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Cloning of cDNA Encoding Thimet Oligopeptidase from *Xenopus* Oocytes and Regulation of the mRNA During Oogenesis

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ABSTRACT—We have isolated a cDNA clone for a zinc-requiring metallopeptidase in *Xenopus* oocytes from *Xenopus* ovary library using oligonucleotides synthesized on the basis of the partial amino acid sequence. The full-length 2,055 bp cDNA encodes a protein of 685 amino acid residues with a predicted molecular mass of 78,136 Da. The deduced amino acid sequence of this protein exhibits high similarity to that of human (74.1%), pig (75.3%) and rat (74.1%) thimet oligopeptidase (TOP) [EC 3.4.24.15]. Expression of the cDNA in bacterial cells resulted in the production of an active metalloenzyme. Thus, we concluded that the metallopeptidase purified from *Xenopus* oocytes is a member of the TOP family.
In Northern blot analyses, one major species of *Xenopus*-TOP (*X*-TOP) mRNA of 3.0 kb was expressed relatively strongly from early stage (III) of *Xenopus* oogenesis, its level decreasing in later stages (V and VI). This result suggests that the expression of *X*-TOP mRNA is regulated during *Xenopus* oogenesis.

INTRODUCTION

Very recently, a novel zinc-requiring metallopeptidase was purified from *Xenopus* oocytes and characterized (Okida *et al*., 1999). However, its chemical structure and biological function have yet to be elucidated. The function is thought to be related to the role of zinc ion, and important for understanding the sequence of events in *Xenopus* oogenesis and oocyte maturation regulated by gonadotropin (Masui and Clarke, 1979; Zhao and Ishikawa, 1994; Falchuk *et al*., 1995; Zhao *et al*., 1997).

The internal amino acid sequence of the enzyme had homology with human (Thompson *et al*., 1995), pig (Kato *et al*., 1994) and rat (Pierotti *et al*., 1990, 1994; McKie *et al*., 1993) thimet oligopeptidase (TOP) [EC 3.4.24.15], thiol- and metal-dependent oligopeptidase (Rawlings and Barrett, 1995). TOP is widely distributed in various cells and tissues, as a subfamily of zinc metallopeptidase containing neurolysin, saccharolysin, mitochondrial intermediate peptidase and bacterial peptidases such as oligopeptidase A and peptidyl-dipeptidase Dcp (Sugiura *et al*., 1992; Kawabata *et al*., 1993; Barrett *et al*., 1995; Rawlings and Barrett., 1995; Chen *et al*., 1998). Very recently, it was reported that TOP has an important function in the pathway of antigen presentation via MHC class I after proteasome-degradation (Portaro *et al*., 1999; Silva *et al*., 1999) and in the processing of amyloid precursor protein *in vivo* (Koike *et al*., 1999; Yamin *et al*., 1999), although the mechanism underlying this function is as yet unknown.

The cDNA cloning of TOP has been completed in human (Thompson *et al*., 1995), pig (Kato *et al*., 1994) and rat (Pierotti *et al*., 1990, 1994; McKie *et al*., 1993), but not in *Xenopus*. Therefore, as the first step to get an insight into the function of *Xenopus*-TOP (*X*-TOP), we carried out cDNA cloning of *X*-TOP, and examined TOP expression in bacterial cells and mRNA expression patterns during oogenesis.

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MATERIALS AND METHODS

Purification and protein sequencing of *X*-TOP

X-TOP was purified from *Xenopus* oocytes as previously described (Okida *et al*., 1999). The amino acid sequences of two peptide fragments of the protein obtained by digestion with lysylendopeptidase were determined with a protein sequencer.

cDNA cloning of *X*-TOP

A PCR fragment was amplified from *Xenopus* ovary cDNA with primers designed on the basis of the amino acid sequences reported for two peptides Nos. 1 and 2 (see the legend of Fig. 1). The amplified PCR fragment was subcloned into pBluescriptII SK(–) vector and was sequenced. The PCR fragment was amplified using digoxigenin (DIG)

