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Amphibian Pepsinogens: Purification and Characterization of *Xenopus* Pepsinogens, and Molecular Cloning of *Xenopus* and Bullfrog Pepsinogens[1]

Masayuki Ikuzawa,* Tomofumi Inokuchi,† Ken-ichiro Kobayashi,* and Shigeki Yasumasu*[2]

**Life Science Institute, Sophia University, Kioi-cho 7-1, Chiyoda-ku, Tokyo 102-8554; and †Department of Biology, Faculty of Education, Utsunomiya University Mine 350, Utsunomiya 321-8505*

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Two pepsinogens (Pg C and Pg A) were isolated from the stomach of adult *Xenopus laevis* by Q-Sepharose, Sephadex G-75, and Mono-Q column chromatographies. Autolytic conversion and activation of the purified Pgs into the pepsins were examined by acid treatment. We determined the amino acid sequences from the NH_2-termini of Pg C, pepsin C, Pg A, and pepsin A. Based on the sequences, the cDNAs for Pg C and Pg A were cloned from adult stomach RNA, and the complete amino acid sequences of the Pg C and Pg A were predicted. In addition, a Pg A cDNA was cloned from the stomach of adult bullfrog *Rana catesbeiana*, and the primary structure of the Pg A was predicted. Molecular phylogenetic analysis showed that such anuran Pg C and Pg A belong to the Pg C group and the Pg A group in vertebrates, respectively. The molecular properties of Pg C and Pg A, such as size, sequences of the activation peptide and active site, profile of autolytic activation, and pH dependency of proteolytic activity of the activated forms, pepsin C and pepsin A, resemble those of Pgs found in other vertebrates. However, the hemoglobin-hydrolyzing activity of *Xenopus* pepsin C is completely inhibited in the presence of equimolar pepstatin, an inhibitor of aspartic proteinases. Thus, the *Xenopus* pepsin C differs significantly from other vertebrate pepsins C in its high susceptibility to pepstatin, and closely resembles A-type pepsins.

Key words: pepsinogen, phylogeny, purification, stomach, *Xenopus laevis*.

In various vertebrates, pepsinogens (Pg) are zymogens for gastric aspartic proteinases, and are classified into four groups; Pg A, Pg C (progastricsin), prochymosin and Pg F. The four types of Pgs differ from each other in their primary structures and the enzymatic properties of their activated forms (*1–3*). So far, Pgs have been purified from the gastric mucosa of various vertebrates, and their primary structures have been reported in some studies; mammals such as human (*4–6*), Japanese monkey (*7*), new world monkeys (*8*), goat (*9*), bovine (*10*), rabbit (*11*), and house musk shrew (*12*), birds such as *Gallus gallus* (*2*, *13*) and Japanese quail *Coturnix coturnix japonica* (*14*), reptiles such as turtle *Trionyx sinensis* (*15*) and snake *Trimeresurus flavoviridis* (*16*), and fishes such as tuna *Thynuus orientalis* (*17*) and trout *Salmo gairdneri* (*18*).

In amphibians, Pg A-like and Pg C-like aspartic proteinases have been partially purified from the digestive tract of adult toad *Caudiverbera caudiverbera* (*19*). In addition, Pg C has been purified from the esophagus of adult bullfrog *Rana catesbeiana* (*20*). Recently, we purified Pg C and Pg A from the stomach of the bullfrog (*21*, *22*). However, studies on such aspartic proteinases in *Xenopus laevis* are scarce. In the present study, we purified Pg A and Pg C from the stomach of adult *X. laevis*, characterized some of their properties, and deduced their primary structures by molecular cloning using reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR).

Recently, a cDNA for bullfrog Pg C was cloned, and its primary structure was predicted (*20*). In the present study, we report a cDNA clone for bullfrog Pg A in addition to *Xenopus* Pg cDNAs. These studies, including the preceding study (*20*), are the first demonstration of the molecular cloning of amphibian pepsinogens.

MATERIALS AND METHODS

Adult South African clawed frog *X. laevis* and adult bullfrog *R. catesbeiana* were obtained from a commercial supplier. We purchased Q-Sepharose fast flow, Sephadex G-75, and a Mono-Q HR5/5 column from Amersham Pharmacia LKB Biotech (Uppsala, Sweden), pepstatin from the Protein Research Foundation (Osaka), porcine pepsin from Sigma (St. Louis, USA), a BCA™protein assay reagent from Pierce (Rockford, USA), molecular marker kit from Daiichi Pure Chemicals (Tokyo), an RT-PCR kit from Toyobo (Osaka), and a marathon cDNA amplification kit from

[1] The nucleotide sequence data reported in the present paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB045379, AB045380, and AB045376.

[2] To whom correspondence should be addressed. Tel: +81-3-3238-4263, Fax: +81-3-3238-3393, E-mail: s-yasuma@hoffman.cc.sophia.ac.jp

Clontech (Palo Alto, USA).

