Molecular Cloning of a Putative Gastric Chitinase in the Toad Bufo japonicus

Authors: Oshima, Hiroyuki, Miyazaki, Rieko, Ohe, Yoshihide, Hayashi, Hiroaki, Kawamura, Kosuke, et al.

Source: Zoological Science, 19(3) : 293-297

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.293

Molecular Cloning of a Putative Gastric Chitinase in the Toad *Bufo japonicus*

Hiroyuki Oshima[1], Rieko Miyazaki[1], Yoshihide Ohe[2], Hiroaki Hayashi[3], Kosuke Kawamura[1] and Sakae Kikuyama[1]*

[1]*Department of Biology, School of Education, Waseda University, Nishi-waseda 1-6-1, Shinjuku-ku, Tokyo 169-8050, Japan*
[2]*Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-0034, Japan*
[3]*Gunma Prefectural College of Health Science, Maebashi 371-0052, Japan*

ABSTRACT—On the basis of our preliminary observation that a crude extract of the stomach of the toad *Bufo japonicus* exhibited a chitinase activity with its optimum pH around 3.0, we undertook molecular cloning of a cDNA encoding this putative gastric chitinase. By use of 2 degenerate oligonucleotide primers derived from the 2 conserved regions of the vertebrate chitinases, a reverse transcription-PCR product was obtained. This product was used as a probe to screen a cDNA library constructed from the toad stomach. The longest positive clone was revealed to contain an open reading frame for a putative chitinase protein of 484 amino acids, which protein exhibited sequence similarity to the known vertebrate chitinases. Our data also revealed this putative gastric chitinase to be distinct from the chitinase that we had previously isolated from the pancreas of the same species. In this putative gastric chitinase, both the N-terminal catalytic domain and the C-terminal chitin-binding domain were perfectly conserved, suggesting this protein to function as chitinase in the toad stomach.

Key words: amphibia, chitin, chitinase, stomach, toad

INTRODUCTION

Chitin is a β-1,4-linked homo polymer consisting of *N*-acetylglucosamine residues that constitutes the cell walls of fungi, the exoskeletons of arthropods including crustaceans and insects and the cuticles of annelids and mollusks (Gooday, 1996; Robyt, 1998). *In vivo* hydrolysis of chitin to acetylglucosamine is performed by the sequential action of a polysaccharidase (chitinase) and an oligosaccharidase (chitobiase; Jeuniaux, 1993). The presence of chitinolytic enzymes has been reported in various invertebrate species. Information about such enzymes in vertebrates, however, is limited. Recently, mammalian chitinases such as chitotriosidase from human macrophages (Renkema *et al*., 1995), acidic mammalian chitinase (AMCase) from the mouse stomach (Boot *et al*., 2001) and chitin-binding protein b04 (CBPb04) from bovine serum (Suzuki *et al*., 2001) have been identified. In lower vertebrates, there are several reports on the presence of chitinase activity mostly in digestive organs (Micha *et al*., 1973; Jeuniaux, 1993; Marsh *et al*., 2001). However, isolation and molecular characterization of chitinases in lower vertebrates have scarcely been attempted. Very recently, we purified a protein possessing a chitinolytic activity from the toad pancreas (Oshima *et al*., 2001). In fact, this chitinolytic protein was the first amphibian chitinase to be identified, as well as the first pancreatic chitinase to be found in vertebrates. During the course of our study on this toad pancreatic chitinase, we noticed the existence of chitinase activity in the stomach of the toad as well. This finding prompted us to perform molecular cloning of this putative gastric chitinase of the toad. Our results are herein reported.

* Corresponding author: Tel. +81-3-5286-1517;
FAX. +81-3-3207-9694.
E-mail: kikuyama@mn.waseda.ac.jp

MATERIALS AND METHODS

Isolation of total RNA and RT-PCR

Total RNA was isolated from the toad stomach by use of Isogen (Nippon Gene, Toyama, Japan). First-strand cDNA synthesis was performed on 3 µg of total RNA by using SuperScript II reverse transcriptase (GIBCO BRL, Rockville, MD) with oligo(dT)$_{12–18}$. After thermal denaturation of the enzyme, the cDNA was precipitated with ethanol and used as template for PCR amplification with degenerate primers. The primers were constructed on the basis of highly conserved regions of known vertebrate chitinases: 5'-CA (AG) TA (CT) (AC) G (ACGT) CC (ACGT) GA (CT) CA (AG) GG-3' as a sense primer and 5'-C (ACGT) A (AG) (AG) TC (AGT) AT (ACGT) GCCCA (ACGT) ACCAT-3' as an antisense primer. The conditions of the PCR reaction were denaturation at 94°C for 5 min followed by denaturation (94°C, 30 sec), annealing (55°C, 1 min) and extension (72°C, 1 min) reactions for 30 cycles. The amplified cDNA fragment was subcloned into plasmid pT7-blue (Novagen, Darmstadt, Germany). The plasmid containing the cDNA encoding toad gastric chitinase was used to transform JM109 competent cells (TaKaRa, Shiga, Japan) and was subjected to sequence analysis.

