ELISA based quantification of Pax6 expression in the developing Zebrafish embryos

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KEY WORDS

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ABSTRACT

Background: Transcription factors are the key regulators of metabolic pathways in the cells, tissues and organ development during embryogenesis. Pax6 is a transcription factor involved in vertebrate eye, brain and central nervous system formation during development.
Purpose: A reliable and sensitive assay for the spatiotemporal expression, quantification and detection of Pax6 is not available so far in zebrafish as a developmental model, hence the objective of this work is to develop quantitative assay in zebrafish embryos.
Methods: The Pax6 transcription factor was purified by heparin agarose affinity chromatography and DEAE cellulose chromatography techniques from the developing zebrafish embryos. The purity was confirmed by SDS-PAGE and western blotting using Pax6 mouse monoclonal antibody. The standard graph was plotted for Pax6 and the expressions in seventeen developmental stages were quantified by indirect ELISA.
Results: The maximum expression of Pax6 was detected at 8 hpf (hours post fertilization) and it was quantified as 179 ng/embryo from the average total protein of 9.5 μg/embryo. The zebrafish Pax6 protein was detected as 48 kDa and confirmed by western blotting.
Conclusions: This study paves way to quantify the level of expression of proteins or transcription factors during early embryonic and larval development or embryogenesis using zebrafish as model system.

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Introduction

The zebrafish, *Danio rerio* has been established as an excellent developmental model for understanding the developmental genetics, organogenesis or any biological processes. Enzyme Linked Immunosorbant Assay (ELISA) is a powerful tool to study the expression level of transcription factors involved in the neuronal pathways using zebrafish as developmental model. Generally, the transcription factors are involved in developmental processes such as morphogenesis, cell fate determination, cellular differentiation, regulating the gene expression specific to tissue during development.[1] Pax6 is transcriptional protein with highly conserved and paired homeodomain in the DNA binding regions, mainly involved in brain development and its maintenance. They are expressed in the adult and embryonic neural stem cells mainly in the astrocytes, neurons in the regions of olfactory bulb, thalamus and cerebellum.[2,3] There are several examples of transcription factors can be found among the members of the *Pax* family of vertebrate genes contain paired box conserved sequence motif which encodes the DNA-binding domain.[4] Pax6 promotes the neurogenic cell fates during development by acting as a transcriptional activator, and is said to be a Pax6-transactivation domain.[5,6] Moreover, Pax6 expression in the embryonic day 8 (E8) mouse starts from the earliest stage of CNS development, and the neural plate consists entirely of proliferating neuroepithelial cells. Pax6 expression was reported in forebrain, hindbrain, and spinal cord during the regionalization of neural tube at E10 stage in mouse.[7] Also there are reports that reduced Pax6 level in heterozygotes for a *Pax6* null allele resulted in ocular abnormalities like Aniridia in humans[8] *Small eye* mutation effects in mice and rat.[9,10]

The expression level of Pax6 in each stage was still unknown and there is no reliable assay available for the quantification of Pax6 transcription factor during embryonic development stages. Hence the main objective of this work was to develop an assay for the detection and quantification of Pax6 in different developmental stages of Zebrafish embryos by indirect ELISA. In this study, we have reported first time on the quantification of Pax6 by ELISA method during the embryonic development in zebrafish.

Methods

Breeding and maintenance of Zebrafish embryos

Zebrafishes were bred and maintained in 30 L tanks at 28°C with 14:10 hour light:dark cycles. After breeding, the eggs were subsequently collected from the bottom of the tanks. Embryos were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$ and 0.16 mM $MgSO_4$ in 1 L $D.H_2O$. Dead or obviously poor quality embryos were removed after observing them in dissection microscope. Embryos were raised in HEPES (10 mM) buffered E3 medium in a dark incubator at 28°C. All protocols were reviewed and approved by Institutional Ethical committee (Approval number for animal usage: MSU/Ethical/2009/5).