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DNA labeling mixture (Boehringer Mannheim) to use as a probe. *Xenopus* ovary cDNA library (Uni-ZAP XR library) (Stratagene) was screened using the DIG-labeled probe. A total of 3×10^5 plaques were blotted onto the Hybond N^+ nylon membranes (Amersham) and hybridized at 60°C in hybridization solution (5×SSC (20×SSC: 330 mM NaCl / 330 mM sodium citrate, pH 7.0) / 0.5% blocking reagent (Boehringer Mannheim) / 0.1% N-lauroyl sarcosine / 0.02% SDS) with the DIG-labeled probe. Through two rounds of hybridization the positive plaques were isolated. Plasmid DNA was prepared by the *in vivo* excision protocol using the ExAssist/SOLR system (Stratagene). DNA sequencing was performed using a 377A DNA sequencer (Perkin Elmer ABI) with the Dye Terminator Cycle Sequencing Kit (Perkin Elmer ABI).

Amplification of 5' region of *X*-TOP

Two oligonucleotide primers, anti-sense upper primer (GSP2; 5'-TGGGAGACCTCCGAGTTC-3') and lower primer (GSP1; 5'-CATCAT-ATCCTCCTGCCAG-3'), were designed from the partial cDNA sequence and used to clone the full length cDNA for *X*-TOP by 5' rapid amplification of cDNA ends (RACE) reactions. 5' RACE reactions were performed using a Marathon cDNA amplification kit (Clontech) according to the manufacturer's protocol. In 5' RACE reaction, adaptor-ligated cDNA was generated from *Xenopus* ovary polyA(+) RNA and first PCR amplification was carried out using lower gene-specific primer and linker primer (AP-1; 5'-CCATCCTAATA-CGACTCACTATAGGGC-3') by touchdown PCR (Don *et al*., 1991; Roux, 1995), then second PCR amplification using upper primer and linker primer (AP-2; 5'-ACTCACTATAGGGCTCGAGCGGC-3') by 30 cycles each cycle consisted of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 60 sec. The resulting PCR product was subcloned into pBluescript KS(+) (Stratagene) and sequenced. To minimize the possibility that artificial mutations were introduced during PCR amplification, at least five independent clones were sequenced to confirm the cDNA sequences.

Isolation of total RNA from *Xenopus* oocytes

Total RNA from oocytes was isolated using ISOGEN (Wako) according to the manufacturer's instructions.

Northern blot analysis

Fifteen µg of denatured total RNA from *Xenopus* oocytes were electrophoresed using the formaldehyde-denatured gel method, then blotted onto Hybond N^+ nylon membrane (Amersham). A DIG-labeled cRNA probe was synthesized using RNA Labeling Kit according to the manufacturer's instruction (Boehringer Mannheim). The membrane was immersed in hybridization solution (50% formamide / 10 mM Tris-HCl, pH 7.6 / 1×Denhardt's solution / 0.6 M NaCl / 0.25% SDS / 1 mM EDTA / 100 µg yeast tRNA /ml) and pre-hybridized for 4 hr at 65°C. The cRNA probe was added and allowed to hybridize for 14 hr at 65°C. The membrane was then washed with SSC. A final wash was performed with 0.2×SSC / 0.1% SDS at 65°C. Chemiluminescence detection was carried out using CDP-star substrate according to the manufacturer's methods (Boehringer Mannheim).

Subcloning and expression of *X*-TOP in bacteria

The full-length open reading frame (ORF) of *X*-TOP was amplified by PCR with primers designed to produce NdeI and XhoI sites at the 5' and 3' ends, respectively. PCR fragments were inserted into the pET21b expression vector (Stratagene) between NdeI and XhoI sites. Bacterial cultures containing the plasmid pET21b with insert were grown overnight at 37°C in 3 ml of LB medium with antibiotic selection (+ampicillin). The overnight culture was added to 500 ml LB medium with antibiotics as above and incubated for 3 hr at 37°C. At this point the production of TOP was induced by the addition of isopropyl beta-D-thiogalactopyranoside (final concentration, 1 mM) and cells were grown for 3 hr at 25°C. The cells were centrifuged and re-suspended in 30 ml of lysis buffer (50 mM Tris-HCl, pH 8.0 / 100 mM NaCl). The suspension was frozen and thawed at least one cycle following sonication treatment (for 10 min containing half time intervals on ice) to obtain the extract. This extract was centrifuged and the pellets were removed. Expression of the recombinant TOP protein in the supernatant was analyzed by both enzyme activity and immunoblotting.

