CHARACTERISATION AND HOMOLOGY MODELLING OF FINFISH NF-KAPPA B INHIBITOR ALPHA USING *IN SILICO* ANALYSIS

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ABSTRACT

NFκB plays an important role in the immune system in all organisms. In this study, physicochemical properties and modelling of finfish NFκBIα protein was analysed using *in silico* approach. Finfish species: guppy (*Poecilia reticulate*), Bicolor damselfish (*Stegastes partitus*), Channel catfish (*Ictalurus punctatus*), Coelacanth (*Latimeria chalumnae*), and Australian ghostshark (*Callorhinchus milii*) were used in this study. Physicochemical characteristics, molecular weight (Mol. Wt.: 33160.2 – 42977.9), theoretical isoelectric point (pI: 4.37 – 5.09), extinction coefficient (EC: 17920– 37650 / 17420 – 36900), aliphatic index (AI: 85.88 – 98.83), instability index (II: 39.83 – 49.09), total number of negatively charged residues (-R: 47 – 55) and positively charged residues (+R: 19 – 29), and grand average of hydropathicity (GRAVY: -0.611 – -0.241) were obtained. The results showed that except NFκBIα from *P. reticulate* all were defined as soluble proteins. Possible pairing and pattern of cysteine residues were found in all protein sequences and the most probable pattern of pairs of cysteine. Secondary structure analysis revealed approximately equal parts of random coils and helices, followed by strands. Three dimensional homology modelling for NFκBIα from finfish was performed and evaluated as credible models based on PROCHECK's Ramachandran plot, ProQ and ProSA analysis.

Keywords: *in silico*, finfish, NfκBIα.

Định tính chất và mô hình tương đồng yếu tố nhân kappa B alpha của cá sử dụng phương pháp mô phỏng máy tính

TÓM TẮT

Yếu tố nhân kappa B (NFκB) đóng vai trò quan trọng trong hệ miễn dịch của các loài sinh vật. Trong nghiên cứu này, tính chất hóa lý và cấu trúc phân tử protein NFκBIα củamột số loài cá như cá bảy màu (*Poecilia reticulate*), cá rạn (*Stegastes partitus*), cá nheo Mỹ (*Ictalurus punctatus*), cá vây tay (*Latimeria chalumnae*), cá mập ma (*Callorhinchus milii*) được phân tích bằng phương pháp *in silico*. Kết quả nghiên cứu đã ghi nhận điểm đẳng điện lý thuyết (pI: 4.37 – 5.09), hệ số tắt (EC: 17920– 37650 / 17420 – 36900), chỉ số béo (AI: 85.88 – 98.83), chỉ số bất ổn định (II: 39.83 – 49.09), tổng số dư lượng điện tích âm (-R: 47 – 55) và điện tích dương(+R: 19 – 29), hệ số GRAVY (-0.611 – -0.241) của phân tử protein NFκBIα trên cá. Ngoại trừ protein của cá bảy màu, tất cả các protein còn lại là protein dễ tan. Axit amin cysteine và các liên kết cysteine dược ghi nhận hiện diện trên tất cả các chuỗi protein. Ở cấu trúc bậc hai, số lượng dạng xoắn (helices) và xoắn ngẫu nhiên (radom coils) với số lượng tương đương chiếm ưu thế và tiếp sau là dạng sợi (strands). Cấu trúc 3D của phân tử protein được thực hiện và được đánh giá là phù hợp cho phân tử protein NfκBIα thông qua kết quả kiểm định bằng chương trình PROCHECK Ramachandran, ProQ và ProSA.

