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Preparation and Application of Polyclonal Antibody against PKZ from *Ctenopharyngodon idellus* (CiPKZ)

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Abstract: The grass carp (*Ctenopharyngodon idellus*) PKZ full-length cDNA (GU299765) had been cloned and identified recently. Within its N-terminal part of the protein there are two Z-DNA binding domains called Zα1 (1~67aa) and Zα2 (81~152aa). Zα domain is unique to PKZ and distinguishes them from other translation Initiation Factor 2 alpha (eIF2α)-kinase. To obtain polyclonal antibody against CiPKZ Zα, the *PKZ Zα* gene was amplified by PCR from the template obtained in the earlier research and identified by DNA sequence analysis. Then, it was digested by BamHI, XhoI and ligated with pET-32a vector which was by the same treatment. Sequenced and blasted with the NCBI GenBank, the recombinant plasmid pET-32a-PKZ Zα was obtained. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and induced by 1 mmol L^{-1} IPTG. Researchers obtained CiPKZ Zα polypeptide via *E. coli* prokaryotic expression and purified with Ni-NTA His-Bind Resin affinity chromatography. Rabbit Polyclonal Antibody (Pab) against CiPKZ was raised using the purified N-terminal fragment of CiPKZ containing its Zα1 and Zα2 domains. Western blot analysis showed that the antibody had high affinity and specificity and higher titer. Immunohistochemistry assay identified that expression of PKZ could be detected in liver tissue.

Key words: *PKZ* gene, Zα, polyclonal antibody, grass carp, immunohistochemistry, China

INTRODUCTION

PKZ (Protein Kinase containing Z-DNA binding domain) (Hu *et al*., 2004; Rothenburg *et al*., 2005) is a kind of unique protein kinase newly found in fish which belongs to not only the family of Z-DNA binding domain proteins but also the translation Initiation Factor 2 alpha (eIF2α)-kinase (Bergan *et al*., 2008). The N-terminal part of the protein is regulatory domain, consists of Zα1 and Zα2 two parts of Z-DNA binding domains instead of dsRNA motifs (Rothenburg *et al*., 2005). The C-terminal part of the protein is functionally distinct translation Initiation Factor 2 alpha (eIF2α)-kinase domain (Hu *et al*., 2004). To date, cDNAs encoding PKZ (PKR-like) eIF2a-kinases have been isolated from goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), rare minnow (*Gobiocypris rarus*), atlantic salmon (*Salmo salar*) and grass carp (*Ctenopharyngodon idellus*), successively (Hu *et al*., 2004; Rothenburg *et al*., 2005; Bergan *et al*., 2008; Su *et al*., 2008; Yang *et al*., 2011). The study on the function of PKZ has just been under investigation recently but some achievements have been obtained. Earlier studies indicated that PKZ in fish transcript levels are known to be upregulated by IFN, the synthetic dsRNA polyI:C (Bergan *et al*., 2008; Hovanessian, 1989), viral infection and heat shock (Hu *et al*., 2004; Yang *et al*., 2011), etc. So, PKZ in fish shares the similar feature with that of mammalian PKR, the results support a role for PKZ, like PKR, in host defense against virus infection but also act as an adaptor in cells to response to many stimulis.

An established method (Rothenburg *et al*., 2005; Cai and Williams, 1998; Kaufman *et al*., 1989) was used to assess the function of zebrafish PKZ to detect the ability of PKZ which inhibit the expression of a cotransfected reporter gene in cultured cells. The result demonstrated that wild-type drPKZ could strongly down-regulated luciferase activity in both HEK293T and CHO cell lines which were cotransfected with the luciferase reporter plasmid pGL3 promoter and with several drPKZ expression vectors containing a myc-His tag (Rothenburg *et al*., 2005; Hovanessian, 1989).