Construction and screening of a cDNA library from the toad stomach

A cDNA library of the toad stomach was constructed by using EcoRI-digested Lambda ZAP II (Stratagene, La Jolla, CA). The PCR-amplified chitinase cDNA fragment was labeled with [α-^{32}P]dCTP by the random-priming method using a BcaBEST Labeling Kit (TaKaRa) and was used to screen the cDNA. Hybridizations were performed at 60°C for 16 hr with the labeled cDNA encoding a partial sequence of toad chitinase. Clones giving positive signals

AG	-1
ATGGCAAAGCTTATCCTGTTCACCGGACTGGCATTGCTGCTGAACGCTCAGATAGGCTCTGCCTACGTGCTGTCA	75
M A K L I L F T G L A L L L N A Q I G S A Y V L S	25
TGCTATTTCACCAACTGGGCCCAGTACAGACCTGGACTGGGGAAGTTCAAGCCTGACAATATTGACCCATGTCTA	150
C Y F T N W A Q Y R P G L G K F K P D N I D P C L	50
TGTACTCACCTGATTTATGCCTTTGCTGGCATGTCAAACAACCAAATTGCCACAATTGAATGGAATGATGTAACT	225
C T H L I Y A F A G M S N N Q I A T I E W N D V T	75
TTGTACAGCTCTTTCCAAAACTTAAAAAATCAAAATGGTAACCTGAAGACTCTGCTGGCTATTGGTGGTTGGAAC	300
L Y S S F Q N L K N Q N G N L K T L L A I G G W N	100
TTTGGCACTGCACCTTTCACCACGATGGTCTCTACTGCTCAGAACCGCCAAACCTTCATCTCATCTGTGATCACA	375
F G T A P F T T M V S T A Q N R Q T F I S S V I T	125
TTCCTGCGTCAGTATGGTTTTGATGGGCTTGACATTGATTGGGAATACCCTGGCTCAAGAGGCAGCACTCCTCAG	450
F L R Q Y G F D G L D I D W E Y P G S R G S T P Q	150
GACAAAGCTCTATTTACCACTTTGGTTCAGGAAATGAGGGCAGCTTTTGAGACAGAGGCTTCACAATCAAATAAG	525
D K A L F T T L V Q E M R A A F E T E A S Q S N K	175
CCAAGACTCATGGTTACTGCTGCTGTGGCTGGTGGAATTTCCAACATTCAGTCAGGTTACCAGATTCCACAACTG	600
P R L M V T A A V A G G I S N I Q S G Y Q I P Q L	200
GCTCAGGCTTTGGATTACTTCCACGTTATGACCTATGATCTGCATGGTTCTTGGGAAGGATATACAGGAGAGAAC	675
A Q A L D Y F H V M T Y D L H G S W E G Y T G E N	225
AGCCCACTGTATTCTAACCCTTCTGCCACTGGTGCCAACTCTTACTTGAATGTGGATTATGTCATGAACTACTGG	750
S P L Y S N P S A T G A N S Y L N V D Y V M N Y W	250
CTTAACAATGGTGCCCCAGCTTCTAAACTCATTGTTGGATTCCCAACTTATGGACACACTTTCATCCTGAGCAAC	825
L N N G A P A S K L I V G F P T Y G H T F I L S N	275
CCATCTAACACTGCTATTGGTGCCCCTACTTCTGGACCTGGACCTGAAGGACCTTACACTAGGCAGTCTGGATTC	900
P S N T A I G A P T S G P G P E G P Y T R Q S G F	300
TGGGCCTACTATGAAATTTGTACTTTCCTGAAGAATGGAGCTACTAATGTGTGGTCTTCTGCTGAAGATGTCCCC	975
W A Y Y E I C T F L K N G A T N V W S S A E D V P	325
TATGCCTACCAGGGAAATGAGTGGTTGGGATATGACAATCAGAAGAGTTTCCAGATAAAGGCTCAGTGGCTGATG	1050
Y A Y Q G N E W L G Y D N Q K S F Q I K A Q W L M	350
AAGAACAACTTTGCAGGTGCTATGGTCTGGGCAATTGATTTGGATGATTTTACTGGTACTTTCTGTAACGAGGGC	1125
K N N F A G A M V W A I D L D D F T G T F C N E G	375
AAATATCCTCTGATATCCACACTGAAGAATACTTTAGGCGTTCAAGCTTCTGGATGCACACCACCTGCAATTCCT	1200
K Y P L I S T L K N T L G V Q A S G C T P P A I P	400
GTTGCTCCAATCACAGCTGCACCACAAACTGTGCCCAGTGGAGGTGGGTCCAGCGGAGGCTCAAGTGGTAGCTCA	1275
V A P I T A A P Q T V P S G G G S S G G S S G S S	425
GGTGGTAGCTCAGGAGGTAGTGGATTCTGCGTCGGCAAAGCCAGTGGACTGTACCCTGTGGCTGGAAACACGAAT	1350
G G S S G G S G F C V G K A S G L Y P V A G N T N	450
GCTTTCTGGCACTGCTTGAATGGCGTCACCTATGAACAATATTGCCAGGCTGGCCTTGTTTTTGACCCCAGCTGT	1425
A F W H C L N G V T Y E Q Y C Q A G L V F D P S C	475
GAGTGCTGCAACTGGCCATCATCTGTGTAGTGTTAGTAAAGGCTACACTGAAAATGTCAAACTGAATTGTTACAA	1500
E C C N W P S S V *	484
GTGCTAAAAAA**AATAAA**CAACATTCAGCATATTCAAAAA	1541

