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A Novel Pro-apoptosis Protein PNAS-4 from *Xenopus laevis*: Cloning, Expression, Purification, and Polyclonal Antibody Production

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Abstract—Human PNAS-4 was identified as a novel pro-apoptotic protein in mammalian cells. Here we report the cloning, expression, purification, and antibody production of a PNAS-4 homolog (named xPNAS-4) from *Xenopus laevis*, an extensively used model organism in exploring gene functions during embryonic development. Recombinant histidine-tagged xPNAS-4 protein was expressed in *Escherichia coli* as insoluble inclusion bodies. The inclusion bodies were subsequently dissolved in 8 M urea and purified to near homogeneity by Ni^{2+} affinity chromatography. The resulting denatured protein was refolded by stepwise dilution of urea concentration via dialysis. This procedure yielded about 4 mg refolded protein per liter of *E. coli* culture with a purity of 95%. The purified protein was identified by liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-Q-TOF-MS) and used to raise anti-xPNAS-4 polyclonal antibodies that were suitable for detecting the expression of PNAS-4 in *X. laevis* embryos by Western blotting. The availability of recombinant protein and specific polyclonal antibodies will provide a valuable tool in studying apoptotic mechanisms of this protein. To our knowledge, this is the first report to demonstrate the presence of PNAS-4 in *X. laevis*.

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Key words: xPNAS-4, apoptosis, *Escherichia coli*, protein purification, antibody production

Apoptosis is recognized as an important process in both embryogenesis and organ development [1]. During the process of normal embryogenesis, apoptosis plays important roles in (i) sculpting structures, (ii) deleting unneeded structures, (iii) controlling cell numbers, (iv) eliminating abnormal, misplaced, non-functional, or harmful cells, and (v) producing differentiated cells without organelles [2]. Furthermore, studies have revealed that apoptosis also contributes to a variety of developmental processes of organs, such as cardiac development, neural development, lymphoid development, cardiovascular development, and lung development [3-7].

Most understandings of the relationship between apoptosis and development are mainly dependent on isolation, identification, and characterization of novel apoptosis-related proteins in various model organisms, such as mouse, zebrafish (*Brachydanio rerio*), *Xenopus*, *Drosophila*, *C. elegans*, etc. Thus far, many components of the apoptotic pathway including p53-dependent and p53-independent pathways have been identified, such as the caspase family proteins, the Bcl-2 family proteins, and the IAP family proteins [8, 9].

Recently, a report showed that human PNAS-4 (hPNAS-4) is a novel pro-apoptotic protein activated during the early response to DNA damage and likely plays a critical role in maintaining genomic integrity [10]. Furthermore, *PNAS-4* was also predicted as one of the

Abbreviations: ABTS) 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid); BCIP) 5-bromo-4-chloro-3-indolyl phosphate; hPNAS-4 and xPNAS-4) human and *Xenopus laevis* PNAS-4, respectively; IPTG) isopropyl-β-D-thiogalactopyranoside; NBT) nitroblue tetrazolium chloride; Ni-NTA) Ni-nitrilotriacetic acid; PVDF) polyvinylidene fluoride.

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targets of p53 tumor suppressor protein via mathematical modeling, quantitative data, and advanced data analysis [11]. Study of its apoptotic mechanisms and functions is of importance for the discovery of new drug targets for the development of effective therapeutic approaches to cancer.

Xenopus laevis is an extensively used model organism in exploring gene functions during embryonic development. With the intensive pursuit for characterization of genes that are involved in apoptosis, more and more genes with homology to mammalian apoptosis regulators have been identified in *X. laevis*. Investigation on functions of PNAS-4 protein involved in embryonic development will help us to better understand its apoptotic mechanisms and the relationship between apoptosis and development. Given the importance of the purified protein and the antibody in functional investigations, we here present the results of cloning, expression, purification, and production of polyclonal antibodies of xPNAS-4 protein.

MATERIALS AND METHODS

In the present work we used the following reagents and chemicals: *E. coli* host strain XL1-Blue MRF′ was purchased from Stratagene (USA); host strain BL21(DE3), the plasmid vector pQE30, and Ni-NTA agarose were from Qiagen (USA). Yeast extract and tryptone for bacterial media were obtained from Oxoid Ltd (UK). Anti-His monoclonal antibody was obtained from Sigma (USA). pGEM-T Easy vector system was purchased from Promega (USA).