Standard Assay Method for Aspartic Proteinase—The proteolytic activity of pepsin was measured by the previous method (*22*) with a slight modification. The method is based on hemoglobin digestion. An adequate amount of enzyme sample was mixed with 0.4 ml of medium consisting of 2.5% bovine hemoglobin and 0.25 M sodium formate buffer (pH 3.0). After incubation at 37°C for 30 min, the reaction was stopped by adding 2.5 ml of 3.0% trichloroacetic acid (TCA). The mixture was allowed to stand in an ice-cold water bath for 10 min, and centrifuged at 1,600 g for 10 min. The absorbance of the supernatant at 280 nm was measured. One unit of activity was defined as a change of absorbance of 1.0 during an incubation time of 30 min.

In general, pepsinogen is a precursor form of pepsin, and is considered to have little or no proteolytic activity. However, under acidic conditions such as pH 1 to 3, it is autolytically converted into an active form, pepsin. Therefore, this assay method is also used to estimate the potential activity of pepsinogen.

Determination of Protein Amount—The amount of protein was estimated by the BCA method with bovine serum albumin as a standard according to the instructions of the manufacturer (Pierce). In column chromatography for purification, amount of protein in the eluant was monitored by absorption at 280 nm.

Purification Procedure for Pepsinogens—All procedures except for step 4 described later were performed at 4°C.

Step1. Preparation of crude extract: Adult *Xenopus* were anesthetized on ice and the spinal cords were cut. The abdomen was incised with scissors and ten stomachs (total weight 3.3 g) were obtained. The stomachs were washed, minced and homogenized in 50 ml of 0.15 M NaCl-20 mM Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 100,000 $\times g$ for 2 h. The supernatant, crude extract, was used as a starting material for the purification of Pgs.

Step 2. Q-Sepharose column chromatography: The crude extract was diluted about 2-fold with 20 mM Tris-HCl buffer (pH 7.5) and applied to a column (1 × 13 cm) of Q Sepharose equilibrated with 20 mM Tris-HCl buffer (pH 7.5). After washing the column with the same buffer, elution was performed with 120 ml of a solution consisting of a linear gradient of 0.1 to 0.5 M NaCl buffered with 20 mM Tris-HCl (pH 7.5).

Step 3. Sephadex G-75 gel filtration: The fractions obtained in step 2 were pooled and concentrated by CENTRIPULUS (Millipore, MA, USA). Then the samples were applied to a column (2.6 × 100 cm) of Sephadex G-75 equilibrated with 0.15 M NaCl-20 mM Tris-HCl (pH 7.5). Elution was performed with the same buffer.

Step 4. Mono-Q column chromatography: The fractions obtained in step 3 were applied to a column (5 × 50 mm) of Mono-Q equilibrated with 0.075 M NaCl–20 mM Tris-HCl (pH 7.5), and eluted with a linear gradient of NaCl from 0 to 0.3 M in a total volume of 60 ml. This step was performed at room temperature.

Electrophoresis—Polyacrylamide gel electrophoresis (PAGE) was carried out by the previous method (*21*) using a 7% slab gel. After electrophoresis, the gel was incubated with 1% hemoglobin in 0.1 M formate buffer (pH 3) for 30 min at 37°C, and then incubated for 20 to 30 min without substrate at 37°C. After that, the gel was stained with 1% Amido Black 10B in 7% acetic acid and destained with 7% acetic acid. A zymogram such as the one shown in Fig 2A showed bands on the gel with acid proteinase activity. Protein bands were detected by staining the gel with Coomassie Brilliant Blue R-250 (CBB).

Sodium dodecylsulfate (SDS)–PAGE was carried out according to Laemmli (*23*) using a 10% gel. An adequate amount of sample was added to the sample buffer. The final concentrations of SDS, 2-mercaptoethanol, and Tris-HCl (pH 6.8) in the mixture were 1.0%, 0.5%, and 30 mM, respectively. The mixture was incubated for 5 min in a boiling water bath. After electrophoresis, the gel was stained with CBB.

Characterization of the Proteolytic Activity of Pepsin—The pH of a 0.1 ml solution containing purified Pg was adjusted to 2.0 by adding 0.1 N HCl. After incubation at 20°C, aliquots of the solution were withdrawn at appropriate intervals and subjected to SDS-PAGE. The gel was stained with a silver stain kit. Such acid treatment converted Pg into an activated form, pepsin.

After acid treatment of Pg, some of the enzymatic properties of the pepsin were analyzed. The pH dependency of the activity was examined over a range of pH 1.0 to 5.0 adjusted with 1.0 N HCl using 2.5% hemoglobin as the substrate.

The inhibitory effect of pepstatin on the pepsin activity was examined by the method of Inokuchi *et al.* (*24*).

Effect of Treatment of Pepsinogens with Glycopeptidase F—Pg was incubated with 1 milliunit of glycopeptidase F (Takara Shuzo, Otsu) for 15 h at 37°C in 0.1 mM Tris-HCl buffer, pH 8.6, containing 0.1% SDS, 0.1% 2-mercaptoethanol, and 1% nonidet P-40. The molecular mass of Pg was analyzed by SDS-PAGE.