Inhibition of protein synthesis in fish cells by over-expression AsPKZ (Bergan *et al*., 2008) was also demonstrated. Vector constructs of wild-type AsPKZ or the variant K217R, T405A and T410A of AsPKZ fused to the Enhanced Ereen Fluuorescence Protein (EGFP) for transient transfection into CHSE-214 cells to monitor the

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ability of AsPKZ which inhibit the expression of a cotransfected reporter gene. The investigation showed that wild-type AsPKZ could strongly inhibited β-gal expression. The K217R variant displayed almost normal expression whereas the T405A and T410A variants suppressed the β-gal expression only next to that of the wt AsPKZ. Furthermore, the same inhibitory effects were also apparent by detecting the intensity of EGFP fluorescence in transfected cells (Bergan *et al*., 2008).

The earlier study (Yang *et al*., 2011) has shown that PKZ could respond to the stress in cell, it inhibited protein synthesis and made cell apoptosis finally by phosphorylated eIF2α. PKZ in fish shares the typical feature of the translation Initiation Factor 2 alpha (eIF2α)-kinase (Bergan *et al*., 2008). However, the exact role of CiPKZ in the Stress/Z-DNA/Z-RNA/PKZ/eIF2α/ apoptosis pathway still remains unclear. Identification of the precise location of PKZ within the cell may provide some clues to its role as a regulator of the antiviral state as well as of cell apoptosis and other processes (Jeffery *et al*., 1995). In the present study, researchers introduce primarly the methods and the results which researchers have obtained.

MATERIALS AND METHODS

E. coli DH5α was kindly provided by Guijianfang's lab of Institute of Hydrobiology, Chinese Academy of Sciences; BL21 strains were purchased from Promage; pET-32a (+) was purchased from Novagen; Taq DNA polymerase, BamHI, XhoI Restriction enzyme and T4 DNA ligase were all purchased from TAKaRa; Freund's complete adjuvant and Freund's uncomplete adjuvant were purchased from Sigma; His-Bind Purifiction kit purchase from Novagen; Male New Zealand albino rabbits were purchased from Animal Department of Medical College of Nanchang University. Goat against rabbit were purchased from Sigma. Anti-rabbit goat MAb conjugated with peroxidase.

Construction of recombinant plasmids: Sequence anlaysis indicated that the gene sequence of CiPKZ shares very highly homology with that of CaPKZ. Based on the full length cDNA of CaPKZ which had identified, researchers designed the primer, upperstream primer F1(5'-ATCGGATCCATGGAGAGGAAGATCATTGAT-3', downstream primer R1(5'-GCTCGAGTCACTCGC CTTCCAGAAGC-3'). Ampify PKZ Zα coding gene domain, N-terminal part of the protein regulated domain which consist of amino acid positions 1-67 and (81-152), PCR products incised by BamHI, XhoI enzymes were inserted into the vector which also were digested by BamHI, XhoI enzymes. Recombinant plasmid pET-32a-PKZ Zα was generated. Above-mentioned PCR amplification condition is: PCR was performed with an initial denaturation step of 5 min at 94°C and then 30 cycles were run as follows: 30 sec of denaturation at 94°C, 30 sec of annealing at 58°C and 30 sec of extension at 72°C. Taq DNA polymerase used for amplification by PCR. Recombinant plasmid sequence assayed by universal primer in vector.

Prokaryotic expression and purifing of His-tag fusion protein of CiPKZ: The recombinant (pET-32a-PKZ Zα) expression vector was transformed into *E. coli* BL21 (DE3). Bacteria were grown at 37°C in Luriae Bertani medium to A600 of 0.6-0.8 and then induced with 1 mM Isopropyl Thio-b-D-Galactoside (IPTG) for 4 h. The cells were harvested and suspended in binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9) and broken by sonication and centrifuged for 30 min (4°C, 14,000 g). The supernatant was collected and purified with Ni-NTA His-Bind Resin affinity chromatography. Pooled fractions containing PKZ Zα were dialyzed overnight against dialysis buffer (20% glycerol, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 20 mM HEPES, pH 7.5) and then analyzed by 12% SDS-PAGE. Samples were stored at 80°C.