Electrophoresis and immunoblotting

Electrophoresis was performed according to the method of Laemmli (1970). Electroblotting and immunodetection were conducted as described previously (Okida *et al*., 1999).

Detection of catalytic activity

The enzyme activity of a recombinant TOP preparation was measured fluorometrically by the use of the quenched fluorescence substrate, MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 (Knight *et al*., 1992). For determination of hydrolyzing activity, recombinant enzyme was purified from a bacterial lysate by Ni-affinity chromatography. Fractions enriched in TOP were dialyzed against 10 mM Hepes-NaOH (pH 7.5) and identified by fluorometric assay as described previously (Okida *et al*., 1999).

RESULTS AND DISCUSSION

Zinc-requiring metallopeptidase was purified from *Xenopus* oocytes as previously described (Okida *et al*., 1999) and two peptide fragments (Nos. 1 and 2) of the protein obtained by digestion with lysylendopeptidase were sequenced (see the legend of Fig. 1). To isolate a cDNA clone for the zinc-requiring metallopeptidase, a DIG-labeled cDNA probe was generated by a PCR using degenerated oligonucleotide primers. A positive clone was isolated from *Xenopus* ovary cDNA library (Stratagene) and pBluescript plasmid was excised. The DNA sequencing was performed as described above. Here, to determine the 5'-terminal sequence of the cDNA clone, 5' RACE reaction was performed as mentioned above. The resulting PCR product was subcloned into pBluescript KS(+) (Stratagene) and five independent clones were sequenced. The nucleotide sequence of a full-length cDNA constructed from the sequences of the first cDNA clone and the product obtained by 5' RACE reaction, and deduced amino acid sequence are shown in Fig. 1. The sequence of cDNA encoding *X*-TOP started at positions 7–9 with an initiating codon, ATG, and terminated at positions 2,062–2,064 with a stop codon, TGA. This 2,055 b ORF codes for a protein of 685 amino acid residues with a predicted molecular mass of 78,136 Da. A sequence, AATAAA, for the polyadenylation of mRNA transcript is found at positions 2,813–2,818. The motif HEXXH which represents the active site of zinc-requiring metallopeptidase (Pierotti *et al*., 1990; Kato *et al*., 1994; Barrett *et al*., 1995; Rawlings and Barrett, 1995; Thompson *et al*., 1995; Chen *et al*., 1998) was found at residues 470–474 (HEFGH) of *X*-TOP. The amino acid sequence deduced from the *X*-TOP cDNA was compared with those of the human (Thompson *et al*., 1995), pig (Kato *et al*., 1994), and rat (Pierotti *et al*., 1990, 1994; McKie *et al*., 1993) TOPs (Fig. 2). The extent of amino acid identity between *X*-TOP and the human, pig, and rat enzyme is 74.1, 75.3, and 74.1%, respectively. These high values clearly indicate that zinc-requiring metallopeptidase

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                                                                                        AATGAT     6
ATGCTTTACATTACACAAGATCACACGATGAATCCCCAGTTTAAGAATGATGGAAACCGTCTCAGATGGGATCTGACTCCGAAACAGATT    96
M  L  Y  I  T  Q  D  H  T  M  N  P  Q  F  K  N  D  G  N  R  L  R  W  D  L  T  P  K  Q  I      30

GAGATACTGACTGGTGAGGTCATTGAGCAAACAAAGAGAGTTTATGACCAGGTTGGGAGCCTAGACCTGAGCAGTGTCACCTATGACAAC   186
E  I  L  T  G  E  V  I  E  Q  T  K  R  V  Y  D  Q  V  G  S  L  D  L  S  S  V  T  Y  D  N      60