Determination of Amino Acid Sequence of the NH_2-Terminal Regions of Pepsinogen and Pepsin—The amino acid sequence of purified Pg was determined by Edman degradation using an automatic amino acid sequencer (Protein Sequencer PSQ-1, Shimadzu, Kyoto). The purified Pg was converted to pepsin by acid treatment as described above and subjected to Mono-Q column chromatography. The conditions of the chromatography were similar to those for purification step 4 (Data not shown). The amino acid sequence of the pepsin isolated by chromatography was determined using PSQ-1.

Cloning of cDNAs for Xenopus Pepsinogens—Total gastric RNA was extracted using an RNA isolation kit and subjected to RT-PCR. Based on the amino acid sequences of the NH_2-terminal regions of Pgs and pepsins, we designed the following primers:

5′-GTNCCNYTNAARAARTTYAARWSNATG-3′: *Xenopus* Pg C
5′-TCRTGRTARTANSWCATRTCCAT-3′: *Xenopus* pepsin C
5′-GTNAARGTNCCNYTNMGNAARGGNGA-3′: *Xenopus* Pg A
5′-GTNCCRTARTAYTCNATRTCCAT-3′: *Xenopus* pepsin A

A 99-base fragment for Pg C and a 126-base fragment for Pg A were obtained by RT-PCR, and subjected to 3′-RACE and 5′-RACE (*25*) using an Advantage cDNA PCR kit. The amplified cDNA fragments were ligated into T4 easy vector, and the nucleotide sequences of the cDNAs were determined by a DNA sequencer (377 DNA Sequencer, ABI) according to the dideoxynucleotide chain termination method using a Big dye cycle sequencing kit.

Cloning of a cDNA for Bullfrog Pepsinogen A—Recently, we purified Pgs C and A from the stomach of adult bullfrog,

characterized some their properties, and determined the amino acid sequences of the NH_2-terminal regions of the Pgs and pepsins (*22*). Using the sequences of Pg A and pepsin A, we designed the following primers for RT-PCR:

5′-AARAARCAYCAYTAYAAYCC-3′: bullfrog Pg A
5′-TCDATRTCCATRTARTTYTG-3′: bullfrog pepsin A

Forty-nine-base fragments were obtained from total gastric RNA by RT-PCR. The 5′-end of the cDNA encoding Pg A was amplified according to the method for single strand ligation of cDNA (*26*), and the 3′-RACE method. Using the primer and an oligo (dT) primer, a cDNA of Pg A was amplified by RT-PCR. The isolated fragment was ligated to pGEM-T Vector (Promega, WI, USA) and sequenced.

RESULTS

Purification of Xenopus Pepsinogens—The hemoglobin-hydrolyzing activities of a crude extract prepared from adult *Xenopus* stomach were visualized on a PAGE gel. Two major bands were detectable in the zymogram (Fig. 2A), and were considered to be those of gastric aspartic proteinases and/or their zymogens because the activities became apparent at acidic pH. Based on the similarity of electrophoretic mobility of bullfrog Pg (*21*), we tentatively defined the two major proteinases as *Xenopus* Pg C and Pg A as shown in Fig. 2A.

We purified the Pg C and Pg A by sequential column chromatography steps as described in "MATERIALS AND METHODS." Two peaks of hemoglobin-digesting activity were detected by the Q-Sepharose column chromatography in purification step 1 (Fig. 1). A zymogram showed that the first and second peak contained mainly Pg C and Pg A, respectively. The fractions in each peak were pooled and subjected to Sephadex G-75 gel filtration. Finally, Pg C or Pg A was purified by Mono-Q Sepharose chromatography. A typical case of purification is summarized in Table I. The specific activities of purified *Xenopus* Pg C and A were compared with that of porcine pepsin (425 units/mg); the activity of *Xenopus* Pg C was almost equal, while that of Pg A was about one-half. Figure 2 shows the electrophoretic patterns of purified *Xenopus* Pg C and Pg A; both purified samples give single bands on SDS-PAGE and native PAGE.

Amino Acid Sequences of Pepsinogens and Pepsins—In the present study, we cloned cDNAs for Pg C and Pg A using RT-PCR and 3′- and 5′-RACE-PCR methods. As shown in Fig. 3A, the nucleotide sequence of *Xenopus* Pg C cDNA consists of an open reading frame from the initiation codon (ATG) to the stop codon (TGA) and a 3′ non-coding sequence containing a polyadenylation signal (AATAAA) and a poly A tail. The 5′ non-coding sequence was missing from the clone. As shown in Fig. 3B, we obtained a cDNA for *Xenopus* Pg A comprising a 5′ non-coding sequence, an open reading frame of ATG to TAA, a polyadenylation signal (AATAAA), and a poly A tail. The Pg A cDNA clone was characteristic of a long 3′ non-coding sequence. Figure 3C shows the nucleotide sequence of bullfrog Pg A cDNA consisting of a 5′ non-coding sequence, an open reading frame (ATG to TAA), a polyadenylation signal (AATAAA), and a