Từ khóa: *in silico*, cá, NFκBIα

1. INTRODUCTION

Nuclear factor kappa B (NFκB) was firstly identified in binding to the decameric oligonucleotide "GGGACTTCC" which was known as a lymphoid specific protein present in the intronic enhancer element of the immunoglobulin κ light chain (Igκ) gene (Sen and Baltimore 1986; Verma et al., 1995). NFκB is a homodimeric or heterodimeric complex formed by monomers, comprising Rel regions and IκB inhibitors. The Rel region is a conserved region consisting of 300 amino acids that is responsible for DNA binding and interaction with IκB inhibitors. The IκB inhibitors with 5-7 ankyrin repeat domains contain about 30 amino acids in length for each, which interact with the Rel regions (Verma et al., 1995; Baeuerie and Baltimore, 1996). The Rel/NFκB family comprises two subfamilies: NF-κB proteins (NFκB1/p50 and NFκB2/p52) and the Rel proteins (RelA/p65, c-Rel/Rel and RelB) (Thanos and Maniatis, 1995). Theoretically, NFκB resides inactively in the cytoplasm of an organism and its activation and regulation are associated with IκB proteins (Thanos and Maniatis, 1995; Verma et al., 1995; Karin, 1999). NFκB plays a key position in activating immune responses to exogenous stimuli like bacteria, viruses, and indigenous stimulation i.e. inflammatory cytokines. In this scenario, NFκB proteins translocate into the nucleus to perform its functions (Thanos and Maniatis, 1995). NFκB expression was found in almost all cell types and tissues (Oeckinghaus and Ghosh, 2009). Furthermore, NFκB is known associating with many processes, including immune and inflammatory responses, stress responses and regulation of cell proliferation and apoptosis (Oeckinghaus and Ghosh, 2009). In aquatic animals, NFκB plays an important role in the innate immune response. Indeed, there are a few reports on NFκB from Japanese flounder (*Paralichthys olivaceus*) (Yazawa et al., 2007; Kong et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Sangrador-Vegas et al., 2005), Pacific oyster (*Crassostrea gigas*) (Zhang et al., 2011), and freshwater prawn (*Macrobrachium rosenbergii*) (Arockiaraj et al., 2012). Multiple structure and function studies of proteins have been reported (Singh et al., 2011; Hossain, 2012; Sahoo et al., 2012 Vidhya et al., 2012; Goel et al., 2013; Verma and Singh, 2013; Qi et al., 2014). However, the NFκB inhibitor alpha (NFκBIα) protein from finfish species has not been characterised so far. Five amino acid sequences of the finfish NFκBIα protein were carefully searched and retrieved from NCBI database where the structural information of these protein sequences are still limited. The aim of the current study was to extend understanding of the physiochemical properties and structures of NFκBIα in finfish. In this study, *in silico* approach, a method performed by employing on computeror *via* computer simulation, was used to characterise the physicochemical characteristics, structural features and the molecular functions of the protein from finfish to provide basic information on the NFκBIα proteins from finfish, which can facilitate the utilisation of molecular tools for further analyses, for example, docking studies providing the potential ligand molecules that respond to invasive pathogens.

2. MATERIALS AND METHODS

2.1. Protein sequence and physiochemical characterisation

NFκBIα protein selected from 5 finfish species was retrieved from the NCBI (National Center for Biotechnology Information) protein database (http://www.ncbi.nlm.nih.gov/) under the FASTA format for analysis. The general information of NfκBIα from fish species was shown in table 1. Physiochemical properties of the protein, including molecular weight (Mol. wt.), amino acid composition, theoretical isoelectric point (pI), total number of positive (Arg + Lys) and negative (Asp + Glu) residues (+R/-R), extinction coefficient (EC), instability index (II), aliphatic index (AI), and grand average of hydropathicity (GRAVY) were performed using Expasy's ProtParam prediction server (Gasteiger et al., 2005).

Table 1. Finfish NFκBIα protein sequences used in this study

Scientific name	Accession number
Poecilia reticulata	XP_008398057
Stegastes partitus	XP_008300227
Ictalurus punctatus	AHH43005
Latimeria chalumnae	XP_005989233
Callorhinchus milii	XP_007891530

2.2. Functional analysis

The server SOSUI (Hirokawa et al., 1998) was performed to identify the types of protein. The CYS_REC (http://linux1.softberry.com/) was used to predict the presence of disulphide bonds and their bonding patterns, which are crucial in defining the functional linkage and the stability of a protein.

2.3. Protein structure prediction

Secondary structure predictions were made using POLYVIEW-2D server (Porollo et al., 2004). Homology modelling was constructed using a server SWISS-MODEL (Schwede et al., 2003; Arnold et al., 2006) that is operated by aligning an input target template and generating a series of predicted models. The modelled structures were selected on the basis of sequence identity (Fiser, 2004). The stereo chemical quality and accuracy of the predicted models were analysed by using Ramachandran plot analysis (Ramachandran et al., 1963) with the PROCHECK program (Laskowski et al., 1996). The structural model analysis was represented with the Swiss PDB Viewer (Guex and Manuel, 1997). Selection of the best models based on criteria of overall G-factor, number of residues in the favoured, allowed, generously allowed and disallowed regions. The best selected model of three dimensional structures was further evaluated using online servers, ProQ (Cristobal et al., 2001) and ProSA (Sippl, 1993; Wiederstein and Sippl, 2007).

