Shikoku Island, Japan.

2.2. Cloning of cDNA encoding Contractin A

The shell containing the globiferous pedicellariae of *T. pileolus* was flash frozen in liquid nitrogen and ground to form a powder. Total RNA was extracted from the powdered shell (about 100 mg) using Isogen solution (Nippon Gene, Tokyo, Japan). Poly(A) RNA was collected using the Oligotex-dT30 mRNA Purification Kit, and cDNA was synthesized using the SMARTer cDNA Cloning Kit. A DNA fragment corresponding to the N-terminal region of Contractin A was amplified by polymerase chain reaction (PCR) using two degenerate primers, DF1: 5′-(A/T)(C/G)IGTIAT(A/C/T)AA(C/T)TT(C/T)GGITGGATG-3′ and DR1: 5′-CC(A/G)AAICC(A/G)CA(A/G)TA(A/G)CAICC(A/G)TA-3′, where “I“ represents deoxyinosine, and the ones in parentheses represent mixed bases. An amplified DNA fragment of approximately 100 bp was cloned into a pTAC-2 vector using *E. coli* JM109 cells, and was sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The amino acid sequence of 13 residues deduced from this DNA fragment was consistent with that determined from the N-terminal sequence of the purified protein. Therefore, 3′- and 5′-rapid amplification of cDNA ends (3′-RACE and 5′-RACE) were performed with the primer IR1, which was newly designed from this region, and primers F1, F2, and F3 using the SMARTer cDNA Cloning Kit (Fig. 1). The full amino acid sequences of Contractin A deduced from the obtained cDNA sequence were compared with the sequences in UniProt database (www.uniprot.org) using BLAST (Basic Local Alignment Search

Tool) (Altschul et al. 1990). Multiple sequence alignments were performed using Clustal Omega (Sievers et al. 2011). These nucleotide sequences were deposited in DDBJ/EMBL/GenBank (accession number: LC034582). Chemical and physical parameters of Contractin A were calculated from the deduced sequence using the ProtPram tool in ExPASy Bioinformatics Resource Portal (www.expasy.org) (Artimo et al. 2012; Gasteiger et al. 2005).

2.3. Expression of recombinant Contractin A in E. coli cells

The coding region of mature Contractin A was amplified by PCR using two primers (forward: 5′-AAGGAGATATACATATGTCAGTTATCAATTTTGGCTG-3′ and reverse: 5′-GTTAGCAGCCGGATCCGTGAAGATAACTCGGATTGC-3′), and inserted into a pET-3a vector at *Nde*I and *Bam*HI restriction sites using the In-Fusion HD Cloning Kit. The plasmid was amplified in *E. coli* JM109 cells, and the protein was expressed in *E. coli* BL21(DE3)pLysS cells. Recombinant Contractin A expression was induced with 0.4 mM isopropylthiogalactoside, and the cells were incubated for an additional 18 h at 37°C. Because the recombinant proteins were obtained as inclusion bodies after the induction and disruption of cells, they were solubilized in solubilization buffer (50 mM Tris-HCl pH 8.0, 0.2 M NaCl, 1 mM ethylenediamine tetraacetate [EDTA], 6 M guanidine hydrochloride), and the protein was refolded in the refolding buffer (0.1 M Tris-HCl pH 8.0, 0.8 M L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione). Next, the refolded protein was dialyzed against Tris-buffered saline (TBS; 10 mM Tris–HCl pH 7.5, 0.15 M NaCl). Protein concentrations were determined from the molar absorption coefficients at 280 nm calculated from the amino acid compositions of the proteins. The N-terminal amino acid sequence of the expressed protein was determined using a protein sequencer, PPSQ-21 (Shimadzu, Kyoto, Japan).

2.4. CF-leakage assay for phospholipid-degrading activity of Contractin A

Liposomes containing CF were prepared following a previously described method (Hatakeyama et al. 1999); egg yolk phosphatidyl choline (1 mg) was dissolved in 1 mL of chloroform/methanol (1:1, v/v) and dried using a rotary evaporator in a conical glass tube. After the addition of 1 mL 10 mM Tris-HCl, pH 8.5, 0.15 M NaCl containing 0.1 M CF, the lipid was hydrated by mixing with a vortex at room temperature. The suspension was then sonicated for 5 min at 45°C using a Taitec Ultrasonic Processor VP-5T at 10 W intensity. The formed liposomes were then separated from free CF by gel filtration using a Sephadex G-75 column (1 × 20 cm) equilibrated with 10 mM Tris-HCl, pH 8.5, 0.15 M NaCl. To measure phospholipid-degrading activity, the liposome solution (50 μL) was mixed with 150 μL of the sample solution in 10 mM Tris-HCl, pH 8.5, 0.15 M NaCl, 10 mM $CaCl_2$, and incubated at 25°C. After an appropriate time, the fluorescence intensity at 518 nm following excitation at 490 nm was recorded using a Hitachi F-3010 Fluorescence Spectrophotometer. To obtain 100% CF leakage Triton X-100 was added to a final concentration of 0.1%.

