Molecular cloning of cDNA coding for a matrix metalloproteinase from Atlantic salmon

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To isolate cDNA coding for matrix metalloproteinases (MMPs) from fish, we first investigated Ca^{2+}-dependent gelatinolytic activities in conditioned media of eight fish cell lines using gelatin-zymography with and without Ca^{2+}. As a result, we found the major activity at positions of 60, 66, 70 and 80 kDa, respectively in some cell lines. RT-PCR was performed using total RNA from ASE cells, which were derived from Atlantic salmon and expressed the gelatinolytic enzyme of 66 kDa, and using degenerate primers that were designed on the basis of the conserved amino acid sequences corresponding to cysteine switch and catalytic domain of MMP. Amplified DNA was 329 bp long and its deduced amino acid sequence was highly homologous to known MMPs. Based on this nucleotide sequence, we performed 5' and 3' RACE and finally obtained 1714 bp long cDNA coding for a protein of 462 amino acid residues. The amino acid sequence showed higher homologies (54-56% identities) to MMP13s (collagenase 3) from several species than other MMPs, suggesting that the cloned cDNA coded for a salmon counterpart of MMP13.

Key Words:Matrix metalloproteinase, cDNA, Fish cell line, Zymography

Introduction

Matrix metalloproteinase (MMP) is the general term for a group of metalloenzymes that hydrolyzed component proteins of extracellular matrix (ECM) including various types of collagen, fibronectin, laminin and so on. ECM has been considered to be involved in morphological formation through the proliferation, differentiation and migration of cells. MMP that plays an important role in the degradation of the ECM, therefore, have been attracting a great deal of attention.

At present, at least 14 types of MMPs are known in humans and others. These enzymes are composed of essential domains that exist in all MMPs (including signal peptide, propeptide, catalytic region and zinc-binding region) and specific domains that exist in some kind of MMPs (including fibronectin-like region, haemopexin-like region and collagen-like region). Based on the molecular structure and the substrate specificity, MMPs can be divided into four main subfamilies including

Abbreviations: MMP, matrix metalloproteinase; CBB, coomassie brilliant blue R250; PBS, phosphate buffered saline; RT, reverse transcription; ECM, extracellular matrix; RACE, rapid amplification of cDNA ends; Mr, molecular weight

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB028240.

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collagenases, gelatinases, stromelysins and membrane-type MMPs, and the other MMPs that do not belong to any subfamilies.

Although MMP and its cDNA have been analyzed in several species, to our knowledge, there are only a few reports about fish MMPs so far. Hence, we intended to analyze fish MMPs and isolate their cDNA for a comparative study and for applications in food processing. Here we report about the detection of gelatinolytic activities in conditioned media from fish cell lines, and about cloning of cDNA coding for a salmon MMP.

Materials and Methods

Cell lines and Culture.

Fish cell lines were cultured as described by Yoshimizu et al. (1988). Briefly, ASE derived from Atlantic salmon (*Salmo salar*), SE from chum salmon (*Oncorhynchus keta*), and RTG-2 and RTT from rainbow trout (*Salmo gairdneri*) were cultured in MEM containing 10% FCS at 20℃. AF-29 from ayu (*Plecoglossus altivelis*) are cultured in Leibovitz-15 medium containing 10% FCS at 20℃. EPC from carp (*Cyprinus carpio*) and EPG from goldfish (*Carassius auratus*) were cultured in MEM and L-15 medium containing 10% FCS, respectively, at 30℃. The pH of these media is adjusted to 7.5 with Tris-HCl buffer. EO-2 from eel (*Anguilla japonica*) are cultured in MEM (adjusted to pH 7.5 with HEPES buffer) containing 10% FCS at 30℃.

Zymography.

Zymography was performed basically accodring to the method of Heussen (1980) as follows: for gelatin or casein zymography, conditioned media were incubated in the Laemmli's sample buffer without 2-mercaptoethanol for 30 min at 37℃ and electrophoresed through SDS-7.5% polyacrylamide gel (Laemmli, 1970) containing 0.1% gelatin from bovine or salmon, or 0.1% casein at 4℃. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 hour at room temperature and incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM Ca^{2+} or 5 mM EDTA for 2 hours at 37℃. Active bands were developed by staining the gel with CBB.

Bovine gelatin was purchased from Wako Co. Ltd. (Osaka Japan) and salmon gelatin was prepared from chum salmon skin as described by Shimizu *et al.* (1999).