Isolation of RNA and RT-PCR amplification. The total RNA was isolated from *X. laevis* adult testis using the Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. Briefly, the testis tissues were cut into pieces and ground by a mortar and pestle. Powder was collected and lysed in Trizol. The lysate was centrifuged and the supernatant was transferred to a new Eppendorf tube. Then the total RNA was precipitated, washed, and dissolved in RNase-free water. The RNA solution was immediately used in RT-PCR amplification or stored at −80°C.

The xPNAS-4 protein-coding region was amplified from total RNA prepared as above based on the sequence of the *X. laevis* PNAS-4 gene (GenBank accession No. BC087412). The following primers were designed to obtain the full-length cDNA sequence. The sense primer (pQE30-F, 5′-GGATCCATGGCCAACCAGCCCATCATC-3′) starts at the initial codon ATG of the open-reading frame (ORF) and contains a *BamH*I restriction site (underlined) at the 5′-end. The anti-sense primer (pQE30-R, 5′-AAGCTTCTATAGTTTTGTGTGGCGCCCAGG-3′) contains a *Hind*III restriction site (underlined). One step RT-PCR for the amplification was performed in 25 μl mixture containing 1 μg of total RNA, 1 μl (10 μM) of each primer, 2.5 μl RT-PCR buffer, 5 μl $MgCl_2$ (25 μM), 2.5 μl dNTP (10 mM), 0.5 μl RNase inhibitor (40 U/μl), 0.5 μl AMV-Optimized Taq (5 U/μl), and 0.5 μl AMV-RNase XL (5 U/μl). The reaction was carried out in a MycyclerTM thermal cycler (Bio-Rad, USA) using a procedure of incubating at 50°C for 40 min and denaturing at 94°C for 2 min, followed by a standard touchdown PCR regime of 94°C for 30 sec, 60°C for 30 sec, 48°C for 30 sec, and 72°C for 40 sec (15 cycles); and 94°C for 30 sec, 48°C for 30 sec, and 72°C for 40 sec (35 cycles). A 5 μl sample of RT-PCR product was analyzed by agarose gel electrophoresis.

Cloning in pGEM-T Easy vector and subcloning in pQE30 vector. The RT-PCR product containing the ORF was directly used to clone into pGEM-T Easy vector in accordance with the manufacturer's instructions. The ligation product was transformed into *E. coli* XL1-Blue MRF′ cells, screened by blue white colony selection, and confirmed by DNA sequencing (Invitrogen). The ORF cloned in pGEM-T Easy vector was digested with the restriction endonucleases *BamH*I and *Hind*III. The 589 bp insert (ORF) was cut and purified by using a gel extraction and purification kit (MBI; Fermentas, Lithuania). The ORF was finally cloned into pQE30 vector, providing the pQE30-xPNAS-4 recombinant plasmid.

Recombinant protein expression and identification of protein solubility. The BL21(DE3) cells were transformed with the recombinant construct pQE30-xPNAS-4 and selected on LB plate containing 100 μg/ml of ampicillin. The transformants were inoculated in 5 ml of Luria's broth (LB medium) containing corresponding antibiotic and grown at 37°C in a shaker at 200 rpm. Cultures in logarithmic phase (at OD_{600} of 0.5~0.6) were induced for 5 h with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C. After induction, proteins were analyzed on 12% SDS-PAGE. To determine the solubility of the recombination protein, the induced cells were suspended in phosphate buffered saline (PBS) and lysed via sonication. The resulting cell lysate was centrifuged at 14,000 rpm at 4°C for 10 min. The clear supernatant (soluble fraction) was collected and the pellet (insoluble fraction) was dissolved in the same volume of PBS buffer containing 8 M urea. Soluble and insoluble fractions were then analyzed in parallel on 12% SDS-PAGE.

Purification and refolding of xPNAS-4 protein. One liter LB medium containing 100 μg/ml of ampicillin was inoculated with 10 ml overnight precultures of *E. coli* BL21 freshly transformed with pQE30-xPNAS-4 plasmid, and the cells were grown at 37°C up to an absorbance at OD_{600} of about 0.6. Recombinant protein synthesis was induced by adding 0.5 mM IPTG and incubating for an additional 5 h at 37°C. Cells were harvested and resuspended in 30 ml suspension buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). After the cells were lysed via sonication, the lysate was centrifuged at 15,000 rpm for

30 min at 4°C. The supernatant was discarded and the pellet (inclusion bodies) was washed twice with 100 ml washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.1% Triton-100, pH 8.0). Then, the pellet was dissolved in 30 ml binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.0). The dissolved inclusion bodies were loaded onto a column containing 2 ml of pre-equilibrated nickel-nitrilotriacetic acid (Ni-NTA) agarose resin. The conjugated proteins were eluted from the resin using a gradient imidazole concentration (25, 50, 100, or 200 mM) in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, gradient concentration imidazole, pH 8.0). The purity of the protein was assessed by densitometry of polyacrylamide gel stained with Coomassie brilliant blue, and the protein concentration was estimated with an ultraviolet spectrophotometer (Bio-Rad).