ACACTAAAGGTCCTGGCTGATGTGGAAGTGGAGTATTCAGGGAAGAGGAGCATGCTTGATTTTCCACAGCATGTATCTCCCTCAAAAGAA   276
T  L  K  V  L  A  D  V  E  V  E  Y  S  G  K  R  S  M  L  D  F  P  Q  H  V  S  P  S  K  E      90
         No.1
ATCCGTGCAGTAAGCACTGCGGCTGATAAGAAACTGTCAGAGTTTGATGTTGAACAAAGCATGAGGGAGGATGTTTACAGAAGAATTGAA   366
I  R  A  V  S  T  A  A  D  K  K  L  S  E  F  D  V  E  Q  S  M  R  E  D  V  Y  R  R  I  E     120

CATTTGCAGACCATTATACCATCTAATACTCTAAAACCTGAAGCAAAGCGATGGCTAGAAAGAGTGATCAAACTGAGCCAGAGGAATGGA   456
H  L  Q  T  I  I  P  S  N  T  L  K  P  E  A  K  R  W  L  E  R  V  I  K  L  S  Q  R  N  G     150
                                                                          No.2
CTGAATCTCCCTTCAGACACCCAGGAGAAAATTAAATCCATTAAGAAAAAGATGAGTACACTCTCCATTGATTTCAACAAGAATTTAAAT   546
L  N  L  P  S  D  T  Q  E  K  I  K  S  I  K  K  K  M  S  T  L  S  I  D  F  N  K  N  L  N     180

GAAGATATCACATTCCTCACCTTCACAAAGGAAGAACTCGGAGGTCTCCCAGATGATTTTCTGAATTCCCTGGAGAAGGCAGGTGATGGG   636
E  D  I  T  F  L  T  F  T  K  E  E  L  G  G  L  P  D  D  F  L  N  S  L  E  K  A  G  D  G     210

AAGTTAAAAATTACATTAAAATATCCACATTATTTCCCCCTGATGAAAAAGTGTTTTATTCCTAATACTCGACAGAAGGTGGAAGCTGCT   726
K  L  K  I  T  L  K  Y  P  H  Y  F  P  L  M  K  K  C  F  I  P  N  T  R  Q  K  V  E  A  A     240

TTCAATTCCCGTTGCAAAGAGGAAAATAGCAGGATACTTCAAGAGTTAGTGCAACTAAGGGAACAAAATAGCAGTATTCTTGGCTTTAAA   816
F  N  S  R  C  K  E  E  N  S  R  I  L  Q  E  L  V  Q  L  R  E  Q  N  S  S  I  L  G  F  K     270

ACACATGCTGATTTTGTTCTGGAGATGAATATGGCAAAGAACAGTGTGACTGTGGCAAGTTTTTTGGAGGAATTGTCTCAAAAATTGAAG   906
T  H  A  D  F  V  L  E  M  N  M  A  K  N  S  V  T  V  A  S  F  L  E  E  L  S  Q  K  L  K     300

CCGCTTGGTGAGCAGGAAAGGTCTATCATGTTAGAACTTAAGAAGAATGAGTGTAAAAAACGTGGGCTGAATTTTGATAACCAGATCAAT   996
P  L  G  E  Q  E  R  S  I  M  L  E  L  K  K  N  E  C  K  K  R  G  L  N  F  D  N  Q  I  N     330

GCCTGGGATATGCGTTATTATATGAACCAAGTTGAGGAGACTTCATACAATGTGGATCAGAACTTCTTGAAGGAATTTTTTCCAATTGAG  1086
A  W  D  M  R  Y  Y  M  N  Q  V  E  E  T  S  Y  N  V  D  Q  N  F  L  K  E  F  F  P  I  E     360

GTAGTTACTACTGGTCTCCTGGGCATATATCAGGAGTTGTTGGGATTGAACTTTGTCTTAGAAAAGGGTGCTGCTGTTTGGCATGAAGAT  1176
V  V  T  T  G  L  L  G  I  Y  Q  E  L  L  G  L  N  F  V  L  E  K  G  A  A  V  W  H  E  D     390

GTGTCTTTGTACTCAGTAAGGGATGCCACAACCGGACAACTCATAGGAAAGTTCTATTTGGACTTATATCCACGGGAAGGGAAATATTCA  1266
V  S  L  Y  S  V  R  D  A  T  T  G  Q  L  I  G  K  F  Y  L  D  L  Y  P  R  E  G  K  Y  S     420