Cloning of cDNA coding for MMP from ASE cell.

Total RNA from ASE cells was prepared as described by Kiyono *et al.* (1989). About 1 μg of total RNA was reverse-transcribed with oligo-d(T) as primer using a cDNA Synthesis System Plus (Amersham, NJ, USA) and the resulting cDNA was used as template for a PCR. Degenerate primers for the PCR were designed on the basis of the conserved amino acid sequences corresponding to the cysteine switch and catalytic domain of MMP, respectively (Takino *et al.*, 1995); those sequences are 5'-CCIMGITGIGGIGTICCIGAT-3' and 5'-TCATCAA AATGIGI RTCICC-3'. The reaction mixture (50 μl) contained 2.5 U of Taq DNA polymerase (Promega Corporation, MD, USA), 1/10 vol of 10×reaction buffer (attached to the enzyme), 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.2 μM of each nucleotide. PCR reaction was performed with the following program: initial denaturation of 94℃ for 3 min, 40 cycles of 94℃ for 1 min, 42℃ for 2 min, 72℃ for 3 min, and a final extention of 72℃ for 7 min. Amplified DNA was cloned on a TA-cloning vector, pCR II (In Vitrogen Co., CA, USA) and sequenced for both strands using Dye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA).

5' and 3' RACE were performed using the 5' RACE System from Gibco Co. Ltd. (MD, USA) and the RNA LA PCR Kit from Takara Co. Ltd. (Kyoto, Japan), respectively. The same cDNA as above was used as a template. Nested primers were designed from the sequence of the degenearate PCR product (see Fig. 2): 5'-ATTTCCAGGGGGGTAAG CAT-3' and 5'-GAGCAAGAAGCCC GTCTG GT-3' for 5' RACE, and 5'-GTGGCCGAATATAATCA CTT-3' and 5'-CAC TTTCCAAATGACCTC-3' for 3' RACE were used. Amplified DNA in 5' RACE or 3' RACE was cloned on the pCR II vector and sequenced as

described above.

Software for computer analysis

Computer analysis of DNA and protein sequences were performed with a MacVector software program (Oxford Molecular Ltd. , UK) .

Results and Discussion

Detection of gelatinolytic activities in conditioned media of fish cell lines.

To isolate cDNAs coding for MMPs from fish, we first performed zymographic analysis of conditioned media from eight fish cell lines. In the zymography using salmon gelatin (Fig. 1), we detected major active bands that depended on the presence of Ca^{2+} (Compare Fig. 1A and Fig. 1B) at position of 60 kDa in regard to EPG and AF-29 cell lines (Fig 1B, lanes 3 and 5, respectively), 66 kDa in regard to EPC and ASE cell lines (lanes 4 and 6, respectivly), 70 kDa in regard to EO-2 cell line (lane 2) and 70 kDa and 80 kDa in RTT cell lines (lane 7) . RTG-2 and SE cell lines seemed to express little gelatinolytic activities (lanes 8 and 9, respectively). The same result was obtained in the case of the zymography using bovine gelatin (data not shown). These bands were not detected in casein-zymography (data not shown). Judging from their molecular weights and gelatinolytic activities, it seems likely that the proteins of 60, 66, 70 and 80 kDa are fish counterparts of human MMP3 (stromelysin 1; Mr. 60 kDa; Wilhelm et al., 1987), MMP13 (collagenase 3; Mr. 65 kDa; Freije et al., 1994), MMP2 (gelatinase A; Mr. 72 kDa; Collier et al., 1988) and MMP9 (gelatinase B; Mr. 92 kDa; Wilhelm et al., 1989), respectively.