The protein obtained in a denatured state was refolded by dialysis against binding buffer containing progressively lower concentrations of urea (6, 4, 2, 1, 0.5, and 0 M) and 1 mM EDTA at 8 h interval at 4°C. The refolded protein was finally concentrated by ultrafiltration using Millipore CX-10.

Protein identification. The purified protein was subjected to digestion using Trypsin Gold (Promega) according to the manufacturer's instruction. Peptide samples were analyzed using a LC-ESI-Q-TOF mass spectrometer (Micromass, UK). Protein database search was performed with MASCOT (http://www.matrixscience.com).

Production of antibodies against xPNAS-4 protein. The refolded recombinant protein was used for raising antibodies in two New Zealand white rabbits according to the following procedure. Briefly, each rabbit was first immunized using 100 μg of recombinant protein in Freund's complete adjuvant. Two weeks later, the rabbits were boosted three times with 100 μg recombinant protein each time in incomplete Freund's adjuvant at weekly interval. Before each immunization, 1 ml of sera were taken from the marginal vein of the rabbit's ears in order to detect antibody titer. After four immunizations, blood was taken from the rabbit's hearts, and sera were isolated through conventional method.

Enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis. The purified recombinant antigens were diluted with coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) and incubated overnight at 4°C in 96-well plates (1 μg/well). The plates were blocked with PBST (PBS containing 0.1% Tween-20) plus 5% non-fat dried milk for 1 h, and then washed three times with PBST for 30 min. A series of diluted sera (1 : 100, 1 : 200, 1 : 400, ..., 1 : 204,800) with PBST were added to the wells and incubated for 2 h at room temperature. Following three further washes with PBST, the wells were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Merck, Germany) at 1 : 5000 dilution for another 2 h at room temperature. The optical density of each well was measured at 450 nm in an ELISA plate-reader after incubation for 15 min with 100 μl/well of detection buffer (50 mM Na_2HPO_4, 25 mM citric acid, 0.5 mg/ml ABTS (2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid), 0.04% H_2O_2, pH 4.2).

Immunoblot analysis was used to analyze multimers of xPNAS-4 protein. Both reduced protein (20 μl) with 100 μM dithiothreitol (DTT) and non-reduced protein (20 μl) from 50 mM imidazole elution were separated on 12% SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membranes using a wet blotting apparatus (Bio-Rad). Mouse anti-His-Tag antibody conjugated with HRP was used to detect the proteins. Western blot analysis was also used to examine the efficacy of antibodies. The prokaryotic proteins from lysate of the induced cells and the total proteins extracted from *X. laevis* embryos were separated on 12% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with immune or pre-immune sera (1 : 100 dilution) as primary antibody. Goat anti-rabbit IgG conjugated with alkaline phosphatase (Merck) were used as the secondary antibody (1 : 30,000 dilution). The membranes were developed by NBT/BCIP (Stratagene, USA).

RESULTS

Gene cloning and homology between xPNAS-4 and hPNAS-4. To obtain the homologous gene of human *PNAS-4* (*hPNAS-4*), homology search was performed using the blast program (http://www.ncbi.nlm.nih.gov/blast) with the amino acid sequence of hPNAS-4 (GenBank accession No. CAH70880) against the *Xenopus* GeneBank. A *Xenopus* cDNA coding for a protein highly homologous to hPNAS-4 was obtained (GenBank accession No. BC087412). The *Xenopus* homolog (named xPNAS-4) possesses 87% identity with the hPNAS-4 (Fig. 1). The high amino acid identity suggests that xPNAS-4 may likely exert similar effects as hPNAS-4 protein. This assumption was proved by our later experiments. By flow cytometry and DNA ladder assay, we found that overexpression of xPNAS-4 could result in significant apoptosis in human epithelial lung cells (A549). In addition, overexpression of xPNAS-4 could induce small eyes or loss of eyes in *Xenopus* and zebrafish embryos via mRNA microinjection (unpublished data).