CATGCAGCATGCTTTGGGCTCCAGCTTGGATGTTTACTGCCTGATGGCACCAGGCAGATCTCTGTGGCTGCTATGGTTGCCAACTTTACT  1356
H  A  A  C  F  G  L  Q  L  G  C  L  L  P  D  G  T  R  Q  I  S  V  A  A  M  V  A  N  F  T     450

AAGCCCACACAAGATGCTCCCTCTCTACTACAACATGATGAAGTAGAGACATATTTCCATGAGTTTGGACACGTTATGCACCAGTTATGT  1446
K  P  T  Q  D  A  P  S  L  L  Q  H  D  E  V  E  T  Y  F  H  E  F  G  H  V  M  H  Q  L  C     480

GCTCAGGCAGAATTTGTACTGTTCAGTGGAACAGGTGTAGAGAGAGATTTTGTAGAAGCTCCTTCTCAGATGCTGGAAAACTGGGTTTGG  1536
A  Q  A  E  F  V  L  F  S  G  T  G  V  E  R  D  F  V  E  A  P  S  Q  M  L  E  N  W  V  W     510

GAAAGGGAATCTTTGCAACGTATGTCACAACATTACAAATCTGGTCAAGCAATTCCTAAAGAGGTATTGGAGCATCTTATAAAATCCCGG  1626
E  R  E  S  L  Q  R  M  S  Q  H  Y  K  S  G  Q  A  I  P  K  E  V  L  E  H  L  I  K  S  R     540

CTGGCAAACACAGGTCTCCTAAACCTTCGACAGATCGTTCTTGCCAAGATAGATCAGGTGCTGCACACACAGATTGGCGTAGATCCTGTT  1716
L  A  N  T  G  L  L  N  L  R  Q  I  V  L  A  K  I  D  Q  V  L  H  T  Q  I  G  V  D  P  V     570

GAGGAATATAGCAAGTTATCCAATGAGATACTTGGAATCCCAGCTACACCTGGTACAAATATGCTAGCCAGCTTTGCACATCTGGCAGGA  1806
E  E  Y  S  K  L  S  N  E  I  L  G  I  P  A  T  P  G  T  N  M  L  A  S  F  A  H  L  A  G     600

GGATATGATGCTCAGTATTATGGATACCTATGGAGTGAGGTATATTCTATGGACATGTTCTACACACGTTTTAAACAGGAAGGAATCATG  1896
G  Y  D  A  Q  Y  Y  G  Y  L  W  S  E  V  Y  S  M  D  M  F  Y  T  R  F  K  Q  E  G  I  M     630

AGCAAGAAGGTTGGTGCAGATTATAGGAACTGTATTCTGAAGCCAGGGGGATCACTGGATGCCACTGAAATGCTAAGGAACTTCCTAGGT  1986
S  K  K  V  G  A  D  Y  R  N  C  I  L  K  P  G  G  S  L  D  A  T  E  M  L  R  N  F  L  G     660

CGTGACCCAAAGCCAGAAGCATTTCTCCAAAGTAAAGGACTGGCTAATGTTAATGCTCCTGCACCCCATGCTAGCTGA              2064
R  D  P  K  P  E  A  F  L  Q  S  K  G  L  A  N  V  N  A  P  A  P  H  A  S  *                685

