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Review

Comparative biochemical properties of vertebrate deoxyribonuclease I

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ABSTRACT

Deoxyribonuclease I (DNase I, EC 3.1.21.1) is an endonuclease that preferentially attacks double-stranded DNA in a Ca^{2+}-dependent manner to produce oligonucleotides with 5'-phospho and 3'-hydroxy termini. This review deals with the biochemical properties and molecular evolution of DNase I. A comparative study of vertebrate DNnase I from Chondrichthyes to *Homo sapiens* has been carried out. The optimal pH stability, the role of N-glycosylation, actin inhibition, thermal stability, pH stability, and structure stability are discussed. Moreover, a phylogenetic analysis was performed. The levels of DNase I activity in serum have been suggested to be a critical factor in the initiation of human and rat SLE. Moreover, as shown above, DNase I is utilized in the treatment of patients with cystic fibrosis. Our comparative study of the biochemical properties and molecular analysis of DNase I will be helpful in the use of DNase I for clinical use.

Key words: deoxyribonuclease I; molecular evolution; vertebrate; substitution mutant

1 Introduction

Deoxyribonuclease I (DNase I, EC 3.1.21.1) is an endonuclease that preferentially attacks double-stranded DNA in a Ca^{2+}-dependent manner to produce oligonucleotides with 5'-phospho and 3'-hydroxy termini (Yasuda et al., 1990). DNase I has been regarded as a digestive enzyme. However, other functions *in vivo* have been clarified. DNase I has been shown as a candidate nuclease that is responsible for internucleosomal DNA degradation during apoptosis (Rauch et al., 1997, Mannherz et al., 1995). DNase I has been reported to be related to several diseases, such as systemic lupus erythematosus (Valle et al., 2008) and acute myocardial infarction (AMI)（Kawai et al., 2004; Kuribara et al., 009; Yasuda et al., 2009).

Comparative studies have been performed on human DNase I purified from various organs and body fluids (Yasuda et al., 1990; Yasuda et al., 1993; Nadano et al., 1991) showing similar physicochemical (Kishi et al., 1990) and catalytic properties (Yasuda et al., 1993).

Moreover, we have performed biochemical, molecular, and evolutionary studies of vertebrate DNases I (Yasuda et al., 1990, 1997; Takeshita et al., 1995, 1997; Mori et al., 2001; Kaneko et al., 2003; Ueki et al., 2007; Nakashima et al., 1999; Takeshita et al., 2001a; Takeshita et al., 2003; Yasuda et al., 2004; Yasuda et al., 2004). In addition, recent studies of DNases I from other vertebrate classes and from mammalian tissues other than pancreas have contributed new information, which will be summarized in this review.

2. Function and biochemistry of DNase I

2.1. DNase I function

DNase I is present principally in organs associated with the digestive system, such as the pancreas and parotid glands, from which it is secreted into the alimentary tract to hydrolyze

exogenous DNA (Moore, 1981; Nadano et al., 1993; Takeshita et al., 2000). The physiological roles of DNase I, other than as a digestive enzyme, have been elucidated. Napirei et al. (2000) demonstrated the presence of autoreactive antibodies and the occurrence of glomerulonephritis in a DNase I-level-dependent manner, suggesting DNase I may protect against autoimmunity by digesting extracellular nucleoprotein. Serum DNase I activity in SLE patients has been reported to be lower than that in healthy subjects (Yasutomo et al. 2001), suggesting that the maintenance of DNase I activity in serum may be an essential factor in the prevention of SLE. Other studies found that serum DNase I activity levels were transiently reduced by somatostatin through their effect on gene expression (Yasuda et al., 2001) and were elevated at the onset of acute myocardial infarction (Kawai et al., 2004). A remarkable increase in serum DNase I activity has been observed immediately after the onset of chest pain in AMI patients, peaking at 3 h after onset and gradually declining to basal levels within 12 h, suggesting that serum DNase I activity is a useful novel marker for AMI.

2.2. Clinical application of DNase I

DNase I is utilized in the treatment of patients with cystic fibrosis (CF). In such individuals, high levels of DNA make the sputum viscous and difficult to expectorate. Recombinant human DNase *in vitro* has been shown to reduce the viscoelasticity of the sputum in CF patients（Ranasinha et al., 1993).

2.3. Biochemical properties

DNase I is one of the most studied enzymes, with a known active site structure and mechanism of DNA hydrolysis. DNase I is sensitive to conformations of the double helix (Suck, 1994; Jones et al., 1996). The rate of hydrolysis depends strongly on the structure:

the B-form of DNA of mixed composition is the best substrate for DNase I, whereas extended A-T or G-G sites are relatively resistant to hydrolysis.

Mammalian DNase I is characterized by neutral optimal pH, a bivalent metal ion requirement for catalytic activity, inhibited by G-actin, and the formation of oligonucleotides with phosphate (Moore, 1981; Sierakowska and Shugar, 1977). Comprehensive comparisons of the enzymes among vertebrates have shown that the biochemical and molecular characterizations of mammalian (Yasuda et al., 1990, 1997; Takeshita et al., 1995, 1997; Mori et al., 2001; Kaneko et al., 2003; Ueki et al., 2007), avian (Nakashima et al., 1999), amphibian (Takeshita et al., 2001a), reptilian (Takeshita et al., 2003), piscine (Yasuda et al., 2004), and shark (Yasuda et al., 2004) DNase I differ (Table 1). An analysis of these differences is shown below.

2.4. Tissue distribution of vertebrate DNase I

DNase I is one of the digestive enzymes secreted into the alimentary tract by exocrine glands, such as the pancreas and /or parotid glands. Previously, we conducted a comparative study to investigate the tissue distribution of the mammalian DNase I (Takeshita et al., 2000). These results are summarized in Table 1. As a result, a mammalian enzyme can be classified into three types: (1) pancreas, (2) parotid, and (3) pancreas-parotid or mixed (Fig. 1). The tissue distribution of DNase I may reflect eating habits: humans and pigs (pancreas-type) are classified as omnivores, whereas rats and mice (parotid-type) and bovines and rabbits (pancreas-parotid-type) are classified as herbivores (Takeshita et al., 2000). We then examined the acid sensitivity of the mammalian enzymes (Fig.2). Pancreas-type enzymes are more sensitive to acidic conditions (pH 5 ± 3) than are the parotid- and pancreas-parotid types. Mammalian gastric juice is usually maintained at about pH 3 ± 6.5 by nutrients. Our findings suggest that the DNase I of the parotid and

pancreas-parotid types, which have to pass through the stomach, are more acid-stable than that of the pancreas type, which is secreted into the small intestine at neutral pH . It is plausible that the DNase I activities of both the parotid and pancreas-parotid types are preserved as they pass through the stomach by changing their primary structure into an acid-stable form.

Pancreatic- type DNase I is secreted into the duodenum as a component of pancreatic juice (Keller et al., 1958). Since trypsin and chymotrypsin are concomitantly present in pancreatic juice, it is postulated that pancreas-type DNase I is not vulnerable to proteolysis by these proteases in order to fully maintain their digestion function activity. On the other hand, if parotid- and mixed-type DNase I secreted into the oral cavity can pass through the stomach intact, then it must be resistant to proteolysis by pepsin.

The susceptibilities of the vertebrate DNase I to proteolysis (trypsin or chymotrypsin or pepsin) were examined (Ueki et al., 2007). Nine mammalian DNases I (horse, *Equus caballus*; rat, *Rattus norvegicus*; mouse, *Mus musculus*; pig, *Sus scrofa domesticus*; dog, *Equus caballus* ; human, *Homo sapiens*; chimpanzee, *Pan troglodytes*; cow, *Bos taurus* and rabbit, *Oryctolagus cuniculus*) and other vertebrate DNase I (hen, *Gallus gallus domesticus;* reptile, *Elaphe quadrivirgata; Elaphe climacophora; Agkistrodon blomhoffii;* amphibian, *Bufo vulgaris*; *Rana catesbeiana* and Tilapia, *Oreochromis mossambica*), examined so far, are predominantly present in the pancreas, being classified as pancreatic-type enzymes) were separately subjected to proteolysis. As a result, the human, porcine, and canine (pancreatic-type) DNases I are more resistant to proteolysis by trypsin and chymotrypsin than the horse, rat, and mouse (parotid-type) enzymes (Table 1).

As for pepsin, the bovine and rabbit enzymes (mixed-type) exhibited relatively high resistance to proteolysis. The activities of humans, mice, and rats (parotid-type) were sensitive; the equine, rat and mouse enzymes were also highly sensitive to pepsin-proteolysis

when compared to bovine and rabbit enzymes.

These results demonstrated a marked correlation between tissue distribution and sensitivity/resistance to proteolysis; pancreatic-type DNase I shared properties of resistance to proteolysis by trypsin and chymotrypsin, whereas parotid-type DNase I did not. In contrast, pancreatic–parotid-type DNase I exhibited resistance to proteolysis by pepsin, whereas the other enzyme types did not.

In contrast to the pancreatic-type DNases I of mammal, hen, reptile, amphibian, and fish enzymes of were more labile to proteolysis, irrespectively of their classification as pancreatic-type enzymes. It can be postulated that pancreatic-type DNases I have acquired resistance protease in pancreatic juice at the stage of evolutionary transition from reptiles to birds. A site-directed mutagenesis analysis revealed that a single amino acid substitution could not account for the acquisition of proteolysis resistance.

As for body fluids, DNase I activity can be detected in urine, seminal fluid, and serum. Among these body fluids, urine shows extremely higher activity: ~10000 x 10^3 units/mg proteinl was found in urine, and 1-2 x 10^3 units/mg protein was found in serum (Nadano et al., 1993).

3. Structure of vertebrate DNase I

3.1. Amino acid sequences of DNase I

Figure 3 shows the entire aa sequence of DNase I for *Homo sapiens* (Yasuda et al., 1995), *Equus caballus* (Ueki et al., 2003), *Canis lupus familiaris* (Kaneko et al., 2003), *Bos taurus* (Chen et al., 1998), *Sus scrofa domesticus* (Mori et al., 2001), Sheep: *Ovis aries*; *Oryctolagus cuniculus* (Yasuda et al., 1997), *Mus musculus* (Takeshita et al., 1997), *Rattus norvegicus* (Polzar et al., 1990), *Gallus gallus domesticus* (Nakashima et al., 1999), snake (*Elaphe quadrivirgata*: Shima-hebi in Japanese; *Elaphe climacophora:*Aodaisho;

Agkistrodon blomhoffii: Nihon-mamushi) (Takeshita et al., 2003), reptile (frog: *Xenopus laevis* and *Rana catesbeiana;* newt: *Cynopus pyrrhogaster*; toad: *Bufo vulgaris japonicas*) (Takeshita et al., 2001a), teleost (tilapia,*Oreochromis mossambica*: carp, *Cryprus carpio*; eel, *Anguilla japonica: Pagrus major*: sea bream) (Yasuda et al., 2004), and elasmobranch (Japanese bullhead shark: *Heterodontus japonicus* and Banded hounds shark: *Triakis scyllia*) (Yasuda et al., 2004). These sequences were obtained from the respective database accession numbers shown in Table 1.

The four aa residues (Glu78, His134, Asp212, and His252) responsible for the catalytic activity (Jones et al., 1996) and two Cys residues at positions 173 and 209 that form the disulfide bond responsible for structural stability of the enzyme (Oefner and Suck, 1986) were conserved in all vertebrates.

Mammalian DNases I possess two potential N-glycosylation sites, Asn18 and Asn106. These two sites were both well conserved in mammalian DNase I (Asn18 (Asn-Asp-Thr) and Asn106 (Asn-Asp-Thr), except for the rabbit enzyme, which has only one potential site at Asn^{18} -^{19}Ala-^{20}Thr, and in bovine DNase I, in which Thr^{108} is replaced by Ser^{108}).The corresponding amphibian residues are replaced by 18Asp and 106Thr, respectively and, in piscine DNase I, the latter site is replaced by Thr or Gln. In shark, these sites are not conserved.