Enzyme Linked Immunosorbant assay of Pax6

ELISA was carried out for the 17 developmental stages of zebrafish and to quantify the Pax6 expression for the developmental stages from 8 hpf (hours post fertilization) to 8 dpf

(days post fertilization). 100 embryos were homogenized in 50 mM Tris-HCl with pH 8.0 containing 100 mM NaCl (TBS) and centrifuged at 10,000 rpm for 5 min at 4˚C. The microtitre plate was coated with 100 μL of embryo extracts for all the 17 developmental stages in triplicates. The titer plate was covered and incubated at 4˚C overnight. The plate was inverted and dried by shaking in order to remove the antigen solution. 200 μL of the blocking buffer (1% BSA in TBS) was added to each well and incubated overnight at 4˚C. The blocking buffer was removed and the plate was washed twice for 3 min with 200 μL wash buffer (0.1% Tween 20 in TBS) per well. The Pax6 primary antibody AD 1.5 sc-53106 (Santa Cruz, USA) was diluted (1:1000 in blocking buffer) and added 100 μL per well with 1 h incubation at room temperature. The plates were washed thrice with 200 μL wash buffer per well and 100 μL of the Goat anti mouse IgG labeled peroxidase secondary antibody (Genei) was diluted (1:2500 in blocking buffer) and added in each well, for 1 hr incubation in room temperature with shaking. The plates were washed five times for 3 min with 200 μL wash buffer. 100 μL substrate solution TMB/H_2O_2 was added and incubated for 5 min at room temperature. After color development, 90 μL of 1N HCl (stop solution) was added in each well. The results were read at 450 nm in the Microplate reader (Molecular Device).

Preparation of standard protein for Pax6

Extraction of crude protein

100 embryos from 8 hpf were homogenized in the buffer containing 10 mM Tris-HCl, pH 8.0 with 1 mM EDTA and centrifuged at 10,000 rpm for 5 min to minimize lipid or lipoprotein content. The pellet was suspended in the buffer with 10 mM $MgCl_2$ and 7 mM β-mercaptoethanol. The protein pellet was extracted with 200 mM NaCl in 20 mM Tris (pH 8). This was repeated twice and the pooled extract was precipitated with 70% ammonium sulfate. The sample was finally stored at -20˚C in equilibration buffer containing 10 mM Tris-HCl, pH 8.0 with 1 mM EDTA.

Heparin agarose - affinity chromatography

The column (Sigma) was equilibrated with 10 mL of equilibration buffer at 3–8°C. 1 mg/mL of the protein solution was loaded onto the column. The column was washed with 10 mL of equilibration buffer to remove unbound protein at a flow rate of 100 μL/min. The bound protein was eluted with 5 mL of elution buffer twice (1 M NaCl in 10 mM Tris-HCl, pH 8.0 with 1 mM EDTA). The presence of Pax6 was analyzed in both bound and unbound elution, the elution containing Pax6 was further used for purification. The elution product was precipitated by ammonium sulphate (30%) precipitation method. The precipitated protein was recovered by centrifugation at 10,000 rpm (Eppendorf 5418R, Germany) and the pellet was dissolved in 500 μL of Tris buffer (pH 8) dialysized against Tris buffer (pH 8). After purification, the sample was centrifuged at 4000 rpm at 4˚C; the supernatant was collected and stored at –40˚C for further purification.

Anion exchange chromatography

1 mL of the supernatant was applied to a column (3 x 9 cm) of DEAE cellulose (HiMedia) equilibrated with 10 mM Tris buffer (pH 8) at a flow rate of 1 mL/min. The non-adsorbed proteins were eluted with 10 mM Tris buffer (pH 8), followed by a step wise gradient of NaCl in Tris buffer (0.1, 0.2, 0.3 to 1M) at a pH of 8.0. After applying 1 mL of supernatant to the column, fractions were collected at a flow rate of 1 mL/min with a fraction size of 3 mL. The fractions were individually measured for their Pax6 concentration by ELISA at 450 nm in ELISA plate reader.

Gel filtration chromatography

Sephadex G-75 matrix was suspended in 10 mM Tris buffer (pH 8.0). The matrix was packed gently in glass column with sintered stopper at the bottom. The ion exchange column purified Pax6 was centrifuged at 3000 rpm for 5 min at 4°C. 1 mL of the supernatant was applied to the column. Tris buffer was used for the elution of the protein at a flow rate of 0.3 mL/min with a fraction size of 2 mL. The fractions were individually analyzed for Pax6 by ELISA.