TCTACTATCATGCAGCACTATCTGTAATGACTTGGAAGTAGACACATGACCAGGGTTATGGAACACAACACTTTACAACATTTTAGGGAA  2154
CAGTTCAGTGTAAAAATAAAAACTTGGTAAATAGATAGGCTGTGCAAAATAATAAATGTTTCTAATATAGTTAGTTAGCCAAAAATGTAA  2244
TGTATAAAGGCTGGAGTAACTGAATGTCTAATATAATAGCCAGAACACCACTTTCTGCTTTTCAGCGGTAAGTCAGCTAGAAGTTTTTTA  2334
AAAAAATGTATTGATAAATAAGGGGAAATAACCTGTGCGGATCTGGTCGGAGTATATTTCAGAAAATAATGAAATAAATTCAGATTTTTA  2424
TAAGTAACCCCCCCCCCCCCAATGTATAATAATTCATACTTTTAAGTTGTGCTCCCCTCTTTGTAGGAACATCTGATACAGTTTTCCATT  2514
TGCAACAGAGGATTGTCAGCTATTCCCATTGCTAGTTGTATCTATTCGAATTAAATGAAGCTTGAGGGCCCCCATATCCTGACTTGTTCT  2604
GGACCACTGAAATATTCTTTTATTTAGGTCTAGCCATCACAACATTTTTAAATAACAATAGTTTAAAAAACAGGAAGCTTTACATTTGGT  2694
TTTTTTTTGTGGGTCATAGCCTAATGTTTGATTAATAGCTGGATAGCAACCCTGATATGTGAAGCAAGCAATAAGTGACTGTTGTTATGA  2784
TTGTAAAAGAAACTGGAGCAGGCGCTCAAATAAAGGCAATCTTCCATCAAAAAAAAAAAAAAAAAA                          2850
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Fig. 1. Nucleotide and deduced amino acid sequences of full-length *X*-TOP cDNA. The nucleotide (bold type) and amino acid position numbers are shown on the right. The cDNA consisted of a total of 2,850 nucleotides, with six nucleotides upstream of the initiating methionine codon (ATG), a coding region of 2,055 nucleotides encoding 685 amino acid residues of approximately 78 kDa-protein and a 3'-noncoding sequence of 789 nucleotides including the termination codon TGA (marked with an asterisk *) and the potential polyadenylation signal AATAAA (underlined). Two peptides used to design primers correspond to fragment Nos. 1 and 2 are underlined. The putative zinc-binding sequence (HEFGH) is double underlined. Seven cysteine residues are open-circled. The GenBank accession No. is AB030904.

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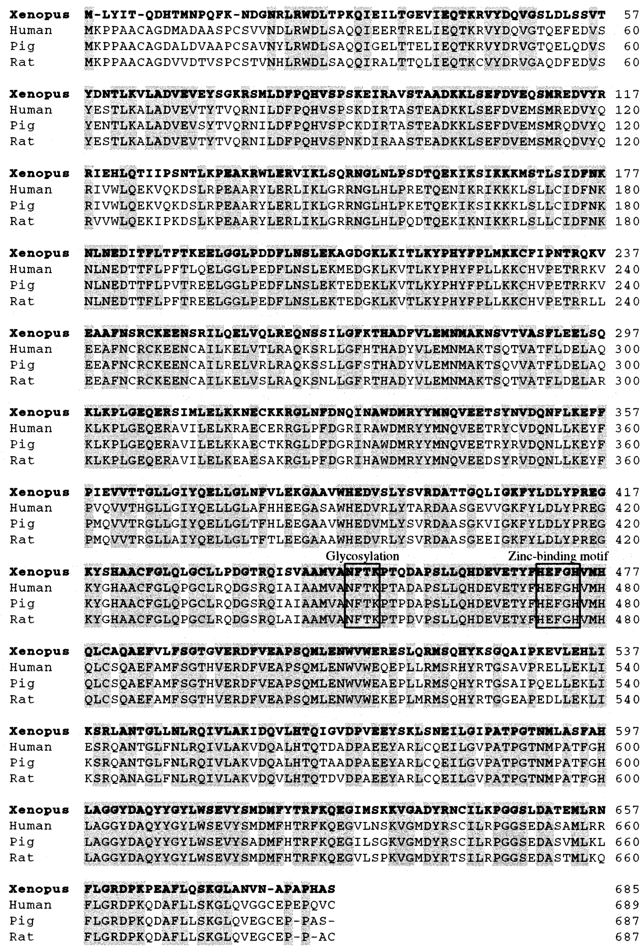

Fig. 2. Alignment of the amino acid sequence of *X*-TOP with those of other homologous mammalian proteins. The deduced amino acid sequence of *X*-TOP (bold type) is aligned with human (Thompson *et al.*, 1995), pig (Kato *et al.*, 1994) and rat (Pierotti *et al.*, 1990, 1994; McKie *et al.*, 1993) TOPs. The amino acid position number is shown on the right. Identical residues among the four sequences are indicated by gray and, additionally, the putative "Glycosylation" site and "Zinc-binding motif" are boxed.