3. RESULTS

3.1. Physicochemical characterisation

The total number of amino acids ranged from 298 to 386. Leucine (11.4 to 14.8%) and tryptophan (0.3 to 1.2%) were identified with

Table 2. Amino acid composition in NFκBIα computed using Expasy's ProtParam

Amino acid	*P. reticulate*	*S. partitus*	*I. punctatus*	*L. chalumnae*	*C. milii*
Alanine	7.8	6.0	7.1	6.7	6.4
Arginine	4.7	2.8	4.5	2.0	4.0
Asparagine	5.7	5.7	4.9	4.7	5.8
Aspartic acid	6.2	8.8	8.4	7.0	8.5
Cysteine	3.1	2.5	2.9	3.7	3.7
Glutamine	5.7	6.6	6.5	6.4	4.9
Glutamic acid	8.0	8.5	6.8	9.4	6.4
Glycine	4.7	5.3	4.9	6.0	7.0
Histidine	5.2	5.3	6.2	2.7	3.7
Isoleucine	4.1	3.8	5.8	4.4	4.6
Leucine	12.7	13.2	11.4	14.8	12.2
Lysine	2.8	3.8	3.2	4.4	3.7
Methionine	1.8	1.6	2.3	1.3	3.7
Phenylalanine	1.8	0.9	2.6	1.3	1.2
Proline	3.1	3.8	2.9	3.0	3.0
Serine	8.8	6.6	7.1	6.7	6.7
Threonine	6.2	6.0	5.2	4.4	4.9
Tryptophan	0.8	0.6	0.3	1.0	1.2
Tyrosine	2.1	3.5	2.6	4.0	3.0
Valine	4.7	4.7	4.2	6.0	5.5

Table 3. Physiochemical characteristics computed using Expasy's ProtParam

Species	No. of aa	Mol. wt.	pI	-R	+R	EC-1	EC-2	II	AI	GRAVY
P. reticulate	386	42977.9	5.07	55	29	29170	28420	40.22	86.97	-0.433
S. partitus	318	35669.3	4.59	55	21	27890	27390	39.83	85.88	-0.611
I. punctatus	308	34698.7	5.09	47	24	17920	17420	49.09	86.49	-0.467
L. chalumnae	298	33160.2	4.37	49	19	35005	34380	47.45	98.83	-0.241
C. milii	328	36479.0	4.71	49	25	37650	36900	46.33	87.71	-0.340

Note: EC-1: assuming all pairs of Cys residues form cystines; EC-2: assuming all Cys residues are reduced

the most and the least number of amino acids in the sequences, respectively (Table 2). The results of the physicochemical characterisation were shown in Table 3. Physicochemical characteristics of all five proteins were measured for the following parameters: Mol. wt. (33160.2 – 42977.9), pI (4.37 – 5.09), -R (47 – 55), +R (19 – 29), EC (17920– 37650 / 17420 – 36900), II (39.83 – 49.09), AI (85.88 – 98.83), and GRAVY (-0.611 – -0.241) (table 3). Methionine was considered as N-terminal of the finfish NFκBIα.

3.2. Functional characterisation

The results showed that except NFκBIα from *P. reticulate* all were defined as soluble proteins by using SOSUI server (Table 4). One transmembrane region in the *P. reticulate* NFκBIα protein was identified (Table 4). Possible pairing and pattern of cysteine residues were found in all protein sequences and the most probable pattern of pairs of cysteine were shown in table 5. These obtained patterns of pairs of cysteine indicated that the protein contains disulphide bonds in its sequence.