2.5. Homology modeling

Homology modeling of Contractin A was performed by SWISS-MODEL server (Arnold et al. 2006) using the automatic modeling mode. To construct the model, the crystal structure of human PLA_2 ($hPLA_2$, PDB code 3ELO) (Xu et al. 2009) was used. The figures for the protein models were drawn using the program PyMOL (DeLano 2002).

3. Results

3.1. cDNA cloning and sequence analysis of Contractin A

cDNA was prepared by reverse transcription using mRNA from the shell with globiferous pedicellariae of *T. pileolus*. To amplify the DNA fragment encoding the N-terminal region of the mature protein, PCR was performed using the degenerate primers DF1 and DR1 (Fig. 1), which were

designed on the basis of the N-terminal amino acid sequence of Contractin A as determined from the purified native protein (Nakagawa et al. 1991). A DNA fragment of about 100 bp that was amplified in degenerate PCR was then sequenced, and the amino acid sequence that was deduced from the resulting fragment (SCVTCTSTRYNG) was found to correspond to residues 10–21 of Contractin A, in which an undetermined residue designated as X was confirmed to be cysteine (or half-cystine). Based on the sequence of this DNA fragment, a new primer (IR1) was prepared and used for 5′-RACE to determine the 5′-terminal sequence of the cDNA. Amplification of the 3′-terminal portion of cDNA was performed by 3′-RACE using the primers F1, F2, and F3. Sequencing of the entire 1298 bp cDNA revealed that Contractin A is encoded by 498 bp, corresponding to 166 amino acid residues. The N-terminal portion of 31 residues was assumed to be the signal sequence, and the mature protein contains 135 amino acid residues with a molecular mass of 14,997.5 kDa.

Proteins homologous to Contractin A were searched using BLAST on the UniProt database (Consortium 2012). As shown in Fig. 2, homology was found with members of the secreted PLA_2 family (Murakami et al. 2011). As listed in Table 1, the amino acid sequences of Contractin A and the other PLA_2, which showed relatively high homologies, share 35–40% identities. Identical residues among these proteins are relatively abundant in the middle region around residues 54–81 of Contractin A (Fig. 2). Cysteine residues in Contractin A are also highly conserved with those found in other proteins, strongly suggesting that they adopt similar tertiary structures via the formation of internal disulfide bonds.

3.2. Expression, purification, and enzymatic activity of Contractin A

Recombinant Contractin A was expressed in *E. coli* cells. The expressed protein was exclusively found in the precipitate as inclusion bodies after centrifugation of the disrupted cells (Fig. 3A). Therefore, they were solubilized using 6 M guanidine hydrochloride and then refolded in buffer containing arginine to promote refolding in a soluble form. As shown in Fig. 3B, recombinant

Contractin A was found in the soluble fraction after refolding as a band of 15 kDa on SDS-PAGE. This protein was then subjected to N-terminal amino acid sequence analysis. As a result, up to 10 N-terminal amino acids were identified (SVINFGWMSS), which was consistent with the N-terminal sequence of Contractin A. The initiator methionine residue, which was introduced to the recombinant gene, was cleaved by methionine aminopeptidase during its expression in *E. coli* cells, since the next amino acid, serine, has a relatively small side chain (Moerschell et al. 1990). To assess PLA_2 activity of the recombinant Contractin A, a lipolytic assay was performed using CF-containing liposomes (CF-leakage assay). As shown in Fig. 4, when the liposomes were incubated with the solubilized Contractin A in the presence Ca^{2+}, the fluorescence intensity at 518 nm increased with time, while it was much lower in the absence of Ca^{2+} (in the presence of 5 mM EDTA). These results indicate that recombinant Contractin A degraded the phospholipid vesicles, presumably by its Ca^{2+}-dependent PLA_2 activity (Murakami et al. 2011), leading to the release of CF from the liposomes.