Four aa residues (Glu13, Tyr65, Val67, and Ala114) are thought to be involved in the binding of G-actin to human and bovine DNases I. It has been suggested that two aa residues (Val-67 and Ala-114) are mainly responsible for actin binding in human and bovine DNase I (Mannherz, 1992; Ulmer et al., 1996). In comparison to mammalian enzymes, the viper snake enzyme has the same aa residue at 67 but a different residue at 114. However,

in the rat snake and African clawed frog, 67-Val is replaced by 67-Ile. 114-Ala, which is retained in most mammalian enzymes, is replaced by 114-Phe in reptilian and amphibian enzymes.

Ile130 and Met166 of *Homo sapiens, Bos taurus , Sus scrofa domesticus , Oryctolagus cuniculus , Rattus norvegicus , Mus musculus ,* and *Gallus gallus domesticus* were replaced by Leu130 and Leu166, respectively, in snakes. The former were observed in all the lower vertebrates studied, i.e., four amphibia and one fish, which are all classified as cold-blooded vertebrates.

Amphibian DNases I had two main, unique structural alterations. First, they all possessed an additional stretch region containing 70 (*X. laevis*), 71 (*C. pyrrhogaster*), or 73 (*B. vulgaris* and *R. catesbeiana*) residues in their C-terminal regions and were, therefore, considerably longer (331±334 residues) than the enzymes from other vertebrates (258±262 residues). A particular feature of this C-stretch region was its high Cys content (about 15%). Secondly, all the amphibian DNases I tested had an additional 205Ser residue in their mid-region, located in an area that has been postulated to form an essential Ca2+-binding site in other vertebrate enzymes and corresponding to a position between 204Ala and 205Thr in the human enzyme. Insertion of Asn205 and Thr206 residue into an essential Ca(2+)-binding site are also shown in shark enzymes.

Cys residues at position 101 and 104 form a disulfide bond that contributes to the structural stability of the enzyme protein in addition to another disulfide bond formed by Cys173 and Cys209. These residues are substituted at positions 102 and 105 of eel DNase I and at positions 100 and 103 of tilapia DNase I, with Ala and Ser and with Pro and Thr, respectively. In shark, these residues were replaced by Ala100 and Thr103 in *H. japonicus* DNase I and by Ser101 and Ser104 in *T. scyllia*.

3.2. Phylogenetic analysis

Based on the aa sequences of the mature enzyme region in 36 DNases I available from various databases, a phylogenetic tree was constructed (Fig. 4).

The aa sequences of the DNases I derived from *Homo sapiens* , *Mus musculus* , *Rattus norvegicus* (Polzar et al., 1990), *Oryctolagus cuniculus* , *Sus scrofa domesticus* , *Bos taurus* (Chen et al., 1998), *Canis lupus familiaris* , *Gallus gallus domesticus* , *Ovis aries* (Paudel and Liao, 1986), *Pan troglodytes*;, *Macaca Rhesus* (Rhesus monkey), *Equus caballus*, *Ailuropoda melanoleuca* (giant panda), *Didelphis virginiana* (opossum), *Ornithorhynchus anatinus* (platypus*), Gallus gallus domesticus* , *Meleagris gallopavo* (turkey), *Taeniopygia guttata* (Zebra finch), *Elaphe quadrivirgata*, *Agkistrodon blomhoffii*, *Xenopus laevis*, *Rana catesbeiana*, *Bufo vulgaris japonicas*, *Cynopus pyrrhogaster* , *Oreochromis mossambica* , *Cyprinus carpio* (carp), *Takifugu rubripes* (Japanese puffer fish), *Pagrus major* (Sea bream), *Danio rerio* (zebra fish), *Anguilla japonica*, *Heterodontus japonicas*, *Triakis syllia*,*Branchiostoma Floridae* (Florida lancelet), *Halocynthia roretzi* (sea squirt), *Anthocidaris crassispina* (purple sea urchin), *Actinia equina* (sea anemone), and *Trilchoplex adhaerens* were obtained from each reference**.**
Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) from the aa sequences corresponding to the mature enzyme part of each DNase I by use of GENETYX-MAC Ver. 10.1.2 (GENETYX Co. Ltd., Tokyo, Japan).
Invertebrates form the same cluster as the amphibians, and fish form one cluster. Reptiles and birds form another cluster. As for mammals, the platypus and opossum form independent clusters.

4. Mutant enzyme analysis of DNase I

4.1. Optimal pH of mammals

As reported above, the optimal pH differs among the pancreas, parotid, and pancreas-parotid types. There are two differences in the amino acid sequence between human and rat DNases I: positions 110 and 178 of the amino acid of human DNase I (pancreas type) are asparagines (N) and proline (P), respectively, whereas these positions in rat DNase I (parotid type) are serine (S) and serine(S), respectively. To investigate the involvement of amino acid replacements at the aa position 110 and 178 in the optimal pH of mammalian DNase I, mutant enzyme (N110S and p178S were constructed.

A human mutant enzyme produced in COS-7 cells by transfection with a N110S substitution construct becomes as acid-stable as the rat enzyme, whereas another mutant produced by transfection with a P178S substitution construct is as acid-labile as the wild type of the human enzyme (Fig.5A). Conversely, a rat mutant enzyme produced by transfection with the S110N or S110A substitution constructs becomes as acid-labile as the human enzyme (Fig.5B). Therefore, it is likely that the rat enzyme obtained its acid stability by a N110S substitution during the course of evolution.

4.2. N-glycosylation site

DNase I is known to be a glycoprotein, and two potential N-linked glycosylation sites (N18 and N106) are known for the mammalian enzymes. We have previously investigated the biological role of N-linked glycosylation in three mammalian (human, bovine, and equine) DNases I by constructing substitution mutants (N18Q, N106Q, and N18Q/N106Q) using site-directed mutagenesis (Fujihara et al., 2008).

The enzyme activities of N18Q and N106Q were lower than those of the wild type, and those of the double mutant (N18Q/N106Q) were lower than those of single mutants. In addition, all mutant enzymes were unstable to heat, suggesting that both sites are required for heat stability. Moreover, in human and equine enzymes, the N18Q and N106Q mutant

enzymes were less resistant to trypsin, while N18Q/N106Q was most sensitive to trypsin. As for bovine DNase I, the trypsin resistance of N18Q and N106Q was similar to that of the wild type, but that of N18Q/N106Q decreased in a time-dependent manner. On the other hand, N-linked glycosylation was not related to pH sensitivity.

4.3. Actin inhibition

Actin, which binds to DNA-rich fibers, potently inhibits the enzymatic activity of recombinant human DNase (Zahm et al., 2001). Actin-binding capacities have been shown to differ among species. DNase I activity in human (Yasuda et al., 1990), cow (Paudel et al., 1986), and mouse (Takeshita et al., 1997) is inhibited by G-actin. In contrast, the rat DNase I activity has been shown to be much more resistant to G-actin (Kreuder et al., 1984), and that of the pig (Mori et al., 2001), chicken (Nakashima et al., 1999), and amphibian (toad, frog, African clawed frog, and newt) (Takeshita et al., 2001a) is unaffected by G-actin (Table 1).

Amino acid residues (Val-67 and Ala-114) have been suggested as being mainly responsible for actin binding in human and bovine DNase I. To investigate the role of aa at 67, mutants of rat snake (Ile67Val) and viper snake (Val67Ile) enzymes were constructed (Fujihara et al., 2006). After substitution, the rat snake was inhibited by actin, while the viper snake was not. For the role of aa at 114, mutants of viper snake (Phe114Ala), rat snake (Phe114Ala), African clawed frog (Phe114Ala), and porcine (Ser114Ala/Ser114Phe) enzymes were constructed. Strikingly, the substitute mutants for viper snake, rat snake, and African clawed frog expressed no protein. The porcine (Ser114Ala) enzyme was inhibited by actin, but the porcine (Ser114Phe) enzyme was not. These results suggest that Val-67 may be essential for actin-binding, Phe-114 may be related to the folding of DNase I in reptiles and amphibians, and Ala-114 may be indispensable for actin-binding in mammals.

4.4. Thermal stability of reptile DNase I

Wild-type snake DNases I is more thermally unstable than wild-type mammalian and avian DNases I. When the primary structures of the enzymes of three snakes, *Elaphe quadrivirgata* (Shima-hebi in Japanese), *Elaphe climacophora* (Aodaisho), and *Agkistrodon blomhoffii* (Nihon-mamushi), were compared with those of other vertebrates, only two aa residues, Leu130 and Leu166, were different. These two residues were Ile130 and Met166, respectively, in human and bird. To investigate the biological significance of these amino acids in snake, substitution mutants were constructed, and their thermal stabilities were investigated (Takeshita et al., 2003) (Fig. 6). *E. quadrivirgata*, *E. climacophora*, and *A. blomhoffii*, wild-type DNases I were all less thermally stable than human, rat, and mouse wild-type enzymes. The mutant enzymes, *E. quadrivirgata* (Leu130Ile), *E. quadrivirgata* (Leu130Ile/Leu166Met), and *A. blomhoffii* (Leu130Ile) were all more thermally stable than the corresponding wild-type DNases I (Fig.6A). On the other hand, *E. quadrivirgata* (Leu166Met) and *A. blomhoffii* (Leu166Met) were as thermally unstable as their wild-type counterparts. Conversely, human (Ile130Leu) and human (Ile130Leu/ Met166Leu) mutants were more thermally unstable than their wild-type counterparts, whereas human (Met166Leu) was not (Fig.6B). The same was true for the mutants of rat and mouse DNase I. These findings demonstrate that the nature of the amino acid at position 130 may generally and markedly affect the thermal stabilities of vertebrate DNases I.

The 3D structure of DNase I based on the X-ray structure analysis of the bovine enzyme showed that the aa residue at position 130 is located in the central core, whereas that at position 166 is not. Accordingly, it could be predicted that a substitution of the former residue might induce some alterations in the structural stability of DNase I (Lahm and Suck, 1991; Keenan et al., 2001).

In reptiles, a single amino acid (aa) substitution of Leu130Ile in the DNase I of the viper snake and two types of rat snakes made the DNase I more thermal-stable than mammal and avian DNase I (Takeshita et al., 2003).

4.5. Thermal stability of amphibian DNase I

As reported above, amphibian DNase I is characterized by a C-terminal end with a unique cysteine-rich stretch and by insertion of a Ser residue into the Ca^{2+}-binding site, which results in thermal instability (Takeshita et al., 2001a).

The biochemical significance of the C-stretch in amphibian DNases I was examined. The two deletion mutants, *R. catesbeiana*-del (C-stretch) and *X. laevis*-del (C-stretch), yielded no immunoreactive bands on SDS/PAGE. This suggests that the mutant proteins might be rapidly degraded in the transfected cells. Although all the residues responsible for catalytic activity were situated outside the C-stretch region, the C-stretch might be required for the formation and/or protection of the active form.

To examine the biochemical role of the Ser^{205} insertion in amphibian DNase I, insertion mutants [human-ins(S205) and rat-ins(S205)] and a deletion mutant [*R. catesbeiana*-del(S205)*X. laevis*-del(S205)] were constructed, and their heat stability was compared with that of the corresponding wild-type enzymes (Fig.7). The insertion of a Ser residue between Ala^{204} and Thr^{205} of the human and rat DNases I rendered these enzymes heat-labile (Fig.7A, B); their heat stability was similar to that of *X. laevis* wild-type DNase I (Fig.8A). An X-ray structural analysis of bovine DNase I (Oefner and Suck, 1986) has shown that a Ca^{2+}-binding site coordinated with the main-chain oxygens of Thr^{203}, Thr^{205}, and Thr^{207} and the two oxygens of the Asp^{201} side chain seem to be responsible for conformational changes and protect the enzyme from inactivation. Both the human-ins (S205) and rat-ins (S205) mutants were heat-labile, similarly to the amphibian enzymes.

