Cellular Expression of the Gut Chitinase in the Stomach of Frogs *Xenopus laevis* and *Rana catesbeiana*

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**ABSTRACT**

Gut chitinase, originally identified as acidic mammalian chitinase, is secreted from the salivary gland and stomach in humans and mice, but exclusively from the liver in cattle. Since the organs producing gut chitinase differ depending on the species, here we determined the expression site of this enzyme in the gastrointestinal tract of frogs, *Xenopus laevis* and *Rana catesbeiana*, which eat a large amount of chitin-coated animals, using *in situ* hybridization and immunohistochemistry. The chitinase was detected only in the oxyntic peptic cells of gastric glands and was accumulated in secretory granules in the cytoplasm, suggesting that the enzyme may be released into the gastric lumen directly. In addition, the enzyme was not found in the stomach of larvae, but was detected at stage 62 of metamorphosis and later, when the frogs changed from a herbivorous to a carnivorous diet.

Chitin is the most abundant glycopolymer in the integument of many species, such as insect exoskeletons, shells of crustaceans, and fungal cell walls (4, 11). Chitin-fragmenting hydrolases, chitinases, are also expressed by a vast array of organisms, including even those that do not contain chitin in the body (3). It was long generally believed that man and other mammals did not possess an enzyme analogous to chitinase (5, 9). However, Boot *et al.* (2) demonstrated that human macrophages produce and secrete chitinase (chitotriosidase). They also found the presence of a second mammalian chitinase, referred to as acidic mammalian chitinase (AMCase), in the salivary gland and the stomach of man and rodents (1). We recently found the presence of a chitin-binding protein (CBPb04) in bovine serum and identified it as an AMCase homologue. Interestingly, the bovine chitinase is only expressed in the hepatic parenchymal cells (12). In addition, we identified chicken AMCase (CBPch04) which is expressed in both the stomach and liver, and thus proposed calling this type of enzyme “gut chitinase” (13). These mammalian and avian gut chitinases are highly homologous to each other, with more than 75% amino acid sequence homology.

Gut chitinase may be one of the most important enzymes in animals that eat chitin-coated animals, such as insects. Recently, Oshima *et al.* (8) have cloned a putative gastric chitinase cDNA from the toad stomach as a counterpart of mammalian gut chitinase. However, the precise production site of the gut chitinase in the anura and its developmental expression pattern remain unknown. In this study, we examined the cellular and subcellular localization of gut chitinase in two frog species, *Xenopus laevis* and *Rana catesbeiana* by *in situ* hybridization and immunohistochemical analysis, and also exam-
ined the expression of the enzyme in the stomach of *X. laevis* during development.

**MATERIALS AND METHODS**

Partial cDNA of gut chitinases in *R. catesbeiana* and *X. laevis*. Total RNA was extracted from the stomach of *R. catesbeiana* or *X. laevis* by the guanidium isothiocyanate method. One µg of the RNA was reverse-transcribed using 60 units of Maloney leukemia virus reverse transcriptase (Gibco BRL, Grand Island, NY), 50 pmol of poly (dT) primer, and 2.5 nmol of dNTP in a total volume of 10 µl at 37°C for 1 h. The first-strand cDNA (1 µL) was then used as a template for polymerase chain reaction (PCR) amplification with 2.5 units Taq DNA polymerase (Promega, Madison, WI), 12.5 nmol of dNTPs, 3 mM MgCl₂, and 50 pmol of respective primer in a total volume of 50 µL. The forward primer was 5'-ATGCTATTTCAACACTGGG-3', which corresponds to the chicken gut chitinase sequence (ref. 13, Genbank accession number AB071038). The reverse primer was 5'-CCCAGTTGGTGAAATAGCAT-3', which is complementary to the human gut chitinase sequence (ref. 1, AF290004). PCR was conducted for 30 cycles, each consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and DNA extension at 72°C for 1 min. The PCR products were subcloned into pGEM-T vector (Promega), and their nucleotide sequence was analyzed by the cycle-sequence method. The partial sequences encoding frog chitinases were submitted to Genbank with the accession numbers AF447579 (*R. catesbeiana*) and AF447580 (*X. laevis*).

**Probes for in situ hybridization.** The following non-overlapping antisense oligonucleotides (for each frog) complementary to the gut chitinase cDNA were used for in situ hybridization: 5'-AGCCTGACAAAATTGATCCATGCTTGCTACTCACTCAGATCTATGC-3' and 5'-GGAGGTGGAACCTTGGAAATGCGGAGGTTGGAACCTTGGAATGC-3' for *R. catesbeiana* gut chitinase cDNA, and 5'-CAATGCTCTTTTGCATAGTCTCAGTCTGCGCCTGGAATGTCC-3' and 5'-GGAGATGGAACCTTCCCAGTGACTGACACCATTGCTAGTGTGCG-3' for *X. laevis* gut chitinase cDNA. All of these oligonucleotides were labeled with 32P-dATP using terminal deoxynucleotidyl transferase (Promega).