3.3. Homology model of Contractin A

The homology model of Contractin A constructed on the SWISS-MODEL server (Biasini et al. 2014) using PLA_2 from human pancreas (hPLA2) (Xu et al. 2009) as a template is shown in Fig. 5A. As shown in Fig. 5B, the alignment of these two proteins indicates that the residues involved in the enzymatic activity, Ca^{2+} binding, and the catalytic residues, are well conserved. In the homology model (Fig. 5A), these residues are located in the central region of the protein. Based on the sequence alignment, putative Ca^{2+}-binding residues in Contractin A are Tyr26, Gly28, Gly30, and Asp47, which correspond to Tyr28, Gly30, Gly32, and Asp49 of hPLA2. The region including these residues is highly conserved in both proteins and in the other homologous proteins (Fig. 2). Importantly, active site residues in hPLA2, His48 and Asp99, are also conserved in Contractin A (His46 and Asp109). It therefore seems reasonable to assume that the active site residues are located at positions appropriate to exert catalytic action. A notable difference between these two proteins is

in residues 77–86, in which there is an insertion in Contractin A. This insertion corresponds to a long extension of a loop located on the opposite side of the active site (Fig. 5A). Based on its location, this loop does not seem to have a direct effect on catalytic activity, and might be involved in other functions of Contractin A.

4. Discussion

Venom from the globiferous pedicellariae of *T. pileolus* contains several toxins, which have various biological activities (Nakagawa et al. 2003). One of these toxins, Contractin A, was purified using an assay to measure smooth muscle-contraction activity (Nakagawa et al. 1991). This activity was inhibited by a phospholipase C inhibitor, suggesting that Contractin A has phospholipase C-like activity or can activate cellular phospholipase C to induce its biological effects, although the N-terminal amino acid sequence has some similarity with PLA_2. As shown in the present study, the amino acid sequence of Contractin A shares homology with that of secreted PLA_2 family proteins from various organisms. As seen in Fig. 2, there are highly conserved amino acid residues, which are involved in Ca^{2+}-binding and catalytic reactions, and cysteine residues, which may be important for correct folding through the formation of internal disulfide bonds. Although the molecular weight of Contractin A was originally estimated to be 18,000 kDa based on SDS-PAGE analysis, the size calculated from the deduced amino acid sequence was 14,997.5 kDa. This discrepancy may be due to the presence of an oligosaccharide chain attached to native Contractin A, which was identified as a glycoprotein by its ability to bind to a lectin (concanavalin A) column (Nakagawa et al. 1991). As shown in Fig. 5A, a potential N-glycosylation site of Contractin A is the asparagine residue in the sequence of Asn14-Ser15-Thr16, which is the only site containing the “Asn-X-Thr/Ser” motif (X: any amino acid) (Hunt and Dayhoff 1970) in Contractin A. Based on the homology model, this site is expected to be located on the opposite side of the putative catalytic site (Fig. 5A). Therefore, it seems likely that even if a relatively large oligosaccharide chain was attached to Contractin A, it would not

interfere with the active site.

Contractin A was successfully expressed in *E. coli*. However, the expressed protein was recovered in inclusion bodies as an insoluble form after disruption of the cells, and thus the protein was refolded after complete denaturation with guanidine hydrochloride. Although part of the protein remained insoluble after dialysis against the buffer (Fig. 3), solubilized Contractin A showed Ca^{2+}-dependent lipolytic activity in a CF-leakage assay, indicating that it was correctly folded. CF-containing liposomes have been used in various studies to assess the activity of proteins and peptides that can degrade or disrupt lipid bilayers, including PLA_2 (Hatakeyama et al. 1999; Mukherjee et al. 2014; Niidome et al. 2004; Nobuhisa et al. 1997; Sorochkina et al. 2013). These proteins or peptides can directly interact with phospholipid vesicles, leading to the leakage of CF from inside the vesicles. As a result, a marked increase in fluorescence could be observed due to dequenching of CF. Owing to its high sensitivity, this method was adopted in the current study to detect potential lipolytic activity of recombinant Contractin A. After incubation with Contractin A, a Ca^{2+}-dependent increase in fluorescence was observed, suggesting that Contractin A had secreted PLA_2-like activity (Schaloske and Dennis 2006). As well as revealing conserved residues that have potential structural and catalytic roles, results from homology modeling further support the similarity between Contractin A and $hPLA_2$, a secreted PLA_2 that is involved in the digestion of dietary lipids. This enzyme is also associated with hypertension, obesity, and diabetes (Frossard and Lestringant 1995; Huggins et al. 2002; Labonté et al. 2006). Similarity of amino acid residues between Contractin A and $hPLA_2$ strongly suggests that they have a similar catalytic mechanism.

