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Molecular Cloning, Expression, and Enzymatic Analysis of Protein kinase C βI and βII from Inshore hagfish (*Eptatretus burgeri*)

Hyeon-Kyeong JO* · Jun-Young CHAE* · Hyung-Ho LEE†
*Pukyong National University(student) · †Pukyong National University(professor)

먹장어 유래의 Protein kinase C βI 과 βII의 분자생물학적 클로닝, 발현, 효소학적 분석

조현경* · 채준영* · 이형호†
*부경대학교(학생) · †부경대학교(교수)

Abstract

Inshore hagfish (*Eptatretus burgeri*) belongs to chordate and cyclostomata, so it is considered to be an important organism for the study of embryology and biological evolution. Protein kinase C (PKC) performs a wide range of biological functions regarding proliferation, apoptosis, differentiation, motility, and inflammation with cellular signal transduction. In this study, PKC beta isoforms, a member of the conventional class, were cloned. As a result, *EbPKCβI* and *EbPKCβII* showed the same sequence in conserved regions (C1, C2, C3, and C4 domain), but not in the C-terminal called the V5 domain. The ORFs of *EbPKCβI* and *EbPKCβII* were 2,007 bp and 2,004 bp, respectively. In the analysis of tissue specific expression patterns by *qPCR, EbPKCβI* was remarkably highly expressed in the root of the tongue and the spinal cord, while EbPKCβII was highly expressed in the gill, liver, and gut. The *EbPKCβI* and *EbPKCβII* expressed in *E. coli* revealed PKC activity according to both qualitative analysis and quantitative analysis.

Key word : PKCβI, PKCβII, Cloning, Expression, Enzymatic analysis, Hagfish

Ⅰ. Introduction

Inshore hagfish (*Eptatretus burgeri*) lives 10~270m under the seabed near Jeju Island in South Korea, South Sea, and the vicinity of Pacific Northwest. It belongs to chordate and cyclostomata, the lowest groups among vertebrates. Hagfish has atrophied eyes, tentacles, a well-developed tongue, mucous glands, and only one caudal fin, except for other fins, such as dorsal fin. Studying fish that are considered to be the early stages of vertebrates could help elucidate the embryogenic system and biological evolution (Das, 2012). Hagfish is considered a commercially viable species because of its edibility and utility in leather industry. However, it has been reported that the hagfish population is declining due to overfishing in the littoral sea. In order to maintain the hagfish population, it is

† Corresponding author : 051-629-5864, hyunghl@pknu.ac.kr
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necessary to study their spawning ecology, and it is thought that molecular biological research data on inshore hagfish must be evaluated.

Protein kinase C (PKC) is a family of enzymes involved in a wide range of biological functions. PKCs have 11 subtypes, which have been categorized into conventional (α, βI, βII and γ), novel (δ, ε, θ and η) and atypical (ζ and ι/λ) classes (Newton, 1995). The regulatory domains of conventional PKC (cPKC) isoforms contain a C1 domain that functions as a DAG-/PMA-binding motif (Baneyx and Mujacic, 2004). cPKC regulatory domains also contain a C2 domain that binds anionic phospholipids in a calcium-dependent manner (Blakey et al., 2005). Novel PKCs (nPKCs) also have twin C1 domains as well as a C2 domain. Importantly, nPKC C2 domains lack the critical calcium-coordinating acidic residues. Atypical PKCs (aPKCs) lack a calcium-sensitive C2 domain; they contain an atypical C1 domain that binds PIP3 or ceramide instead. PKCβI and βII activated by diacylglycerol (DAG) and calcium ions perform a wide range of biological functions regarding proliferation, apoptosis, differentiation, motility, and inflammation with cellular signal transduction (Kawakami et al., 2002; Newton, 1995; Wightman and Raetz, 1984).

PKCβI and βII have four identical conserved (C1-C4) regions, but they have about 50 different amino acids in the C-terminal called V5 domains. V5 domains have 50-70 amino acid sequences; COOH-terminal to the catalytic core of the enzyme (C3 and C4 domains) that contain the highly conserved turn and hydrophobic phosphorylation motifs as well as an additional 7-21 residues at the extreme COOH terminus (beyond the hydrophobic motif), that are highly variable in both length and sequence (Schreiber et al., 2001). These regions were generally ignored in early studies exploring the structural determinants of PKC isoform function (Steinberg, 2008). However, V5 domains have recently emerged as structures that impart important determinants of PKC isoform-specific targeting and function, suggesting that V5 domains may represent novel targets for pharmaceuticals designed to regulate PKC isoform-specific signaling in cells (Bobeszko et al., 2004; Cole and Igumenova, 2015; Newton, 1995).

PKCβI and PKCβII are expressed in a tissue-specific and developmentally regulated manner (Gopal and Kumar, 2013). RACK1 anchors PKCβII (but not PKCβI) to the perinuclear region; a PKCβI selective RACK protein is yet to be identified (Ono et al., 1989). While RACK1 binding sites were initially mapped to the PKCβ C2 domain, C2 domain RACK1 binding sites (which are common to PKCβI and PKCβII) do not explain the in vivo specificity of RACK1 for PKCβII (Ohno et al., 1968). Rather, the RACK1-binding specificity has been attributed to protein-protein interaction motifs in the V5 domain that are unique to PKCβII ($_{621}$ACGRNAE^{627}, $_{645}$QEVIRN^{650}, and $_{660}$SFVNSEFLKPEVKS^{673}) and not found in PKCβI (Donoghue and Purnell, 2005).

Thus, we carried out the molecular research on PKCβI with wide physiology function in inshore hagfish.

Ⅱ. Materials and Methods

1. cDNA cloning for the complete coding sequence of *Eptatretus burgeri* PKCβI and βII

The total RNA was obtained from 11 tissues (brain, tentacle, gill, root of the tongue, spinal cord, heart, liver, gut, muscle, skin, and mucous gland) of inshore hagfish using GeneAll® Hybrid-R™ Total RNA (GeneAll Biotechnology Co., Ltd., Korea) as described by previous manuscripts. The RNA was reverse-transcribed by oligo (dT)18 using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Korea) and random hexamer primers. Several PKCβ nucleotide sequences were collected from other species. Degenerated primers were designed around the highly conserved parts of the sequences using BioEdit Sequence Alignment Editor version 5.0.9 (sense primers, DgPKCβ-F1 and DgPKCβ-F2; antisense primer, DgPKCβ-R1 and DgPKCβ-R2,

<Table 1> Oligonucleotide primers used in PCR amplification of PKCβI and βII genes of *E. burgeri* (F, Forward; R, Reverse)