Preparation of polyclonal antibody of CiPKZ: For injection of two rabbits, rabbit Polyclonal Antibody (PAb) against CiPKZ was raised using the purified N-terminal fragment of CiPKZ containing its Zα1 and Zα2 domains (Pham *et al*., 2006). For the native form of the PKZ-fusion protein sample (normally 500 μg in 1 mL) is mixed with equal volume adjuvant injecting. The 1 mL of antigen solution into the Freund's adjuvant and vortexing vigorously for 2 min to get a good suspension. Inject 500 μL of inoculum. The process for the other rabbits was repeatted. Injections were done every 2 weeks with bleeds 7~10 days after each injection. Collect the blood by allowing it to drop into a sterilized tube. Take the collected blood and place at 37°C for 30 min, place the tube at 4°C overnight to contract. Loosen the clot from the tube wall with a spatula and decant the blood into a plastic centrifuge tube. Centrifuge at 4°C 4000 g for 10 min, decant or pipet off the supernatant. The antiserum was stored at -80°C.

Detection of titer of PKZ Zα polyclonal antibody (Zhu *et al*., 2006): His tag-PKZ Zα fusing protein (100 ng/hole) was used to coat Enzyme-linked plate, different dilution diploid preimmune rabbit serum and immunization rabbit serum were used as the primary

antibody, respectively and indirect method (ELISA) to detect the titer of polyclonal antibody against PKZ was also adopted.

Western blot analysis (Zhu *et al*., 2006): Western blot analysis was performed to confirm the specificity of polyclonal antibody against CiPKZ. In brief, Pzα was separated on 12% SDS-PAGE gels. Protein was electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween20). The blocked membrane was incubated with the rabbit antiserum at a dilution of 1:400 in TBST buffer containing 1% milk at room temperature for 1 h. The membrane was washed three times with TBST buffer, each for 10 min and then incubated with 1:1000 dilution of alkaline peroxidase -conjugated goat anti-rabbit IgG. After another three washes with TBST buffer, each for 10 min, detection was performed using BCIP/NBT staining.

Immunohistochemical staining of paraffin embedded tissues: Grass carp came from Jiangxi Provincial Fisheries Research Institute were injected 10 μg g^{-1} bodyweight Poly I:C (Pharmacia Biotech) 7 days later. The control group fish were injected with PBS. Liver, spleen, from every group were harvested. Tissues of liver and spleen were embedded by paraffin. Section paraffin blocks at the desired thickness (5 μm) on a microtome. Before deparaffinization, the slides was in a 60 oven for 10 min to melt the paraffin. Deparaffinize slides in 2 changes of xylene or xylene substitute for 5 min each. Transfer slides to 100, 95, 90, 85 and 80% alcohol for 5 min each. Rinse in PBS for 5 min each time. Antigen retrieval to unmask the antigenic epitope was performed. Block endogenous peroxidase activity by incubating sections in 3% H_2O_2 solution in methanol for 10 min. The slides was blocked with 5% non-fat dry milk at room temperature for 1 h then incubated with 1:400 dilution primay antibody at 4°C overnight. Then incubated with 1:400 dilution primay aitibody at room temperature overnight. DAB staining was performed for 5 min. Hematoxylin stained again for 45 sec.

RESULTS AND DISCUSSION

PCR amplification of *PKZ Zα* gene and identification of recombinant plasimd of pET-32a-PKZ Zα: PCR amplification product we obtained is 500 bp, the result of electrophoresis of product of CiPKZ Zα is shown in Fig. 1. The PCR product was recycled. The recycled PCR product was digested with BamHI and XhoI and ligated into the pET32a (+) expression vector to obtain pET-PKZ Zα. After transformation of *Escherichia coli*, identification of bacterial colony PCR was performed. Clones containing the expected fragments in the correct orientation were identified putative positive clones were verified by sequencing.