The cDNA fragment coding the xPNAS-4 was amplified by RT-PCR based on the total RNA from adult testis using the primers as mentioned in "Materials and Methods". The amplified product, which is in accordance with the theoretical length of *xPNAS-4* gene (591 bp) (Fig. 2a), was first cloned into the pGEM-T Easy vector and confirmed by DNA sequencing (data not shown). The resulting plasmid was digested with the restriction endonucleases *BamH*I and *Hind*III. The insert fragment was then purified and ligated to the pQE30 vector digested with the same enzymes. The ligation production was transformed

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hPNAS-4    MGANQLVVLNVYDMYWMNEYTSSIGIGVFHSGIEVYGREFAYGGHPYPFSGIFEISPGNA 60
xPNAS-4    -MANQPIILNVYDMYWINEYTSSLGIGVFHSGIQVYGREFAYGGHPYPFSGVFEISPGDS 59
             *** ::*********:******:**********:******************:******::

hPNAS-4    SELGETFKFKEAVVLGSTDFLEDDIEKIVEELGKEYKGNAYHLMHKNCNHFSSALSEILC 120
xPNAS-4    TELGDTFKFKEAIALGSTDFTENDIEKIIEELGKEYKGNAYHLMHKNCNHFSSALSEILC 119
           :***:*******:.****** *:*****:********************************

hPNAS-4    GKEIPRWINRLAYFSSCIPFLQSCLPKEWLTPAALQSSVSQELQDELEEAEDAAASASVA 180
xPNAS-4    GKEIPRWVNRLAYFSTCVPFLQSCLPKEWLTPAALQSSISQELQDELEEAEDAAASASTS 179
           *******:*******:*:*********************:*******************.:

hPNAS-4    STAAGSRPGRHTKL 194
xPNAS-4    TTAMP-RPGRHTKL 192
           :**    ********
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Fig. 1. Multiple sequence alignment of hPNAS-4 and xPNAS-4. Identical residues in the two sequences are indicated by a star, conserved residues by two dots, and similar residues by a single dot. The alignment was done using CLUSTAL W software, version 1.83.

into *E. coli* XL1-Blue competent cells. Eventually, the constructed plasmid (pQE30-xPNAS-4) was verified by digestion with the restriction endonucleases *BamH*I and *Hind*III (Fig. 2b) and DNA sequencing (data not shown).

Expression of xPNAS-4 and identification of solubility. The BL21(DE3) competent cells were transformed with the recombinant expression plasmid pQE30-xPNAS-4. Small-scale cultures of the positive clones were incubated with 0.5 mM IPTG to express the recombinant protein. Analysis of SDS-PAGE showed that expressed recombinant protein (23 kD) could be detected in induced cell lysate but not in cell lysate before induction (Fig. 3a). The results confirmed that the ORF was expressed selectively in the transformed *E. coli* cells and a large fraction (over 10%) of the total protein was apparently constituted.

To examine the relative distribution of the expressed recombinant protein in the soluble or insoluble fractions, both the supernatant and the pellet of the lysate from induced cells were examined by SDS-PAGE. Results showed that expressed recombinant protein was mainly found in the insoluble fraction as inclusion bodies (Fig. 3b). Changes in expression conditions, such as lowering the temperature to 16°C, reducing induction time, or changing the IPTG concentrations from 0.05 to 0.5 mM, did not improve the amount of soluble xPNAS-4 protein (data not shown).

Purification and refolding of the xPNAS-4 protein. Taking the insolubility of this protein into account, the inclusion bodies were washed with washing buffer containing 0.1% Triton-100 (w/v). This procedure removed