Primer name	5'-3' sequence	Information
DgPKCβ-F1	GGC AAG GTS CTG YTG GCA GAR GAG	
DgPKCβ-F2	CAC TTC CTB ACV TCG CTC TAC TGC GCC	
DgPKCβ-R1	CCA STT CTG GGY TTG AAV GGH GGC	
DgPKCβ-R2	GCM GGG TKC TTB GTS AGG AGB CC	Primers for
3'GSP-EbPKCβ-F1	GTG GAA CTC CAG ACT ATA TCG CTC C	cDNA library screening
3'GSP-EbPKCβ-F2	GGC TGG AGA GCC GCC ATT TGA TGG TG	
5'GSP-EbPKCβ-R1	GCG CCT GAC CTG CTG AAT CTG GTA CAT G	
5'GSP-EbPKCβ-R2	GCA AGA ACA CGC TTC TCC ACC ATG GTG C	
Eb18s rRNA-RT-For	CTG CAC GCG CGC CAC ACT GAC TGG	
Eb18s rRNA-RT-Rev	CAA CGA GTG CCA CGG ACG GCC CG	
EbPKCβI-RT-For	GAC CGC TGT GAT GCT GGT AAC	Primers for
EbPKCβI-RT-Rev	CAT GGA TAA TGA ACT CAG GGT TAG TG	RT-PCR and real-time PCR
EbPKCβII-RT-For	GAG ATG CGG TCA ACT TCG ACA AG	
EbPKCβII-RT-Rev	TCA GAG AGA GGG CCC AAA AGC	
EbPKCβI-Full-F	ACG CGG GGA GAT TGA GAA AGA G	
EbPKCβI-Full-R	TAG CGG TAG CCT TGC ATC TTT ATC ATC	
EbPKCβI-ORF-F	ATG TCG GAG ATG GAC AGC GAG	
EbPKCβI-ORF-R	CTA AAC ATG GAT AAT GAA CTC AGG GTT AGT GA	
EcoRI-EbPKCβI-F	GGA ATT CGA TGT CGG AGA TGG ACA GC	Primers for the construction
XhoI-EbPKCβI-R	CCC TCG AGC TAA ACA TGG ATA ATG AAC TCA G	of pET32
EbPKCβII-Full-F	ACG CGG GGA GAG AAA AGA GAA GCG	
EbPKCβII-Full-R	ACA GCT GCC AAT ATC AAC TCT AAA GCC TAG C	
EbPKCβII-ORF-F	ATG TCG GAG ATG GAC AGC GAG CAT TTG	
EbPKCβII-ORF-R	TCA GAG AGA GGG CCC AAA AGC CGG	
XhoI-EbPKCβII-R	CCT CGA GTC AGA GAG AGG GCC C	

<Table 1> and employed in order to amplify cDNAs from the inshore hagfish cDNA library.

3′ cDNA library screening was conducted with 3′ GSP (gene specific primer) (sense primer, 3'GSP-EbPKCβ-F1 and 3'GSP-EbPKCβ-F2, Table 1) and Universal primers. In order to isolate the full-length EbPKCβ, 5′ RACE-PCR was conducted using the GeneRacer™ Kit (Invitrogen, Korea). 5′ RACE-PCR clone was amplified with gene specific primer (antisense primer, 5'GSP-EbPKCβ-R1 and 5'GSP-EbPKCβ-R2, <Table 1> and Universal primers. Next, the PCR product was recovered by GeneAll® SV gel (GeneAll, Korea). The purified products were then ligated into pGEM T-Easy vector (Promega, Korea). Finally, database searches were performed using the BLAST (Basic Local Alignment Search Tool) at the NCBI (National Center for Biotechnology Information).

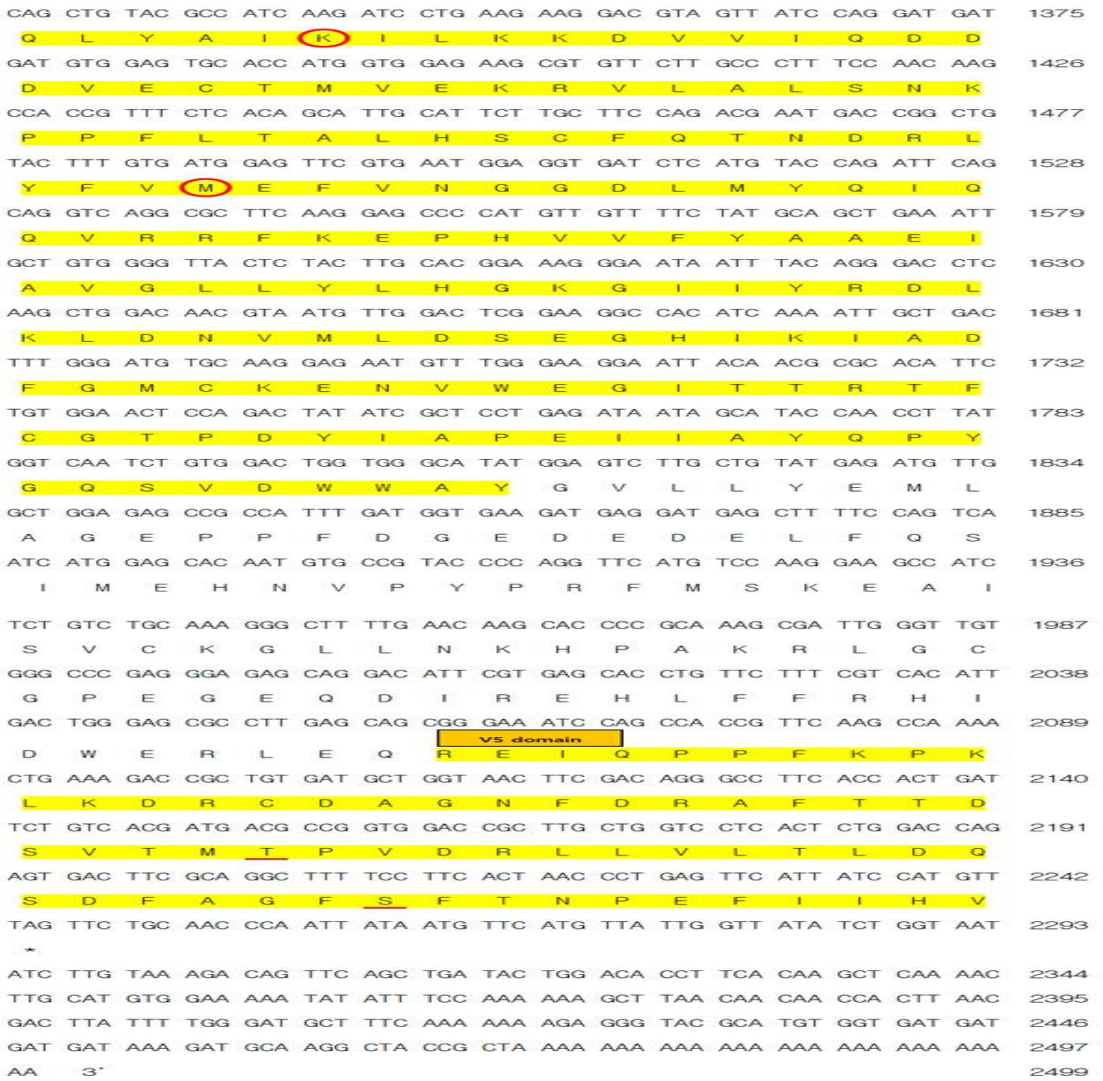
2. Sequence and phylogenetic analysis

Nucleotide and predicted peptide sequences of *E. burgeri PKCβI* and *βII* (*EbPKCβI* and *EbPKCβII*) were analyzed using DNASIS for Windows version 2.5 (Hitachi software engineering Co., Japan), BioEdit Sequence Alignment Editor, and BLAST programs in the non-redundant databases of the NCBI (http://www.ncbi.nlm. nih.gov\/BLAST/). Multiple alignments of *EbPKCβI* and *βII* amino acid sequences were analyzed using CLUSTAL W version 1.8. The identities and homologies between amino acid sequences were analyzed using BioEdit Sequence Alignment Editor version 5.0.9. The multiple sequence alignment obtained was used in order to generate a phylogenetic tree using neighbor-joining methods, and the reliability of the trees was evaluated using the bootstrap method

with 1,000 replications. In order to identify possible phylogenetic clade, a neighbor-joining tree was generated based on this genetic distance matrix using Kimura 2-parameter model included in MEGA 6.