Phospholipase A_2 are contained in various animal venoms as a major component. As demonstrated in this study, Contractin A from the venomous sea urchin, *T. pileolus* was also found to be a PLA_2-like toxin. Therefore, the implication of PLA_2-like activity in the biological role of Contractin A seems very interesting. Additionally, it is also intriguing to investigate the presence of this or related proteins in different tissues in *T. pileolus* to reveal the evolutionary origin of this

protein. One of the Contractin A-like protein UT841 has been identified as a toxic component of the venom of *T. pileolus* (Zhang et al. 2001). In fact, we have identified the cDNA with the similar N-terminal sequence, in which tyrosine residue was identified at position 21 instead of asparagine. Although it is not yet clear whether this cDNA actually encodes UT841 or not, the presence of Conrtactin A-related protein, including UT841, in the venom or other tissues seems very probable. Data suggesting a role for PLA_2 in various cellular functions such as signal transduction have accumulated recently. Elucidating the mechanisms of Contractin A action would be very informative because of the common mechanisms underlying various cellular functions that are mediated by lipid metabolism.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. Nucleotide and deduced amino acid sequences of Contractin A. The N-terminal amino acid sequence determined from the purified protein (Nakagawa et al. 2003) is indicated by a broken line. The N-terminal amino acid of the mature protein is numbered as “+1”, and the initiator methionine residue is numbered as “-31”. The primers used in PCR analysis are indicated by horizontal arrows. An asterisk indicates the stop codon.

Fig. 2. Comparison of the amino acid sequences of Contractin A with PLA_2 from other organisms. PLA_2 showing high similarities in a BLAST search on the UniProt website (http://www.uniprot.org/) were aligned by the Clustal Omega program (Sievers et al. 2011). The sequences used were from the following species: bovine (*Bos taurus*) (Tanaka et al. 1987); Uruguayan coral snake (*Micrurus altirostris*) (Rey-Suárez et al. 2011); crown-of-thorns starfish (*Acanthaster planci*) (Ota et al. 2006); rabbit (*Oryctolagus cuniculus*) (Kumar 1993); Hardwick's spine-bellied sea snake (*Hydrophis hardwickii*); and human pancreas (*Homo sapiens*) (Xu et al. 2009). UniProt accession numbers are listed in Table 1. Asterisks, colons, and periods indicate the positions of identical, strongly similar, and weakly similar residues, respectively. Residues are numbered consecutively from the initiator methionine. N-terminal amino acids are marked in red. The amino acids conserved among all of the sequences are marked in yellow. Short vertical arrows and inverted triangles indicate the residues assumed to be involved in Ca^{2+} binding and the catalytic reaction, respectively, based on the structure of bovine PLA_2 (Dijkstra et al. 1981).

Fig. 3. Expression of Contractin A. A, SDS-PAGE of the soluble fraction (lane 1) and precipitate (lane 2) from the *E. coli* cells induced by isopropylthiogalactoside. B, the solubilized protein (lane 1) and precipitate (lane 2) after refolding of the expressed Contractin A.

Fig. 4 CF-leakage assay to determine the phospholipid-degrading activity of Contractin A. Changes in the fluorescence intensity at 518 nm after Contractin A (15 μg/ml) was mixed with egg yolk liposomes (100 μg/ml) containing CF were measured in 10 mM Tris-HCl (pH 8.5), 0.15 M NaCl at 25°C in the presence of 10 mM $CaCal_2$ (●) or 5 mM EDTA (○). The CF-leakage values were calculated from the increase in the fluorescence intensity taking the value after addition of Triton X-100 (0.1%, v/v) as 100%.