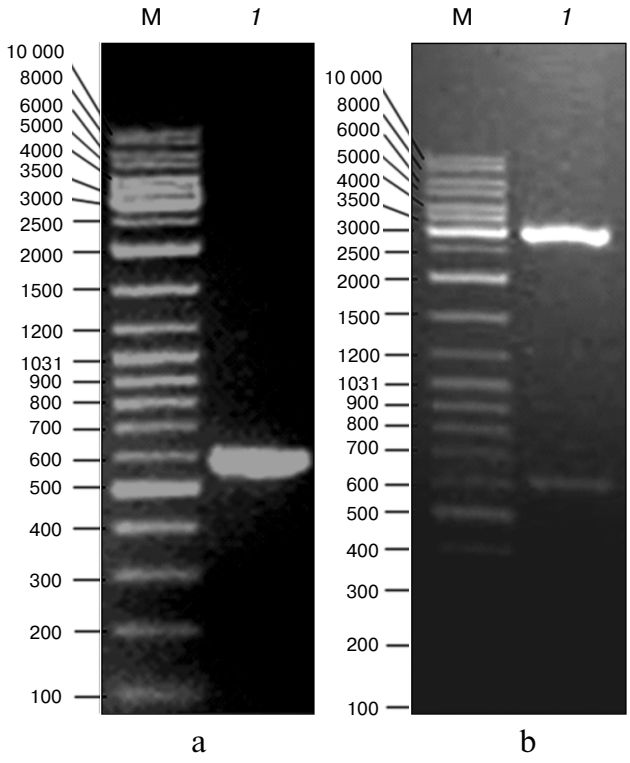


Fig. 2. Amplification of the *xPNAS-4* gene and identification of recombinant plasmid. a) 1% agarose gel electrophoresis of RT-PCR amplification product of the *xPNAS-4* gene. Lanes: M) DNA markers; *1*) the amplified product (591 bp). b) 1% agarose gel electrophoresis of digested product of pQE30-xPNAS-4 using *BamH*I and *Hind*III restriction endonucleases. Lanes: M) DNA markers; *1*) digestion product of recombinant plasmid.

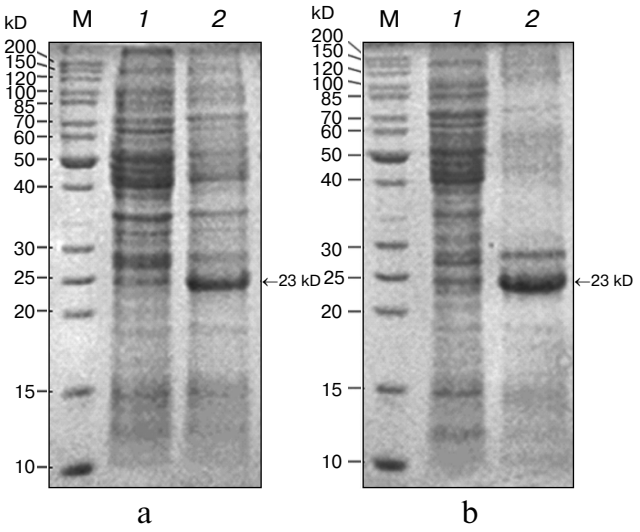


Fig. 3. Expression and identification of solubility of the recombinant protein. a) Proteins were separated on 12% SDS-PAGE gel and stained with Coomassie brilliant blue. Lanes: M) protein molecular weight markers; *1*) *E. coli* BL21(DE3)/pQE30-xPNAS-4 whole cell proteins before induction; *2*) *E. coli* BL21(DE3)/pQE30-xPNAS-4 whole cell proteins after induction. b) Proteins were separated and stained with Coomassie brilliant blue. Lanes: M) protein molecular weight markers; *1*) soluble fraction from induced cell lysates; *2*) insoluble fraction from induced cell lysates.

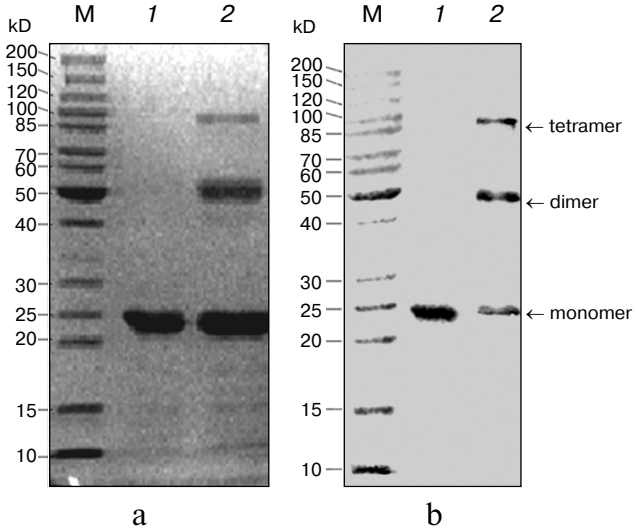


Fig. 5. DTT could diminish the multimers of xPNAS-4. a) SDS-PAGE analysis of xPNAS-4 eluted from 50 mM imidazole fraction with or without DTT. Lanes: M) protein molecular mass weight markers; *1*) reduced sample of xPNAS-4 with 100 mM DTT; *2*) non-reduced sample of xPNAS-4 without DTT. b) Western blot analysis using anti-His monoclonal antibody. Lanes: M) protein molecular mass markers from the same acrylamide gel as using for Western blot; *1*) reduced sample of xPNAS-4 with 100 mM DTT; *2*) non-reduced sample of xPNAS-4 without DTT.