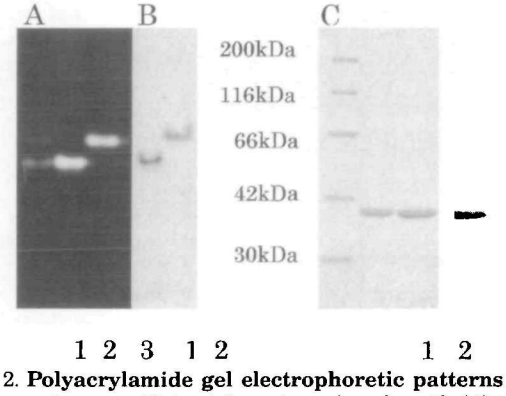


Fig. 2. **Polyacrylamide gel electrophoretic patterns of *Xenopus* pepsinogens.** Extract from stomach and purified Pgs were analyzed by polyacrilamide gel electrophoresis (PAGE) using a 7% slab gel. A: After electrophoresis, proteolytic activities were visualized on the gel as described in "MATERIALS and METHODS." Lanes 1, 2, and 3 indicate crude extract (2 μg), Pg C (2 μg) and Pg A (2 μg), respectively. B: After electrophoresis, the gel was stained with Coomassie Brilliant Blue R 250. Lane 1, purified Pg C (10 μg); lane 2, purified Pg A (10 μg). C: Purified Pgs were analyzed by SDS-PAGE. Lane 1, purified Pg C (10 μg); lane 2, purified Pg A (10 μg).

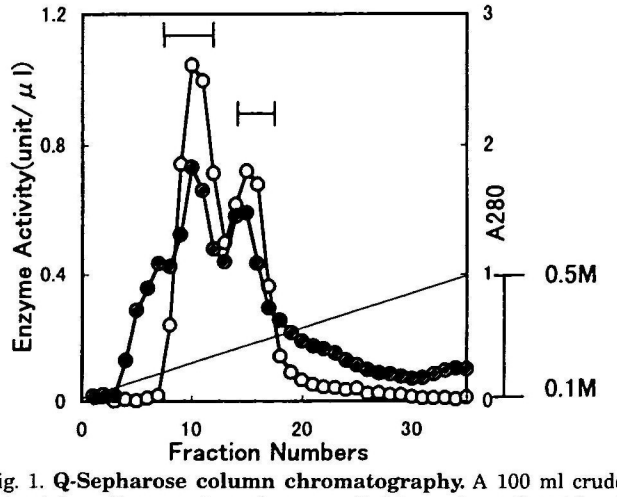


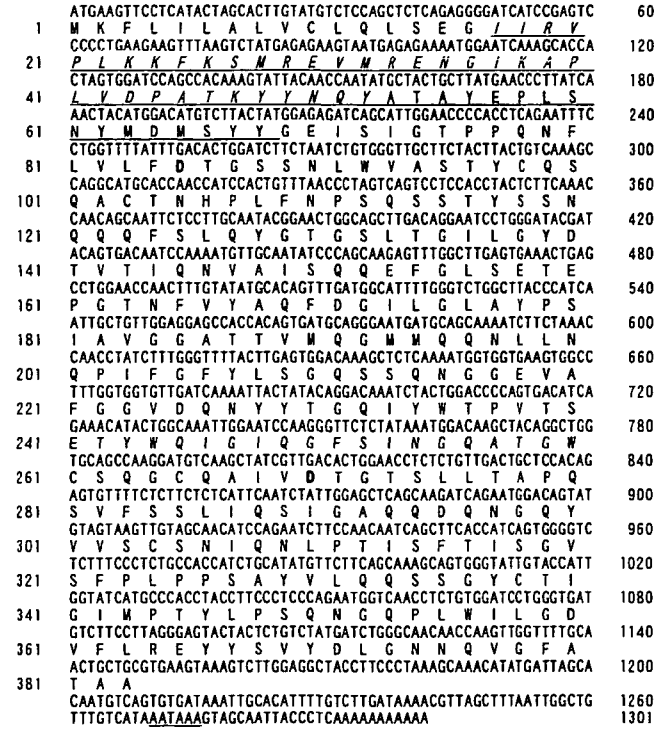
Fig. 1. **Q-Sepharose column chromatography.** A 100 ml crude extract from *Xenopus* stomach was applied to a column (1 × 13 cm) of Q-Sepharose equilibrated with 20 mM Tris-HCl (pH 7.5). After the column was washed with about 50 ml of the same buffer, elution was performed with a buffer consisting of a linear gradient of 0.1 to 0.5 M NaCl buffered with 20 mM Tris-HCl (pH 7.5): flow rate, 30 ml/h, fraction volume, 4 ml. ●, absorbance at 280 nm; ○, enzyme activity. The fractions indicated with bars were pooled and subjected to the next step.