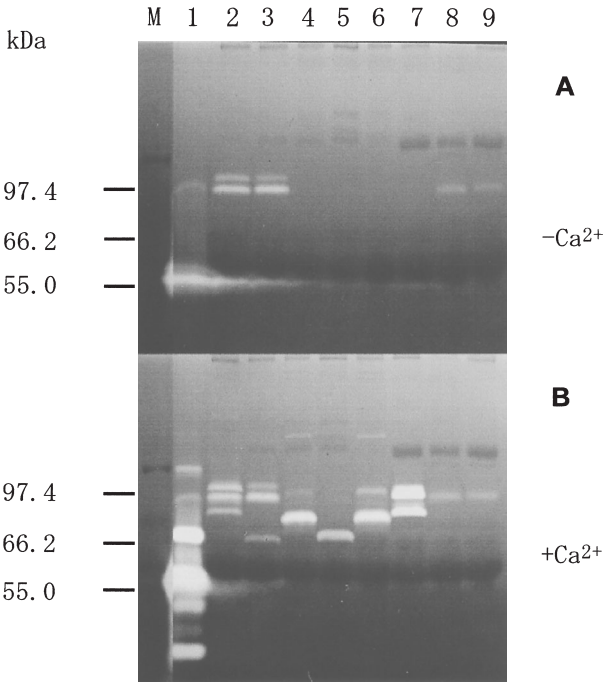

Fig. 1　Gelatin zymography of conditioned media from fish cell lines.

Conditioned medium of EO cells (lane 2), EPG (lane 3), EPC (lane 4), AF29 (lane5), ASE (lane 6), RTT (lane 7), RTG2 (lane 8) or SE (lane 9) was electrophoresed through SDS-polyacrylamid gel containing the salmon gelatin. After electrophoresis, the gel was incubated in the buffer with (B) or without (A) Ca^{2+} as described in the "Materials and Methods" section. Lane 1, collagenase from *Vibrio* sp. ; Lane M, molecular weight markers.

Cloning and structural analysis of cDNA coding for MMP from ASE cell.

First of all, we intended to isolate cDNA coding for a fish MMP from ASE cells. Total RNA was converted to cDNA by oligo-d(T) primed reverse-transcription followed by PCR using degenerate primers. When the degenerate primers corresponding to the cysteine switch and Zn^{2+}-binding domain were used, the expected size of fragment was not amplified. While, when the degenerate primer corresponding to catalytic domain instead of Zn^{2+}-binding domain was used, about 300 bp fragment was amplified. This DNA fragment (designated ASE-1) was cloned on pCR II vecter and sequenced. A deduced amino acid sequence (109 aa) of ASE-1 (329 bp long) showed 62 to 65% identity to MMP13 (collagenase 3) and MMP3 (stromelysin 1) in a similarity search using NCBI BLAST server. We, therefore, concluded that ASE-1 was a partial cDNA coding for MMP of ASE cell.

To isolate a full-length cDNA, 5' and 3' RACE were performed using nested primers that were designed on the basis of the nucleotide sequence of ASE-1. DNA fragments of 550 bp (designated as ASE-2) and 1407 bp (designated as ASE-3) long were amplified in 5' and 3' RACE, respectively. Nucleotide sequences of ASE-2 and ASE-3 showed

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*3
ATTCATCCGGTCAAC ATGGAGCTCACAGCT GTGGTATTGCTGGTG ATTGCTGCACACGCT TTAGCAAAACCAATT   75
                M  E  L  T  A   V  V  L  L  V   I  A  A  H  A   L  A  K  P  I

TCAAGTGAGGAGAAA GACAAGGTGTTATTC GCTGAGAAATATCTT CGAAGGTACTATGGC ATGCCAGCTGGTCTA  150
S  S  E  E  K   D  K  V  L  F   A  E  K  Y  L   R  R  Y  Y  G   M  P  A  G  L

CAAGGAAAAGAAAAA ACATCCGATGTGATG TATAAGAAGAAAATT CAAGAGATGCAAGAG TTTTTTAAGTTGAAC  225
Q  G  K  E  K   T  S  D  V  M   Y  K  K  K  I   Q  E  M  Q  E   F  F  K  L  N
                                                *1
GTTACGGGAAAGCTG GACGACGACACTTTG GAGCTAATGGAAATG GCTCGCTGCGGAGTT CCTGATGTGGCCGAA  300
V  T  G  K  L   D  D  D  T  L   E  L  M  E  M   A  R  C  G  V   P  D  V  A  E
                                                <---------------------->
    *5
TATAATCACTTTCCA AATGACCTCAAGTGG AAAACCACAGAGGTG ACCTTCAGGATTCTG AATTACACTCCTGAC  375
Y  N  H  F  P   N  D  L  K  W   K  T  T  E  V   T  F  R  I  L   N  Y  T  P  D

CTGAGGAAGGCGGAT GTGGACAGAGCCGTC CGAAATGGTTTAAAT GTCTGGAGCAGTGTA ACTCCGCTGAAGTTT  450
L  R  K  A  D   V  D  R  A  V   R  N  G  L  N   V  W  S  S  V   T  P  L  K  F