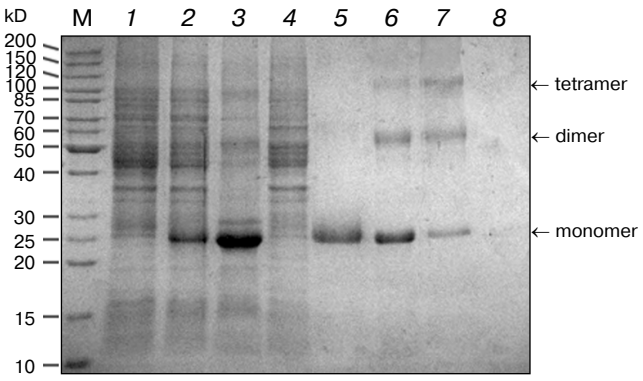


Fig. 4. Purification of xPNAS-4. Proteins were separated using 12% SDS-PAGE and stained with Coomassie brilliant blue. Lanes: M) protein molecular mass markers; *1*) cell lysates before induction; *2*) cell lysates after induction; *3*) washed inclusion bodies; *4*) flow-through fraction; *5-8*) 25, 50, 100, and 200 mM imidazole elution fractions, respectively.

most contaminating proteins and resulted in a higher purity of the protein (>70%). After inclusion bodies were dissolved with binding buffer, we performed a chromatographic step using Ni^{2+}-NTA chelating Sepharose column based on 6×His-tag. The conjugated protein was gradually eluted with elution buffer containing 25, 50, 100, or 200 mM imidazole, respectively. Interestingly, the SDS-PAGE analysis of the gradual imidazole eluted proteins showed a migration of multimers with molecular weight of 23, 46, and 92 kD corresponding to monomers, dimers, and tetramers of this protein (Fig. 4). To prove that multimerization was a result of intermolecular disulfide bond, 100 mM DTT was added to the 50 mM imidazole eluted fraction to disrupt the formation of intermolecular disulfide bonds. The result showed only one band corresponded to the monomers appeared in the reduced sample (Fig. 5a). The Western blot analysis using anti-His antibody further supported this postulation (Fig. 5b).

The xPNAS-4 protein obtained in a denatured state was refolded by dialysis against binding buffer containing 1 mM EDTA and progressively reduced concentrations of urea (6, 4, 2, 1, 0.5, and 0 M). This procedure resulted in about 25% refolding efficiency with a final yield of near 4 mg of xPNAS-4 protein (Table 1).

Protein identification. The refolding protein of xPNAS-4 was further verified by LC-ESI-Q-TOF mass spectrometer and four peptides were successfully identified, covering 30% of xPNAS-4 protein sequence (Fig. 6). The result further confirmed that the purified protein was, in fact, xPNAS-4.

Production of polyclonal antibodies against the recombinant protein and Western blot analysis. The refolded recombinant protein was used for raising antibodies in two New Zealand white rabbits. The titers of antibodies in