TABLE I. **Summary of the purification of *Xenopus* pepsinogens C and A.**

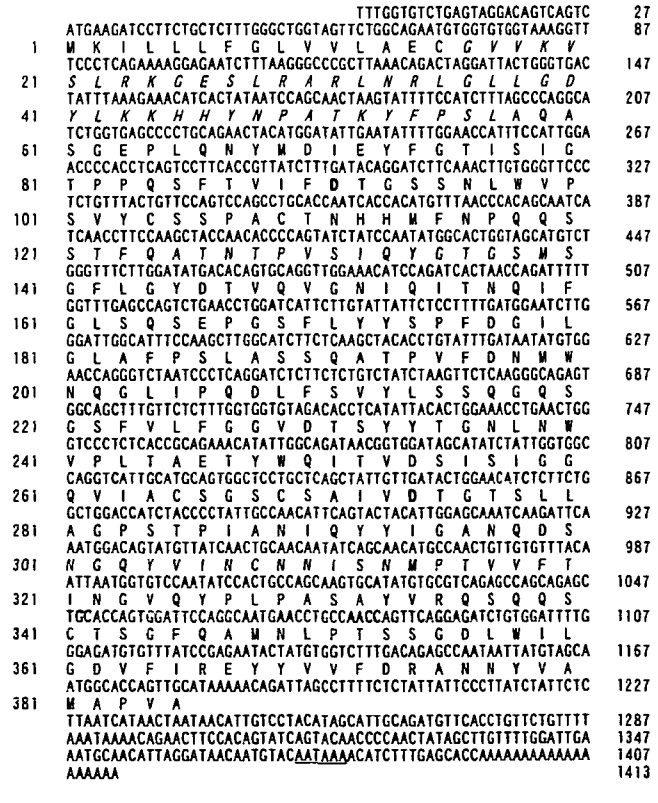
	Protein (mg)	Activity (unit)	Specific activity (unit/mg protein)	Yield (%)
Crude extract	130	11,500	88.5	100
Q-Sepharose column chromatography				
Pg C	23	7,980	347	69.4
Pg A	20	1,420	70.8	12.3
Sephadex G-75 column chromatography				
Pg C	11.4	5,130	450	44.6
Pg A	4.2	540	129	4.7
Mono-Q column chromatography				
Pg C	1.6	750	457	6.5
Pg A	0.8	191	227	1.7

poly A tail. The 3′ non-coding sequence of the bullfrog Pg A cDNA is shorter than that of the *Xenopus* Pg A cDNA. The complete amino acid sequences of Pg C and A were predicted from the nucleotide sequences of the respective cDNAs (Fig. 3).

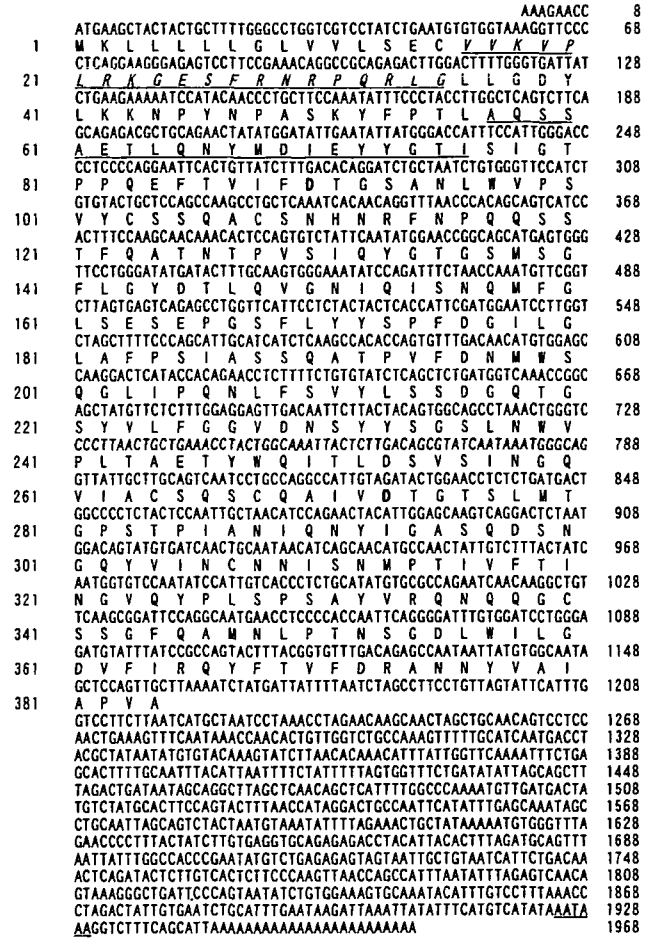
In the present study, we determined the amino acid



(A)



(C)



(B)

Fig. 3. **Nucleotide sequences of cDNA clones and deduced amino acid sequences of pepsinogens (A, *Xenopus* Pg C; B, *Xenopus* Pg A; C, bullfrog Pg A).** The top and second lines represent the nucleotide sequence of cDNA and the predicted amino acid sequence, respectively. ATG, TGA (A) or TAA (B and C), and AATAAA underlined in the nucleotide sequences indicate initiation codon, stop codon, and polyadenylation signal, respectively. The amino acid sequences underlined represent those of purified Pgs and pepsins determined by Edman degradation. Aspartic acid residues indicated by a bold letter **D** (Asp^{55} and Asp^{270} in *Xenopus* Pg C; Asp^{90} and Asp^{273} in *Xenopus* Pg A; Asp^{91} and Asp^{274} in bullfrog Pg A) are highly conserved among vertebrate Pgs, and are considered to be catalytic residues. The full sequences of Pgs contain sequences identical to the NH_2-terminal regions of purified Pgs and their activated forms, pepsins.