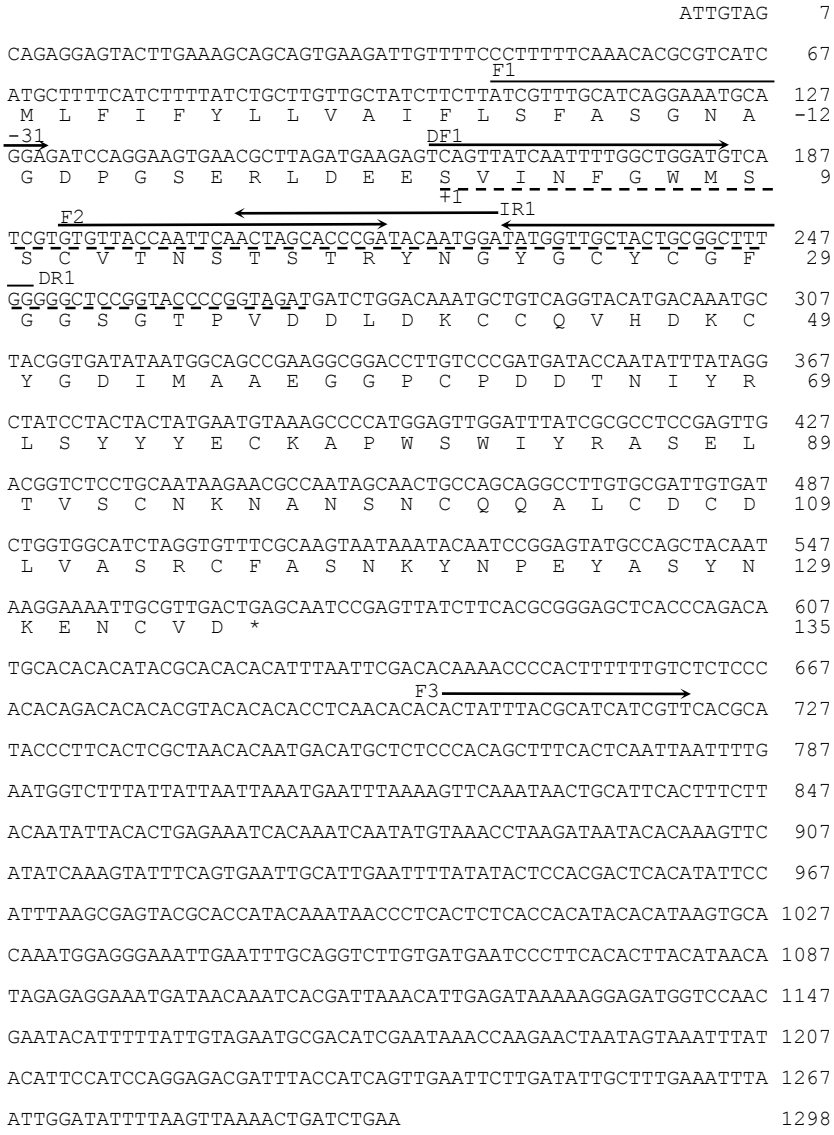
Fig. 5. Homology model of Contractin A constructed using $hPLA_2$ as a template model. A, Comparison between the homology model of Contractin A and hPLA2. The homology model of Contractin A (*green*) was constructed by the Swiss-Model server (http://swissmodel.expasy.org/) and superposed with $hPLA_2$ (PDB code 3ELO) (*orange*) using the program PyMOL. B, Sequence alignment of Contractin A and $hPLA_2$. The alignment was carried out using the Clustal Omega program (Sievers et al. 2011). The residues are numbered from the N-terminus of the mature proteins. The Ca^{2+}-binding and catalytic residues, based on those of $hPLA_2$, are enclosed in red and blue boxes, respectively. A long insertion sequence found in Contractin A (residues 77–86) is indicated by a horizontal bar. Asterisks, colons, and periods indicate the positions of identical, strongly similar, and weakly similar residues, respectively.

415

416 Table 1. Similarities in the amino acid sequences of Contractin A and other PLA_2 proteins

Species	Accession number	Identity (%)
Bos taurus (Bovine)	P00593	38
Micrurus altirostris (Uruguayan coral snake)	F5CPF0	39
Acanthaster planci (Crown-of-thorns starfish)	Q3C2C1	35
Oryctolagus cuniculus (Rabbit)	Q7M334	37
Hydrophis hardwickii (Hardwick's spine-bellied sea snake)	Q8UW08	40
Homo sapiens (Human pancreas)	P04054	35