AAGAAACTGTATGAG GGAAATGCTGACATC ATGATCAGCTTCGGA GCAAGAGAACATGGA GACTTCAACCCGTTT  525
K  K  L  Y  E   G  N  A  D  I   M  I  S  F  G   A  R  E  H  G   D  F  N  P  F
                            *4                                              *2
GATGGACCAGACGGG CTTCTTGCTCATGCT TACCCCCCTGGAAAT GGCATTGGTGGTGAT ACACACTTTGATGAG  600
D  G  P  D  G   L  L  A  H  A   Y  P  P  G  N   G  I  G  G  D   T  H  F  D  E
                                                         <---------------------->

GACGAAACGTGGACC AAAGACTTTCATGAA TTCAATCTCTTCTTG GTCGCGGCCCATGAG TTTGGTCATGCTCTC  675
D  E  T  W  T   K  D  F  H  E   F  N  L  F  L   V  A  A  H  E   F  G  H  A  L

GGCATGGCCCATTCC TCTGACCCTGGGTCA CTGATGTACCCGGTT TACTCCTACGGCAAG GGTTACCCGCTCTCT  750
G  M  A  H  S   S  D  P  G  S   L  M  Y  P  V   Y  S  Y  G  K   G  Y  P  L  S

GAGGATGACATTAAG GGGATCCAGTCTCTC TATGGTGAAAACCCA AACCATAGAAGGATC AAGCCCAAACCCGAC  825
E  D  D  I  K   G  I  Q  S  L   Y  G  E  N  P   N  H  R  R  I   K  P  K  P  D

GCTCCAAGCAAGTGT GACCCTGAACTGAGT TTTGATGCCGTCACT GAACTCCGTGGAGAG ACAATCATTTTCAAA  900
A  P  S  K  C   D  P  E  L  S   F  D  A  V  T   E  L  R  G  E   T  I  I  F  K

GACAGGTTTTACTGG CGGCTACATTCACAA ATACCTGAGCCTGAA CAAACCCTCATTAAA AGTACCTGGCCTGAA  975
D  R  F  Y  W   R  L  H  S  Q   I  P  E  P  E   Q  T  L  I  K   S  T  W  P  E

ATACCAAACAAAGTA GATGCTGCCTATGAG AACCCAGAAAAAGAT GTTGTCATCATATTC AGTGGAATCAAAATG 1050
I  P  N  K  V   D  A  A  Y  E   N  P  E  K  D   V  V  I  I  F   S  G  I  K  M

TGGGCTCTCAATGGT TACAATCTTGTTGAC GGCTATCCAAAATAC ATTCACAAACTGGGT CTCCCGAAGACCGTT 1125
W  A  L  N  G   Y  N  L  V  D   G  Y  P  K  Y   I  H  K  L  G   L  P  K  T  V

CGCAAAATAGATGCA GCTGTAAACATCCGA GATACTGGCAAGACT CTACTTTTTGTAGAA GAGGAATACTGGAGT 1200
R  K  I  D  A   A  V  N  I  R   D  T  G  K  T   L  L  F  V  E   E  E  Y  W  S

TATGATGAGCGGACA GGCACCATGGATAGT GGATACCCACGATCC ATTGAGGAGGACTTT CCCGGAATTGGAGAT 1275
Y  D  E  R  T   G  T  M  D  S   G  Y  P  R  S   I  E  E  D  F   P  G  I  G  D

GAAGTGGATGCCGCT GCTTATCATTTTGGA TATTTGTACTTCTAC CATGAGCATATTCAG TTTGAATACAGTTAC 1350
E  V  D  A  A   A  Y  H  F  G   Y  L  Y  F  Y   H  E  H  I  Q   F  E  Y  S  Y

AACTCAAGGAAAGTC ATGCGTATCATGAGG GCCAACTCTATTCTC AACTGTTGAGCACTT ACAATGTGGATGTGC 1425
N  S  R  K  V   M  R  I  M  R   A  N  S  I  L   N  C  .