purified from *Xenopus* oocytes (Okida *et al.*, 1999) is the *Xenopus* version of TOP. In addition, the deduced amino acid sequence of *X*-TOP exhibits also relatively high similarity to that of pig (Sugiura *et al.*, 1992) (63.6%) and rabbit (Kawabata *et al.*, 1993) (64.2%) oligopeptidase M. The potential N-linked glycosylation site (NXT/SX) (Pierotti *et al.*, 1990; Barrett *et al.*, 1995; Rawlings and Barrett, 1995; Thompson *et al.*, 1995) was found at residues 448–451 (NFTK). The sequence of *X*-TOP contains seven cysteine residues (Fig. 1), five (C-228, -245, -424, -431, -480) of which are conserved. C-480 near the catalytic site has been suggested to be responsible for the thiol dependence of TOP (Pierotti *et al.*, 1990), but the fact raises a doubt about that (Chen *et al.*, 1998).

To confirm that the isolated cDNA indeed encodes an enzyme with metallopeptidase activity, the cDNA of the first positive clone (deficiency of nine amino acid residues, MLYITQDHT, in N-terminal of the full-length sequence, see Fig. 1) was expressed in bacterial cells. Recombinant *X*-TOP was purified using Ni-affinity chromatography from cell extracts, and its apparent molecular size of 78 kDa estimated by SDS-PAGE using 12% gel (Fig. 3-panel A). Both crude and purified recombinant *X*-TOPs crossreacted with antibody raised against zinc-requiring metallopeptidase purified from *Xenopus* oocytes (Fig. 3-panel B). These results confirm that the cDNA isolated in this study encodes a member of the TOP family (Pierotti *et al.*, 1990, 1994; McKie *et al.*, 1993).

The metallopeptidase activity in purified recombinant *X*-TOP was measured using fluorogenic peptide substrate and the effects of *o*-phenanthroline or zinc ion on it were tested as described previously (Okida *et al.*, 1999) (Table 1). When an *X*-TOP construct containing the histidine-tag was expressed,

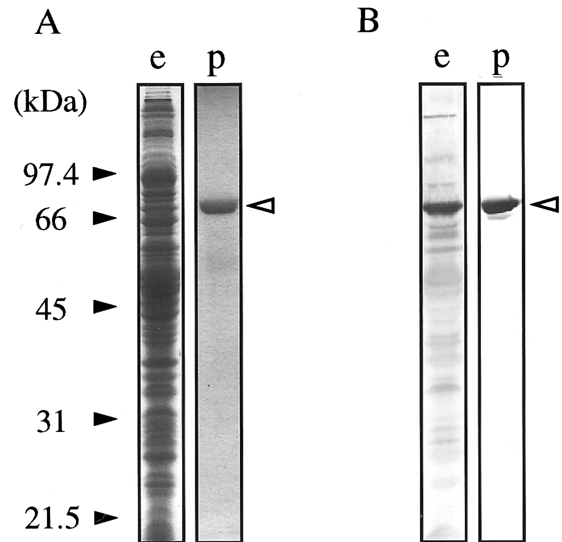

Fig. 3. Expression and purification of recombinant *X*-TOP. Bacterial extracts containing recombinant *X*-TOP (lane e) and recombinant *X*-TOP purified by Ni-affinity chromatography (lane p) were subjected to SDS-PAGE followed by Coomassie staining (panel A) and Western blotting (panel B). The opened arrowhead shows a distinct band indicating *X*-TOP, predicted molecular mass = 78 kDa, and size markers (kDa) are indicated on the left of the panel (closed arrowheads).

Table 1. Effects of *o*-phenanthroline or zinc ion on metallopeptidase activity of the purified recombinant *X*-TOP.

Reagent	Concentration (mM)	Relative activity (%)
Control	—	100
o-Phenanthroline	1.0	67
	2.0	44
	5.0	19
	10.0	–2
$ZnSO_4$	0.01	96
	0.1	136
	1.0	121
	10.0	22
	100.0	3

Peptidase activities in 0.37 µg of recombinant *X*-TOP purified from cell extracts were determined at different concentrations of *o*-phenanthroline or $ZnSO_4$ as described in the text. Values (%) are the mean of five determinations.