417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CAGAGGAGTACTTGAAAGCAGCAGTGAAGATTGTTTTCCCTTTTTCAAACACGCGTCATC 67
F1
ATGCTTTTCATCTTTTATCTGCTTGTTGCTATCTTCTTATCGTTTGCATCAGGAAATGCA 127
M L F I F Y L L V A I F L S F A S G N A -12
-31
DF1
GGAGATCCAGGAAGTGAACGCTTAGATGAAGAGTCAGTTATCAATTTTGGCTGGATGTCA 187
G D P G S E R L D E E S V I N F G W M S 9
T1
F2
IR1
TCGTGTGTTACCAATTCAACTAGCACCCGATACAATGGATATGGTTGCTACTGCGGCTTT 247
S C V T N S T S T R Y N G Y G C Y C G F 29
DR1
GGGGGCTCCGGTACCCCGGTAGATGATCTGGACAAATGCTGTCAGGTACATGACAAATGC 307
G G S G T P V D D L D K C C Q V H D K C 49
TACGGTGATATAATGGCAGCCGAAGGCGGACCTTGTCCCGATGATACCAATATTTATAGG 367
Y G D I M A A E G G P C P D D T N I Y R 69
CTATCCTACTACTATGAATGTAAAGCCCCATGGAGTTGGATTTATCGCGCCTCCGAGTTG 427
L S Y Y Y E C K A P W S W I Y R A S E L 89
ACGGTCTCCTGCAATAAGAACGCCAATAGCAACTGCCAGCAGGCCTTGTGCGATTGTGAT 487
T V S C N K N A N S N C Q Q A L C D C D 109
CTGGTGGCATCTAGGTGTTTCGCAAGTAATAAATACAATCCGGAGTATGCCAGCTACAAT 547
L V A S R C F A S N K Y N P E Y A S Y N 129
AAGGAAAATTGCGTTGACTGAGCAATCCGAGTTATCTTCACGCGGGAGCTCACCCAGACA 607
K E N C V D * 135
TGCACACACATACGCACACACATTTAATTCGACACAAAACCCCACTTTTTTGTCTCTCCC 667
F3
ACACAGACACACACGTACACACACCTCAACACACACTATTTACGCATCATCGTTCACGCA 727
TACCCTTCACTCGCTAACACAATGACATGCTCTCCCACAGCTTTCACTCAATTAATTTTG 787
AATGGTCTTTATTATTAATTAAATGAATTTAAAAGTTCAAATAACTGCATTCACTTTCTT 847
ACAATATTACACTGAGAAATCACAAATCAATATGTAAACCTAAGATAATACACAAAGTTC 907
ATATCAAAGTATTTCAGTGAATTGCATTGAATTTTATATACTCCACGACTCACATATTCC 967
ATTTAAGCGAGTACGCACCATACAAATAACCCTCACTCTCACCACATACACATAAGTGCA 1027
CAAATGGAGGGAAATTGAATTTGCAGGTCTTGTGATGAATCCCTTCACACTTACATAACA 1087
TAGAGAGGAAATGATAACAAATCACGATTAAACATTGAGATAAAAAGGAGATGGTCCAAC 1147