Electrophoresis

Sodium dodecyl sulphate (SDS) Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Weber and Osborn[11] with 10% acrylamide gel. The gel was stained with standard silver staining procedure.[12]

Western Blotting

10% SDS PAGE was run using 3 lanes and subsequently electrotransferred on the Nitrocellulose paper. The electro transfer process was carried out for 5 hrs at 50V. Then the blotting membrane was carefully removed and incubated in 20 ml of blocking buffer for 1 hr at room temperature with shaking. Incubation was carried out overnight at 4°C. The primary antibody Pax6 was diluted in 1:2500 blocking buffer for 2 hr at room temperature according to the manufacturer's specifications. The membrane was washed twice with wash buffer for 5 min each. Secondary antibody conjugate was diluted in 1:2500 ratio of 1% BSA in PBS diluent buffer and incubated for 1 hr at room temperature with shaking. The membrane was washed four times with wash buffer for 10 min each. 10 ml of 2% TMB/H_2O_2 of freshly prepared substrate was incubated in 15 min at room temperature with shaking and the bands were visualized.

BCA assay

2 mg/mL BSA sample (Pierce, USA) was prepared and diluted in a range of 25–2000 μg/mL and loaded onto microplate. 200 μL of BCA reagent was added and allowed to incubate at room temperature for 30 min. The absorbance of each sample was measured at 562 nm using Microplate reader. The standard curve was plotted and the concentration of total protein was calculated for the 17 developmental stages of the zebrafish.

Statistical analysis

Each experiment was performed at least three times, and all values presented are the means ± SD of triplicate assays. *T*-test was used to analyze the statistical significance of the results. P values <0.05 were considered statistically significant.

Results

Pax6 purification from zebrafish embryos were carried out by three steps of chromatography (affinity, ion exchange and gel filtration techniques). The total protein extracts were prepared from 8 hpf (hours post fertilization) of the zebrafish embryos for 100 mg in one mL (100 embryos). ELISA results revealed high level of Pax6 expression at 8 hpf, since the total protein for Pax6 purification was taken from this stage. Pax6 is purified using heparin agarose affinity chromatography and the obtained absorbance values are shown in Fig. 1a. The first elution showed

more immunoreactivity in ELISA and hence it was concentrated by ammonium sulfate precipitation and followed by dialysis. The elution was purified in DEAE cellulose chromatography and the absorbance values are shown in Fig. 1b. Fraction D3 from ion exchange showed maximum level of Pax6 activity and this fraction was purified through gel filtration, in which, the elution G6 showed more immunoreactivity in ELISA (Fig. 1c) which