The structural change caused by the insertion of a serine residue into this Ca^{2+}-binding site might have induced the instability (Fig.8). In contrast, although the *R. catesbeiana*-del (S205) and *X. laevis*-del (S205) mutants yielded immunoreactive bands at the same positions as the wild-type enzymes on SDS/PAGE Western blot analysis, they showed no enzyme activity. These findings strongly suggest that Ser^{205} in amphibian DNases I is essential for the generation of the active enzyme conformation irrespectively of whether this induces instability of the enzyme protein.

The same results were obtained in shark DNase I: the second structural alteration in *T. scyllia* and *H. japonicus* DNases I was the insertion of Asn205 and Thr206, corresponding to the position between Ala204 and Thr205 in human DNase I. Two mutants, human-ins (Thr205) and *H. japonicus*-del (Thr206), in which a Thr residue was inserted or deleted in the human and *H. japonicus* enzymes, respectively, were constructed, and their thermal stability was compared with that of the corresponding wild types.

4.6. pH stability

Vertebrate DNases I could be classified into two groups: a low-pH group (with a pH optimum of 6.5-7.0), such as mammalian enzymes, and a high-pH group (with a pH optimum of approximately 8.0), such as reptile, amphibian, and piscine enzymes. This indicates that their catalytic properties are suited to environments with relatively higher pH.

The amino acid sequences of the five piscine DNases I were compared with those of other vertebrates, and the five amino acid residues (Asp44, Met118, Gln134, Glu190, and Met236 in eel DNase I) corresponding to those commonly observed in all piscine enzymes were found to be different from the residues of the mammalian enzymes. These five residues were replaced by His44, Arg/Lys117, Leu133, Ser189, and Gln236, respectively. Any of the five amino acid substitutions listed above may be responsible for the shift of optimum

pH for DNase I activity. Therefore, we constructed mutants of eel DNase I (eel-Asp44His, -Met118Arg, -Gln134Leu, -Glu190Ser, and -Met236Gln) and the amino acid residues in eel DNase I were replaced by their human enzyme counterparts. Their optimum pH activities were compared to those of the wild-type eel enzyme (Fig. 9A). The eel-Asp44His mutant showed a shift of optimum pH from 8.0 to 6.5–7.0. On the other hand, the pH–activity profiles of the four mutants, eel-Met118Arg, -Gln134Leu, -Glu190Ser, and -Met236Gln, had an optimum pH of 8.0, similar to that of the wild-type eel enzyme. These results indicate that Asp44 is involved in its catalytic properties, while the four residues, Met118, Gln134, Glu190, and Met 236, of eel DNase I are not. Identical results were obtained in a mutant of tilapia DNase I; the optimal pH of tilapia-Asp44His also shifted to 6.5–7.0 (Fig. 9B). The optimum pH shift of DNases I to a more alkaline range is similar to that which occurs in reptile (Takeshita et al., 2003) and amphibian (Takeshita et al., 2001) enzymes. Replacement of Asp44 of piscine, reptile, and amphibian DNases I by His decreased their optimum pH to a value similar to that of the low-pH group. Asp44His might be involved in an evolutionarily critical change in the optimum pH for the activity of vertebrate DNases I.

4.7. Structure Stability

Chen et al. (1998) have reported that the disulfide bond is important for the structural integrity of bovine DNase I. Cys residues at positions 101 and 104 form a disulfide bond that contributes to the structural stability of the enzyme protein, in addition to another disulfide bond formed by Cys173 and Cys209 (Suck et al., 1984; Lahm and Suck, 1994). In the shark, these residues were replaced by Ala100 and Thr103 in *H. japonicus* DNase I and by Ser101 and Ser104 in *T. scyllia*. The substitution mutant human-sub (Cys101Ala/Cys104Thr), in which the two Cys residues of human DNase I were substituted

by their counterparts from *H. japonicus*, exhibited lower thermal stability than the corresponding wild type, whereas double-substitution mutant I, *H. japonicus*-sub(Ala100Cys/Thr103Cys), made the enzyme more thermally stable than the wild type (Fig. 10A). These findings suggest that the formation of the disulfide bond between Cys101 and Cys104 may have been acquired during the evolutionary stage in Osteichthyes, resulting in the production of a more stable form of the enzyme.

Identical results have been shown in piscine DNase I. Cys at 101 and 104 residues are substituted at positions 102 and 105 of eel DNase I and at positions 100 and 103 of tilapia DNase I with Ala and Ser and Pro and Thr, respectively. Two mutants, eel-Ala102Cys/Ser105Cys and human-Cys101Ala/Cys104Ser, were constructed, and their thermal stability was compared with that of the wild-type enzymes (Fig.10B). Incorporation of two Cys residues into eel DNase I enhanced its thermal stability, whereas removal of two Cys residues from the human enzyme diminished its thermal stability.

5. Molecular evolution of DNase I

Figure11 shows the scheme of molecular evolution of DNase I. To maintain DNase I activity, vertebrates acquire enzyme and thermal stability, optimal pH, and proteolysis resistance.

Two Cys residues at positions 101 and 104, which are well conserved in the vertebrate DNases I, are substituted in shark and piscine DNase I. Therefore, the disulfide bond formed between Cys101–Cys104 may also contribute to the stability of the enzyme. Shark and piscine species enzymes are thermally labile in comparison to reptiles, amphibians and mammals. The formation of the disulfide bond between Cys101 and Cys104 may have been acquired during the evolutionary stage in Osteichthyes, resulting in the production of a

more stable form of the enzyme.

The unique cysteine-rich-terminus (C-terminal end with a unique cysteine-rich stretch of approximately 70 amino acid residues; the reptile and piscine enzymes possess no such C-terminal stretch) and the insertion of a Ser residue at position 205 (in a domain containing an essential Ca^2+-binding site) were observed in amphibian DNase I.

There are at least two mechanisms that might be involved in the thermal stability of vertebrates. One is Ser205 in amphibians; the other is the substitution of Ile130Leu in snakes. It is interesting that DNases I of warm-blooded vertebrates (*Homo sapiens, Sus scrofa domesticus*, *Oryctolagus cuniculus*, *Rattus norvegicus*, and Gallus *gallus domesticus*) are all thermally stable, while those of cold-blooded vertebrates (*Elaphe quadrivirgata***,** *Elaphe climacophora***,** *Agkistrodon blomhoffii***,** *Xenopus laevis***,** *Rana catesbeiana***,** *Cynopus pyrrhogaster, Bufo vulgaris japonicas*) are all thermally unstable. It could be postulated that the thermally stable DNases I of the vertebrates must have been produced principally by the Leu130Ile substitution in avian enzymes first at the evolutionary stage from reptiles to birds after deletion of Ser205 from the enzymes of amphibians as they evolved into reptiles.

With regard to the optimum pH for activity, vertebrate DNases I can be classified into a low-pH group (the mammalian enzymes: optimum pH of 6.5–7.0) or a high-pH group (the reptile, amphibian, and piscine enzymes: optimum pH of 7.5–8.0). A His residue is commonly found at position 44 in the former group, whereas an Asp residue is found in the latter group. The pH of mammalian blood is known to be lower than that of fish blood. The enzymological properties of the DNases I of the two groups appear to be matched to the physiological conditions. It could be postulated that low-pH group DNases I, such as that in mammals, must have been produced by a single Asp44His substitution in the high-pH group during the course of evolution.

6. Conclutions

In the present review, compatrative biochemical properties (the optimal pH stability, the role of N-glycosylation, actin inhibition, thermal stability, pH stability, and structure stability) and molecular evolution of vertebrate DNase I have been discussed.

Levels of DNase I activity in serum have been suggested to be a critical factor in the initiation of human and rat SLE (Bruch et al., 1989; Napirei et al., 2000). Serum DNase I activity levels were elevated at the onset of AMI, suggesting that serum DNase I activity is a useful novel marker for AMI (Kawai et al., 2004). Moreover, as shown above, DNase I is utilized in the treatment of cystic fibrosis. Our comparative study of the biochemical properties and molecular analysis of DNase I will be helpful to advance the use of DNase I in clinical settings.

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Figure legends

Fig. 1. Distribution patterns of mammalian DNases I

Fig. 2. Comparison of acid sensitivities among pancreas type, pancreas-parotid type and parotid type DNases I.

Fig. 3. Aligment of amino acid sequences of DNases I from *Homo sapiens* (Yasuda et al., 1995), *Equus caballus* (Ueki et al., 2003), *Canis lupus familiaris* (Kaneko et al., 2003), *Bos taurus* (Chen et al., 1998), *Sus scrofa domesticus* (Mori et al., 2001), *Ovis aries*, *Oryctolagus cuniculus* (Yasuda et al., 1997), *Mus musculus* (Takeshita et al., 1997), *Rattus norvegicus* (Polzar et al., 1990), *Gallus gallus domesticus* (Nakashima et al., 1999), snake (*Elaphe quadrivirgata*: Shima-hebi in Japanese; *Elaphe climacophora*:Aodaisho; *Agkistrodon blomhoffii*: Nihon-mamushi) (Takeshita et al., 2003), reptile (frog: *Xenopus laevis* and *Rana catesbeiana*; newt: *Cynopus pyrrhogaster*; toad: *Bufo vulgaris japonicas*) (Takeshita et al., 2001a), teleost (*Oreochromis mossambica*: tilapia; *Cryprus carpio*: carp; *Anguilla japonica*: eel; *Pagrus major*: sea bream) (Yasuda et al., 2004), and elasmobranch (Japanese bullhead shark: *Heterodontus japonicus* and Banded hounds shark: *Triakis scyllia*) (Yasuda et al., 2004). Sequence of each DNase I was taken from each refarences. GenBank accession numbers for these amino acid sequences are shown in Table 1.

Fig. 4. Phylogenetic analysis of the vertebrate DNase I family based on their amino acid sequences. The amino acid sequences encoding the mature DNase I proteins of various

species were subjected to phylogenetic analysis by the neighbor-joining method (Saitou N, Nei M). The numbers represent evolutionary distance. References and accession numbers for the amino acid sequences of DNase I are in part described in the Table 1 and those of other species (*Pan troglodytes, Macaca Rhesus , Ailuropoda melanoleuca,* opossum, *Ornithorhynchus anatinus, Meleagris gallopavo , Taeniopygia guttata, Takifugu rubripes, Branchiostoma Floridae, Halocynthia roretzi, Anthocidaris crassispina, Actinia equina,* and *Trilchoplex adhaerens*) are obtained from the KEGG database (http://www.genome.jp/kegg/).

Fig. 5. Changes of acid sensitivity of DNases I in wild types and mutants derived from t *Rattus norvegicus* (A) and *Homo sapiens* (B).

Fig. 6. Thermal stability of wild-type and mutant DNases I derived from *Elaphe quadrivirgata*; *Elaphe climacophora*; *Agkistrodon blomhoffii*:(A) and *Homo sapiens* (B).

Fig. 7. Effects of insertion of S205 in *Homo sapiens* (A) and *Rattus norvegicus* (B) DNases I on thermal stability at 50˚C

Fig. 8. Conformational alterations accompanied by insertion and deletion of Ser205.

Fig. 9. Optimal pH of wild-type and mutant DNases I derived from *Oreochromis mossambica*.

Fig. 10. Heat stability of shark (*H. japonicas*) (A) and human (B) DNases I and their mutant constructs.