GAATACATTTTTATTGTAGAATGCGACATCGAATAAACCAAGAACTAATAGTAAATTTAT 1207
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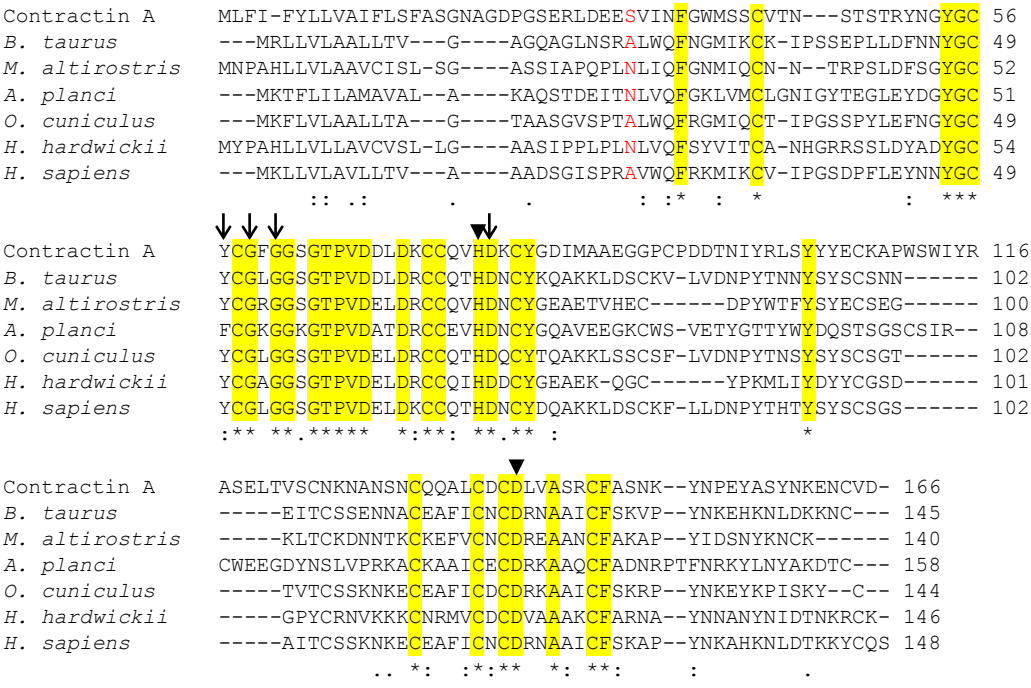
Fig. 1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B. taurus ---MRLLVLAALLTV---G----AGQAGLNSRALWQFNGMIKCK-IPSSEPLLDFNNYGC 49
M. altirostris MNPAHLLVLAAVCISL-SG----ASSIAPQPLNLIQFGNMIQCN-N--TRPSLDFSGYGC 52
A. planci ---MKTFLILAMAVAL--A----KAQSTDEITNLVQFGKLVMCLGNIGYTEGLEYDGYGC 51
O. cuniculus ---MKFLVLAALLTA---G----TAASGVSPTALWQFRGMIQCT-IPGSSPYLEFNGYGC 49
H. hardwickii MYPAHLLVLLAVCVSL-LG----AASIPPLPLNLVQFSYVITCA-NHGRRSSLDYADYGC 54
H. sapiens ---MKLLVLAVLLTV---A----AADSGISPRAVWQFRKMIKCV-IPGSDPFLEYNNYGC 49
Contractin A YCGFGGSGTPVDDLDKCCQVHDKCYGDIMAAEGGPCPDDTNIYRLSYYYECKAPWSWIYR 116
B. taurus YCGLGGSGTPVDDLDRCCQTHDNCYKQAKKLDSCKV-LVDNPYTNNYSYSCSNN------ 102
M. altirostris YCGRGGSGTPVDELDRCCQVHDNCYGEAETVHEC------DPYWTFYSYECSEG------ 100
A. planci FCGKGGKGTPVDATDRCCEVHDNCYGQAVEEGKCWS-VETYGTTYWYDQSTSGSCSIR-- 108
O. cuniculus YCGLGGSGTPVDELDRCCQTHDQCYTQAKKLSSCSF-LVDNPYTNSYSYSCSGT------ 102
H. hardwickii YCGAGGSGTPVDELDRCCQIHDDCYGEAEK-QGC------YPKMLIYDYYCGSD------ 101
H. sapiens YCGLGGSGTPVDELDKCCQTHDNCYDQAKKLDSCKF-LLDNPYTHTYSYSCSGS------ 102
Contractin A ASELTVSCNKNANSNCQQALCDCDLVASRCFASNK--YNPEYASYNKENCVD- 166
B. taurus -----EITCSSENNACEAFICNCDRNAAICFSKVP--YNKEHKNLDKKNC--- 145
M. altirostris -----KLTCKDNNTKCKEFVCNCDREAANCFAKAP--YIDSNYKNCK------ 140
A. planci CWEEGDYNSLVPRKACKAAICECDRKAAQCFADNRPTFNRKYLNYAKDTC--- 158
O. cuniculus -----TVTCSSKNKECEAFICDCDRKAAICFSKRP--YNKEYKPISKY--C-- 144
H. hardwickii -----GPYCRNVKKKCNRMVCDCDVAAAKCFARNA--YNNANYNIDTNKRCK- 146
H. sapiens -----AITCSSKNKECEAFICNCDRNAAICFSKAP--YNKAHKNLDTKKYCQS 148