3.3. Protein structure prediction

The predicted composition of thesecondary protein structure elements was relatively consistent for all five studied proteins, with strands as the least abundant (0.0–3.4%), while helices (47.0–50.3%) are marginally more abundant than random coils (47.9–50.7%) in all species except *I. punctatus*and *L. chalumnae* (Table 6). In Fig. 1, generated by POLYVIEW-2D,

Table 5. Probable pattern of pairs of disulphide bond predicted by CYS_REC

Species	CYS_REC
P. reticulate	Cys55-Cys236
	Cys204-Cys256
	Cys212-Cys263
S. partitus	Cys135-Cys187
	Cys160-Cys167
I. punctatus	Cys154-Cys161
L. chalumnae	Cys116-Cys258
	Cys173-Cys232
C. milii	Cys14-Cys182
	Cys46-Cys267
	Cys169-Cys175

Table 4. Types of protein and transmembrane region identified by using SOSUI

Species	Type of proteins	Length	Transmembrane region
P. reticulate	Transmembrane	23	VWAEIPSLALLCVCEVILVVSLV
S. partitus	Soluble	-	-
I. punctatus	Soluble	-	-
L. chalumnae	Soluble	-	-
C. milii	Soluble	-	-

Table 6. Secondary structure elements (in %) of finfish NFκBIα predicted by POLYVIEW-2D

Species	Helix	Strand	Random coil
P. reticulate	48.7	3.4	47.9
S. partitus	50.3	0.0	49.7
I. punctatus	47.4	2.3	50.3
L. chalumnae	47.0	2.3	50.7
C. milii	50.3	0.0	49.7

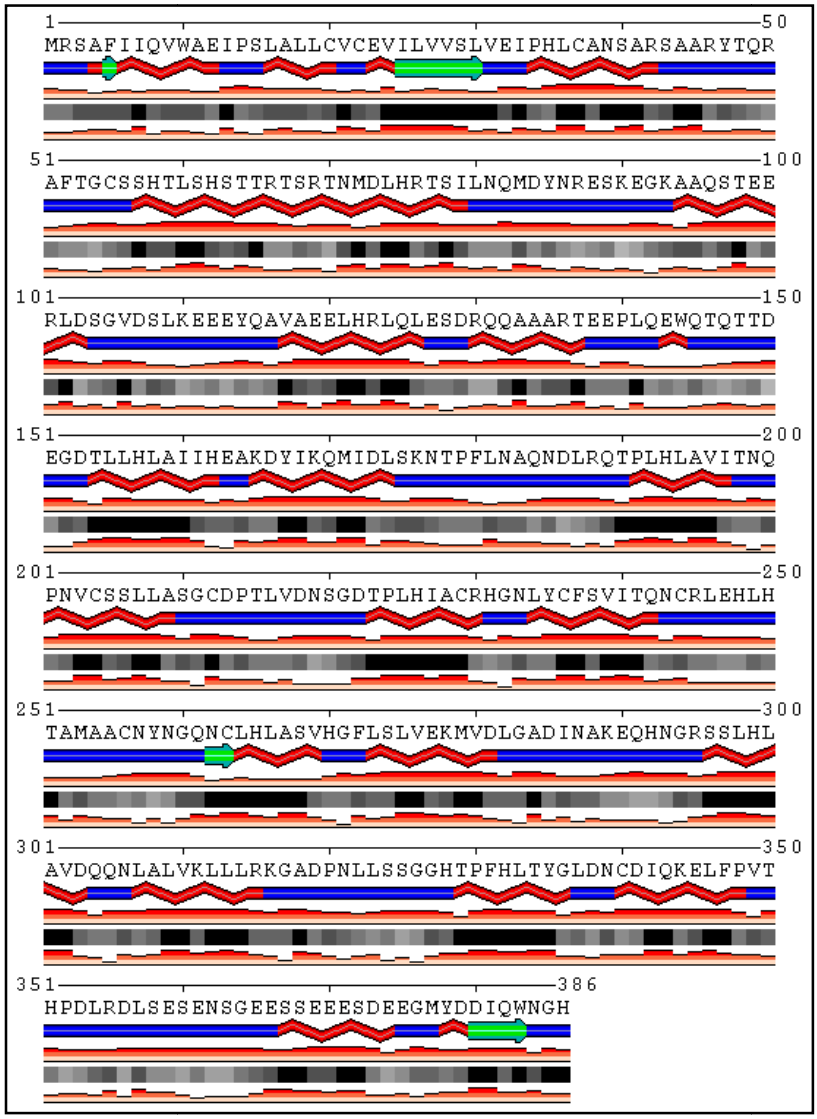

Fig. 1. Protein secondary structure of *P. Reticulate* NFκBIα as predicted by using POLYVIEW server

Note: 1——— amino acid residue numeration; H-alpha and other helices (model 1); H-alpha and other helices (model 2); E-beta-strand or bridge; C-coil; 0123456789 relative solvent accessibility (RSA) (0-completely buried: 0-9% RSA, 9-fully exposed: 90-100% RSA); 0123456789 confidence level of prediction (0-the lowest level, 9-the highest level).

the *P. Reticulate* NFκBIα was used as an example to illustrate the residue numeration, amino acid sequence, graphical representation of secondary structure, confidence level for secondary structure prediction and relative solvent accessibility.