3. Tissue specific expression patterns of EbPKCβI and βII by qRT-PCR analysis

The mRNA distribution of *EbPKCβI* and *βII* were measured by qPCR analysis. The total RNA extraction and reverse transcription was obtained through the same process described above (2). For the tissue distributions of *EbPKCβI* and *βII*, 18s rRNA was used as an internal control gene. The specific primers Eb18s-rRNA-RT-F, Eb18s-rRNA-RT-R, EbPKCβI-RT-F, EbPKCβI-RT-R, EbPKCβII-RT-F, and EbPKCβII-RT-R were used for qPCR(<Table 1>).



[Fig. 1] Nucleotide sequence and deduced amino acid sequence of *EbPKC βI*.
Shaded sequences indicate C1, C2, Kinase, and V5 domains. The four circles are lipid cofactor binding surface inside the C1 domain. The two squares are calcium-binding Asp and the square brackets are RACK binding sites. The three triangles indicate ATP binding site (GXGXXG). In the Kinase domain, the first circle is invariant Lys and the second circle is Met as a gatekeeper residue. An underlined Trp is the turn motif and an underlined Ser is the hydrophobic motif. Asterisk (*) at the end of amino acid sequence shows the stop codon.

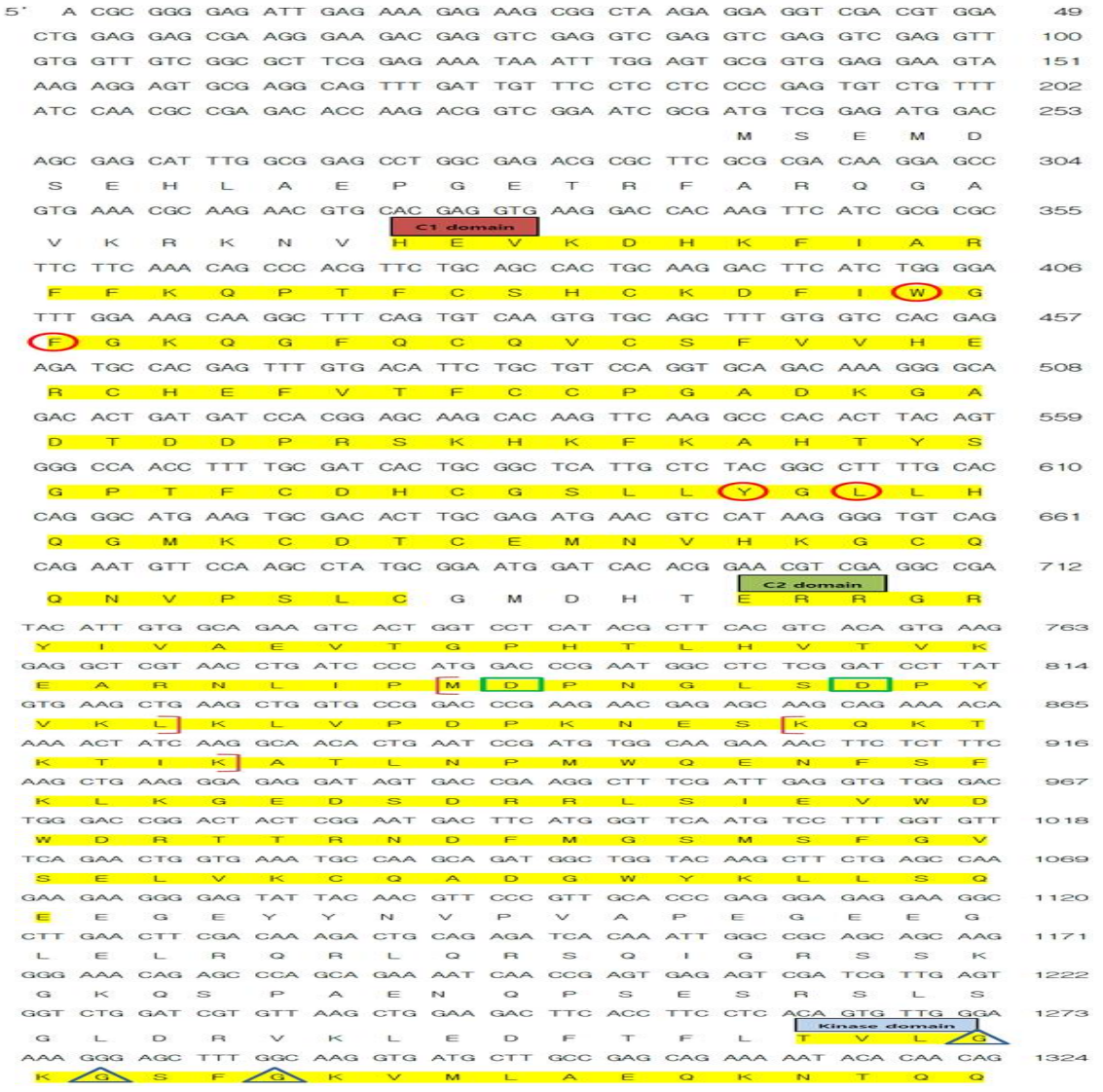
LightCycler® 480 SYBR Green I Master (Roche, Switzerland) was used in order to monitor the quantitative real-time PCR of mRNA transcript abundance on the LightCycler® 480 II Real-Time PCR System (Roche) using the following program: pre-incubation at 95°C for 5 min, 30 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The qPCR mixture was made up of the following

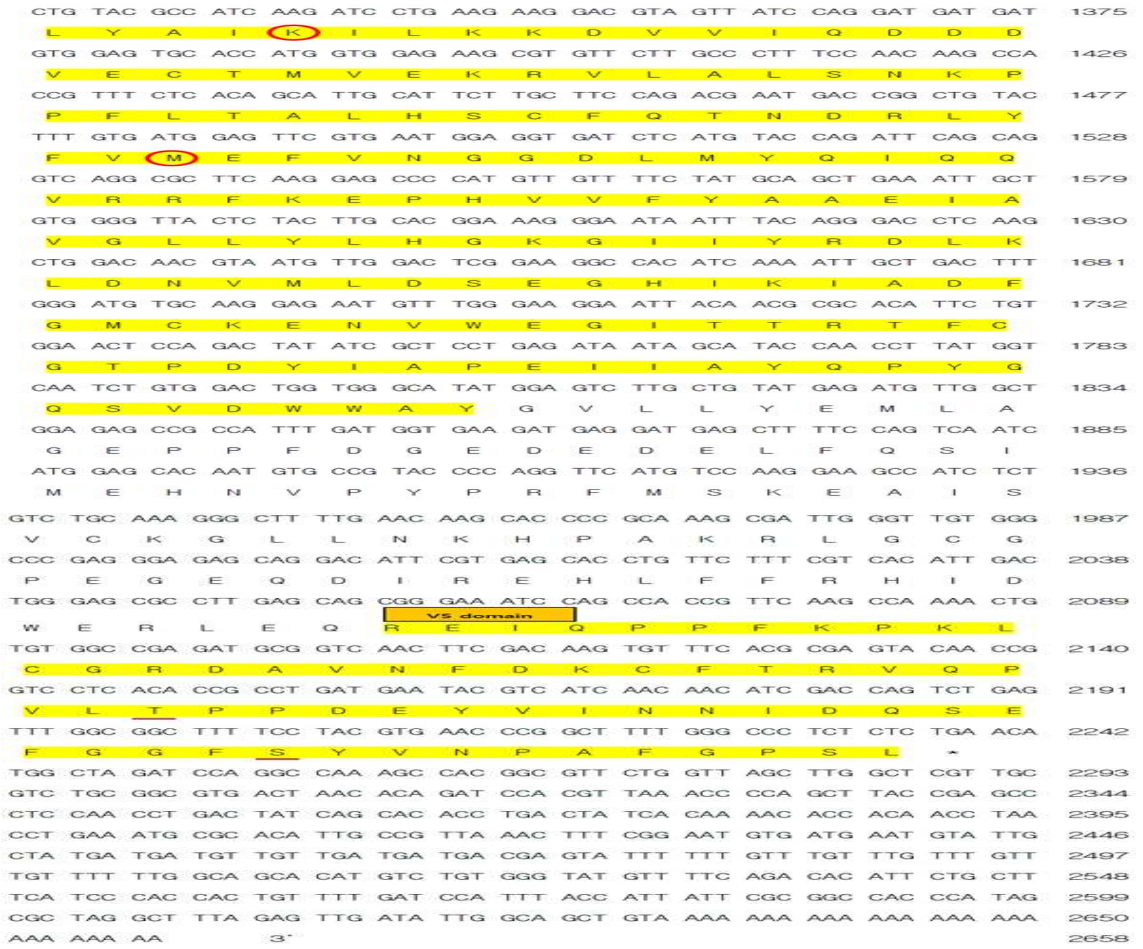
components: 10 μl of 2X SYBR (Roche), 7.5 μl of SYBR water (Roche), 1 μl of sense primer, 1 μl of antisense primer, and 0.5 μl diluted first-strand cDNA (diluted at 1:20)