CATAAACTGTCAATT GTTATCAACTGCTGT GAAAATTAGCATTTC CTTTTCTGTGTTTTC AACTACTGAGCTGGA 1500
AAATCAGTAGAAAGT CTACATTATTTTCTC ATTTATTTATAGACC ATGATTCTGGAATCG TGTCTGGGATATTTT 1575
    ______                                                      ______
GGTAAAATTAACTTC TGTATGATTCTTTAT ATGCATCCAATGGGG AGCAAAGAAAGTGAA ATATATATTGATTGA 1650
                                                                    *6
ACTAAAAACTGTAAA TGTGTTATGCCCTCT ACAATACCATTGTTA AAAAAAAAAAAAAAA AAAA            1714
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Fig. 2 Nucleotide sequence of salmon MMP13 cDNA and its deduced amino acid sequence.

ASE-1, ASE-2 and ASE-3 cDNA contained the sequence from ＊1 to ＊2, that from ＊3 to ＊4, and that from ＊5 to ＊6, respectively. Underlines with arrowheads represent the positions of degenerate primers described in the "Materials and Methods section". Two putative poly(A) signals are presented by lines over their letter. The nucleotide numbers are presented at the right side of each line.

that they overlapped each other with no mismatch over 244 bp long within the ASE-1 sequence. Finally, we obtained a consecutive cDNA of 1714 bp long (designated as ASE-4)(Fig. 2). ASE-4 sequence contained 1386 bp long of an open reading frame encoding a protein of 462 amino acids (calculated molecular weight, 51, 810) that was flanked by 15 bp and 291 bp of 5'- and 3'-noncoding sequences, respectively. Two putative poly(A) signals existed in 54 bp and 108 bp upstream of poly(A) site.

When the overall sequence similarity of the protein coded by ASE-4 was searched using the NCBI BLAST server, this showed higher homologies (54 to 56% identities, see Table 1) to MMP13s from several species than other MMPs. We, therefore, concluded that ASE-4 probably coded for a salmon counterpart of MMP13.

The polypeptide coded by the ASE-4 (salmon MMP13) is considered to be glycosylated because it has two putative N-glycosylation sites (N^{69} and N^{116}, see Fig. 3). Accodringly, its apparent molecular weight ought to be larger than the calculated one (51.8 kDa), which suggest that the 66 kDa gelatinase detected in the ASE conditioned medium (see lane 6 in Fig. 1B) might be salmon MMP13.

*Comparison of amino acid sequences between salmon and known MMP*13s.

Table 1　Homologies of amino acid sequences among MMP13s from various species

	Identities (%)							
	H.sap	*M.mus*	*O.cun*	*E.cub*	*B.fau*	*X.lae*	*C.pyr*	*S.sal*
H. sap		86	90	91	89	66	67	56
M. mus	92		86	86	85	66	66	54
O. cun	94	92		90	88	67	66	54
E. cub	95	93	93		90	68	68	54
B. fau	93	91	93	93		65	65	53
X. lae	78	80	79	80	79		72	54
C. pyr	81	80	80	81	79	86		56
S. sal	71	70	70	70	68	71	70	
	Similarities (%)							

H. sap, human; *M. Mus*, mouse; *O. cun*, rabbit; *E. cub*, equine; *B. fau*, bovine; *X. lae*, frog; *C. pyr*, newt; *S. sal*, salmon

An amino acid sequence alignment of the putative salmon MMP13 (simply called salmon MMP13 after this) and the other known ones were presented in Fig. 3. Based on the knowledge about the domain structure of MMP (Matrisian, 1990; Woessner, 1991 and Knauper et al., 1996), the putative functional domains of salmon MMP13 was assigned as follows: a signal peptide (animo acid No. 1 to 15), a propeptide domain (16 to 95), a core enzyme domain (96 to 257), a hinge domain (258 to 274) and a haemopexin-like domains (275 to 462).

MMP is secreted as an inactive precursor and activated by the digestion of N-terminal peptide (Stricklin et al., 1983; Stetler-Stevenson et al., 1990). Sequence around C^{96} in human MMP13 (C^{89} in salmon MMP13, consensus: PRCGVPD) involved in this latency of MMP (van Wart and Birkedal-Hansen, 1990) through a mechanism called as the cysteine switch was highly conserved in salmon MMP13, in which alanine replaced the first proline. This replacement does not seem to be due to the misincorporation of nucleotides during PCR because ASE-2 cDNAs from two independent clones of *E. coli* had the same nucleotide sequence. By the way, a similar replacement is observed in equine MMP3 (stromelysin 1, accession No. U62529), in which the proline is replaced with serine.