Fig. 11. Acquisition of stability and neutral shift of optimal pH in vertebrate DNases I during evolution.

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Table 1. Summary of amino acid sequences and biochemical properties of DNase I.

Species	Accession number	Typical alterations in amino acid sequence compared with the human DNase I	N-glycosylation site (Asn18 and Asn106)	Inhibition by G-actin	Tissue distribution	Optimal pH	Proteolysis by trypsin or chymotrypsin	Proteolysis by pepsin	Other properties compared with the human DNase I
Mammals									
Homo sapiens	M55983		both	Inhibited	Pancreas	6.5	Resistant	Highly sensitive	
Mus musculus	D83038		both	Inhibited	Parotid glanc	6.5	Sensitive	Sensitive	
Rattus norvegicus	X56060		both	not	Parotid glanc	6.5	Sensitive	Sensitive	
Oryctolagus cuniculus	D82875		both	Inhibited	Parotid glanc	6.5	Resistant	Resistant	
Sus scrofa domesticus	AB048832	Ser110Asn, Ser178Pro, Ala114Ser	present	not	Pancreas	6.5	Resistant	Highly sensitive	
Bos taurus	AJ001538		both	Inhibited	Mix	6.5	H. sensitive	Resistant	
Equus caballus	AB162819	65Phe?	both	Inhibited	Parotid glanc	6.5	H. sensitive	Sensitive	
Canis lupus familiaris	AB113380	Ile130Val	both	Inhibited	Pancreas	6.5	Resistant	Highly sensitive	
Avian									
Gallus gallus domesticus	AB013751		both	not	Pancreas	7.0	Resistant	Highly sensitive	
Reptile (Snake)									More unstable
Elaphe quadrivirgata	AB04545	Ile130Leu, Met166Leu, Val67Ile, Ala114Phe	both	not	Pancreas	7.5	Sensitive	not tested	
Elaphe climacophora	AB058784	Ile130Leu, Met166Leu, Val67Ile, Ala114Phe	both	not	Pancreas	7.5	Sensitive	not tested	
Agkistrodon blomhoffii	AB050701	Ile130Leu, Met166Leu, Ala114Phe	both	not	Pancreas	7.5	Sensitive	not tested	
Amphibian									More unstable
Bufo vulgaris	AB045037	Additional stretch region contains high Cys Ser205 insertion in Ca2+ binding site	Asn106Thr	not	Pancreas	8.0	Sensitive	not tested	
Rana catesbeiana	AB038776	Additional stretch region contains high Cys Ser205 insertion in Ca2+ binding site	Asn106Thr	not	Pancreas	8.0	Sensitive	not tested	
Xenopus laevis	AB030958	Additional stretch region contains high Cys Ser205 insertion in Ca2+ binding site	Asn106Thr	not	Pancreas	8.0	Sensitive	not tested	
Cynopus pyrrhogaster	AB041732	Additional stretch region contains high Cys Ser205 insertion in Ca2+ binding site	Asn106Thr	not			Sensitive	not tested	
Osteichthyes									
Anguilla japonica	AB097843	Ala22 4deletion, Cys102Ala. Cys105Ser	Asn106 Thr/Gln106	?	Pancreas	8.0	Sensitive	not tested	
Oreochromis mossambica	AJ001538	Ala224 deletion, Cys100Pro, Cys103Thr	Asn106 Thr/Gln106		Pancreas	8.0	Sensitive	not tested	
Cyprinus carpio	AB075779	Ala224 deletion, Ser59 insertion	Asn106 Thr/Gln106		Pancreas	8.0	Sensitive	not tested	
Pagrus major	AB097844	Ala224 deletion, Ser59 insertion	Asn106 Thr/Gln106		Pancreas	6.5-8.0	Sensitive	not tested	
Chondrichthyes							not tested	not tested	Less content of α-helics in the signal peptide
Heterodontus japonicus	AB126699	Cys100Ala, Cys103Thr, Asn205and Thr206 insertion		?					
Triakis scyllia	AB126700	Cys100Ala, Cys103Thr, Asn205and Thr206 insertion							

Fig.1

Types	Organs	DNase I activities	Acid stability
Parotid type *Mus musculus,* *Rattus norvegicus* *Oryctolagus cuniculus* *Equus caballus*	Parotid gl. Pancreas		Sensitive
Pancreas type *Homo sapiens* *Sus scrofa domesticus* *Reptile,* *Amphibian* *Fish*	Parotid gl. Pancreas		Stable
Pancreas-Parotid type(Mixed type) *Bos taurus*	Parotid gl. Pancreas		Stable

Fig.2

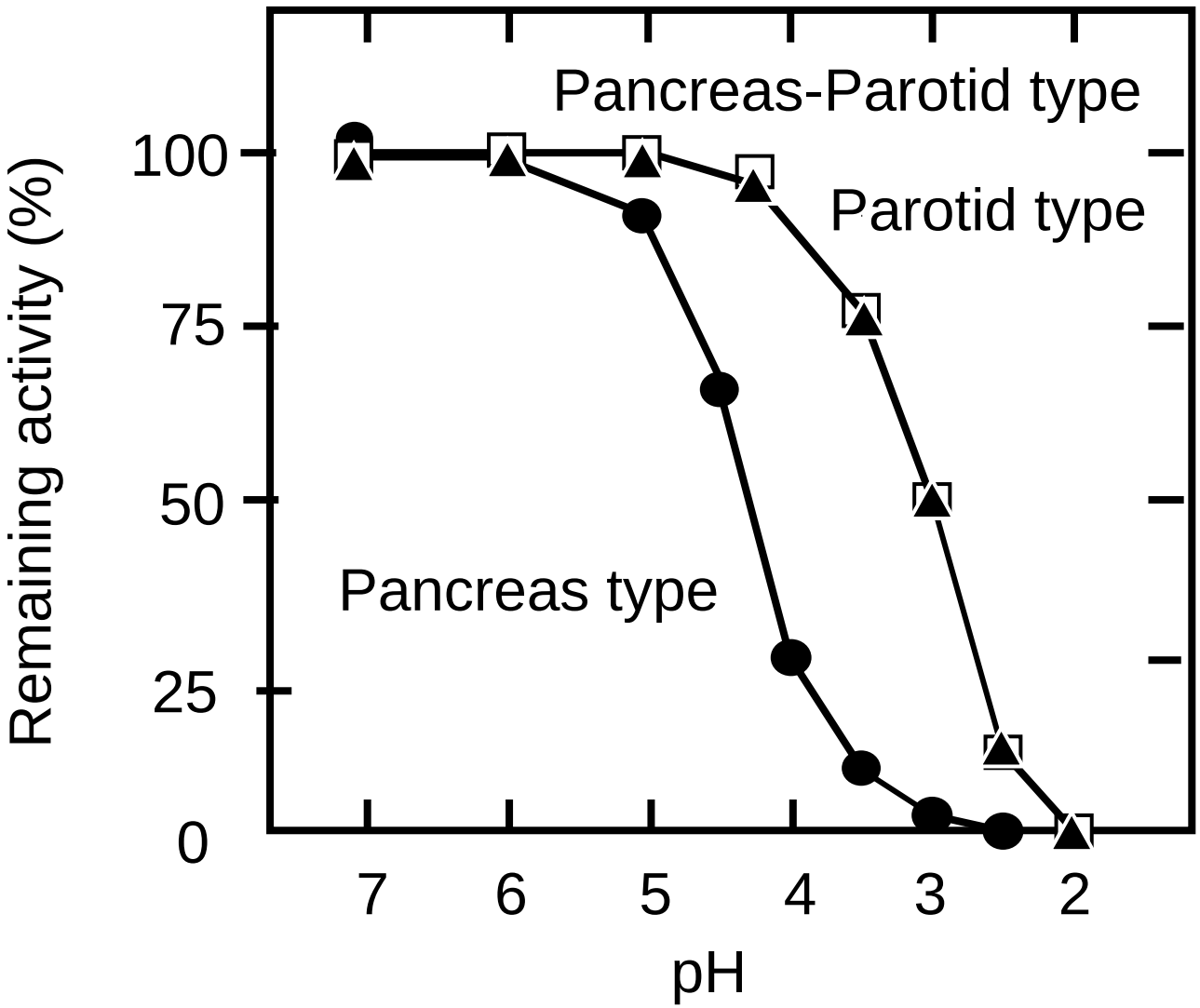

Pancreas-Parotid type
Parotid type
Pancreas type
Remaining activity (%)
100
75
50
25
0
7
6
5
4
3
2
pH