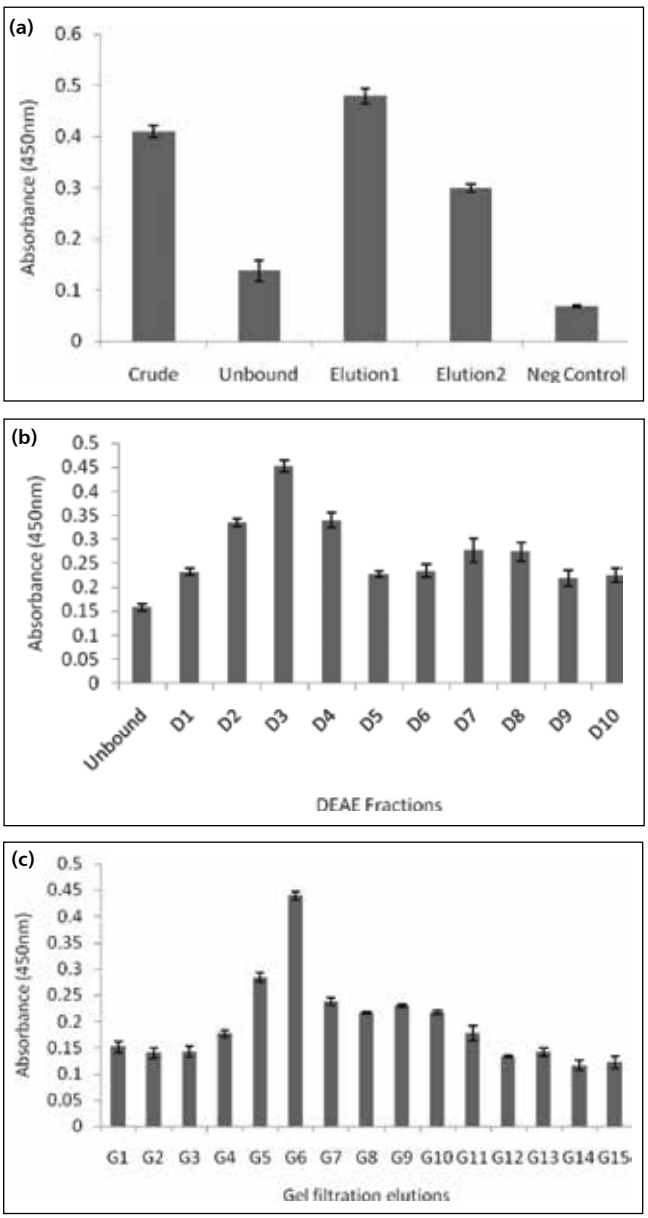

Fig. 1: (a) ELISA of Pax6 eluted using affinity chromatography (Heparin agarose) in the 8 hpf zebrafish embryos (100 embryos/mL) with high immunoreactivity in the first elution and it was used for further purification. All the values presented are the means ± SD of triplicate assays. **(b)** ELISA of Pax6 eluted using DEAE cellulose fractions in the 8 hpf zebrafish embryos with high immunoreactivity in Fraction D3 and it was used for further purification. All the values presented are the means ± SD of triplicate assays. **(c)** ELISA of Pax6 eluted using Sephadex G-75 purification in the 8 hpf zebrafish embryos with high immunoreactivity in Elution G6 and it was used for further assays. All the values are presented are the means ± SD of triplicate assays.

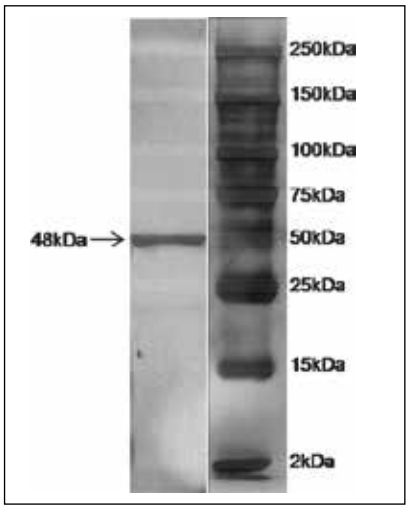

Fig. 2: SDS- PAGE analysis of purified Pax6 (ELISA andWB confirmed).

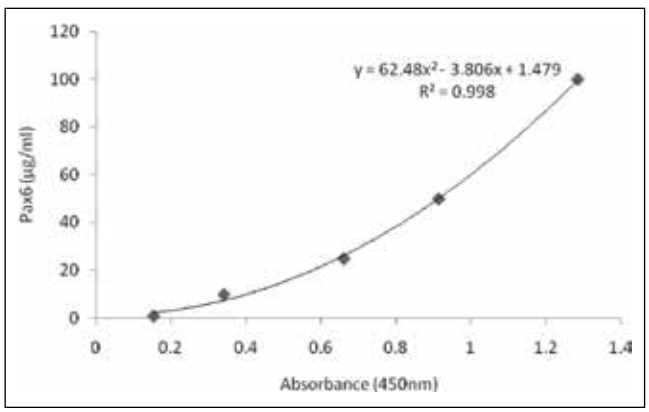

Fig. 3: Standard graph of ELISA using serially diluted Pax6 as standard protein.

confirmed the presence of Pax6. Analysis of SDS PAGE showed a single band with 48 kDa (Fig. 2), which implies a purified protein and this confirms the purity of Pax6 and confirmed by western blotting. The known quantity of this protein had established through BCA assay and was used as standard to plot the standard graph of Pax6 for ELISA. The linearity plot of the concentration dependent activity profile was plotted in Fig. 3.

The expression of Pax6 in all the 17 embryonic stages of the zebrafish has been quantified by an indirect ELISA method (Fig. 4). The level of Pax6 expression had found to be high at 8 hpf embryos and was calculated as 179.59 ± 2.86 ng/embryo and the minimum Pax6 expression is identified at 60 hpf and was found to be 44.43 ± 1.55 ng/embryo (Table 1).