The homology modelling of finfish NfκBIα proteins resulted in the template with PDB ID:1nfi.1.C (Jacobsand Harrison, 1998) at the resolution of 2.7 Å which was picked to build the model for all finfish NFκBIα proteins based on the highest sequence identity, ranging from 48.82% to 68.25%. The three dimensional final structure of the model represented with the Swiss PDB Viewer was shown in fig. 2. PROCHECK's Ramachandran analysis was used to check the stereo chemical quality of predicted models (Table 7). As shown in Table 7, all protein models in the range of 81.8 to 85.2% of residues were found in the most favoured regions, 13.8 to 16.4% of residues were in the additional allowed regions, and 0.5 to 2.1% of residues were in the generously allowed regions; only *C. milii* NFκBIα comprised 0.5% of residues in the disallowed regions, while others consisted of 0.0% of residues in the disallowed regions. The overall average G-factor of dihedral angles and main-chain covalent forces ranged from -0.11 to 0.35. The ProQ validation with Lg score and MaxSub index were performed. The LG score values ranged from 3.577 to 5.269 and MaxSub ranged from 0.257 to 0.438. The Z-scores in ProSA of all models ranged from -7.28 to -6.72, which are within the range of scores typically found for native proteins of similar size (Fig. 3. *-1) and values of single residue energies (window 40) were negative for all models (Fig. 3. *-2).

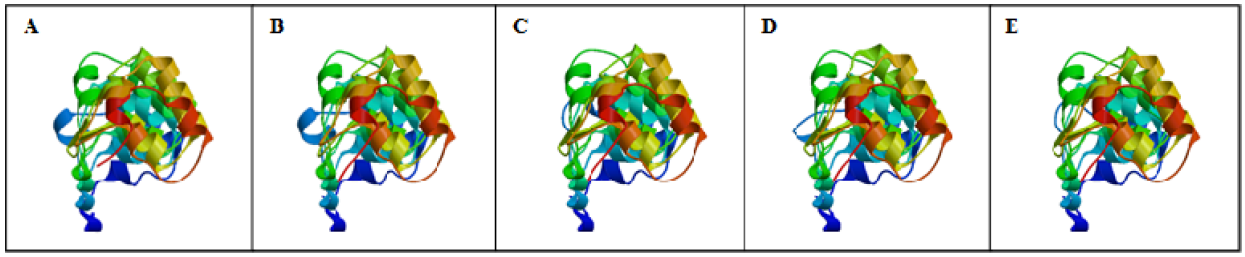

Fig. 2. Three dimensional structures of predicted models for NfκBIα proteins from (A) *P. Reticulate*, (B) *S. partitus*, (C) *I. Punctatus*, (D) *L. chalumnae* and (E) *C. milii* by using SWISS-MODEL

Table 7. Ramachandran plot analysis with PROCHECK programfor finfish NFκBIα using SWISS-MODEL server

	P. reticulate	*S. partitus*	*I. punctatus*	*L. chalumnae*	*C. milii*
Total number of residues	209	211	210	211	211
Most favoured regions (%)	84.9	85.2	83.2	83.1	81.8
Additional allowed regions (%)	14.5	13.8	15.3	16.4	15.6
Generously allowed regions (%)	0.5	1.1	1.6	0.5	2.1
Disallowed regions (%)	0	0	0	0	0.5
Overall G-factor average	0.07	0.07	0.09	0	-0.01
LGscore	3.577	3.809	4.652	5.016	5.269
MaxSub	0.257	0.26	0.347	0.322	0.438
Z-score	-7.17	-7.28	-6.72	-6.79	-7.1