Fig.3

```
Homo sapiens                1  LKIAAFNIQT FGETKMSNAT LVSYIVQILS RYDIALVQEV RDSHLTAVGK LLDNLNQDAP DTYHYVVSEP LGRNSYKERY LFVYRPDQVS AVDSYYYDDG
Equus caballus              1  LRIAAFNIRT FGETKMSNDT LSNYIVQILN RYDIALIQEV RDSHLTAVGK LLDRLNQDDP NTYHFVVSEP LGRNNYKERY LFVFRPDQVS LLDSYQYNDG
Canis lupus familiaris      1  LRMAAFNIRT FGETKMSNAT LSKYIVQILS RYDVAVVQEV RDSHLTAVGK LLDTLNQDDP NAYHYVVSEP LGRSSTKERY LFLFRPDRVS VLDSYQYDDG
Bos taurus                  1  LKIAAFNIRT FGETKMSNAT LASYIVRIVR RYDIVLIQEV RDSHLVAVGK LLDYLNQDDP NTYHYVVSEP LGRNSYKERY LFLFRPNKVS VLDTYQYDDG
Sus scrofa domesticus       1  LRIAAFNIRT FGETKMSNAT LANYIVRILS RYDIALIQEV RDSHLTAVGK LLNELNQDDP NNYHHVVSEP LGRSTYKERY LFVFRPNQVS VLDSYLYDDG
Ovis aries                  1  LKIAAFNIRT FGETKMSNAT LSSYIVRILR RYDIALIQEV RDSHLVAVGK LLDDLNQDDP NSYHYVVSEP LGRNSYKERY LFVFRPNKVS VLDTYQYDDG
Oryctolagus cuniculus       1  LKIAAFNIRS FGETKMSNAT LTSYIVRILQ RYDIALIQEV RDSHLTAVGK LLDKLNEKAA DTYRFVASEP LGRSTYKERY LFVYRPDQVS VLDSYYYDDG
Mus musculus                1  LRIAAFNIRT FGETKMSNAT LSVYFVKILS RYDIAVIQEV RDSHLVAVGK LLDELNRDKP DTYRYVVSEP LGRKSYKEQY LFVYRPDQVS ILDSYQYDDG
Rattus norvegicus           1  LRIAAFNIRT FGDTKMSNAT LSSYIVKILS RYDIAVVQEV RDTHLVAVGK LLDELNRDIP DNYRYIISEP LGRKSYKEQY LFVYRPSQVS VLDSYHYDDG
Gallus gallus domesticus    1  LRISAFNIRT FGDSKMSNQT VAGFIVSIIV QYDITLVQEV RDADLSSVKK LVSQLNSASS YPYSFLSSIP LGRNSYKEQY VFIYRSDIVS VLESYYYDDG
Elaphe                      1  LRIGAFNIRA FGDKKLSNQT ISSSIVRILT TYDLVLIDEV RDADLSAVKK LMQLVSGASP DPFGYLISKP LGHNSYKEQY LFVYRQDRVS PVESYYYDDG
Agkistrodon blomhoffii      1  LRIGAFNIRA FGDKKLSNQT ISSFIVRILT AYDLALIQEV RDADLSAVKK LMHLVNWASP NAFSYLVSKP LGHNSYKEQY LFIYRRDRVS PVESYYYKDG
Xenopus laevis              1  FKIASFNIQR FSMTKVDDPV VLELLIRILS RYGIIAIEEV MNADNTAIIS LVKELSLATK LNYNVLISDH LGRSSTREKY AYVYIEEIVK PTEWYHFDDG
Rana catesbeiana            1  LKIAGFNIER FSATKVDDPV VLNRLIQILR RYELIAVQEV MNKDDTAIIR LVRELNKATG LSYNLLISDH LGRSAYREKY VYVYREDILK PTEWFHYDDG
Cynops carpio               1  FKVASFNIAR FSMSKVTDTA VRDRLVQILR RYELISIQEV MSAENEAIIS LVRELNQATG LPYNVLVSDH LGRSTYREKY AYVYREDILK PTEWYHYDDG
Bufo vulgaris japonicas     1  LKIASFNIER FAVGKVDDPT VLSLLIQILR RYELIAVQEV MSKDNTAIIR LVQELNAATG LHYNLLISDH LGRSSYREKY VYIYREDILK PSEWYHYDDG
Oreochromis mossambica      1  LLLGAFNIKS FGDTKASNAT LMNIITKIVK RYDVILIQEV RDSDLSATQT LMNYVNKDSP -QYKYIVSEP LGASTYKERY LFLYREALVS VVKSYTYDDG
Cyprinus carpio             1  LLIGAFNIKS FGDSKASNAT LLDIITKVVH RYDIVLIQEV RDSDLTATNK LMQSVNGGSSPYEYQYIVSEP LGRSTYRERY LFIYRRPAVS VANSFQYDDG
Anguilla japonica           1  LFIGAFNIRS FGDKKASNAT LVDIIVKIVH MYDILLIQEV RDSDLSATKK LMQNVNNGSSPHKYKYIVSEP LGRNTYQERY LYLYREDSVS VVKNETYDDG
Pagrus major                1  LLLGAFNIKS FGDKKSSNTT LMNIISTIVH RYDIVLIQEV RDSDLSATKR LMEHVNKGSPQFRYSHIVSEP IGRSSYKERY LFLYREQTVS VVKNYYYDDG
Heterodontus japonicus      1  IHISAFNIRA FGDSKMSHPT ITNIIVNLIQ RYDIILIQEV RDADLSVVKI LMNKLNSASA QAFSYITSVP LGHSSYKERY LFVYRNSVVS VVDNYLYSD-
Triakis scyllia             1  IQICAFNIFS FGDSKMSNAT IANIIVNIVQ RYDIILIQEV RDENLSAVKA LMNKLNSPSA HIYSYIASDP LGRSSYKERY LFIYRNTMVS VTNSYLYDDG

Homo sapiens              101 CEPCGNDTFN REPAIVRFFS RFTEVREFAI VPLHAAPGDA VAEIDALYDV YLDVQEKWGL  EDVMLMGDFN AGCSYVRPSQ WSSIRLWTSP TFQWLIPDSA
Equus caballus            101 CEPCGNDTFS REPAIVKFSS PFTQVKEFAI VPLHAAPSDA LAEIDSLYDV YLDVQQKWDM  EDIMLMGDFN AGCSYVTSSQ WPSIRLRRNP AFWWLIPDTA
Canis lupus familiaris    101 CEPCGNDTFS REPAIVRFHS PLTEVKEFAV VPLHAAPLDA VAEIDALYDV YLDVQHKADL  EDIVLMGDFN AGCSYVAASQ WSSIRLRTQP AFQWLIPDTA
Bos taurus                101 CESCGNDSFS REPAVVKFSS HSTKVKEFAI VALHSAPSDA VAEINSLYDV YLDVQQKWHL  NDVMLMGDFN ADCSYVTSSQ WSSIRLRTSS TFQWLIPDSA
Sus scrofa domesticus     101 CEPCGNDTFN REPSVVKFSS PFTQVKEFAI VPLHAAPSDA AAEINSLYDV YLNVRQKWDL  QDIMLMGDFN AGCSYVTTSH WSSIRLRESP PFQWLIPDTA
Ovis aries                101 CESCGNDSFS REPAVVKFSS PSTKVKAFAI VPLHSAPSDA VAEINSLYDV YLDVQQKWDL  NDIMLMGDFN ADCSYVTSSQ WSSIRLRTSS TFQWLIPDSA
Oryctolagus cuniculus     101 CEPCGTDTFS REPAVVRFSS PSTKVREFAI VPLHSAPEDA VAEIDALYDV YLDVQKKWGL  QDVMLMGDFN ADCSYVTSSQ WSSIRLRTNP AFKWLIPDTA
Mus musculus              101 CEPCGNDTFS REPAIVKFFS PYTEVQEFAI VPLHAAPTEA VSEIDALYDV YLDVWQKWGL  EDIMFMGDFN AGCSYVTSSQ WSSIRLRTSP IFQWLIPDSA
Rattus norvegicus         101 CEPCGNDTFS REPAIVKFFS PYTEVREFAI VPLHSAPTEA VSEIDALYDV YLDVRQKWGL  EDIMFMGDFN AGCSYVTSSQ WSSIRLRTSP IFQWLIPDSA
Gallus gallus domesticus  101 CESCGTDIFS REPFIVKFSS PTTQLDEFVI VPLHAEPACA PAEINALTDV YTDVINKWET  NNIFFMGDFN ADCSYVTAEQ WPSIRLRSLS SCEWLIPDSA
Elaphe                    101 CEPCGNGTFS REPFIVKFAV PLAAVEELVL VPLHAAPEAA VTEIDSLYDV YQDVKDRWGV  TDALLLGDFN ADCNYVQAED WRSIRLRSSK DFQWLIPDTA
Agkistrodon blomhoffii    101 CEPCSNTTFS RKPFIVKFAV PQAEVKELVL VPLHAAPEAA VTEIDALYDV YQDVKGRSGA  TDALLLGDFN AGCKYVRVED WPSIRLRSIK DFQWLIPDTA
Xenopus laevis            101 CENCGTDSFI REPFVARFTS LTTVVKDFAL ISIHTSPDYA IMEVDALYDA WVDAKQRLKM  ENILILGDYN AACSYGASRH WPIIRLRHVE ELVWLIGDKE
Rana catesbeiana          101 CENCGTDVFM REPFVARFSS LTTEVKDFAL AVVHTSPDYA VREVDALFDV WEDAKQRLLM  EDIFILGDYN AGCSYVKSAH WPTIRLRQEA SLQWLIGDNA
Cynops carpio             101 CENCGTDVFM REPFVARFSS LTTVVKDLAL VSIHTSPDYA VREVDALFDV WEDVQQRLLT  QNILILGDYN ADCKYVTNKH WPSIRLRHEP QLHWLISDDE
Bufo vulgaris japonicas   101 CENCGTDVFI REPFVARFSS LTTELKDFAL VSIHTSPDYA VREVDGLYDV WEDAKQRLLL  EDILILGDYN AGCSYVKTSH WPNIRLRQES SLQWLIGDTE
Oreochromis mossambica    100 PEETGQDTFS REPFVVMFSS KNTAVKDFTL IPQHTSPDLA VRELNALYDV VLDVRARWNT  NDIVLLGDFN AGCSYVSGSA WQQIRIFTDK TFHWLITDAA
Cyprinus carpio           102 CESCGTDTFN REPFVVMFSS NTAVQQKFAL VPQHTSPEVA VTEIDALHDV VLDTRQRLNT  NNIMLLGDFN AGCSYVSNSD WSKIRLRTDQ SYTWLIPDSA
Anguilla japonica         102 AEESGTDTFN REPFVVMFSS PHTRVPEFAL VPQHTSPDEA VKVIDALYDV IVDIRARWNT  DNIILLGDFN AGCNYVAGSD WQQIRLYVDK SFHWLIPDSA
Pagrus major              101 CEPCGTDTFS REPFVVMFSS RYSAVKNFVL IPQHTSPDSV VKEVNALYDV VVDVRTRWNT  NDIVLFGDFN TDCNYVSGSD WQHIRLFTDK SFRWLIGNHV
Heterodontus japonicus    101 ASNTGQDTFN REPFLVKFSS PYSVIRDFVI MPMHTSPSVA VREIDALYDV FLDAKKKPGT  DNMLIMGDLN ADCSYVKPTD WAHIRLRKDR QFQWLIPDSA
Triakis scyllia           101 SEDSGHDIFS REPFLVKFSS PYSAVHDKEI MPQHTSPSLA IKEIDALYDV FFDARRKLGT  DNMLIMADLN AACSYVKPAD WADIRLRKDS QFQWQIPDSA

Homo sapiens              201 DTTA-TPTHCA YDRIVVAGML LRGAVVPDSA LPFNFQAAYG LSDQLAQAIS DHYPVEVMLK
Equus caballus            201 DTTV-KSTHCA YDRIVVAGTL LQEAVVPDSA VPFDFQAAYG LNDQTAEAIS DHYPVEVTLM
Canis lupus familiaris    201 DTTS-TSTHCA YDRIVVAGSQ LQHAVVPESA APFNFQVAYG LSSQLAQAIS DHYPVEVTLK RA
Bos taurus                201 DTTA-TSTNCA YDRIVVAGSL LQSSVVPGSA APFDFQAAYG LSNEMALAIS DHYPVEVTLT
Sus scrofa domesticus     201 DTTV-SSHTCA YDRIVVAGPL LQRAVVPDSA APFDFQAAFG LSQETALAIS DHYPVEVTLK RA
Ovis aries                201 DTTA-TSTNCA YDRIVVAGSL LQSSVVPGSA VPFDFQAAYG LSNEMALAIS DHYPVEVTLT
Oryctolagus cuniculus     201 DTTA-TSTNCA YDRIVVAGPL LQDAVVPNSA APFNFQAAYG LSNQLAQAIS DHYPVEVTLA
Mus musculus              201 DTTV-TSTHCA YDRIVVAGAL LQAAVVPNSA VPFDFQAEYG LSNQLAQAIS DHYPVEVTLA KI
Rattus norvegicus         201 DTTA-TSTHCA YDRIVVAGAL LQAAVVPSSA VPFDFQAEYR LTNQMAEAIS DHYPVEVTLR KT
Gallus gallus domesticus  201 DTTV-TSTDCA YDRIVACGSA LRQAVEYGSA TVDNFQETLH LTNQMAEAIS DHYPVEVTLR AR
Elaphe                    201 DTTV-TNTICA YDRIVAVGSK LRESILPATA KVDNFQKTLK LSSKDALAVS DHYPVEVTLK ST
Agkistrodon blomhoffii    201 DTTV-TNTVCA YDRIVAVGSK LRESILPATA KVDDFQKTLK LSSKDALAVS DHFPVEVILK ST
Xenopus laevis            201 DTTVSTNTNCA YDRMVAGGEE LQRGIVPDTA KAFNYHVAYD LTYEMAKAVY DHYPVEVELY DDVFYSGQCF EPSASTGISF GLSLNGPCTC EGWDFSSCRG
Rana catesbeiana          201 DTTVSTNTNCP YDRIVVGGSR LQDSVVPGSA KAFNYQEAYG LTTEGTKAAS DHYPVEVELR NDPFTSGQKF EVSASIGISG GLSLNGVCDC LGVDFTSCVG
Cynops carpio             201 DTTVSTNYHCA YDRKVASGVE MLNAIFPETA TAFNYHEAYG LTYEEAKDVS DHYPVEMQLR LDSDYNGERF QTSPTLGING GPSTNGACSC AGVDVTSCQG
Bufo vulgaris japonicas   201 DSTVSTNTHCP YDRLVVGGAR FQDIVIPGTA KAFNYHVAYD LTYEMAKAVS DHYPVEMEIR DDSLYNNQKL PISGSIGISG GLSLNGVCDC VGVDFTSCVG
Oreochromis mossambica    200 DTTV-SQTVCP YDRIVVTTDM MRG-VVQNSA KVYNYMTDLN LKQDLALAVS DHFPVEVKLS
Cyprinus carpio           202 DTTV-THTNCP YDRIVATSDM MKG-VSAGSA QVFDFMQAHG LSQSWGLAVS DHFPAEVQLL
Anguilla japonica         202 DTTV-SHTNCP YDRIVATTTM MEA-VVPHSA SVYDYMTSLK LKLDMALAVS DHFPVEVTLF GP
Pagrus major              201 DSTVSQTTNCA YDRIVVTTDM LKG-VLQGSA QVYNFMTDLK LSHSLTFAVS DHYPVEVELI G
Heterodontus japonicus    202 DTSTTITTKCA YDRIIAVGSE MKNAIIDGSA AIYNFTSVLN LNNKMTAAVS DHYPLEVRLG AV
Triakis scyllia           201 DTTTNIATKCA YDRIIAVGSE MKNAVIDGST AIYNFGKVFN LNNEMTLAVS DHYPVEVSLR AA

Xenopus laevis            302 RCGASGKTYP CNCNASCTN- --CCVDYTSS CKL
Rana catesbeiana          302 RCGANSSSYP CNCNATCSSS KKCCADYAAV CKI
Cynops carpio             302 RCGAYSKTLP CNCNASCGQY GSCCADYKAH C
Bufo vulgaris japonicas   302 RCGANSSSYP CNCNASCSSS NKCCADYGAS CKV
```