Discussion

The Pax6 in each stage are quantified to find the maximum expression in the developmental stages of zebrafish. Recent advances in quantitative mass spectrometry[13,14] helps in the quantification of individual proteins and peptides. They are widely found reliable on immunoassays because of the binding specificity of the antibodies. Hence the immunoassays like enzyme-linked immunosorbent assays (ELISAs) based quantifications of

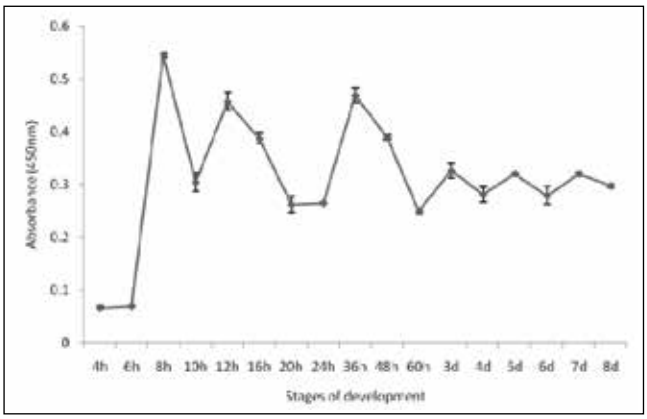

Fig. 4: ELISA absorption values of Pax6 expression in the zebrafish embryos during its various stages of development. Results are expressed as mean ± SD (n = 3).

Table 1: Quantification of Pax6 expression in the zebrafish embryos during its various stages of development. Results are expressed as mean ± SD (n = 3)

Developmental Stage (h = hpf, d = dpf)	Pax6 (ng/embryo)
	Control
4 h	0
6 h	0
8 h	179.59 ± 2.87
10 h	61.24 ± 5.77
12 h	128.29 ± 8.64
16 h	93.71 ± 4.36
20 h	47.85 ± 4.54
24 h	48.49 ± 0.65
36 h	134.55 ± 7.92
48 h	94.81 ± 2.43
60 h	44.42 ± 1.55
3 d	69.12 ± 5.11
4 d	53.61 ± 4.43
5 d	66.79 ± 0.34
6 d	53.04 ± 5.43
7 d	66.61 ± 0.88
8 d	58.76 ± 0.87

transcription factors are more reliable for the detections.[15,16] In this study, we have developed a method for the transcription factor quantifications in the zebrafish embryos or larval developmental stages. There are no reports on simple detection and quantification of lower level of transcription factors like Pax6 from the embryonic tissue extracts. Thus the present study suggested that the development of a sensitive ELISA method is suitable for the determination and quantification of Pax6 in a single zebrafish embryo. Wullimann and Rink[17] studied the expression of Pax6 in zebrafish at various embryonic and post-embryonic stages (i.e., 1, 2, 3, 5, 9 days post fertilization) by immunohistochemistry, but there is no report for the quantifications of the Pax6. The expression of the Pax6 protein at day 1 from the present study corresponds with the zebrafish brain immunohistochemistry, the day 3 showed more similarity with day 2 and day 9.[17] Thus the present study supported the expression level of Pax6 at 8 dpf embryonic zebrafish by showing similar results with the maximum expression of Pax6 by immunohistochemistry.[17] The detection of Pax6 transcription factor in the present indirect ELISA is very sensitive as it showed the detection as 44.42 ± 1.55 ng/embryo. The variation of Pax6 expression during the 17 developmental stages suggesting that the transcription factor might be involved in different developmental pathways of brain, CNS or eye development during embryogenesis and larval development in zebrafish model. There are many upstream regulators and downstream targets are reported for Pax6 during organogenesis, such as *Sox2*, *Six3* and *Prox1* genes in eye,[4] calcium-binding protein by *Necab2*, auto-regulation by *Pax2*, regulation of glucagon expression in the pancreas by *maf,*[18] *Cspg2*, *Mab21l2*, *Olfm3*, *Spag5* and *Tgfb2* genes in lens development and homeostasis,[19] *Smad3* dependent auto-regulation by secreted protein acidic rich in cysteine (SPARC) and *p53* in brain of mice[3] and also in the development of tumor by sonic hedgehog-*GLI1*.[20] Hence the above reports of transcription factors shall be studied for their expression level in the embryonic developmental stages of zebrafish which will help to find the spatiotemporal regulation of Pax6 transcription factor during embryonic development.

In conclusion, the ELISA quantification of Pax6 transcription factor would be helpful to quantify the expression of transcription factors during early embryonic development and in larval zebrafish. This is a simple tool to quantify the proteins involved development genetics to understand the vertebrate development using zebrafish as model system.

Authorship contributions

Samuel GP Vincent, **Rajaretinam R Kannan:** Designed the objectives, **Rajaretinam R Kannan:** Carried out the experiments, **Samuel GP Vincent**, **Rajaretinam R Kannan:** Involved in the data interpretation and manuscript preparation.

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