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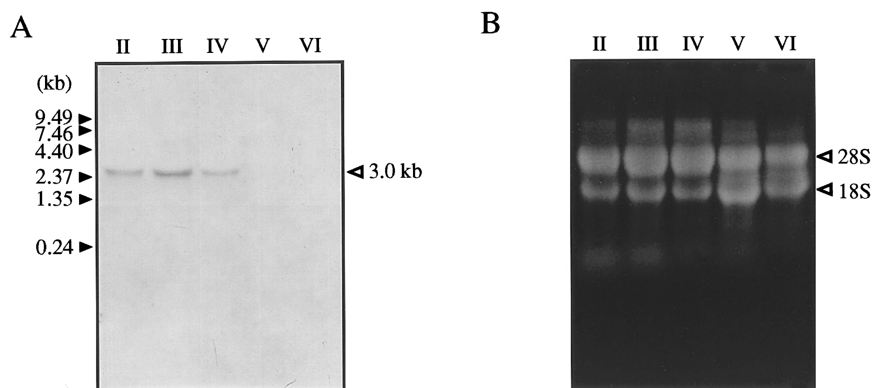

Fig. 4. *X*-TOP mRNA expression during oogenesis in *Xenopus* oocytes. Fifteen µg of total RNA extracted from each stage (II–VI) oocyte (Dumont, 1972) were electrophoresed on 1% agarose, blotted and hybridized with the DIG-labeled cRNA (panel A). The opened arrowhead (3.0 kb) on the right indicates the size of *X*-TOP mRNA. Panel B shows total RNA stained by ethidium bromide after electrophoresis; 28S and 18S rRNAs (opened arrowheads). The migration of RNA size markers (kb) is shown on the left of panel A (closed arrowheads). Roman numerals indicate the stages (II–VI) of oocytes.

the hydrolyzing activity of recombinant *X*-TOP (2.8 nmol/min/mg protein) was significant. However, its activity was reduced approximately 0.7-fold when compared to the enzyme purified from *Xenopus* oocyte extract (4.1 nmol/min/mg protein). First, when this enzyme was preincubated with different concentrations of *o*-phenanthroline, its initial activity was blocked by 56% of the control at 2 mM. A concentration of 10 mM led to complete inhibition of the activity of the control. In addition, we examined the role of additive zinc ion in the enzyme activity. Zinc ion at 0.01 mM had no effect on the enzyme activity, but at 0.1 and 1 mM activated the enzyme. By contrast, 10 and 100 mM zinc ion inhibited the enzyme activity.

Northern analyses were performed on the total RNA extracted from different stages of *Xenopus* oocytes (Dumont, 1972) using the DIG-labeled cRNA as a probe in order to determine the size of *X*-TOP mRNA (Fig. 4). The analysis detected the presence of a single mRNA with a length of approximately 3.0 kb. The mRNA was found between stages II and IV. The maximum expression was at stage III. The signals for *X*-TOP mRNA in stages V and VI were under the detection in the assay conditions.

In this study, it was confirmed that the zinc-requiring metallopeptidase purified previously from *Xenopus* oocytes (Okida *et al.*, 1999) is a TOP (Pierotti *et al.*, 1990, 1994; McKie *et al.*, 1993; Kato *et al.*, 1994; Barrett *et al.*, 1995; Rawlings and Barrett, 1995; Thompson *et al.*, 1995; Chen *et al.*, 1998). Information on the biochemical properties of TOP has been obtained largely from works on the mammals, with other vertebrate species including amphibian yet to be studied. Although the specific functions of TOP during oogenesis remain to be investigated, the cloning of TOP and detailed study of its mRNA expression through these processes would provide valuable information on TOP function in oogenesis. These studies may shed light on the new role of *X*-TOP in addition to the known proteasomes (Tokumoto and Ishikawa, 1995; Coux *et al.*, 1996) in the proteolytic steps of *Xenopus* oocyte maturation.

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