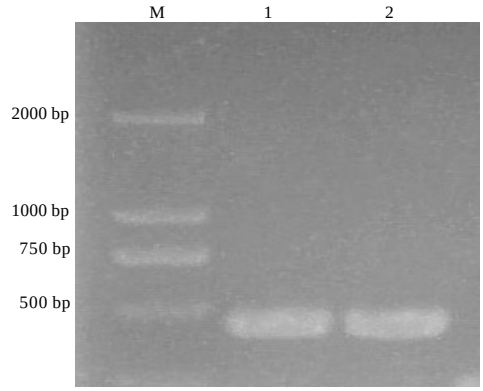

Fig. 1: PCR amplification of *PKZ Zα* gene. M: DNA marker (DL 2 000); Lane 1, 2: PCR amplification product of CiPKZ Zα

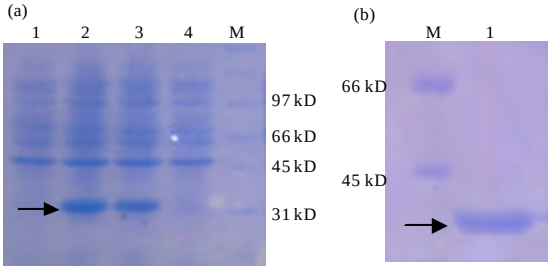

Fig. 2: SDS-PAGE of expression His-PKZ Zα fusion protein with different treatment and purificated PKZ zα protein. a) M: Molecular mass marker; Lane: 1 and 4 total protein of BL21 (DE3) without IPTG-induction; Lane 2 and 3: total protein of BL21 (DE3) with IPTG-induction. b) M: Molecular mass marker, Lane 1: purificated PKZ Zα protein

Expression and purification of His-tag-PKZ Zα fusion protein in *E. coli* BL21 (DE3): The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and induced by 1 mmol L^{-1} IPTG. SDS-PAGE showed that the fusion protein was expressed after affinity purification with Ni-NTA His-Bind Resin affinity chromatography. The result showed (Fig. 2) that a putative protein with molecular mass of 32 kD was expressed by BL21 (pET32a-PKZ Zα).

Identification of PKZ Zα antibody: In order to investigate the specificity of PKZ Zα antibody, recombinant protein acted as antigen was employed. Using ELISA to assay the titer of CiPKZ Zα antibody, the result indicated that the titer is 1:64000. A single protein band with a putative molecular mass of about 32 kD was identified in the SDS-PAGE and Western blot. The CiPKZ antiserum was obtained and characterized by western blotting. It could recognize fusion protein of His-tag-PKZ Zα specially.

Expression of PKZ in cells: In order to analyze tissue distribution and expression of PKZ in grass carp, immunohistochemistry assay was generated. As shown in this study, the PKZ is expressed in all tested tissues and expressed to varying extents relative to the induction hour. PKZ protein is ubiquitously distributed in all examined tissues. Therefore, PKZ might be a ubiquitous kinase. Immunohistochemistry assay detected the expressing of PKZ in liver tissue showed that PKZ was upregulated in liver tissue after induction by polyI:C for 7 days. N-terminal Z-DNA binding (Zα) domain is unique to PKZ and distinguishes them from other translation Initiation Factor 2 alpha (eIF2α)-kinase. (Rothenburg *et al*., 2008; Wu *et al*., 2010). In contrast to the Za of ADAR1 (Herbert *et al*., 1997; Brown *et al*., 2000), ZBP-1 (Schwartz *et al*., 2001), E3L (Kim *et al*., 2003), Za of PKZ harbor the same function that is able to combine and stabilize Z-DNA conformation specifically (Hu *et al*., 2004; Rothenburg *et al*., 2005; Bergan *et al*., 2008). Under normally physiological conditions, the left-handed Z-DNA conformation enhanced by negative supercoiling which is formed by moving RNA-polymerases during transcription (Wolfl *et al*., 1997; Deigendesch *et al*., 2006). Z-DNA can either enhance or repress promoter activity, probably by modulating the local architecture of transcriptional control regions and nucleosome positioning (Deigendesch *et al*., 2006; Nordheim and Rich, 1983; Rothenburg *et al*., 2001a, b; Liu *et al*., 2006). Investigation of the subcellular location of PKZ within the cell has been shown to be essential, it may provide some clues to its role as a regulator of the antiviral state.