Fig. 1. Nucleotide and deduced amino acid sequences of putative tGCase cDNA. Annealing positions of primers for RT-PCR are boxed. The putative signal peptide region is underlined. An asterisk indicates the termination codon. A polyadenylation signal (AATAAA) is indicated by boldface type.

were obtained by *in vivo* excision. The cDNA sequences were analyzed by a cycle sequencing method on a DNA sequencer Model 4000L (LI-COR, Lincoln, NE). By use of a computer program (GENETYX-MAC), the molecular weight and isoelectric point of the putative chitinase predicted from a cDNA encoding the mature protein were calculated.

Northern blot analysis

Total RNAs extracted from various organs were electrophoresed in 1% formaldehyde-agarose gel and transferred to a nylon membrane. The RNAs were fixed on the membrane by UV-cross-linking. Hybridizations with the radiolabeled cDNAs were performed for 16 hr at 60°C following addition of the probe to the pre-hybridization solution (Sambrook *et al*., 1989). The filters were washed for 30 min at 60°C with 0.1-fold standard saline citrate containing 0.1% SDS and placed in contact with X-ray film (Eastman Kodak, Rochester, NY) for 16 h at −80°C.

RESULTS

One PCR product (997 bp) was obtained by using as primers synthetic nucleotides coding for a conserved region of vertebrate chitinase sequences. By employing this product as a probe, we isolated chitinase cDNA from a toad stomach cDNA library. The nucleotide sequence of the longest clone obtained was analyzed. This clone was estimated to be 1541 bp long, and it had an open reading frame of 1452 bp encoding the toad putative gastric chitinase protein consisting of 463 amino acid residues and a putative signal peptide of 21 amino acid residues (Fig. 1). The molecular mass and isoelectric point of the predicted mature protein were calculated to be 50 kDa and 4.89, respectively.