**Tissue samples for in situ hybridization.** Adult *R. catesbeiana* and *X. laevis* were used for the present in situ hybridization. The animals were sacrificed by destruction of the cervical spinal cord after ether anesthesia, and the stomach, lung, intestine, liver, kidney and spleen were rapidly removed and frozen in liquid nitrogen. The stomach was also collected from the larvae of *X. laevis* every 6 days after hatching, and from larvae during metamorphosis every 2 stages according to the classification by Nieuwkoop et al. (7). Cryostat sections, about 10 µm thick, were prepared and mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Shinetsu Chemical Industry, Tokyo, Japan).

**In situ hybridization.** The sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 15 min and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). The sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 8.0), 4 x SSC (1 x SSC=150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% sodium dodecyl sulfate (SDS), 200 µg/mL transfer RNA, 1 mM EDTA, and 10% dextran sulfate. Hybridization was performed at 42°C for 10 h in the prehybridization buffer supplemented with 10,000 cpm/µL of 32P-labeled oligonucleotide probes. The slides were washed at room temperature for 30 min in 2 x SSC containing 0.1% sarkosyl and twice at 55°C for 40 min each in 0.1 x SSC containing 0.1% sarkosyl. The sections were either exposed to BioMax MR film (Kodak, Rochester, NY) for 1 week or dipped in Kodak NTB2 nuclear track emulsion (Kodak) and exposed for 2 weeks.

**Western blot analysis.** The gastric mucosa was excised from *X. laevis*, homogenized and solubilized in ice-cold buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1% Triton X-100 for 30 min. The solubilized proteins were recovered by centrifugation and subjected to SDS-PAGE (12% gcl). The proteins were then transferred to a nitrocellulose membrane (Hybond-P; Amersham, Buckinghamshire, England) and incubated with antibody against bovine chitinase (CDPB04, 90 ng/mL) diluted in 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween 20. The bound antibody was visualized using peroxidase-labeled anti-rabbit IgG (Bio-Rad, California, USA) and the ECL system (Amer- sham) according to the manufacturer's directions.
Immunohistochemistry. The fresh stomach of *X. laevis* was immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 6 h and dipped in 30% sucrose solution overnight at 4°C. Tissues were embedded in Tissue-Tec O. C. T. compound (Sakura Finetechical, Tokyo, Japan) and quickly frozen in liquid nitrogen. Cryostat sections, 10 μm thick, were pretreated with 0.3% Triton X-100-containing PBS (pH 7.4) for 1 h, and with 0.03% H2O2 in methanol to inhibit the endogenous peroxidase activity.

Immunoreactivity for gut chitinase was detected by the avidin-biotin complex (ABC) method. After treatment with normal goat serum for 30 min, the sections were incubated overnight at room temperature with rabbit polyclonal antibody to CBPb04 (45 ng/mL in 0.01 M PBS). The antigen-antibody reactions were detected by incubation with biotin-labeled goat anti-rabbit IgG (Histofine kit; Nichirei, Tokyo, Japan), followed by streptavidin-peroxidase complex (VECTASTAIN; Vector Labs., Burlingame, USA). The peroxidase activity was visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.001% H2O2.

Immunoelectron microscopy. For immunoelectron microscopy, the stomach was processed for a pre-embedding silver-intensified immunogold method. Paraformaldehyde-fixed cryostat sections were incubated with antibody against CBPb04 (45 ng/mL in 0.01 M PBS), and subsequently reacted with goat anti-rabbit IgG covalently linked to 1.4-nm gold particles (BBInternational, Golden Gate, UK). Stained sections were osmicated, dehydrated, and embedded in Epon 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with an aqueous solution of 2% uranyl acetate for observation under an electron microscope (JEM-100SX; JEOL, Tokyo, Japan).

RESULTS

Partial cDNA cloning of frog gut chitinases and Western blot analysis

To determine the cellular localization of gut chitinase in frogs, cDNA cloning of the enzyme was performed using primers designed based on the human and chicken gut chitinase cDNAs. The nucleotide sequences of the cDNAs (both 305 bp in length) obtained from the stomach of *X. laevis* and *R. catesbeiana* (Fig. 1A) were highly homologous to those of avian and mammalian species (>73%), and the sequences of the cDNAs of these two frogs and a toad (*B. japonicus*) showed even higher homology (>83%) (Table 1). The predicted amino acid sequences of the two frog chitinases (Fig. 1B) also showed homology to those of chicken and mammals (>82%), and the sequences of the putative chitinases of the two frogs and the toad showed much higher homology (>95%) (Table 2).