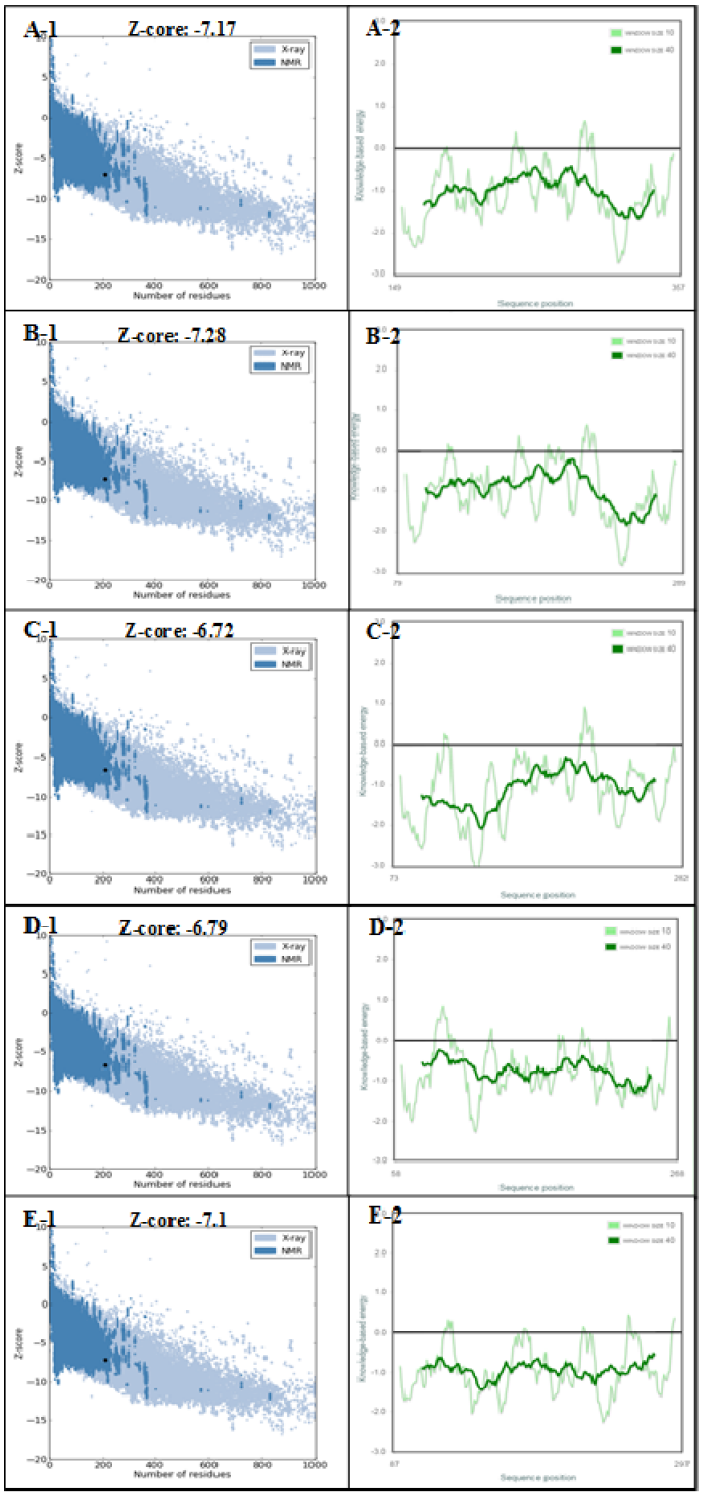

Fig. 3. ProSA-web server analysis results of model for finfish NfκBIα

Note: (A) P. reticulate: (B) S. partitus, (C) I. punctatus (D) L. chalumnae and (E) C. milii with (-1) z-scores of protein chain in PDB determined by X-ray crystallography or NMR spectroscopy with respect to their length, the z-scores of tested protein are highlighted as large dots, and (*-2) a plot of single residue energies. The plot is smoothed by calculating the average energy over each 40-residue fragment is (i,i+39), which is then assigned to the 'central' residue of the fragment at positioni + 19 (thick line). A second line with a smaller window size of 10 residues is shown in the background of the plot (thin line).*