The core enzyme domain contains a zinc-binding site (Jiang and Bond, 1992)(around the region of L^{216} to M^{240} in human MMP13, L^{209} to M^{233} in salmon MMP13), whose sequence is also well conserved in salmon MMP13; three histidine residues involved in zinc chelating were completely conserved. Knauper et al. (1996) have discussed that three amino acid residues in the zinc-binding site corresponding to L^{218}, D^{231} and P^{236} in human MMP 13 may be more responsible for the activity by comparing amino acid sequences in this region among several types of MMPs; L^{218} might be involved in determining a substrate range of MMP, while, D^{231} and P^{236} might be involved in a proteolytic efficiency. L^{218} is conserved in salmon MMP13 and changed to a chemically similar amino acid (valine

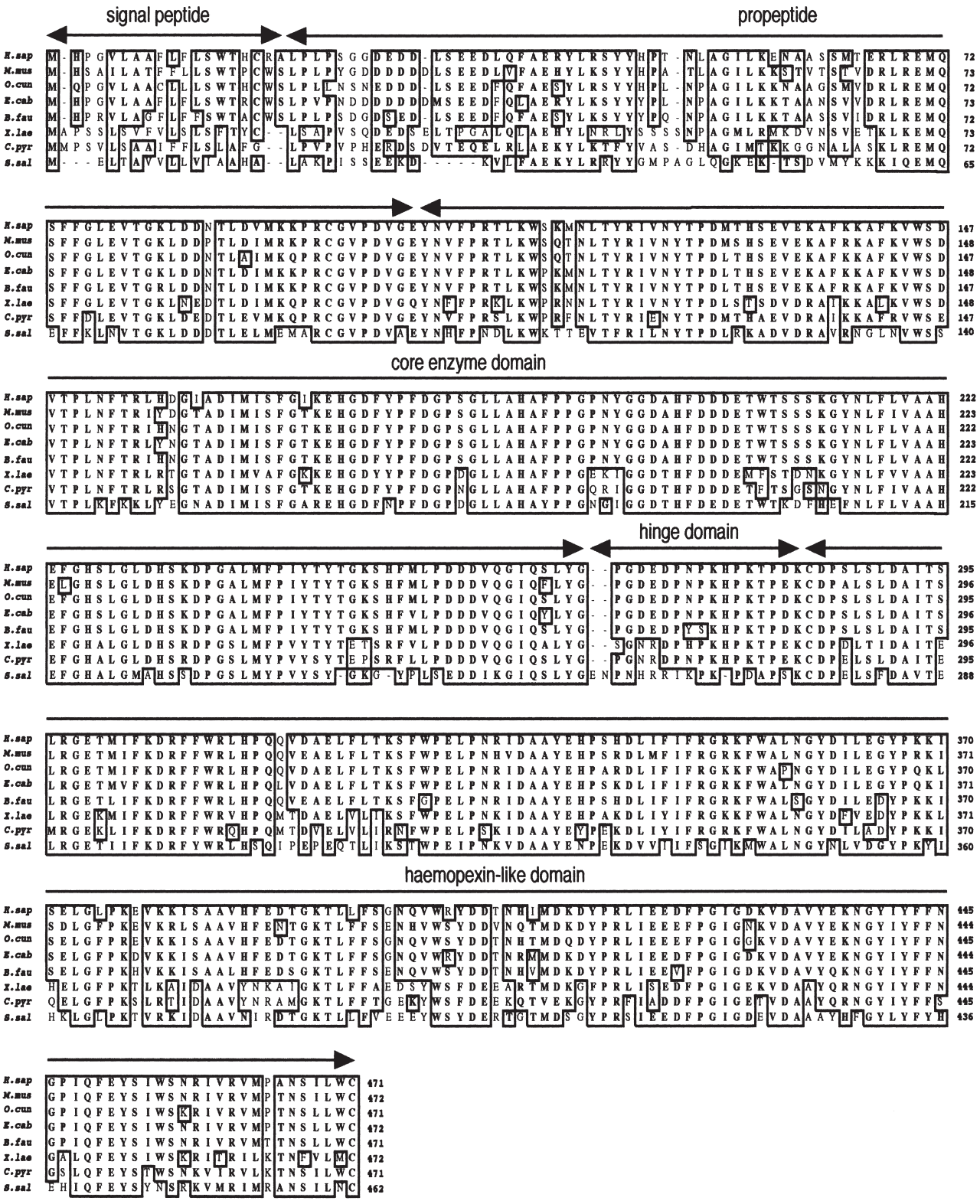

Fig. 1 Amino acid sequence alignment of salmon and known MMP13s.