The $\Delta\Delta CT$ method was adopted in order to calculate the data and the $2-\Delta\Delta Ct$ method was adopted in order to calculate the relative quantitative value (Giulietti et al, 2001).

4. Expression and purification of recombinant EbPKC βI and βII in *E. coli*

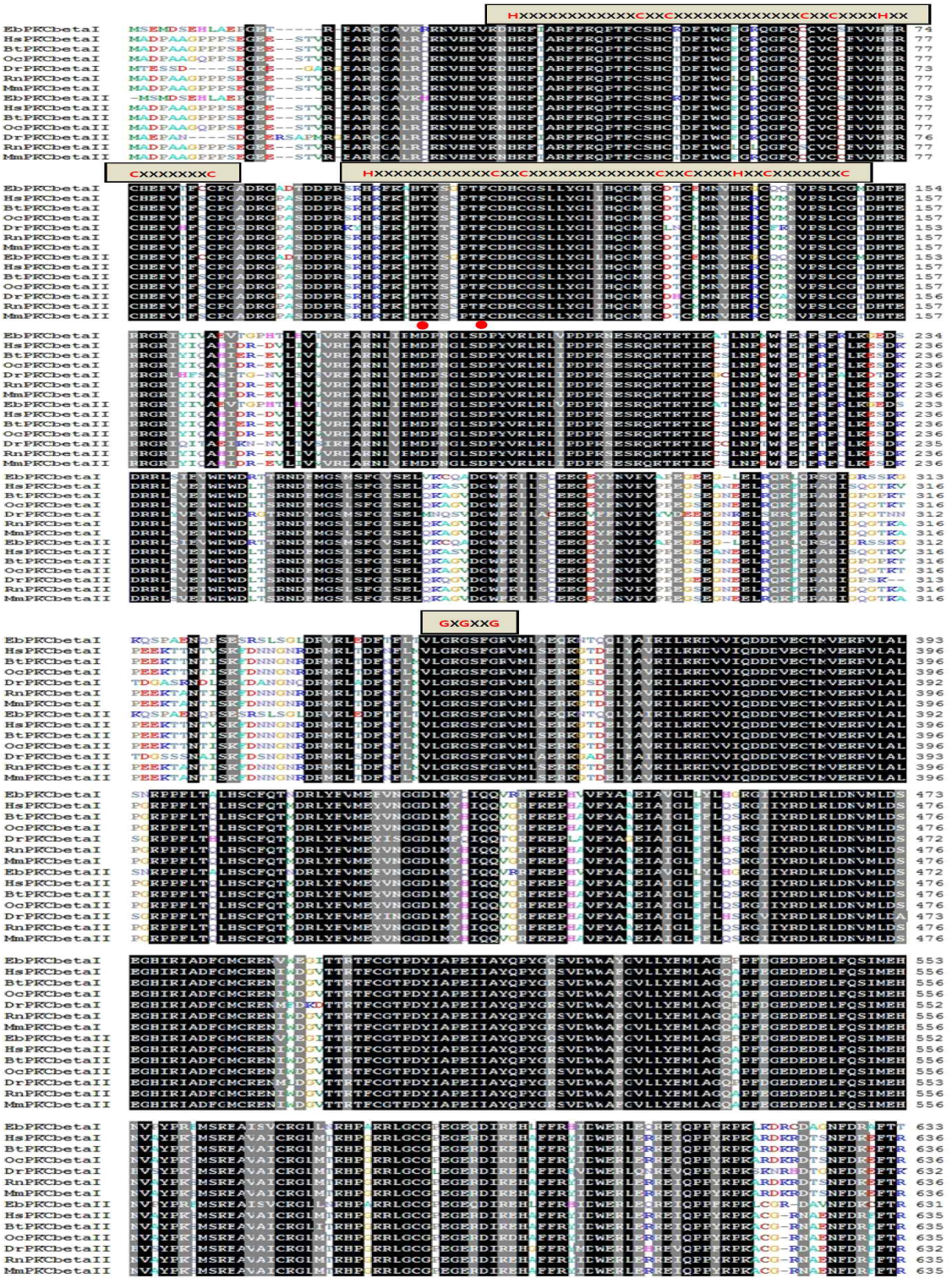
In order to construct an expression vector for the suitable production of recombinant *EbPKC βI* and *βII* in E. coli, the open reading frame (ORF) of *EbPKC βI* was amplified by PCR using the primers (sense primer, EcoRI-EbPKC βI-F; and antisense primer XhoI-EbPKC βI-R, as shown in <Table 1>).