Fig.4

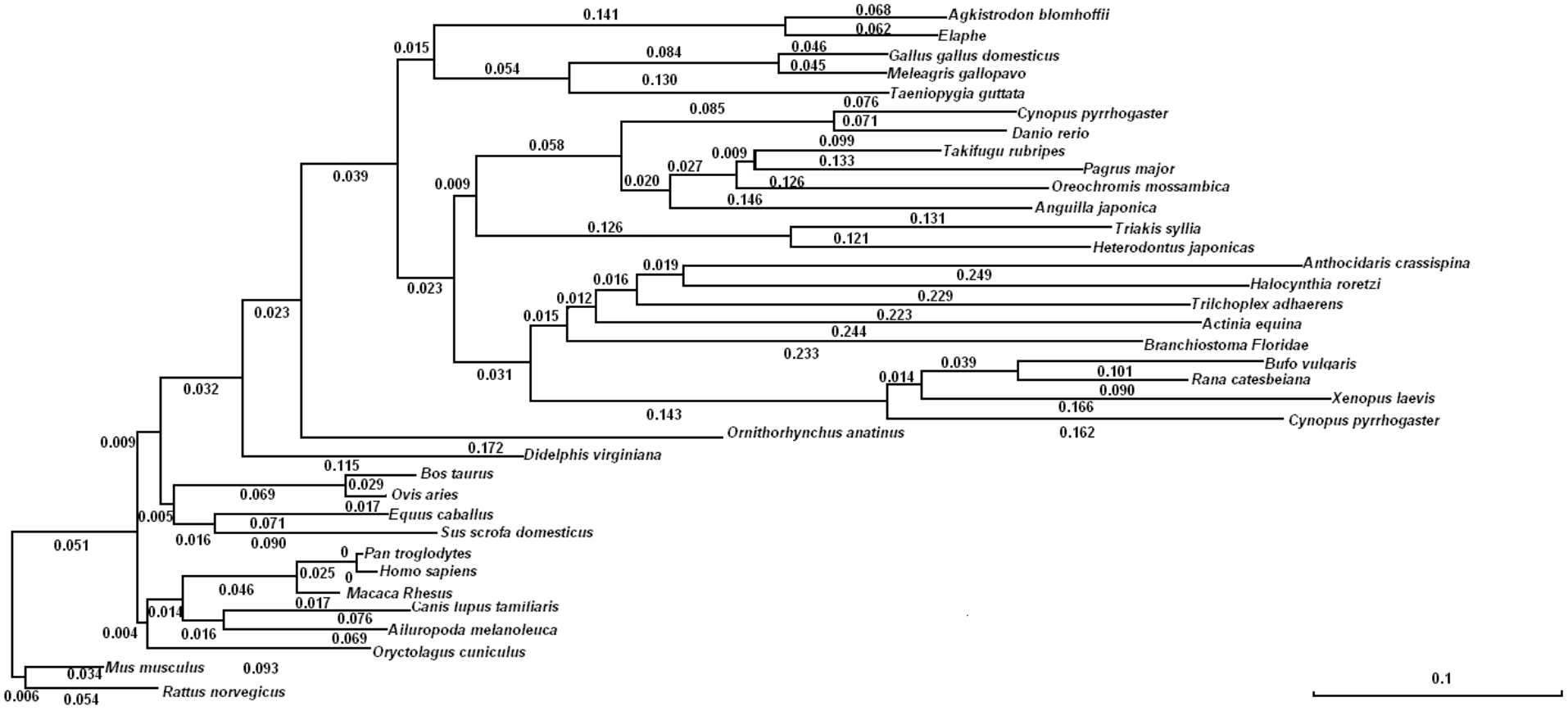

Agkistrodon blomhoffii
Elaphe
Gallus gallus domesticus
Meleagris gallopavo
Taeniopygia guttata
Cynopus pyrrhogaster
Danio rerio
Takifugu rubripes
Pagrus major
Oreochromis mossambica
Anguilla japonica
Triakis syllia
Heterodontus japonicas
Anthocidaris crassispina
Halocynthia roretzi
Trilchoplex adhaerens
Actinia equina
Branchiostoma Floridae
Bufo vulgaris
Rana catesbeiana
Xenopus laevis
Cynopus pyrrhogaster
Ornithorhynchus anatinus
Didelphis virginiana
Bos taurus
Ovis aries
Equus caballus
Sus scrofa domesticus
Pan troglodytes
Homo sapiens
Macaca Rhesus
Canis lupus familiaris
Ailuropoda melanoleuca
Oryctolagus cuniculus
Mus musculus
Rattus norvegicus
0.1

Fig.5

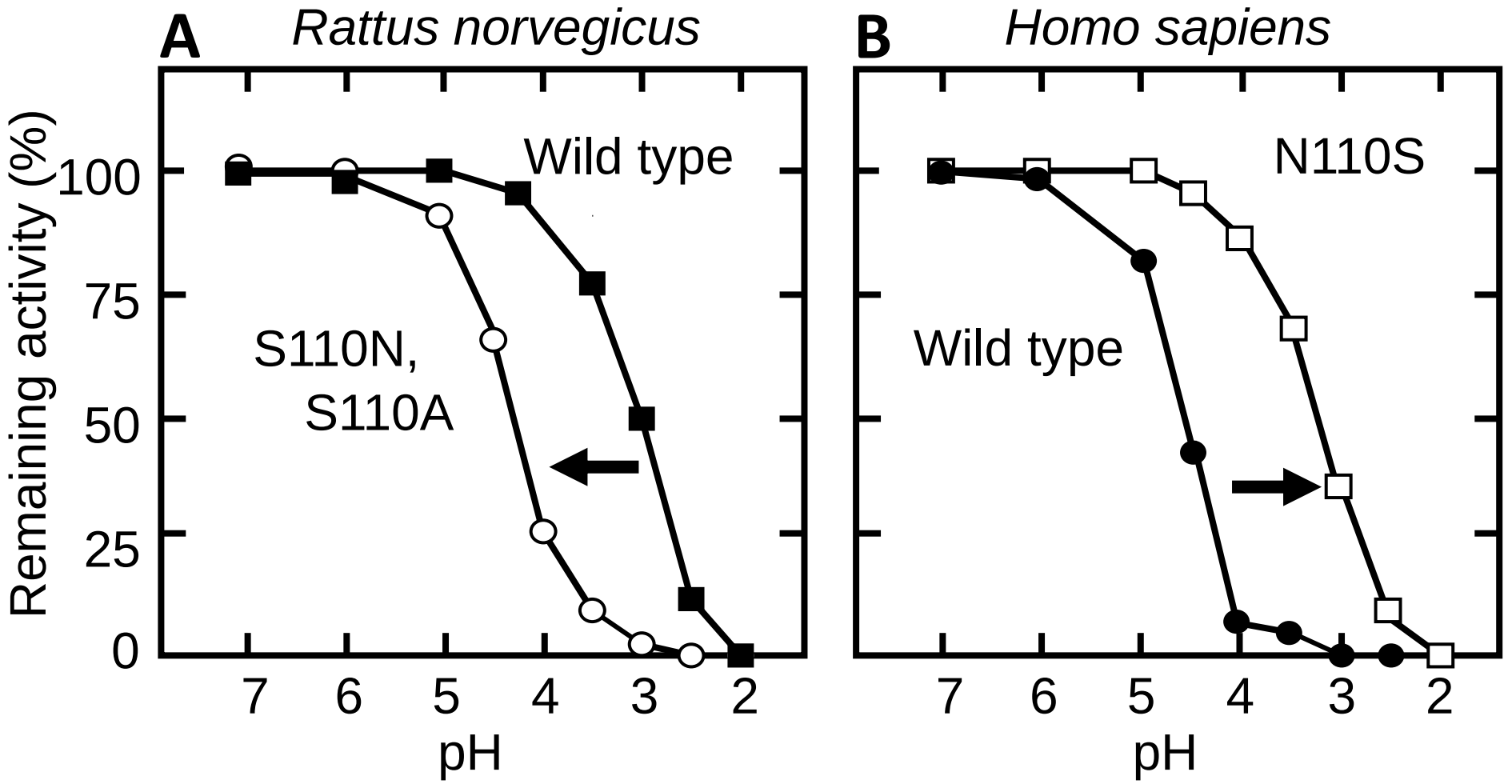

A
Rattus norvegicus
B
Homo sapiens
Remaining activity (%)
100
75
50
25
0
7
6
5
4
3
2
pH
Wild type
S110N,
S110A
N110S
Wild type