The bovine chitinase antibody, which detects three isozymes of the enzyme (~50 kDa), cross-reacted predominantly with a 50-kDa protein in the *X. laevis* stomach extract, and with two minor proteins with molecular weights of 29 kDa and 20 kDa (Fig. 2, lane 2).

In situ hybridization of frog gut chitinases

*In situ* hybridization analysis of various organs in *X. laevis* and *R. catesbeiana* was performed using two non-cross-hybridizing antisense oligonucleotides as probes. X-ray film images of the gut chitinase expression showed intense and selective mRNA expression in the stomach, but no signal in other organs such as the lung, intestine, liver, kidney and spleen (Fig. 3). This result was consistent with the tissue distribution of the toad gastric chitinase (tGCase) mRNA determined by RT-PCR (8). In the stomachs of both frogs, the signals were detected in the gastric glands of the non-pyloric part consisting of oxynticopeptic cells, but not in the mucus cells that line the surface of the gastric mucosa and gastric pit (Fig. 4).

Immunohistochemistry and immunoelectron microscopy of frog gut chitinase

Immunohistochemical staining of the *X. laevis* stomach with antibody against bovine chitinase revealed that the immunoreactivity was localized in the gastric glands (Fig. 5A), in agreement with the distribution of chitinase mRNA detected by *in situ* hybridization. All secretory cells of the gastric gland were moderately stained, and some granular structures with intense reactivity were found in the cytoplasm (Fig. 5B). Immunoelectron microscopic analysis revealed immunogold particles gathered on some secretory granules, especially at the peripheral electron-pale region of the granule termed the halo (Fig. 6). However, various other granules were immunonegative.

Change in chitinase mRNA expression in the stomach of *X. laevis* during its metamorphic development

The structure of the stomach of *X. laevis* changes dramatically from the larval type to the adult type at stages 59–65 of metamorphosis (7). Histologically, the histolysis of mucosal epithelium occurs at stage
**Fig. 1A** Partial cDNA sequences of gut chitinase of the frogs *X. laevis* and *R. catesbeiana*. Partial nucleotide sequences of the cDNAs for frog gut chitinases (Genbank accession number AF447580 for *X. laevis*, and AF447579 for *R. catesbeiana*) were compared with the corresponding regions from *B. japonicus* (GCase, AJ345054), chicken (CBPb04, AB071039), cow (CBPb04, AB051629), mouse (AMCase, AF290003), and man (AMCase, AF290004). Dotted letters (c) represent nucleotides identical to those of *X. laevis*. Underlined regions were used as probes for *in situ* hybridization. Shadowed regions were used for designing PCR primers.

**Fig. 1B** Comparison of predicted amino acid sequences of gut chitinases. Dotted letters represent amino acids identical to those of *X. laevis* gut chitinase. The amino acid (D) marked with asterisks (*) is thought to be involved in the catalytic center for chitinase activity (14).
Table 1  Comparison of homology of the nucleotide sequences of gut chitinases of various species (%)  

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Table 2  Comparison of homology of the deduced amino acids of gut chitinases of various species (%)  

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59 in the non-pyloric part of the larval stomach, accompanied by the onset of mucosal reconstitution to form the adult type of gastric gland.

At stage 60, the chitinase mRNA was not found in the stomach of larvae by in situ hybridization (Fig. 7A). However, at stage 62, when the mucosal remodeling was almost finished, chitinase mRNA was detected in the non-pyloric part of the larval stomach (Fig. 7B). At stage 66, the intensity of the signal for chitinase mRNA was sharply increased and was comparable with that in the adult (Fig. 7C).

**DISCUSSION**

Gut chitinase has been identified and sequenced in man, mouse, cow and chicken (1, 12, 13). High sequence homologies among species (more than 70% for amino acid sequences) suggest the conservation of gut chitinase during evolution. Recently, Oshima et al. (8) cloned and characterized the cDNA of a chitinase from the stomach of the toad *B. japonicus*. Comparison of the amino acid sequence between the toad gastric chitinase and known mammalian chitinase family proteins revealed amino acid identities of 75.9 and 70.3% with mouse gut chitinase (AM-Case) and bovine gut chitinase (BHPb04), respectively. In the present study, we first determined the partial cDNA sequence of gut chitinase from two frog species, and showed that among these three anuran species, the sequence identity of the deduced 101 amino acids was extremely high (more than 95%).