4. DISCUSSION

In this study, we investigated the physicochemical properties and modelled the structure of NFκBIα proteins selected from finfish using *in silico* approach for the first time. Using Expasy's ProtParam prediction server (Gasteiger et al., 2005), the pI ranged from 4.37 to 5.09, indicating the proteins from different fish species are acidic in character. As proteins carry a net positive charge below and negative charge above their pI, this information can be used for purification of the proteins on a polyacrylamide gel by isoelectric focusing. The EC of proteins was measured at 280 nm which was from 17,920 to 37,650 $M^{-1}.cm^{-1}$ (assuming all pairs of cysteine residues form cysteine) and from 17,420 to 36,900 $M^{-1}.cm^{-1}$ (assuming all cysteine residues are reduced), referring a high concentration of cysteine, tryptophan and tyrosine in the proteins of interests. The EC plays a role in quantitating the protein-protein and protein-ligand interactions in solution. Based on the II value, which is a measure to evaluate the stability of proteins in a test tube, excepting for the NFκBIα from *S. partitus*, all are unstable proteins (II >40) (Guruprasad et al., 1990). The AI is a positive factor for the increase of globular proteins thermal stability which is directly relating to the mole fraction of aliphatic side chains (alanine, isoleucine, leucine, and valine) in the protein (Ikai, 1980). The AI of finfish NFκBIα ranged from 85.88 to 98.83. These high AI value indicated a high thermal stability of target proteins. The GRAVY of protein computed as from -0.611 to -0.241 which implies the protein is hydrophilic in the natural condition. The total number of negatively charged residues (Asp + Glu) was from 47 to 55 and the total number of positively charged residues (Arg + Lys) was in the range of 19 and 29. The findings on the analysis results of physicochemical parameters in this current study were also reported inteleost mannose binding lectin MBL homologue proteins (Goel et al., 2013). The results herein provide basic information on the physicochemical characteristics of finfish NFκBIα proteins, which are useful for further studies on the investigation of specific functionality of this protein in these fish species.

This study provided the three dimensional structure of NFκBIα proteins from finfish *via* a homology modeling method. The SWISS-MODEL was used to model the structure of studied proteins. The stereo chemical quality checking was performed by using online server. Ramachandran plot analysis indicates good quality of a model when it comprises over 90% residues in the most favoured regions (Ramachandran et al., 1963), in this study the results showed that all models are acceptable because of their structure consisted of higher number of residues distributing in the most favoured and additional allowed regions. The overall G-factor is an important index to evaluate the quality of stereo chemical property and a high G-factor displays the high probability of predicted model conformation for proteins (Aslanzadeh and Ghaderian, 2012). The overall average G-factor of models ranged from -0.11 to 0.35 which was greater than acceptable cut-off of -0.5, indicating good quality of all five proposed models. Similarly, LG score values ranged from 3.577 to 5.269, indicating very good model (>2.5) of the former two and extremely good model (>4.0) of the latter three models. MaxSub validation measures (0.257–0.438) implied fairly good quality of all models (Cristobal et al. 2001). Both Z-scores, which are within the range of scores typically found for native proteins of similar size, and plots of single residue energies, where positive values correspond to problematic or erroneous parts of the input structure, further corroborate high reliability of all five proposed models (Wiederstein and Sippl, 2007). These obtained data confirmed the reliability of predicted models for finfish NFκBIα. The findings of homology modelling analysis suggested that the predicted models for finfish NFκBIα possessed a three dimensional structure similar to that of human I-kappa-B-alpha. However, the models should be validated further by using other servers or approaches in

order to better understand the structure and function of these proteins from fish species. This work results may be used as the bases for further researches on functional analysis by using experimentally derived crystal structures of proteins. Also, it can be used for molecular docking studies which are helpful in providing potential ligand molecules against pathogen infections.

5. CONCLUSION

By the use of *in silico* approach to analyse the physicochemical properties and modelling for finfish NFκBIα the current study the protein NFκBIα from finfish has been modelled for the first time. Finfish NFκBIα was predicted as a soluble protein except NFκBIα from *Poecilia reticulate*. Cysteine residues and high probability of cysteine in pair were found in all studied protein sequences. An approximately number of helices and random coils were computed dominating, followed by strands in the secondary structure of all proteins. The three dimensional model of five investigated proteins was predicted and validated as good structure for finfish NFκBIα based on quality checking with PROCHECK's Ramachandran plot, ProQ and ProSA analysis results. This study provides a better understanding on the properties, structures and functions of finfish NFκBIα protein by *in silico* analysis approach.

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