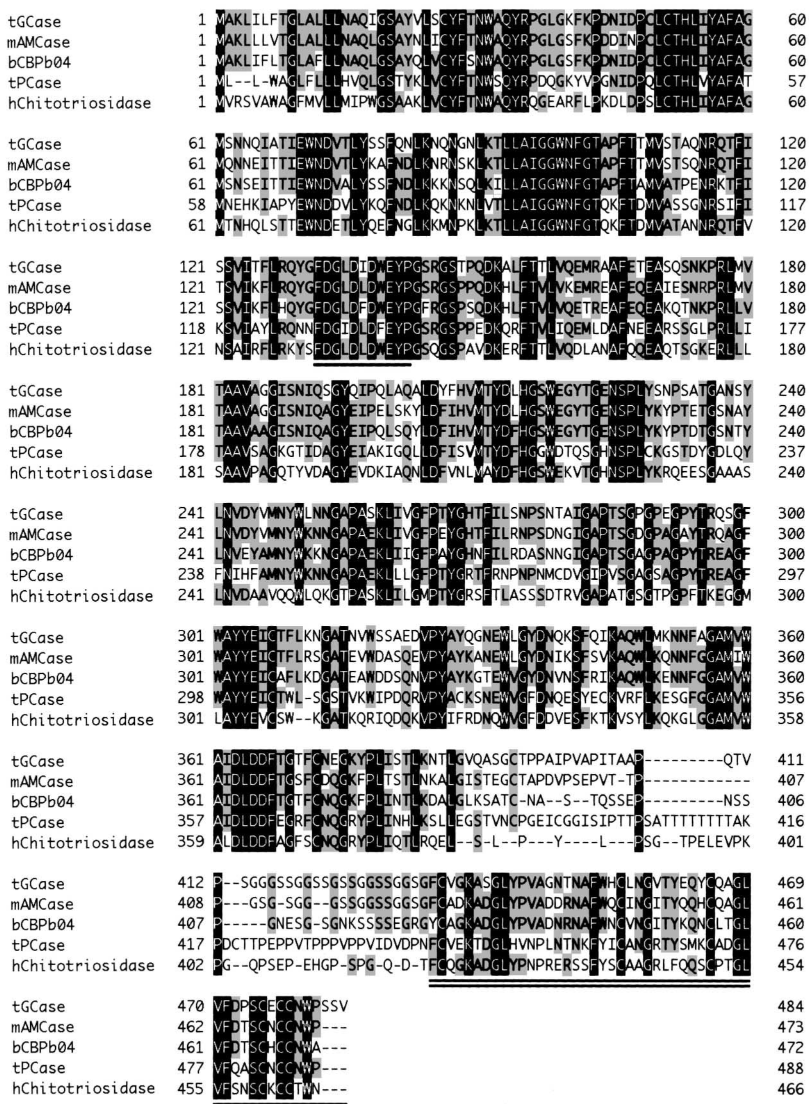

Fig. 2. Amino acid sequence comparison of putative tGCase, mouse (m) AMCase (Boot *et al*., 2001; accession number AF290003), bovine (b) CBPb04 (Suzuki *et al*., 2001; accession number AB051629), tPCase (Oshima *et al*., 2001; accession number AJ345054) and human (h) chitotriosidase (Boot *et al*., 1995; accession number U29615). Identical amino acid residues among the 5 chitinases are shown in white letters. Identical residues among any 3–4 chitinases are shadowed. The catalytic center for chitinase activity is underlined and the chitin-binding site, doubly underlined.

Fig. 3. Northern blot analysis of RNA from various organs of *Bufo japonicus* for detection of mRNA for tGCase. Total RNAs of the stomach (lane 1), brain (lane 2), kidney (lane 3), large intestine (lane 4), liver (lane 5), lung (lane 6), olfactory epithelium (lane 7), pancreas (lane 8) and small intestine (lane 9) were hybridized with the radiolabeled tGCase cDNA. The amount of the applied RNA was 15 µg in each case except for the stomach, where 5 µg RNA was applied.

Comparison of the amino acid sequence between the putative toad gastric chitinase (tGCase) and known vertebrate chitinase family proteins revealed homologies of 75.9, 70.3, 52.1 and 50.2% with mouse AMCase, bovine CBPb04, toad pancreatic chitinase and human chitotriosidase, respectively. Like these vertebrate chitinases, this putative tGCase was predicted to contain an N-terminal catalytic domain and a C-terminal chitin-binding domain (Fig. 2).

Northern blot analysis revealed the toad putative gastric chitinase mRNA to be 1.5 kb long and to be expressed in the stomach but not in other organs so far tested (Fig. 3).

DISCUSSION

Considering that amphibians eat chitin-covered preys, it is highly probable that chitinolytic enzymes would be required for the digestion of the ingested animals. In fact, Micha *et al*. (1973) demonstrated chitinase activity in the gastric mucosa and pancreas of 4 species of amphibians, i. e., *Rana temporaria*, *Bufo marinus*, *Salamandra salamandra taeniata* and *Triturus alpestris alpestinus*. However, it was only very recently that the isolation and molecular characterization of an amphibian chitinase was done. We isolated from the pancreas of the toad *Bufo japonicus* a 60-kDa protein possessing a potent chitinase activity with a considerable amino acid sequence homology (about 50%) with known mammalian chitinases (Oshima *et al*., 2002). Using a cDNA encoding this chitinase as a probe, we found the mRNA for this enzyme to be expressed exclusively in the pancreas. We designated this chitinase as toad pancreatic chitinase (tPCase). The optimum pH of tPCase was 6.0.