sequences from the NH_2-termini of purified *Xenopus* Pg C and Pg A using a Protein Sequencer. In addition, the NH_2-terminal sequence of bullfrog Pg A was determined in our previous study (*22*). As shown in Fig. 3, sequences identical to these sequences were found near the NH_2-terminal regions of the full sequences deduced from the respective cDNAs. The results show that the N-terminal sequences of Met^1 to Gly^{16} in Fig. 3A, Met^1 to Cys^{15} in Fig. 3B, and Met^1 to Cys^{15} in Fig. 3C are missing in purified *Xenopus* Pg C, *Xenopus* Pg A, and bullfrog Pg A, respectively. It is reasonable to conclude that these are hydrophobic signal peptides in the respective Pgs.

We determined some amino acid sequences from the NH_2-termini of the activated forms of Pgs, pepsins, using a Protein Sequencer. As shown in Fig. 3, the full Pg sequences contained sequences identical to the NH_2-terminal regions. The results show that the activation peptides of *Xenopus* Pg C, *Xenopus* Pg A, and bullfrog Pg A consist of Ile^{17} to Tyr^{52} in Fig. 3A, Val^{16} to Leu^{56} in Fig. 3B, and Gly^{16} to Leu^{57} in Fig. 3C, respectively.

The molecular weights of the Pgs and pepsins calculated from the deduced sequences were as follows: 39,962 for *Xenopus* Pg C, 35,682 for *Xenopus* pepsin C, 40,245 for *Xenopus* Pg A, 35,502 *Xenopus* for pepsin A, 40,057 for bullfrog Pg A, and 35,279 for bullfrog pepsin A. These are similar to the molecular masses of the purified Pgs and pepsins determined by SDS-PAGE: 40 kDa for *Xenopus* Pg C, 34 kDa for *Xenopus* pepsin C, 40 kDa for *Xenopus* Pg A, 34 kDa for *Xenopus* pepsin A (Fig. 2C), 40 kDa for bullfrog Pg A, and 36 kDa for bullfrog pepsin A (*22*).

Amino acid sequences of various vertebrate Pgs were obtained by computer-aided search of the nucleotide/protein database. The sequences of Pgs, including *Xenopus* and bullfrog Pgs, were compared and aligned by the program CLUSTAL W ver 1.7 (*27*). Such multiple alignments show that Asp residues at putative catalytic sites are found in the *Xenopus* and bullfrog sequences as shown in Fig. 3, and their positions in the overall lengths of the anuran sequences are similar to those in various Pgs.

Sequence identities of *Xenopus* Pg C to bullfrog Pg C, chicken Pg C, and Japanese monkey Pg C are 92.2, 70.4, and 66.7%, respectively, while the identities of *Xenopus* Pg A to tuna Pg II, bullfrog Pg A, turtle Pg A, chicken Pg A, and Japanese monkey Pg A are 55.8, 85.5, 63.0, 59.6, and 67.5%, respectively.

Enzymatic Properties of Xenopus Pepsins—As is well known for Pgs from various animals (*1*, *30*), *Xenopus* Pgs undergo limited hydrolysis and are converted to activated forms, pepsins. The activation profile of Pg was analyzed at 20°C at pH 2.0 (Fig. 4). The activated form of Pg C, pepsin C, can be observed after 2 min incubation, while pepsin A appears 5 min later. Two types of intermediate forms are found during acid activation. The complete activations of Pg C and Pg A occur after 20 and 30 min, respectively (data

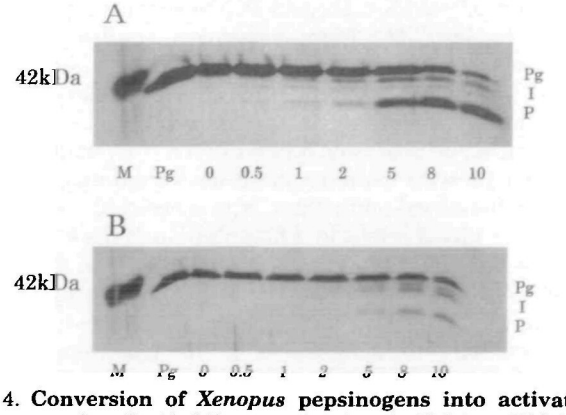


Fig. 4. **Conversion of *Xenopus* pepsinogens into activated forms, pepsins.** Purified Pgs were incubated pH 2.0 at 20°C. The amount of protein applied to each lane is 2 μg. A: PgC, B: PgA. M: Molecular marker, Pg: Pgs, P: pepsins, I: Intermediate forms appearing during the conversion of Pgs into pepsins. The incubation time (in min) is indicated below the two panels.