Amino acid sequences of MMP13s from human (*H. sap*, accession No. X81334), mouse (*M. mus*, P33435), rabbit (*O. cun*, AAC39251), equine (*E. cub*, AF034087), bovine (*B. fau*, AF072685), frog (*X. lae*, L49412), newt (*C. pyr*, D82055) and salmon (*S. sal*, the present paper) were aligned. Conserved residues among the MMP13s (over 51%) were boxed. The putative functional domains are presented. Consensus sequence involved in the cysteine switch was presented by asterisks. Three histidines involved in Zn^{2+}-binding are presented by dots. L^{218}, D^{231} and P^{236} in human MMP13 are presented by closed triangles. The amino acid numbers are presented at the right side of each line.

or isoleucine) in frog, newt or mouse. D^{231} was replaced with alanine in salmon MMP13, which was the same in nine independent clones of ASE-3 cDNA. On the other hand, D^{231} as well as P^{236} was conserved in all other MMP13s presented in Fig. 3. Accoding to the dicussion of Knauper et al. (1996), these facts allow us to speculate that salmon MMP 13 may have a similar substrate range to human MMP13 but a lower proteolytic efficiency than that.

Reference

Collier, I. E, Smith, J., Kronberger, A., Bauer, E. A., Wilhelm, S. M., Eisen, A. Z. and Goldberg, G. I. (1988). The structure of the human skin fibroblast collagenase gene. *J. Biol. Chem.,* **263**, 6579-6587.

Freije, J. M., Diez-Itza, I., Balbin, M., Sanchez, L. M., Blasco, R., Tolivia, J. and Lopez-Otin, C. (1994) . Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J. Biol. Chem.,* **269**, 16766-16773.

Goldberg, G. I. (1989). SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J. Biol. Chem.,* **264**, 17213 -17221.

Heussen, C. and Dowdle, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.,* **102**, 196-202.

Jiang, W. and Bond, J. S. (1992). Families of metalloendopeptidases and their relationships. *FEBS Lett. ,* **312**, 110-114.

Kiyono, T., Nagashima, K. and Ishibashi, M., (1989). The primary structure of major viral RNA in a rat cell line transformed with type 47 human papillomavirus DNA and the transforming activity of its cDNA and E6 gene. *Virology,* **173**, 551-565.

Knauper, V., Lopez-Otin, C., Smith, B., Knight, G. and Murphy, G. (1996) . Biochemical characterization of human collagenase-3. *J. Biol. Chem.,* **271**, 1544-1550.

Laemmli, U. K. (1970) . Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature,* **227**, 680-685.

Matrisian, L. M. (1990) . Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet. ,* **6**, 121-125.

Shimizu, H., Nagashima, K., and Shimizu, J., (1999). Mechanical properties of freeze-thawed trout gelatin gel. *Food Sci. Technol. Res.,* **5**, 129-131.

Stetler-Stevenson, W. G., Brown, P. D., Onisto, M., Levy, A. T. and Liotta, L. A. (1990). Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. *J. Biol. Chem.,* **265**, 13933-13988.

Stricklin, G. P., Jeffrey, J. J., Roswit, W. T. and Eisen, A. Z. (1983). Human skin fibroblast procollagenase: mechanisms of activation by organomercurials and trypsin. *Biochemistry,* **22**, 1173-1178.

Takino, T., Sato, H., Yamamoto, E. and Seiki, M. (1995). Cloning of a human gene potentially encoding a novel matrix metalloproteinase having a C-terminal transmembrane domain. *Gene,* **155**, 293-298.

Wilhelm, S. M. , Collier, I. E., Kronberger, A., Eisen, A. Z. , Marmer, B. L., Grant, G. A., Bauer, E. A. and Goldberg, G. I. (1987). Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. *Proc. Natl. Acad. Sci.* USA, **84**, 6725-6729.

Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and Goldberg, G. I. (1989). SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J. B. C.,* **264**, 17231-17221.

Woessner, J. F. Jr. (1991). Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.,* **5**, 2145-2154.

van Wart, H. E. and Birkedal-Hansen, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential

applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA,* **87**, 5578-5582.

Yoshimizu, M., Kamei, M., Dirakubusarakom, S. and Kimura, T. (1988). "Invertabrate and Fish Tissue Culture.", Japan Scientific Societies Press, Tokyo / Springer-Verlag, Berlin.

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