Due to the fact that there hasn't any commercially available PKZ antibody against fish now a days, this restricts further investigation of the function of *CiPKZ* gene seriously. In order to obtain polyclonal antibody against CiPKZ which has reactionogenicity and specificity against CiPKZ, two part of CiPKZ encoding domains Zα1 and Zα2 were choosed to act as antigen domains in this study, pET-32a prokaryotic expression vector was adoptted which consist of six His polypeptide sequence tag which is convenient to detect and purification of fusing protein. In the study, researchers successfully constructed His-tag-PKZ Zα recombinant plasmids (Fig. 1). After transformation of *Escherichia coli*, clones containing the expected fragments in the correct orientation were identified by restriction mapping. Putative positive clones were verified by sequencing. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and induced by 1 mmol L^{-1} IPTG. A single protein band about 32 kDa was identified in the SDS-PAGE shown in Fig. 2. The CiPKZ antiserum was obtained and characterized by Western blotting (Fig. 3), ELISA assay showed that its titer is 1:64000. It could recognize fusion protein of His-tag-PKZ Zα specially as well as PKZ Zα in tissues. The distribution in liver cells of grass carp (*Ctenopharyngodon idellus*) was detected by using immunohistochemistry technique as well as the expression of PKZ. The result exhibited that the expression of PKZ was upregulated in liver tissue, spleen tissue, kidney tissue after induction by polyI:C for 7 days.

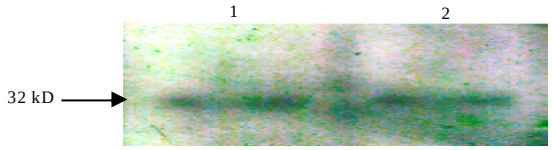

Fig. 3: Identification the specificity of PKZ antibody by Western bolt. Lanel and 2: PKZ Zα fusion protein

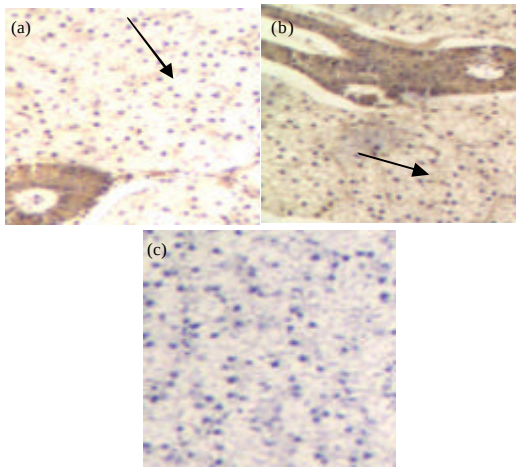

Fig. 4: Immunohistochemistry assay detect the expression of PKZ Zα in tissues. a) liver tissue treated by PBS (uninduction (liver) (100x)); b) liver tissue treated by polyI:C for 7 days (polyI:C induction (7 days) (100x)); c) liver tissue treated by pre-antiserum (control (liver) (100x))

Researchers demonstrated that CiPKZ is a ubiquitous tissue expression gene that had a very low level of constitutive expression but up-regulated in response to Poly I:C as confirmed by immunohistochemistry staining (Fig. 4). The earlier RT-PCR analysis indicated that PKZ RNA exsisted in tissue has constitutive expression which can be upregulated by induction obviously (Yang *et al*., 2011). The result is consistent with the characteristics of recombinant PKZ analyzed by immunohisto-chemistry.

CONCLUSION

With the development of the interaction between antigen and antibody, a series of techniques were applied such as the technique of co-immunoprecipitation, Western blot assay, the technique of immunohisto-chemistry which were employed extensively in the area of protein analyse and function study. In the study, researchers successfully prepared and obtained polyclonal antibody which has high specificity. It could recognize fusion protein of His-tag-PKZ Zα specially as well as PKZ Zα in tissues. The studies provide a favorable tool for further study on relation to expression of PKZ in tissues, sub-cellular localization in cells and any other protein-protein interaction domain in the future.

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