

Original article

# Molecular cloning of a novel chitinase gene from the kidney of Japanese sardine (*Sardinops melanostictus*)

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## Introduction

Chitin is an amino polysaccharide containing *N*-acetyl-D-glucosamine (GlcNAc) units connected with  $\beta$ -1,4 linkages. It is the second most abundant biological resource on earth next to cellulose, and is widely found in the exoskeletons of arthropods, cell walls of fungi, and the epidermis of nematodes [1-3].

Chitinases (EC 3.2.1.14) are enzymes that hydrolyze the glycosidic bonds of chitin and belong to the glycoside hydrolase GH family 18 or 19 based on the amino acid sequence homology within the catalytic domain [4]. The degradation products of chitin exhibit various bioactivities; these include the promotion of bifidobacteria proliferation and immunostimulatory effect in chitooligosaccharides ((GlcNAc)<sub>n</sub>) [5] and improvement of osteoarthritis in GlcNAc [6]. Chitinases are found in many biological species, including mammals [7], fish [8,9], insects [10], plants [11], and fungi [1,10]. It is thought that chitinase has important physiological role in those organisms. For instance, chitinases are thought to play roles of digestion of food in the stomach of mammals [12] and fish [13,14], defense in plants [15] and the lung of mammals [7], and molt in insects [16].

We have been investigating the purification, characteristics, and cDNA cloning of chitinases by using several kinds of aquatics. We reported two types of chitinase (acidic fish chitinase-1, acidic fish chitinase-2) that are active at acidic pH and involved in digestion in the stomach of Actinopterygii [9,13]. In addition, we suggested the existence of the new fish chitinase which probably has a different function from the digestion. In this study, we used *Sardinops melanostictus* in which it has been reported the characteristic and cDNA cloning of chitinase isozymes (SmeChi-1, SmeChi-2) in the stomach [9]. We tried to clone a novel chitinase cDNA from the kidney.

## Materials and methods

### Material

The Japanese sardine *S. melanostictus* of 15 cm in body length and 53 g of body weight was used.

### cDNA cloning of SmeChi-3 from the kidney

Total RNA was extracted from the kidney of *S. melanostictus* using ISOGEN II (Nippon Gene, Tokyo), according to the manufacturer's instructions. First strand cDNA was synthesized using 1.0  $\mu$ g total RNA and Oligo dT primers with PrimeScript™ I Reverse Transcriptase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Three degenerate primers were designed for the reverse transcriptase-polymerase chain reaction from conserved sequences of the GH family 18 chitinases of vertebrates and internal sequence amplification was performed using GoTaq® Green Master Mix (Promega, Madison, WI). RACE method was performed to obtain sequences of the upstream (5') and downstream (3') regions. The full-length chitinase cDNA was confirmed by using PrimeSTAR® Max DNA Polymerase (Takara Bio) with proofreading activity.

### Phylogenetic analysis of SmeChi-3

Phylogenetic analysis of chitinase from several organisms was carried out using the Clustalw2 program (EMBL-EBI: The European Bioinformatics Institute, European Molecular Biology Laboratory) and the tree view program. A bacterial chitinase (GenBank: X03657) was used as outgroup.

## Results and discussion

### cDNA Cloning of SmeChi-3

A gene of approximately 350 bp was obtained after amplification of the internal sequence of chitinase genes from the kidney of *S. melanostictus*. Sequence analysis by NCBI Blast revealed 74% homology with

*Paralichthys olivaceus* chitinase3. Thus, the upstream and downstream regions of chitinase genes were amplified using RACE method. The start and stop codons were found in the upstream and downstream regions of amplified genes. Consequently, the full-length cDNA of *S. melanostictus* kidney chitinase *SmeChi-3* was 1,531 bp in length and contained an ORF of 1,482 bp encoding 495 amino acids. The deduced amino acid sequence of *SmChi-3* revealed that it consisted of a signal peptide, catalytic domain, linker region, and chitin-binding domain. In addition, the unique sequence (DXDXE) specific in the active site of vertebrates was found. DDBJ accession numbers have been obtained for the full-length genetic sequences: LC119087 for *SmeChi-3*. *SmChi-1* and *SmeChi-2* have a serine and glycine-rich linker region like *AMCase*. In contrast, *SmeChi-3* contained no apparent repeat sequence in the linker region.

#### Phylogenetic analysis of chitinases from different species

The phylogenetic analysis of the amino acid sequence of *SmeChi-3* was performed based on its homology with chitinase amino acid sequences across another organism. Consistent with previous reports [9,13], the Osteichthyes stomach chitinase was classified in the two groups, *AFCCase-1* and *AFCCase-2*. *SmeChi-3* was classified in neither *AFCCase-1* nor *AFCCase-2*. As a result, *SmeChi-3* was classified in a novel chitinase group on Osteichthyes. Namely, *SmeChi-3*, *Neolamprologus brichardi* *AMCase*-like, *Oryzias latipes* chitotriosidase-1-like, *Larimichthys crocea* *AMCase*, *Thunnus orientalis* Chitinase3, *P. olivaceus* *fChi-3*, *Sebastes marmoratus* *SmChi-3* formed a new chitinase group, Fish Chitinase-3 (*FCCase-3*).

#### Conclusions

A Full-length cDNA of novel chitinase was obtained from the kidney of *S. melanostictus* (*SmeChi-3*, 1,531 bp), which contained 1,482 bp open reading frame. The domain structure of *SmeChi-3* was presumed according to those of *SmeChi-1* and *SmeChi-2*. *SmeChi-1* and *SmeChi-2* have a serine and glycine-rich linker region, which is characteristic in *AMCase*. In contrast, *SmeChi-3* contained no apparent sequence in the linker region. Phylogenetic analysis revealed the existence of new chitinase group, which was named *FCCase-3*, differed from *AFCCase-1* and *AFCCase-2*.

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