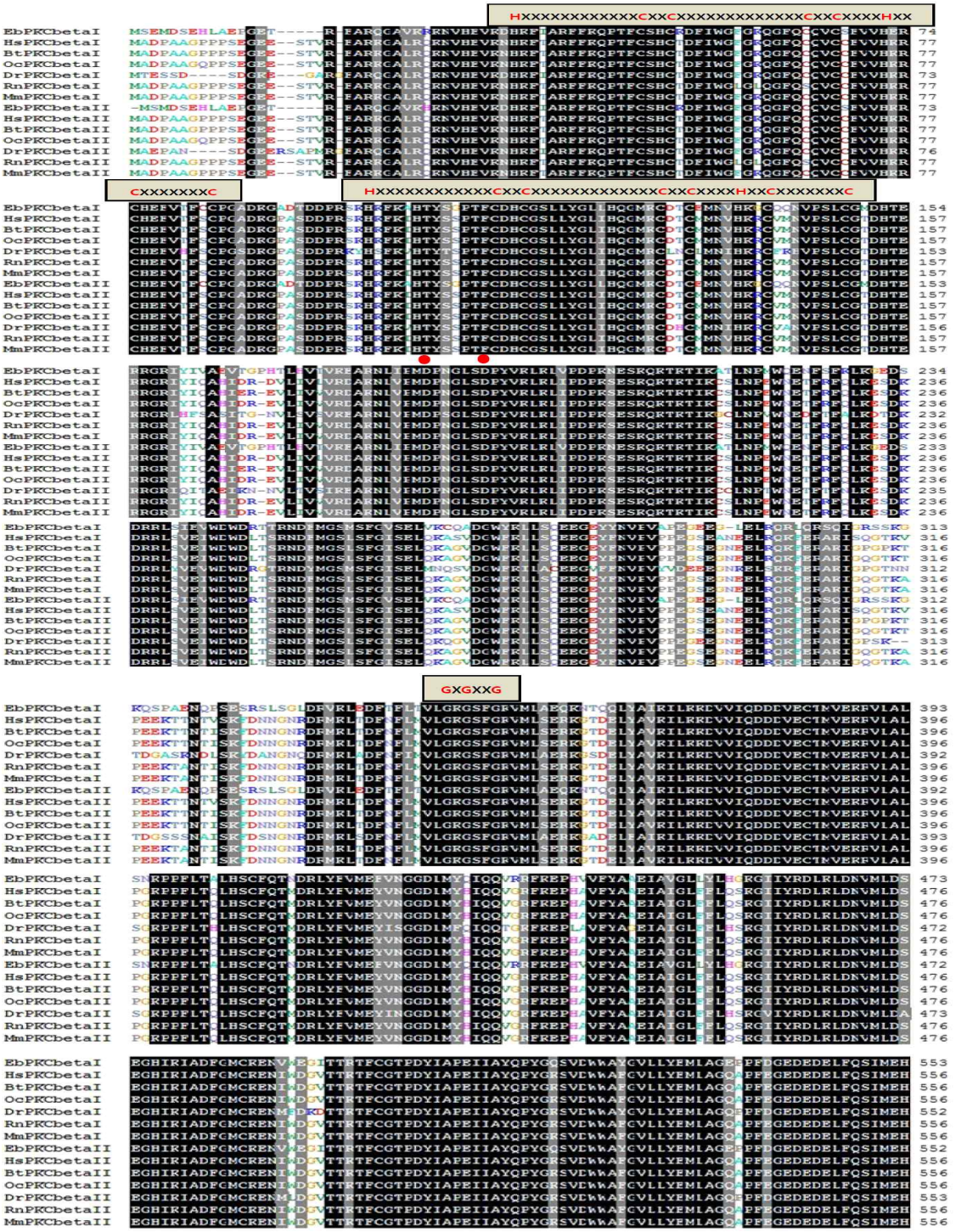


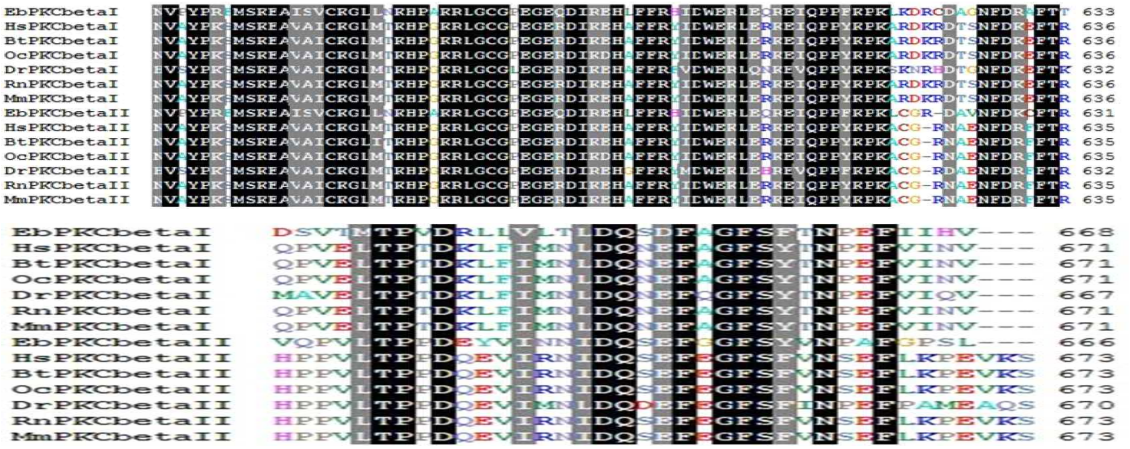


[Fig. 2] Nucelotide sequence and deduced amino acid sequence of *EbPKC βII*.
Shaded sequences indicate C1, C2, Kinase, and V5 domains. The four circles are lipid cofactor binding surface inside the C1 domain. The two squares are calcium-binding Asp and the square brackets are RACK binding sites. The three triangles indicate ATP binding site (GXGXXG). In the Kinase domain, the first circle is invariant Lys and the second circle is Met as a gatekeeper residue. An underlined Trp is the turn motif and an underlined Ser is the hydrophobic motif. Asterisk(*) at the end of amino acid sequence shows the stop codon.

In addition, in the case of *EbPKC βII*, ORF was amplified by PCR using the primers (sense primer, EcoRI-EbPKC βI-F and antisense primer XhoI-EbPKC βII-R, as shown in <Table 1>). They have the same nucleotide sequence at the beginning of ORF, so the same sense primer was used in both cases. The amplified fragment was cloned into the pET32b (Novagen). The recombinant plasmids (*EbPKC βI*/pET32b and *EbPKC βII*/pET32b) were transformed into *E. coli* strain BL21 (DE3). Transformed cells were grown in LB broth (5 ml) containing ampicillin (100 μg/ml) at 37°C for about 12 h, re-inoculated in two LB broths (5ml) containing ampicillin (100 μg/ml), and grown at 37°C until the OD_{600}=0.6. Only one of the two LB broths (5 ml) had IPTG




HXXXXXXXXXXXXCXXCXXXXXXXXXXXXCXXCXXXXHXX
CXXXXXXXC
HXXXXXXXXXXXCXXCXXXXXXXXXXXCXXCXXXXHXXCXXXXXXXC
GXGXXG



[Fig. 3] Multiple amino acids sequence alignment analysis of PKC subtypes in various species.
The GenBank accession numbers used in the alignment are shown in table 2. Identical amino acid residues are darkly shaded, similar amino acids are lightly shaded, unrelated residues have a white background, and amino acid numbers are shown on the right. "$HX_{1\ 2}\ CX_2\ CX_{1\ 3}\ CX_2\ CX_4\ HX_2\ C_7C$" in the first square box is C1 motif, that exists in both C1A and C1B. The two circles above the sequence show the Asp residues binding to calcium. The second square box shows the ATP binding site, "GXGXXG". The first inverted triangle is invariant Lys and the second inverted triangle is Met as a gatekeeper residue.

(Isopropyl-β-D-thiogalactopyranoside) added to it to a final concentration of 1 mM and grown at 20°C about 24 h. The induction of the target proteins was checked by SDS-PAGE (10% running gel, 5% stacking gel) and Western blotting. In order to obtain target proteins (*EbPKC*βI and βII) in large scale, the cells were inoculated in LB broth (500 mL) and grown at 37°C until the OD_{600}=0.6.

5. SDS-PAGE and Western blot

For the electrophoresis procedures, all samples were denatured in buffer containing 60mM Tris/pH 6.8, 25% glycerol, 2% SDS, 14.4mM 2-mercaptoethanol, and 0.1% bromophenol blue, then boiled for 5min. Purified *EbPKC*βI and βII were separated by 10% SDS-PAGE (Bio-Rad, USA). Prestained molecular weight markers (Bio-Rad, USA) were run as standards on each gel. Following electrophoresis, the gels were stained with Coomassie brilliant blue R-250.