Fig.6

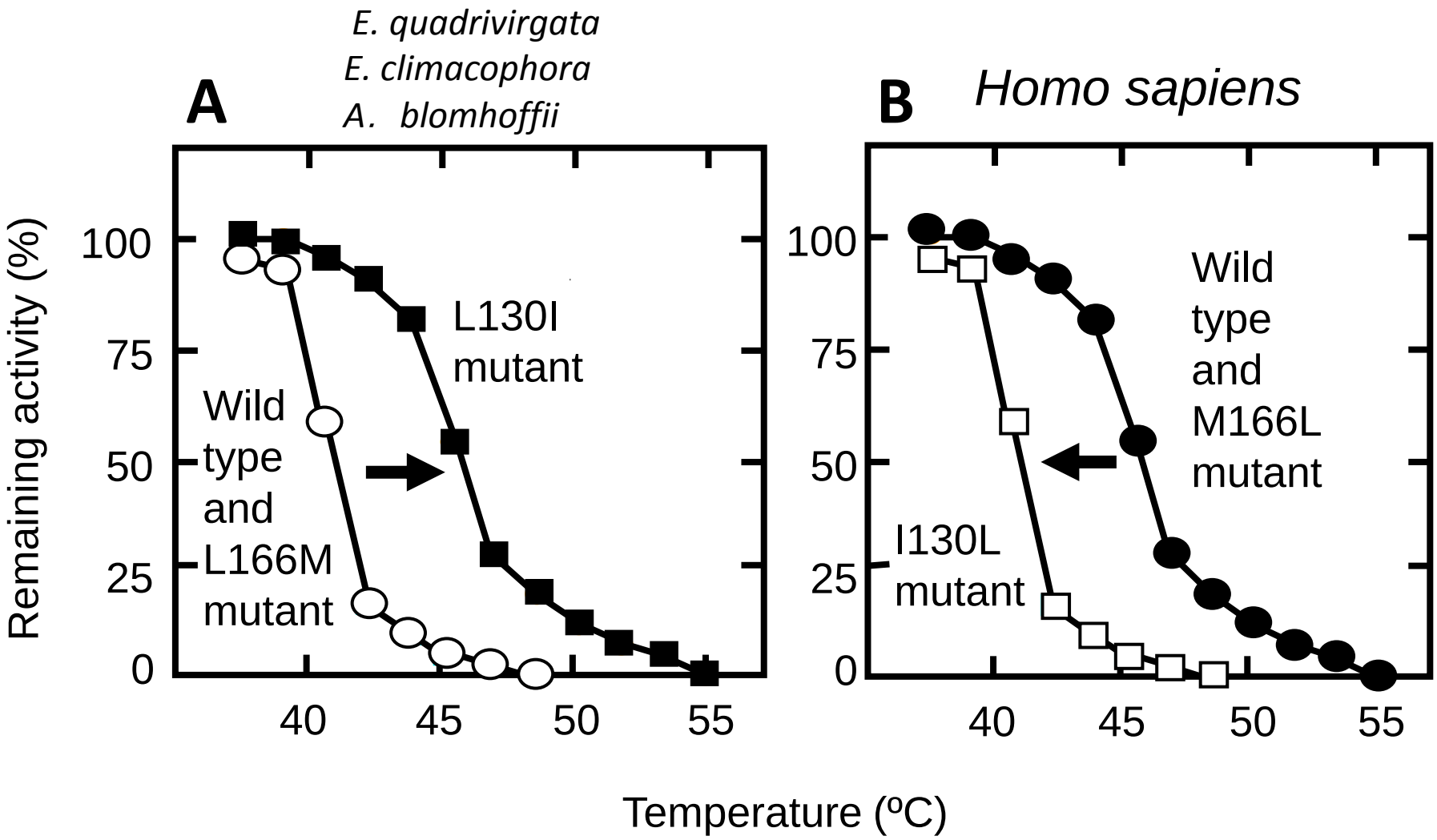

E. quadrivirgata
E. climacophora
A. blomhoffii
A
B
Homo sapiens
Remaining activity (%)
100
75
50
25
0
40
45
50
55
Temperature (ºC)
L130I mutant
Wild type and L166M mutant
Wild type and M166L mutant
I130L mutant

Fig.7

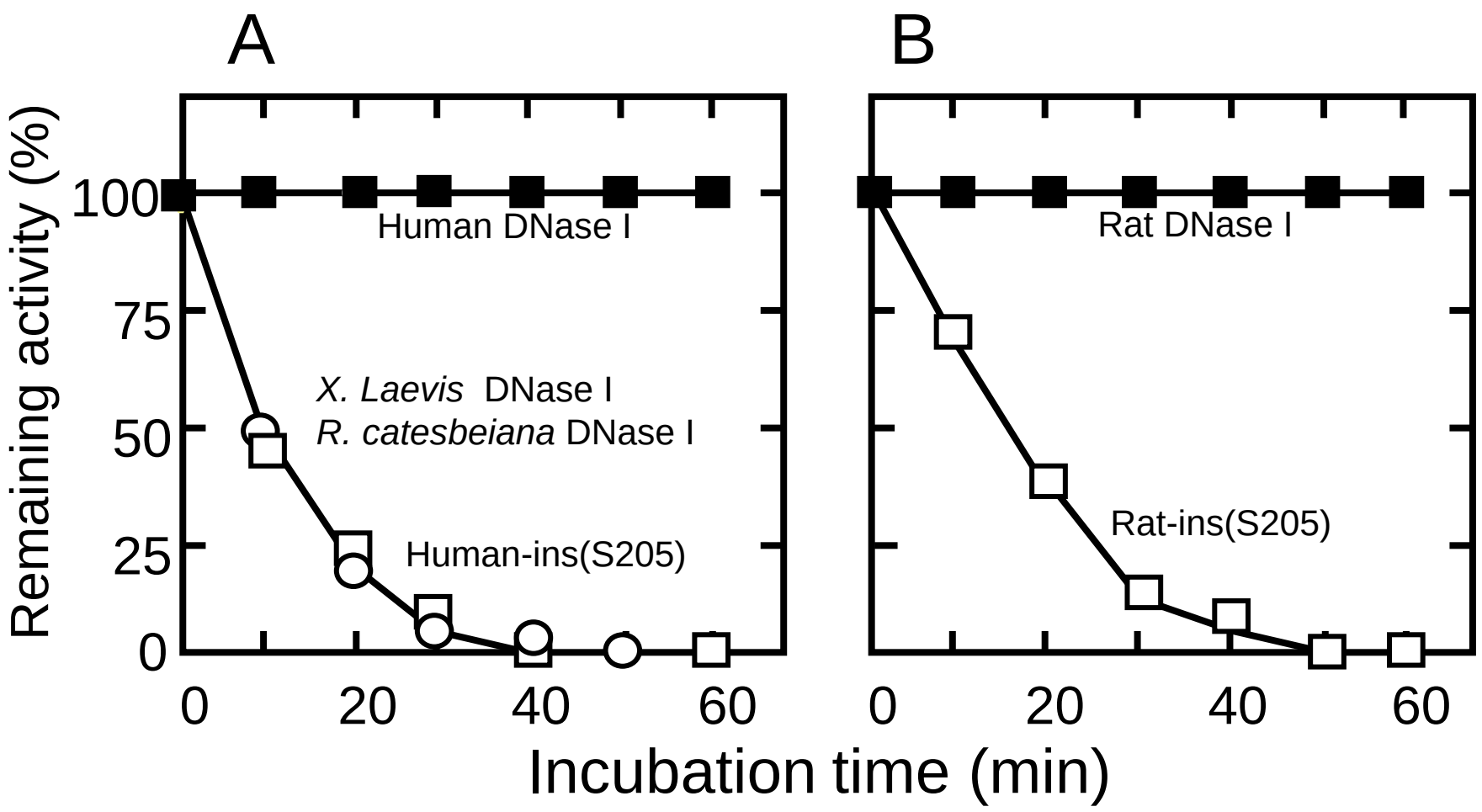

A
B
Remaining activity (%)
100
75
50
25
0
Human DNase I
X. Laevis DNase I
R. catesbeiana DNase I
Human-ins(S205)
Rat DNase I
Rat-ins(S205)
0
20
40
60
Incubation time (min)

Fig.8

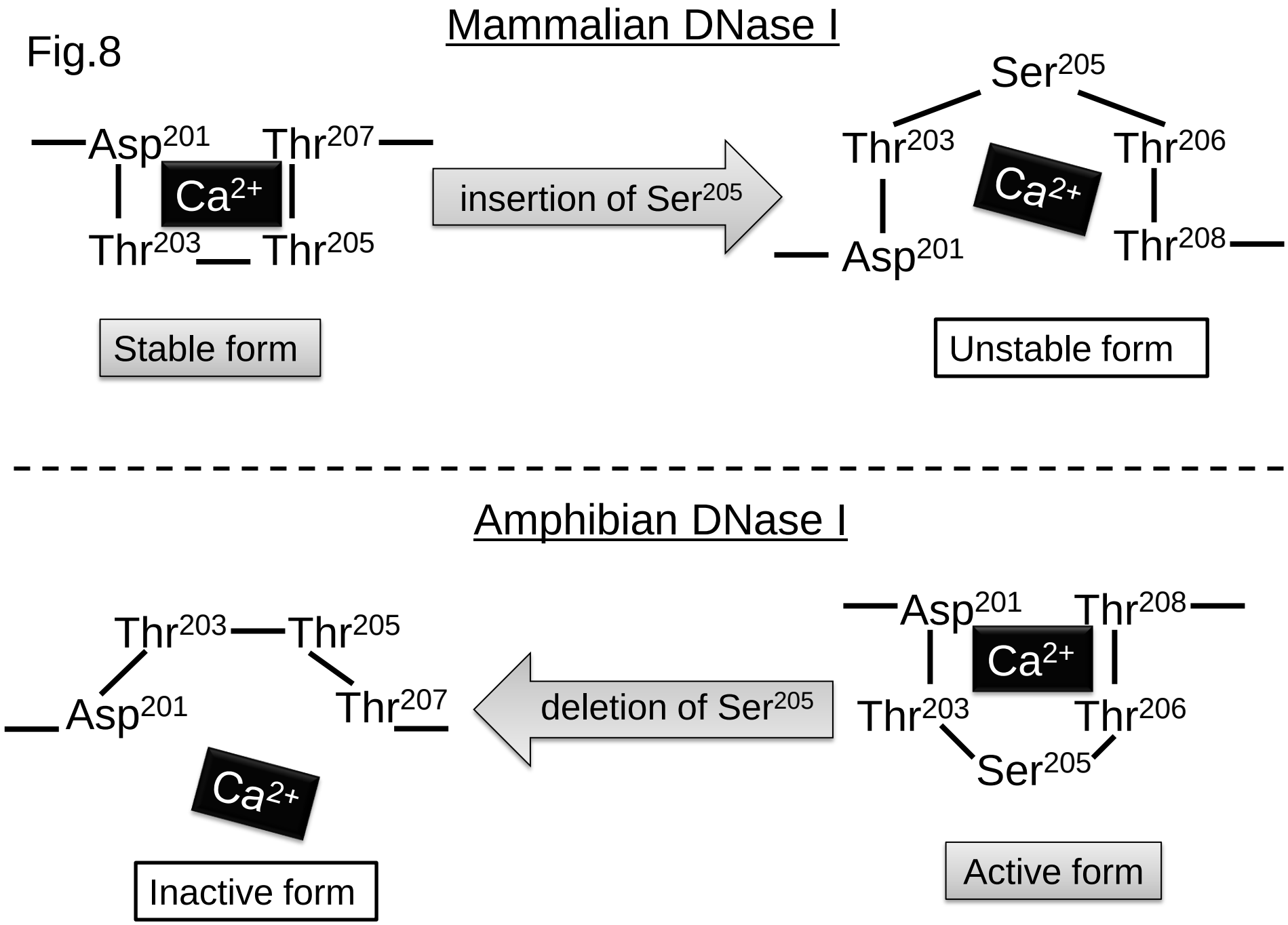

Mammalian DNase I
Asp201
Thr207
Ca2+
Thr203
Thr205
Stable form
insertion of Ser205
Ser205
Thr203
Thr206
Ca2+
Asp201
Thr208
Unstable form
Amphibian DNase I
Thr203
Thr205
Asp201
Thr207
Ca2+
Inactive form
deletion of Ser205
Asp201
Thr208
Ca2+
Thr203
Thr206
Ser205
Active form