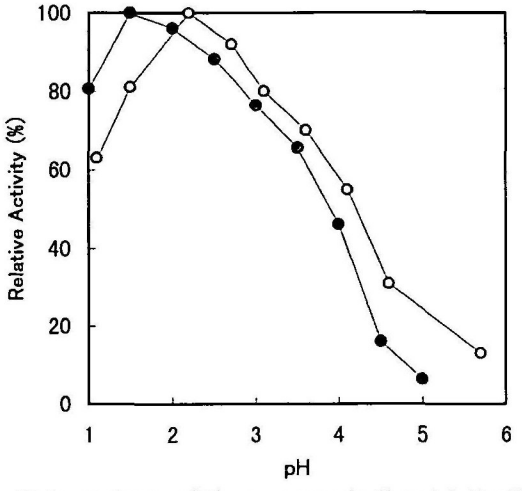


Fig. 5. **pH dependency of *Xenopus* pepsin C and A.** Purified Pg C and Pg A were incubated at pH 2.0 at 20°C for 30 min and for 90 min, respectively. Such acid treatment converted the Pgs into pepsins. An aliquot of pepsin C (2.4 μg) or A (2.5 μg) thus prepared was assayed in 0.4 ml of 2.5% hemoglobin solution at various pHs at 37°C. The incubation of pepsin C or pepsin A was performed for 30 min. The activity is expressed as percentages of the highest activity. ●, pepsin C; ○, pepsin A

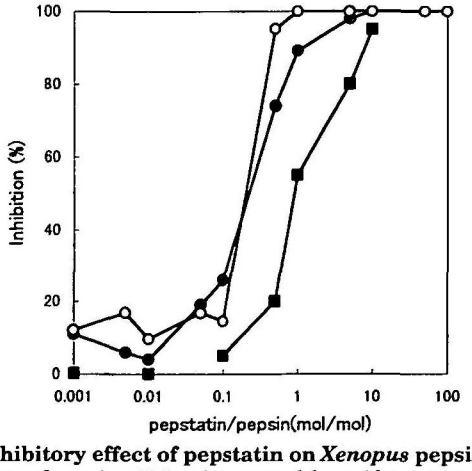


Fig. 6. **Inhibitory effect of pepstatin on *Xenopus* pepsin C and A.** Aliquots of pepsins (6.5 μg) prepared by acid treatment of Pgs were assayed at pH 3.0 in 0.4 ml of a solution consisting of 2.5% hemoglobin and various concentrations of pepstatin at 37°C for 30 min. ●, pepsin C; ○, pepsin A. A profile of the inhibitory effect of pepstatin on bullfrog pepsin C is cited from Inokuchi *et al.* (*21*) and depicted in the figure. The inhibition is expressed as percentages of the highest inhibition.

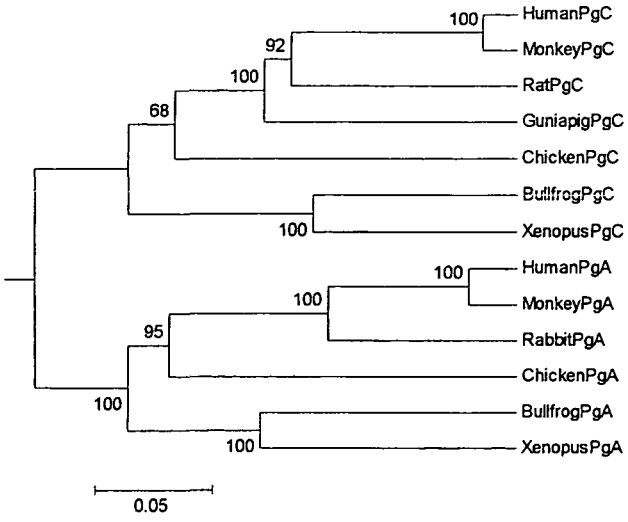


Fig. 7. **Molecular phylogenetic analysis of vertebrate pepsinogens.** Nucleotide sequences of Pgs from various vertebrates including *Xenopus laevis* and bullfrog were aligned using the program CLUSTAL W ver 1.7 (*27*). A tree was constructed by UPGMA (*28*) using the program MEGA (*29*). Bootstrap values are obtained for 1,000 replicates and values higher than 50% are indicated above or below the branches. The evolutionary distance was expressed in terms of substitutions per site. Human Pg C (*6*), Japanese monkey Pg C (*34*), guinea pig Pg C (*35*), rat Pg C (*36*), chicken Pg C (*2*), bullfrog Pg C (*20*), human Pg A (*5*), Japanese monkey Pg A (*34*), rabbit Pg A (*3*), chicken Pg A (*2*),

not shown).

Figure 5 shows the pH dependency of pepsin activity. Optimum activity of *Xenopus* pepsin C was observed around pH 1.5, while that of *Xenopus* pepsin A was around pH 2.2. The activities of both the pepsins were undetectable at pHs above 5.0. Such properties of *Xenopus* Pgs and pepsins are similar to those of various vertebrates including bullfrog (*21*).