Western blotting was performed using rabbit polyclonal anti-His antibody (1:2000, Santa Cruz Biotechnology) and rabbit monoclonal anti-His antibody (1:1000, Santa Cruz Biotechnology). Prestained molecular weight markers (Bio-Rad, USA) were run as standards. The electrophoresed samples were transferred to nitrocellulose membranes (Schleicher & Schuell. Co., USA) using a Hoefer transblotting system (Pharmacia. Co., USA). Following this transfer, the membrane was blocked with 3% BSA in TPBS [200 mM Tris (pH 7.0), 1.37 M NaCl, 1% Tween-20] for 30 min at room temperature. Primary antibody was attached to the target proteins at 4℃ for 16 h. Secondary antibody was attached to the target proteins at 4℃ for 1.5 h.

6. Activity assay

Phosphorylation by PKC of its specific substrate alters the peptide's net charge from +1 to −1. PepTag® Non-Radioactive PKC Assay (Promega) was used to analyze the activities of *EbPKCβI* and *EbPKCβII*. For qualitative analysis, proteins were diluted to 1, 2, 5, and 10 ng/μl, and reacted with substrate at 30℃ for 30 min. After reaction, samples underwent electrophoresis on gel, which was made of 50 mM Tris-HCl(pH 8.0) and 0.8% agarose, over 20 min. Phosphorylated peptide was separated for quantitative analysis. At 95℃, it was completely melted and Gel Solubilization Solution, glacial acetic acid, and distilled water were added. Using a spectrophotometer, we assessed the absorbance at A_{650} and calculated activity using the following equation:

$$A = \varepsilon BC,$$

where: A = absorbance of the sample, ε = the molar absorptivity of the peptide in L/mol • cm^{-1}, B = the width of the light cell, and C = the concentration of the peptide in mol/L of the sample read.

Ⅲ. Results

1. Cloning and sequence analyses of *EbPKCβI* and *βII*

In order to identify the partial sequences of *EbPKCβ*, databases of other PKCs were obtained using NCBI sequence data. These sequences were used to design degenerated primers. The initial partial sequences were obtained through PCR amplification of inshore hagfish cDNA, including the brain, tentacle, gill, root of the tongue, spinal cord, heart, liver, gut, muscle, skin, and mucous gland. In order to isolate full-length inshore hagfish PKCβI and βII, the partial sequences were used as bases for gene-specific primers for RACE PCR.

As a result, the full nucleotide sequences of *EbPKCβI* and *EbPKCβII* were 2,499 bp and 2,658 bp, respectively. The *EbPKCβI* sequence was composed of a 238 bp 5'-untranslated region (5'-UTR), a 2,007 bp coding region, and a 254 bp 3'-untranslated region (3'-UTR) [Fig. 1]. 3'-UTR of *EbPKCβI* had a miR-199-5p binding site from 73 to 78 (5'-TACTGG-3'). The *EbPKCβII* sequence was composed of a 235 bp 5'-untranslated region (5'-UTR), a 2,004 bp coding region, and a 419 bp 3'-untranslated region (3'-UTR) [Fig. 2]. 3'-UTR of *EbPKCβII* had a miR-203a-5p binding site from 328 to 334 (5'-GATCCAT-3'). The *EbPKCβI* codes 668 amino acids, which the molecular weight is approximately 76.43 kDa, and the *EbPKCβII* codes 667 amino acids, which the molecular weight is approximately 76.08 kDa. These sequences were submitted to the NCBI database [PKCβI(**MH350863**), PKCβII(**MH350864**)].

EbPKCβI and *EbPKCβII* have the same sequences in conserved regions (C1, C2, C3, and C4 domain), but not in the C-terminal called the V5 domain. C1 domains were highly conserved DAG/PMA binding sites with a characteristic $HX_{12}CX_2CXnCX_2CX_4HX_2CX_7C$ motif (H, histidine; C, cysteine; X, any other amino acid; n is 13). C2 domains contained a calcium binding loop, which has several highly conserved Asp residues, and a RACK (receptor for activated C-kinase) binding site.

It was confirmed that both *EbPKCβI* and *βII* had these highly conserved motifs. Kinase domains include an ATP-binding site (GXGXXG; G is glycine and X is any other amino acid), invariant Lys, and gatekeeper residue. cPKCs and nPKCs

have Met as a gatekeeper residue, while aPKCs use Ile. It was confirmed that *EbPKCβI* and *βII* have these residues as well. In the case of V5 domains, which are about 50 residues of C-terminus, *EbPKCβI* and *βII* differ significantly. Thus, βI and βII were determined by referencing previous studies [Fig. 3].

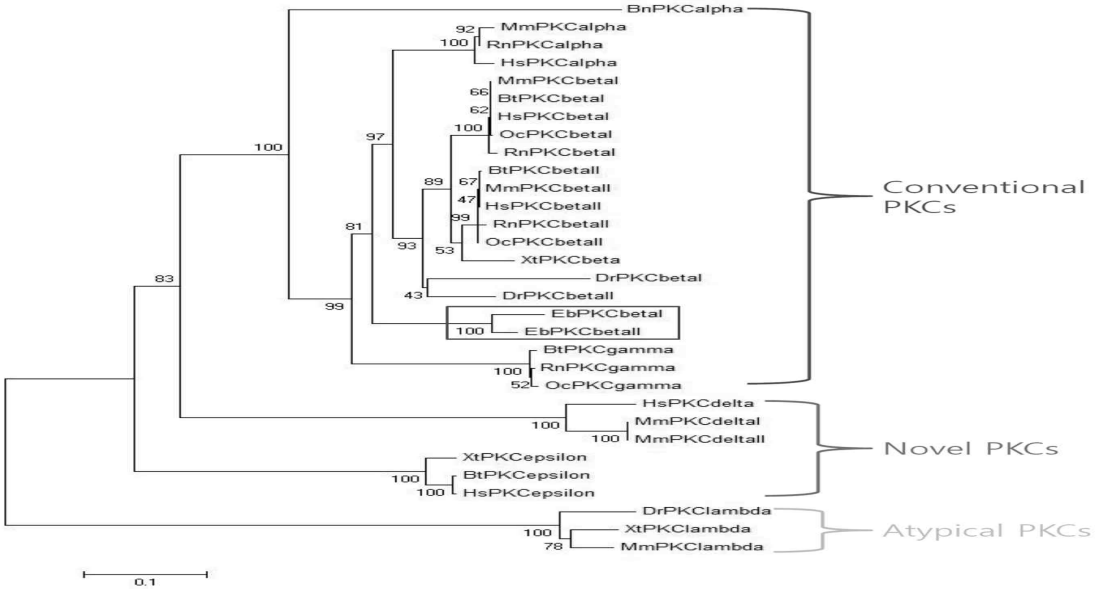
2. Phylogenetic tree of *EbPKCβI* and *βII*

In order to determine the evolutionary relationship of *EbPKCβI* and *βII* with other families of the PKC, a phylogenetic tree was constructed.