Table 1. Purification of recombinant xPNAS-4

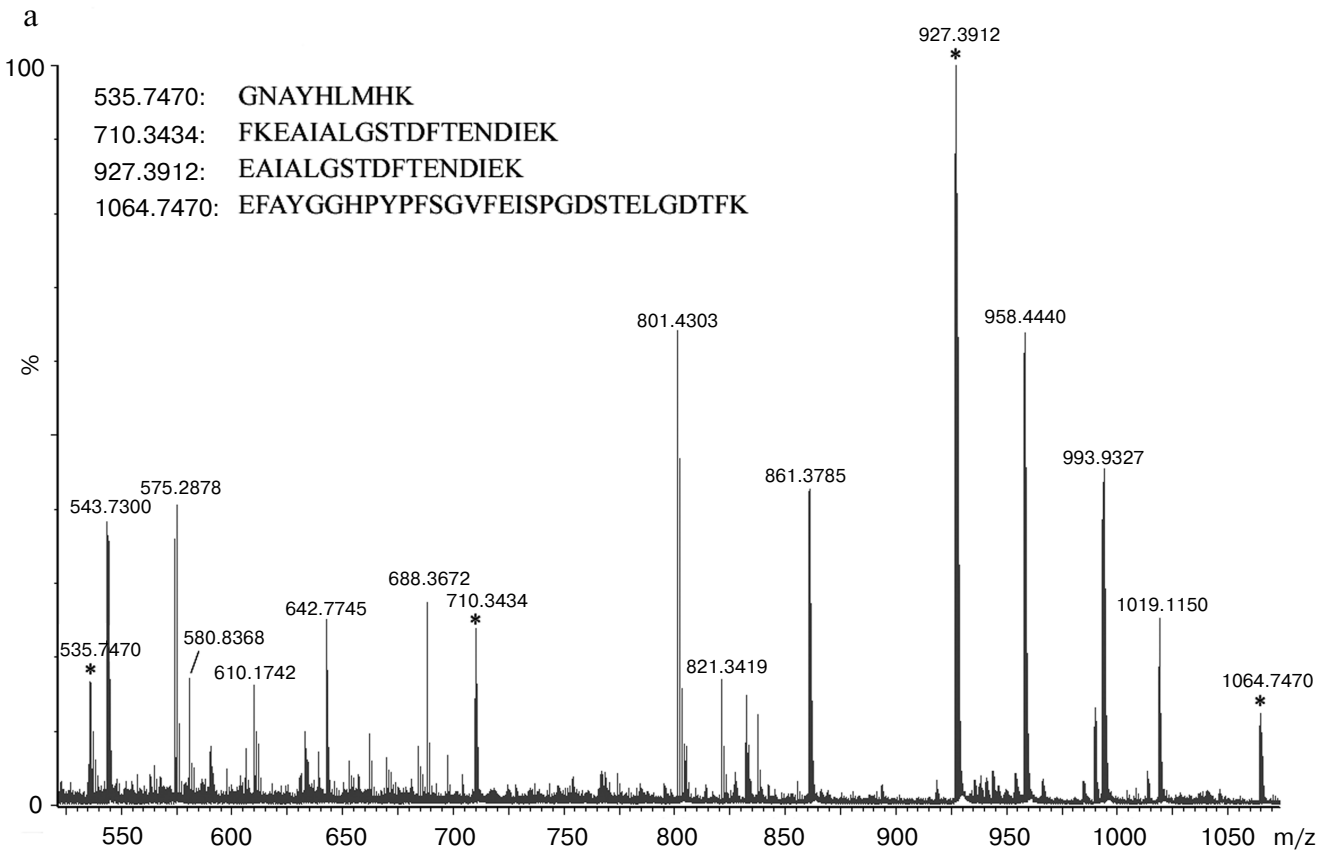
Step	Volume, ml	Total protein, mg	Purity, %
Crude extract	30	499.9	10
Inclusion bodies washing	30	47	70
Ni^{2+} chelating affinity chromatography	56	15.6	95
Refolding and concentration	3	3.9	95

Note: One liter of culture cells (about 3 g wet weight cells) was used for purification. Concentration was detected by optical density meter Smartspec™ 3000 (Bio-Rad), and purity was evaluated by densitometry of polyacrylamide gels stained with Coomassie brilliant blue.

the two rabbits were increased along with the increase in immunization times and finally achieved the highest titer levels of 1 : 51,200 and 1 : 102,400, respectively (Table 2). Repeated immunization (2 or 3 times) resulted in significantly increased antibody production. The Western blot analysis showed that the polyclonal antibodies could react with both the expressed prokaryotic xPNAS-4 protein from *E. coli* and endogenous xPNAS-4 protein from *X. laevis* embryos (Fig. 7).

DISCUSSION

PNAS-4 is a novel recently identified gene related to apoptosis. James and coauthors [13] found that the transcript level of *PNAS-4* was greatly up-regulated in glucocorticoid-treated human lens epithelial cells (HLE B-3), indicating that PNAS-4 is possibly involved in perturba-



b

MANQPIILNVYDMYWINEYTSSLGIGVFHSGIQVYGR**EFAYGGHPYPFSGVF EISPGDSTELGDTFKFKEAIALGSTDFTENDIEK**IIEELGKEYK**GNAYHLMH K**NCNHFSSALSEILCGKEIPRWVNRLAYFSTCVPFLQSCLPKEWLTPAALQSSI SQELQDELEEAEDAAASASTST TAMPRPGRHTKL

Fig. 6. Mass spectrometry analysis of the purified protein. a) ESI mass spectrum of xPNAS-4. Masses are indicated with one asterisk above the peaks and the resulting peptides were shown. b) Matched peptides fragments of xPNAS-4. The matched portions are shown (underlined and bold).