We also showed the presence of immunoreactive proteins in the stomach of *X. laevis* by use of anti-

Fig. 2 Western blot analysis of gut chitinase in the stomach of *X. laevis*. The Triton-extract from the gastric mucosa of *X. laevis* was subjected to SDS-PAGE (12%) and probed using an anti-bovine gut chitinase antibody Lane 1, purified bovine chitinase (0.04 μg); Lane 2, the gastric-mucosa extract (2.75 μg).
Fig. 3  X-ray film images of gut chitinase mRNA expression in various tissues of *X. laevis*. Intense signals of gut chitinase mRNA is found in the stomach (St) but not in the duodenum (D), liver (Li), spleen (Sp) or lung (Lu) (A). The hybridization signals disappear when an excess of cold probe was added to the hybridization fluid (B).

Fig. 4  *In situ* hybridization for chitinase mRNA expression in the stomach of *X. laevis* (bright-field micrograph). Intense and selective expression of chitinase mRNA is detected in the gastric glands but not in the surface mucous cells. N: non-pyloric region, P: pyloric region, bar = 100 μm.

Fig. 5  Immunohistochemistry of gut chitinase in the stomach of *X. laevis*. The immunoreactivity for gut chitinase is diffusely distributed in the secretory cells (oxynticopeptic cells) of gastric glands (A). Note the intense immunoreactivity in granular structures dispersed in the cytoplasm (B). bars = 100 μm (A), 20 μm (B).
body against bovine gut chitinase. The immunoreactive predominant protein, which has a molecular weight 50 kDa, is thought to be the frog gut chitinase, as the enzymes of other species possess similar molecular weights (1, 8, 12, 13), and the predicted amino acid sequences of the frog chitinases are highly homologous to those of other species (Fig. 2, Table 2). The minor bands seen in the blot might be proteolytic products of the enzyme.

In previous reports (12, 13) we identified the expression sites of gut chitinase in the mouse, cow, and chicken by in situ hybridization analysis. The main organs for gut chitinase production are the parotid and gastric glands in the mouse, while in the cow only hepatocytes express the chitinase mRNA. The high level of bovine chitinase in the circulating blood implies that chitinase may be directly released from hepatocytes into the blood. The chicken produces gut chitinase in the glandular stomach (proventriculus) and liver, predominantly in the former. The present in situ hybridization and immunohistochemical analysis demonstrated the selective localization of frog gut chitinase in gastric glands. There was no production of gut chitinase in the liver in contrast to the expression pattern in chickens and cattle. In the frogs, the enzyme was detected in the oxynticopeptic cells, which secrete gastric acid and pepsinogen, but it was not detected in the mucous cells. Micha et al. (6) demonstrated chitinase activities in the gastric mucosa of 4 species of amphibians, i.e., Rana temporaria, Bufo marinus, Salamandra salamandra taeniata and Triturus alpestris alpestris. In the present study, we demonstrated the subcellular localization of the chitinase in the secretory granules, suggesting that the chitinase should be secreted into the gastric lumen, thereby facilitating the digestion of chitin. The enzyme may be active in the lumen, since the optimal pH of the toad chitinase is around 3.0 (8). Reportedly, the rodent and human gut chitinases are also stable in acidic conditions and have a pronounced pH optimum at pH 2.3. In the stomach of frogs (present study) and chickens (13), all secretory cells of the gastric gland intensely expressed chitinase mRNA. In the mouse, on the other hand, a subpopulation of the chief cells express chitinase mRNA; the population of chitinase-expressing chief cells appears to be less than one-third of the pepsinogen-expressing cells (13). The production of gut chitinase by a large proportion of gastric glands, possibly resulting in a very large amount of enzyme, in amphibian and avian species should be related to their feeding habits, i.e., that they eat a lot of insects and other prey coated by an chitin exoskeleton.
Fig. 7 Developmental change of gut chitinase mRNA expression in the stomach of *X. laevis*. No signals are detectable in the larval stomach at stage 60 (A). The first expression in the stomach appears in the non-pyloric part at stage 62 during metamorphosis (B). At stage 66, the signal intensity is as strong as that in adult frog (C). N: non-pyloric region, P: pyloric region, bar = 50 μm

It is interesting to note that gut chitinase was not found in the stomach of larvae, but appeared during the metamorphosis, along with structural changes of the stomach. The larvae of amphibians, including *X. laevis*, are herbivorous. After metamorphosis, the frogs construct an adult type stomach and start to eat prey covered with chitin. During metamorphosis, the stomach drastically changes and reconstructs the
mucosa to produce gastric acid and some digestive enzymes, possibly including gut chitinase. We recently demonstrated that the intensity of gut chitinase mRNA expression in the mouse stomach and parotid gland increased dramatically just before the weaning period (13). In the chicken, which eats prey just 1 day after hatching, significant expression of the enzyme mRNA was found in the stomach before hatching (13). These findings suggest that the expression of gut chitinase may be controlled to meet the demand for the enzyme to digest chitin-coated foods.

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REFERENCES