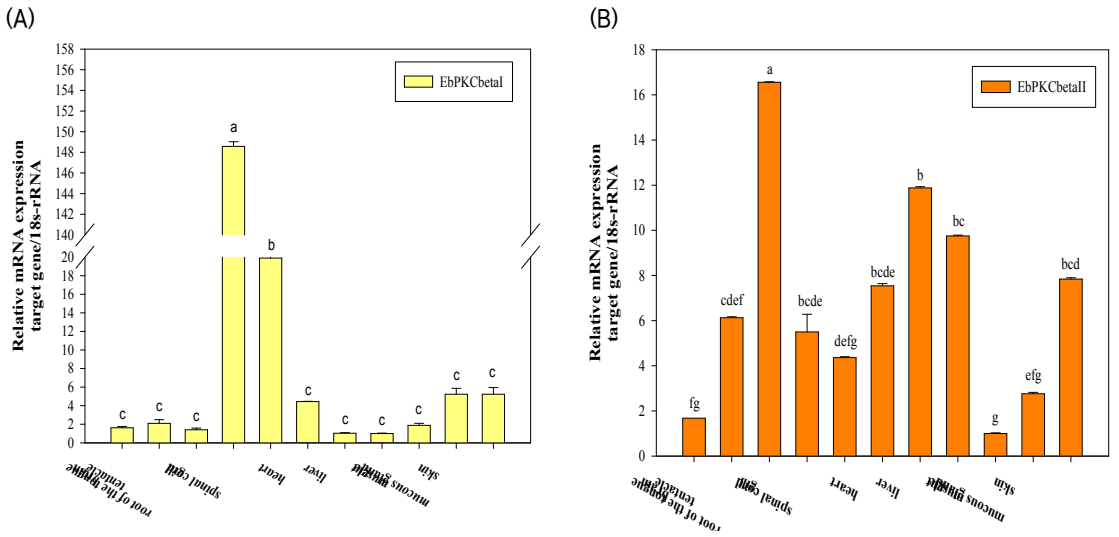
Phylogenetic analysis was performed with the amino acid sequences of human PKCs and the PKCs of other species obtained from GenBank using neighbor-joining methods [Fig. 4]. Based on a comprehensive phylogenetic analysis, cPKCs, nPKCs, and aPKCs were classified. In other species, βI was more closed compared to the βI of another species but not in the case of fish (*DrPKCβI* and *EbPKCβI)*.

3. Tissue distribution of *EbPKCβI* and *βII* by qRT-PCR analysis

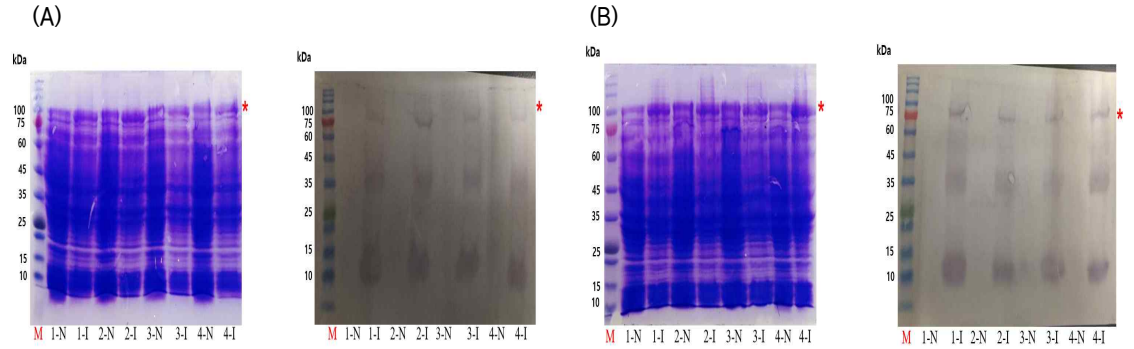
The distributions of *EbPKCβI* and *βII* transcripts in different organs were examined by RT-PCR. The results of qPCR indicated that *EbPKCβI* and *βII* were expressed in different organs, including the brain, tentacle, gill, root of tongue, spinal cord, heart, liver, gut, muscle, skin, and mucous gland. The expression pattern of *EbPKCβI* was found at its highest levels in the root of the tongue and spinal cord [Fig. 5A]. The expression pattern of *EbPKCβII* was found at high levels in the gill, liver, and gut [Fig. 5B].



[Fig. 4] Phylogenetic relationship of *EbPKCβI* and *βII* with other PKC subtypes.
In this neighbor-joining phylogram, all individuals are represented and the branches are based on the number of inferred substitutions, as indicated by the bar. The square indicates *EbPKCβI* and *EbPKCβII*.



[Fig. 5] Tissue-specific distribution of *EbPKC βI* and *βII*
Quantitative real-time PCR of *EbPKC βI* and *EbPKC βII* in various tissues. Mean of mRNA levels in *E. burgeri* tissues were analyzed by real-time PCR, and $2-\Delta\Delta Ct$ levels were calculated relative to the tissue with the lowest expression (PKC βI from gut tissue and PKC βII from muscle tissue) set to 1 and normalized against 18s-rRNA expression. Each experiment was done in triplicate.



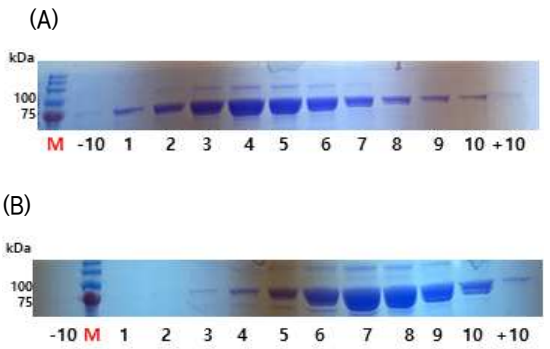
[Fig. 6] SDS-PAGE and Western blot analysis of *EbPKC βI* and *βII*.
10% SDS-PAGE gel and coomassie R-250 blue was used to perform SDS-PAGE. Anti-His antibody was used for Western blotting. Predicted recombinant *EbPKC β*s Molecular weight is approximately 92 kDa, as they include Trx-tag, S-tag, His-tag, and other amino acids. M, standard size marker; N, cell lysate from IPTG-not induced *EbPKC β*-expressing *E. coli* strain BL21 (DE3); I, cell lysate from 1 mM IPTG-induced *EbPKC β*-expressing E. coli strain BL21 (DE3).

4. SDS-PAGE and western blot

In order to select recombinants of *EbPKCβI* and *βII*, transformed cells were grown in LB broth (5 ml) containing ampicillin (100 μg/ml) and had IPTG added to a final concentration of 1 mM. The

sample was checked with induction of target proteins by SDS-PAGE (10% running gel, 5% stacking gel) and Western blotting was performed [Fig. 6A, 6B].

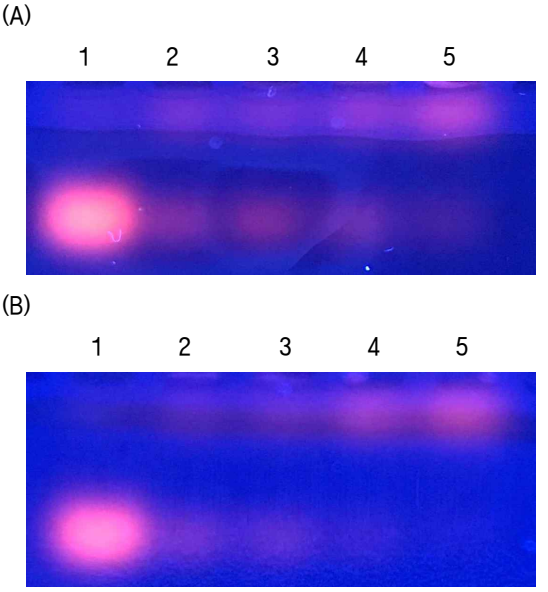
Although the size of *EbPKCβI* and *βII* is approximately 76 kDa in both cases, the target size on SDS-PAGE is about 92 kDa, because pET32b vector expresses other proteins, including Trx tag, S tag, and His tag. Recombinant *EbPKCβI* and *βII* were purified by affinity column with nickel resin at 4°C. SDS-PAGE was onducted with 10% acrylamide gel [Fig. 7A, 7B]. Purified samples were dialyzed in order to check the *EbPKCβ* 's activities.