Table 2. Titer of antibodies against xPNAS-4

Rabbit	Titer of antibodies (immunization times)				
First	0 (0)	1 : 100 (1)	1 : 400 (2)	1 : 12 800 (3)	1 : 51 200 (4)
Second	0 (0)	1 : 200 (1)	1 : 12 800 (2)	1 : 51 200 (3)	1 : 102 400 (4)

Note: Recombinant xPNAS-4 protein (1 μg/ml) was used for coating per well (96-well plate). A series of diluted (1 : 100, 1 : 200, 1 : 400, ..., 1 : 204,800) sera was used for primary antibody to detect the titer of antibodies. An optical density (at 450 nm) of each well was measured. $p < 0.01$ compared with pre-immune control sera.

tion of lens epithelial cell proliferation and differentiation. Furthermore, Filippov and coauthors [10] reported *hPNAS-4* was a novel pro-apoptotic gene able to promote apoptosis in osteosarcoma U2OS cells. By microarray-based gene expression profiling, they found that *PNAS-4* was significantly up-regulated in osteosarcoma U2OSE6 cells treated with the genotoxin mitomycin C. To determine the effect of PNAS-4 on cell viability, they overexpressed *PNAS-4* in U2OS cells. Analysis for cell viability confirmed a significant increase in cell death via apoptosis. Therefore, as a novel pro-apoptotic protein, PNAS-4 is likely an important discovery for new targets for the development of novel and effective therapeutic approaches to cancer. Although there is still a long way to go to eventually apply this gene to true eradication of cancer, a full understanding of its functions in embryonic cell biology is undoubtedly helpful. The xPNAS-4 protein possesses a high identity with the hPNAS-4 protein, which indicated that xPNAS-4 likely plays an important role in embryonic development as an essential component of apoptosis. Indeed, apoptosis in animal development has been extensively studied. It is a widely accepted notion that apoptosis is involved in many embryological processes and plays a significant role in animal development. In our study, we found that overexpression of xPNAS-4 was able to promote apoptotic death in human epithelial lung cells (A549) and resulted in eye developmental defect in *Xenopus* and *Br. rerio* (zebrafish) embryos (unpublished data). We believe the successfully purified xPNAS-4 protein and the polyclonal antibodies produced could facilitate further investigations in cells and embryonic development. The expression and purification procedures used in this study provide a simple and efficient method to obtain pure prokaryotic xPNAS-4 protein in large quantities. The identified xPNAS-4 protein by LC-ESI-Q-TOF-MS can exist as monomers, dimers, and tetramers. The antibodies against this protein were produced with a high titer, which would serve as an experimental tool to identify its subcellular localization and to test its tissue and development expression profile. In conclusion, our presentation opens a door for the understanding of xPNAS-4 function in *X. laevis* development.

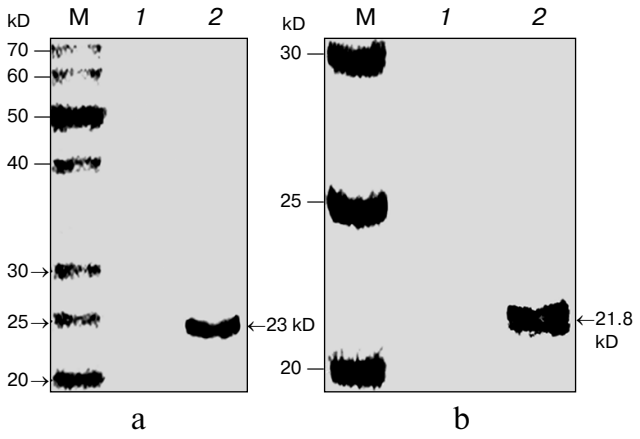


Fig. 7. Western blot analysis with antibodies against xPNAS-4. a) The produced antibodies could recognize prokaryotic expressed xPNAS-4 protein in the lysates from IPTG-induced *E. coli*. The stripped membranes bound with prokaryotic proteins were detected with pre-immune or immune serum from rabbit 2, respectively. Lanes: M) protein molecular mass weight markers; *1*) pre-immune serum (1 : 2000 dilution) from rabbit 2 was used in the Western blot analysis; *2*) immune serum (1 : 2000 dilution) from rabbit 2 was used in the Western blot analysis. b) The produced antibodies could recognize endogenous xPNAS-4 protein in the extracts from *X. laevis* embryos. The stripped membranes bound with eukaryotic proteins from *X. laevis* embryo extracts were detected with pre-immune or immune serum from rabbit 2, respectively. Lanes: M) protein molecular mass weight markers; *1*) pre-immune serum (1 : 100 dilution) from rabbit 2 was used in the Western blot analysis; *2*) immune serum (1 : 100 dilution) from rabbit 2 was used in the Western blot analysis.

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