It is well known that the proteolytic activity of pepsin is strongly inhibited by an aspartic proteinase inhibitor, pepstatin. As shown in Fig. 6, the inhibitory effect of pepstatin on *Xenopus* pepsin A is quite consistent with the effects on various vertebrate pepsins A (*4*, *8*, *9*, *12*, *22*), showing equimolar inhibition: the activity of 1 mol of pepsin A is completely inhibited by 1 mol of pepstatin. In general, pepsin C is known to be less sensitive to the inhibitor than the A-type pepsins. For example, bullfrog pepsin C requires a 10-molar excess of pepstatin for complete inhibition (Fig. 6; *22*). Mammalian pepsins C, such as those from house musk shrew (*12*), goat (*9*) or new world monkeys (*8*), require a 100-molar excess. As shown in Fig. 6, however, *Xenopus* pepsin C is inhibited by equimolar pepstatin. Such high susceptibility to pepstatin is a characteristic of *Xenopus* pepsin C.

Xenopus pepsinogens C and A have potential *N*-glycosilation sites, Asn^{110}-Pro^{111}-Ser^{112}, and Asn^{301}-Ile^{302}-Ser^{303}, respectively. The *Xenopus* Pgs were treated with glycopeptidase F. However, no decrease in their molecular sizes was detected (data not shown).

DISCUSSION

Pepsinogens (Pgs) of an amphibian, bullfrog, have recently been purified from both stomach and esophagus (*20*, *21*). However, the activity of the aspartic proteinase could not be detected in the esophagus of *X. laevis*. Therefore, we purified pepsinogen from the stomach, and isolated Pg C and Pg A in the present study.

Based on amino acid sequences of the NH_2-terminal regions of the purified Pgs and pepsins, cDNAs for the Pgs were cloned by PCR and the 3′- and 5′-RACE-PCR method, and the complete amino acid sequences were predicted from the cDNAs. Some sequences conserved in almost all vertebrate Pgs were found in comparable regions in the *Xenopus* Pgs and bullfrog Pg A: the active site sequences around the putative catalytic sites include Asp^{85} and Asp^{270} in *Xenopus* Pg C; Asp^{90} and Asp^{273} in *Xenopus* Pg A; Asp^{91} and Asp^{274} in bullfrog Pg A and six cysteines probably forming three disulfide bonds.

Differences in susceptibility to pepstatin is suggested to be useful for distinguishing pepsin C from pepsin A (*8*). However, the *Xenopus* pepsin C differed significantly from other vertebrate pepsins C in susceptibility, and was nearly the same as A-type pepsins.

We conducted a tentative molecular phylogenetic analysis of vertebrate Pgs. A tree was constructed from the nucleotide sequences of the open reading frames of vertebrate Pg cDNAs by the UPGMA (*28*) using the program MEGA (*29*). As shown in Fig. 7, two groups were definitely distinguishable; the Pg C group and the Pg A group. The tree suggests that amphibian (*Xenopus* and bullfrog) Pg C and Pg A diverged earlier from chicken and mammalian Pg C and Pg A, respectively. In each group, amphibian Pg diverged earlier than chicken and mammalian Pgs.

Some mammals have a neonate-specific pepsinogen, prochymosin (*1*, *30*). Recently, it has been demonstrated that the rabbit neonatal pepsinogen is different from adult pepsinogen, Pg A, in its primary structure; it is named Pg F. Embryonic pepsinogen, which is classified as prochymosin, is also found in chicken embryos. During development, the expression of embryonic or neonatal Pgs is known to transit that of Pg A/C (*2*, *3*). Such embryonic or larval Pg has not been found in anuran tadpoles. Recently, a cathepsin E-type aspartic proteinase was detected in the foregut of bullfrog tadpoles (*21*, *31*).

In *X. laevis*, although studies on Pgs are scarce, it has been suggested that a pepsin-like activity is detectable in adult stomach, while cathepsin D–like activity is present in tadpole foregut (*32*). Immunochemical studies using an antibody against bullfrog Pg C suggested that *Xenopus* Pg is newly expressed in the digestive tract of froglets immediately after metamorphosis (*33*). The result implies that the Pg-producing cells become differentiated from adult epithelial primodia with metamorphosis-associated apoptotic cell death in the larval epithelium. It is conceivable that apoptosis of the larval cells and differentiation of the Pg-producing cells are induced by thyroid hormone. Thus, the purification, characterization, and cDNA cloning of aspartic proteinases expressed in adult and/or larval digestive tracts will contribute to understanding the molecular mechanisms of metamorphosis.

In the present study, we purified Pg A and Pg C from the stomach of adult *X. laevis*, characterized some of their properties, and deduced their primary structures by molecular cloning. Our preliminary study suggests that cathepsin E–like proteinase is found in the digestive tract of *Xenopus*

tadpoles. In the near future, we will describe the purification, characterization and cDNA cloning of the cathepsin E-like proteinase of *Xenopus* tadpoles.

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