[Fig. 7] SDS-PAGE of *EbPKCβI* and *βII* purified by affinity chromatography.
10% SDS-PAGE gel and coomassie R-250 blue was used to perform SDS-PAGE. Predicted recombinant EbPKCβs Molecular weight is approximately 92 kDa. M, standard size marker; -10, sample obtained for 10 minutes after passing through an elution buffer; 1~10, sample obtained for the next minute; +10, sample obtained during the last 10 minutes.

5. Activity assay

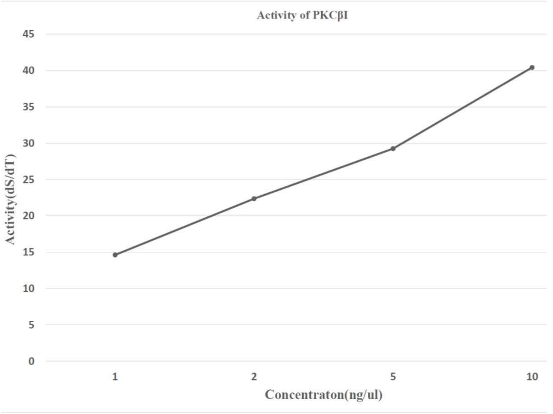
In order to check whether recombinant *EbPKCβI* and *βII* proteins have PKC activity, we conducted activity assay using substrate which could be phosphorylated by PKC. Consequently, we identified that they can indeed phosphorylate substrate [Fig. 8].



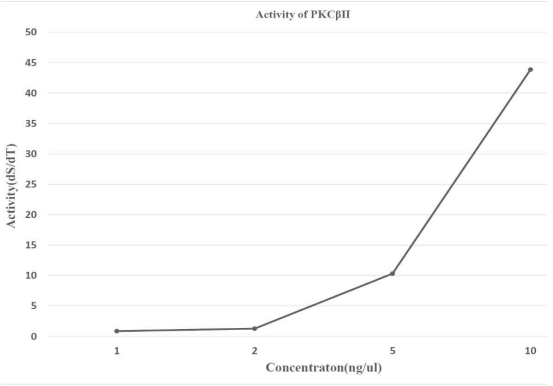
[Fig. 8] Qualitative analysis of *EbPKCβI* and *EbPKCβII*.
Substrate was phosphorylated by purified proteins at 1, 2, 5, and 10 ng/μl, respectively. Lane 1 indicates negative control and lanes 2-5 indicate reactants which contain substrate and diversely diluted purified protein (1, 2, 5, and 10 ng/μl). The upper parts are phosphorylated substrate and the lower parts are non-phosphorylated substrate.

The results of quantitative analysis showed that in the case of *EbPKCβI*, it phosphorylated substrate at 14.52, 22.36, 29.25, and 40.43 pmol/min at 1, 2, 5, and 10 ng/μl, respectively [Fig. 9A]. *EbPKCβII* phosphorylated substrate at 0.86, 1.27, 10.32, and 43.87 pmol/mine at 1, 2, 5, and 10 ng/μl, respectively [Fig. 9B].

(A)



(B)



[Fig. 9] Quantitative analysis of *EbPKC βI* and *EbPKC βII*.
We checked the amount of phosphorylated substrate treated with *EbPKC βI* and *EbPKC βII* 1, 2, 5, and 10 ng/μl. (A) Activity of *EbPKC βI*, (B) Activity of *EbPKC βII*.

4. Discussions

In this study, we identified sequences of *EbPKC βI* and *EbPKCβII,* which originated from one PKCβ gene, and conducted enzymatic analysis. As a result, *EbPKCβI* and *EbPKCβII* were found to encode 668 and 667 amino acids, respectively. They also showed PKC activity.

<Table 2> Sequences used in this study

Sequence for comparison	Species	NCBI accession number
HsPKCα	Homo sapiens (Human)	BAU98542.1
HsPKCβI		NP_997700.1
HsPKCβII		NP_002729.2
HsPKCδ		NP_001303256.1
HsPKCε		NP_005391.1
MmPKCα	Mus musculus (House mouse)	AAA39934.1
MmPKCβI		NP_032881.1
MmPKCβII		NP_001303601.1
MmPKCδI		AAF79208.1
MmPKCδII		NP_035233.1
MmPKCλ		BAA32499.1
MaPKCβII	Monopterus albus (Asian Swamp Eel)	XP_020453442.1
MaPKCβI		XP_020453441.1
MaPKCε		XP_020460965.1
LbPKCβ	Labrus bergylta (Ballan wrasse)	XP_020502596.1
AmPKCβI	Astyanax mexicanus (Mexican tetra)	XP_022530628.1
AmPKCβII		XP_022530629.1
AmPKCη		XP_022540784.1
BpPKCβ	Boleophthalmus pectinirostris (Boleophthalmus)	XP_020775613.1
BpPKCη		XP_020775049.1
SdPKCβ	Seriola dumerili (Greater amberjack)	XP_022612562.1
SdPKCδ		XP_022600899.1
SdPKCη		XP_022598245.1
NfPKCα	Nothobranchius furzeri (Turquoise killifish)	SBS59970.1
NfPKCγ		SBP44224.1
AsPKCα	Aphyosemion striatum (killifish)	SBP12178.1
AsPKCγ		SBP15911.1
ApPKCδ	Acanthochromis polyacanthus (Spiny Chromis)	XP_022046770.1
ApPKCε		XP_022045543.1
ApPKCη		XP_022077382.1
FhPKCδ	Fundulus heteroclitus (Mummichog)	XP_021172777.1
OlPKCδ	Oryzias latipes (Japanese rice fish)	XP_011472964.1
OmPKCε	Oncorhynchus mykiss (Rainbow trout)	XP_021432053.1
OmPKCη		XP_021440637.1
NkPKCα	Nothobranchius korthausae (killifish)	SBQ50761.1
NrPKCα	Nothobranchius rachovii (killifish)	SBR89037.1
DrPKCβI	Danio rerio (Zebrafish)	XP_005170901.1
DrPKCβII		NP_957272.1
DrPKCλ		AAK91291.1
DrPKCδ		NP_999873.1
DrPKCη		NP_001038271.1

In order to check tissue-specific expression, we conducted qPCR. *EbPKCβI was* highly expressed in the root of the tongue and spinal cord, and *EbPKC*

βII was highly expressed in the gill, liver, and gut. In particular, a clearly large amount of mRNA was transcribed in the root of the tongue. In several species, PKCβ is involved in the immune system, such as in immunoreceptor signaling, immunodeficiency, and the development and activation of B cells (Kawakami et al., 2002). Therefore, we predict that the root of tongue can act as not only a predatory organ but also a sensory or immune-related organ through contact with the environment. In the jawed vertebrate, PKC β1 is highly expressed in the brain (Goldberg and Steinberg, 1996; Ohno et al., 1987). However, in hagfish (the jawless vertebrate), PKCβ1 is highly expressed in the spinal cord but not in the brain. It is suggested that the spinal cord of the hagfish is more important than that of a jawed vertebrates in the role of the central nerve system.

In vertebrates, PKCβI and PKCβII are regulated by miR-203 and miR-7, respectively. Hagfish has miR-199 gene and miR-203a gene (Heimberg et al., 2010). In this study, *EbPKCβI* and *βII* contained miR-199 and miR-203a binding sites in their respective 3'-UTR. Therefore, it is possible that Ebu-miR-199 and Ebu-miR-203a regulate *EbPKCβI* and *EbPKCβII*, respectively.

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