Fig. 2

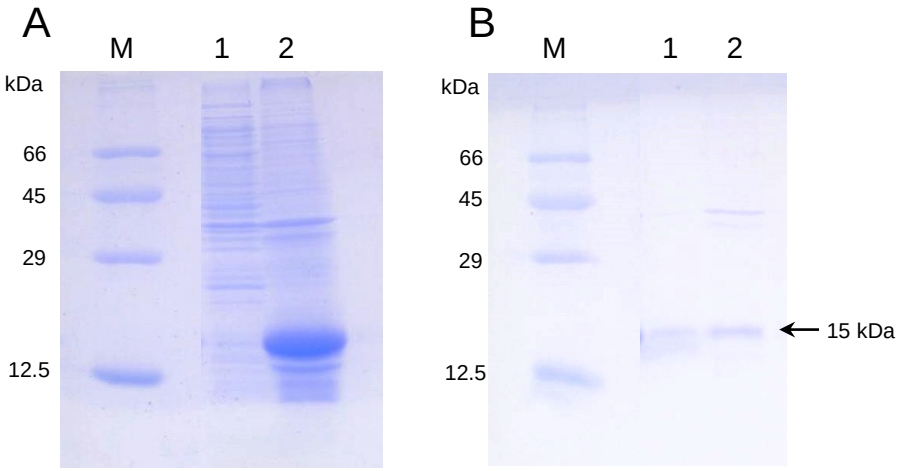


Fig. 3

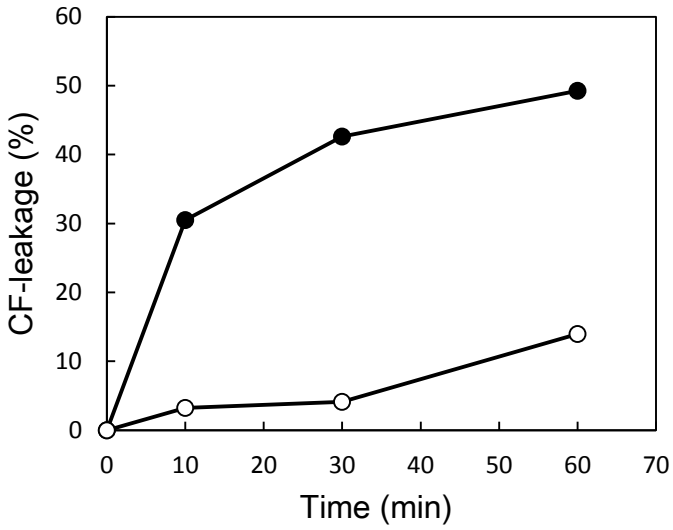
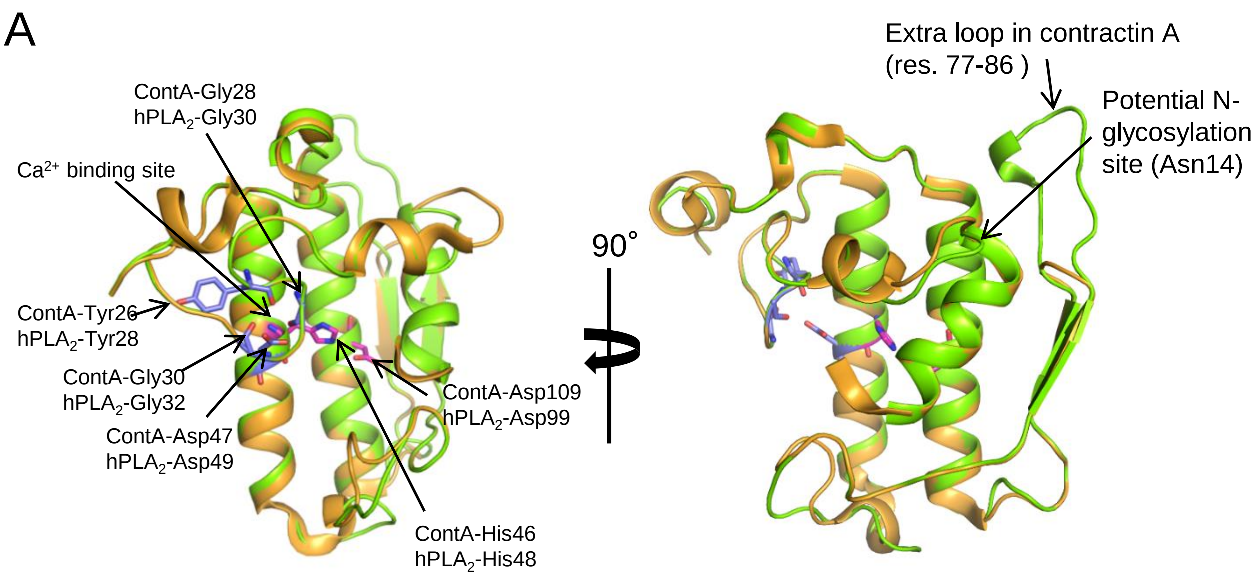


Fig. 4



B

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                           Asn14
                             ↓             26 28 30                 46 47
Contractin A   SVINFGWMSSCVTNS--TSTRYNGYGCYCGFGGSGTPVDDLDKCCQVHDKCYGDIMAAEG 58
Human PLA2     AVWQFRKMIKCVIPGSDPFLEYNNYGCYCGLGGSGTPVDELDKCCQTHDNCYDQAKKLDS 60
               :* :*  * .**  .     .** ******:********:******.**:** :    :.
                                           28 30 32                 48 49

                                 77       86                        109
                                 __________
Contractin A   GPCPDDTNIYRLSYYYECKAPWSWIYRASELTVSCNKNANSNCQQALCDCDLVASRCFAS 118
Human PLA2     CKFLL-DNPYTHTYSYSC----------SGSAITCS-SKNKECEAFICNCDRNAAICFSK 108
                      * *  :* *.*          *  :::*. . *.:*:  :*:**  *: **:.
                                                                  99

Contractin A   NKYNPEYASYNKENCVD- 135
Human PLA2     APYNKAHKNLDTKKYCQS 126
                 **  : . :.::  :
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Fig. 5