On the other hand, we noticed that a crude extract of the stomach from the same species exhibited chitinase activity (unpublished data), indicating that another chitinase, perhaps different from tPCase, exists in the stomach of the toad. In the present experiment, we obtained a cDNA clone encoding a putative tGCase from a cDNA library of the toad stomach, and found the predicted amino acid sequence to be distinct from that of tPCase. This putative tGCase was expressed in the stomach but not in other organs so far studied. It is of interest to note that its amino acid sequence showed higher homology with AMCase from the mouse stomach than with other known vertebrate chitinases of extra-stomach origin.

Chitinases are classified into 2 different families, namely, families 18 and 19, on the basis of the amino acid sequence similarity of their catalytic domain (Davies and Henrissat, 1995). Judging from the predicted amino acid sequence of the putative tGCase, this enzyme seems to belong to the family-18 chitinases. In these chitinases, the catalytic center of the chitinase activity was identified in a study using mutant recombinant chitinase (Renkema *et al*., 1998). In the case of family-18 chitinases, the second Asp (D) and Glu (E) in the DG-D-D-E motif of the N-terminal catalytic domain are considered to be essential for chitinase activity (Bleau *et al*., 1999). In addition, 6 cysteine residues forming 3 sets of disulfide bonds in the chitin-binding domain are reported to be essential for exerting chitinolysis (Tjoelker *et al*., 2000). In our putative tGCase, both of these structures were perfectly conserved (Fig. 3), suggesting that tGCase functions as a chitinolytic enzyme in the toad stomach.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid from the Ministry of Education, Science and Culture, of Japan and by a research grant from Waseda University to SK.

REFERENCES

Bleau G, Massicotte F, Merlen Y, Boisvert C (1999) Mammalian chitinase-like proteins. EXS 87: 211–221

Boot RG, Blommaart EF, Swart E, Ghauharali-van der Vlugt K, Bijl N, Moe C, Place A, Aerts JM (2001) Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J Biol Chem 276: 6770–6778

Davies G, Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. Structure 3: 853–859

Gooday GW (1996) Aggressive and defensive roles for chitinases. In: “Chitin Enzymology Vol 2” European Chitin Society, Ed by Muzzarelli RAA, pp 125–134

Jeuniaux C (1993) Chitinolytic systems in the digestive tract of vertebrates: a review. In: “Chitin Enzymology Vol 1” European Chitin Society, Ed by Muzzarelli RAA, pp 233–244

Marsh RS, Moe C, Lomneth RB, Fawcett JD, Place A (2001) Characterization of gastrointestinal chitinase in the lizard *Sceloporus undulatus garmani* (Reptilia: Phrynosomatidae). Comp Biochem Physiol B 128: 675–682

Micha JC, Dandrifosse G, Jeuniaux C (1973) Distribution and tissue localization of chitinase synthesis in lower vertebrates. Arch Int Physiol Biochim 81: 439–451

Oshima H, Miyazaki R, Ohe Y, Hayashi H, Kawamura K, Kikuyama S (2002) Isolation and sequence of a novel amphibian pancreatic chitinase. Comp Biochem Physiol B (in press)

Renkema GH, Boot RG, Muijsers AO, Donker-Koopman WE, Aerts JM (1995) Purification and characterization of human chitotri-

osidase, a novel member of the chitinase family of proteins. J Biol Chem 270: 2198–2202
Renkema GH, Boot RG, Au FL, Donker-Koopman WE, Strijland A, Muijsers, AO, Hrebicek M, Aerts JM (1998) Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. Eur J Biochem 251: 504–509
Robyt JF (1998) Essentials of Carbohydrate Chemistry, Springer-Verlag, New York
Sambrook J, Fritsch FE, Maniatis T (1989) Molecular cloning. 2nd ed, Cold Spring Harbor Laboratory Press, New York
Suzuki M, Morimatsu M, Yamashita T, Iwanaga T, Syuto B (2001) A novel serum chitinase that is expressed in bovine liver. FEBS Lett 506: 127–130
Tjoelker LW, Gosting L, Frey S, Hunter CL, Trong HL, Steiner B, Brammer H, Gray PW (2000) Structural and functional definition of the human chitinase chitin-binding domain. J Biol Chem 275: 514–520

(Received November 9, 2001/ Accepted November 26, 2001)