Fig.9

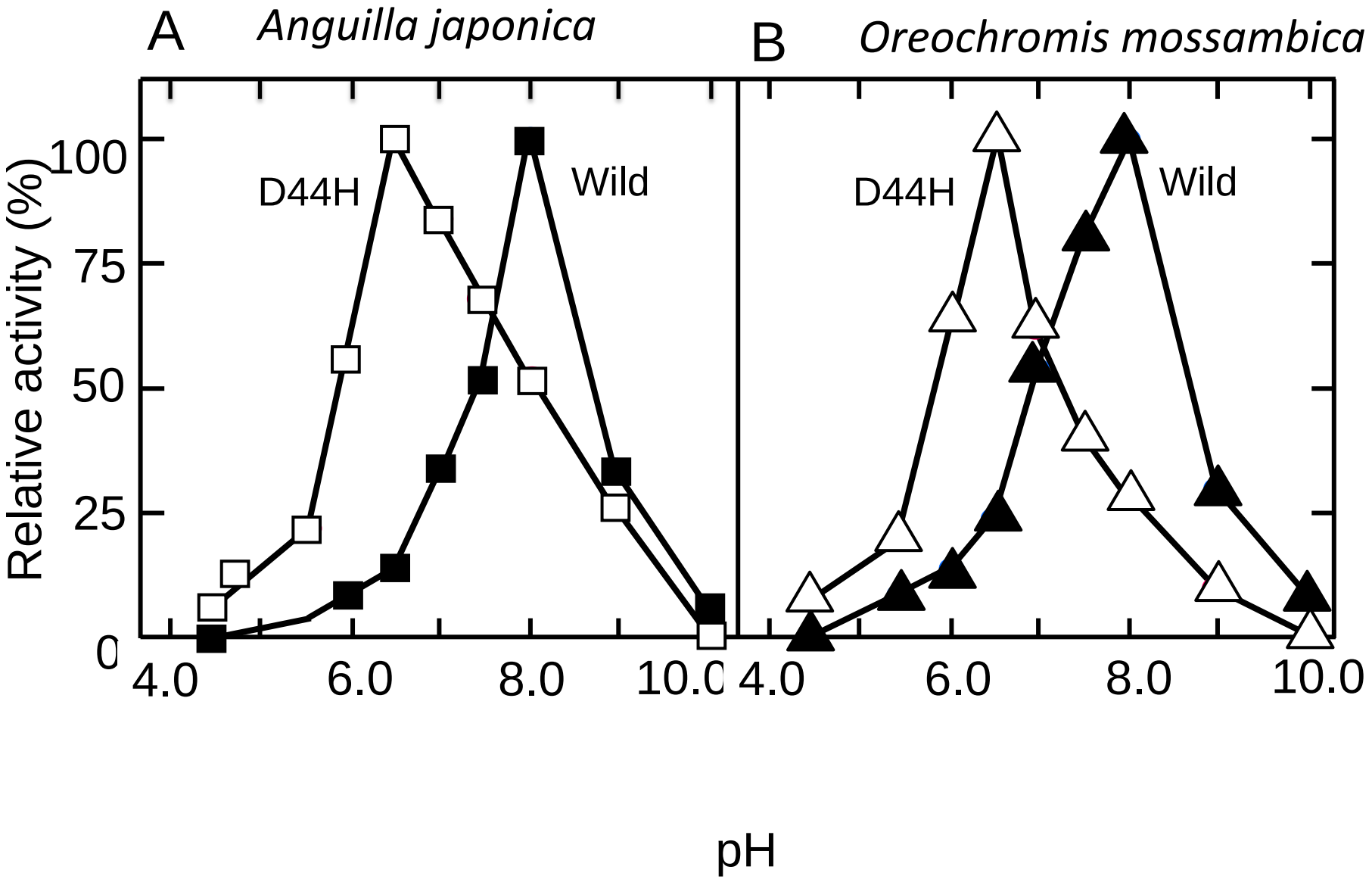

A
Anguilla japonica
B
Oreochromis mossambica
D44H
Wild
D44H
Wild
Relative activity (%)
100
75
50
25
0
4.0
6.0
8.0
10.0
4.0
6.0
8.0
10.0
pH

Fig.10

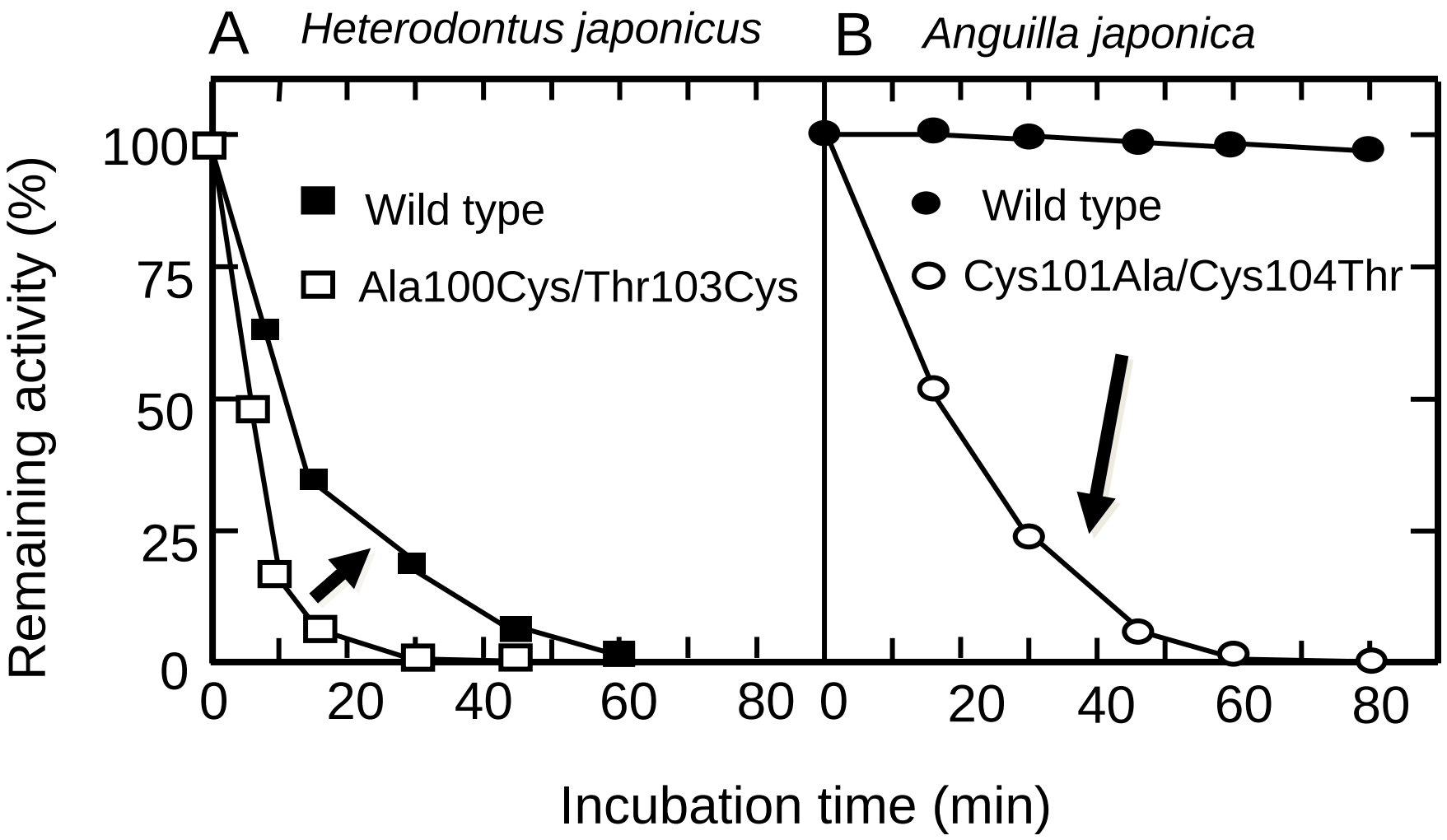
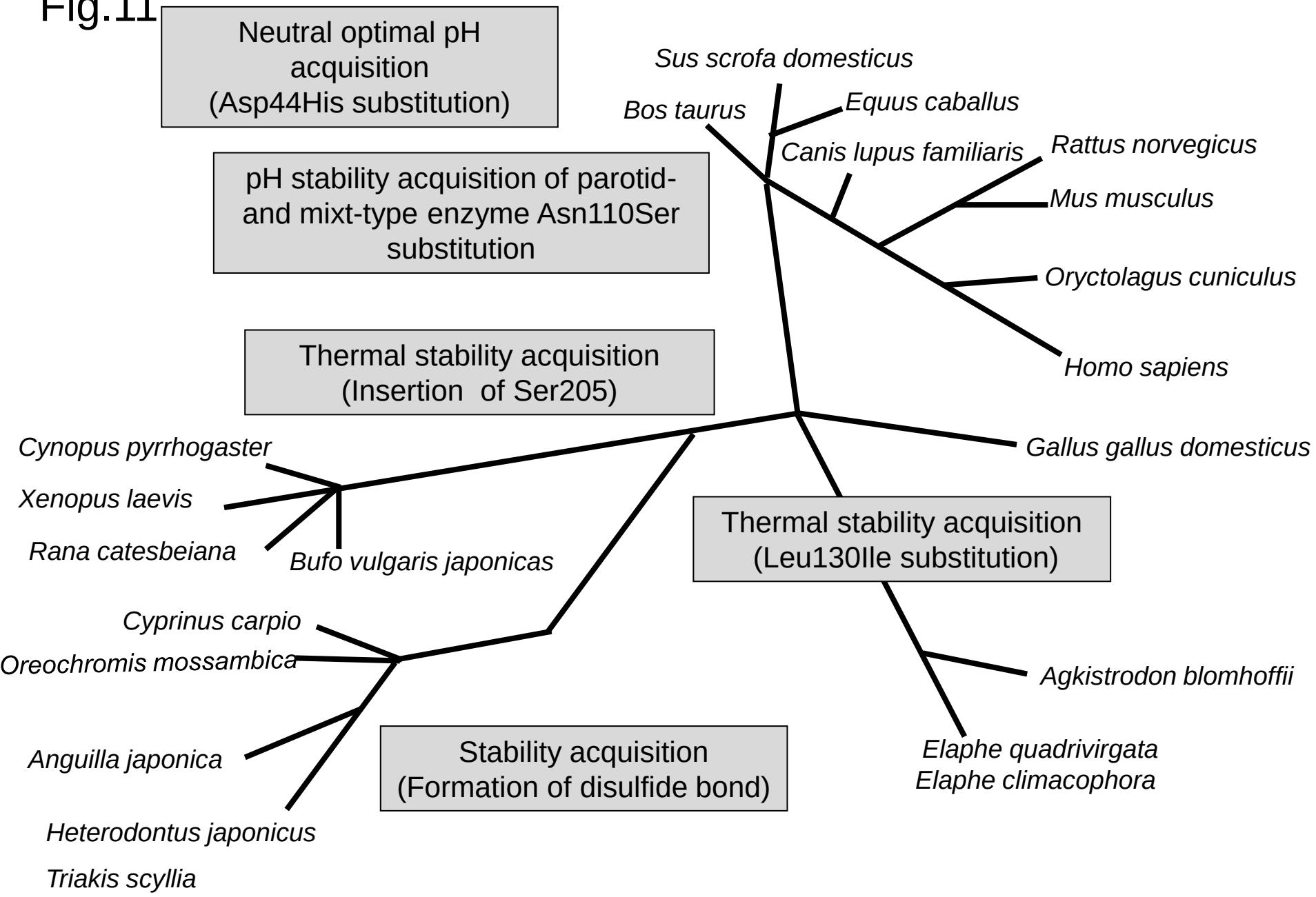

A
Heterodontus japonicus
B
Anguilla japonica
Wild type
Ala100Cys/Thr103Cys
Wild type
Cys101Ala/Cys104Thr
Remaining activity (%)
100
75
50
25
0
0
20
40
60
80
0
20
40
60
80
Incubation time (min)

Fig.11


Neutral optimal pH acquisition (Asp44His substitution)
pH stability acquisition of parotid- and mixt-type enzyme Asn110Ser substitution
Thermal stability acquisition (Insertion of Ser205)
Thermal stability acquisition (Leu130Ile substitution)
Stability acquisition (Formation of disulfide bond)
Sus scrofa domesticus
Bos taurus
Equus caballus
Canis lupus familiaris
Rattus norvegicus
Mus musculus
Oryctolagus cuniculus
Homo sapiens
Cynopus pyrrhogaster
Xenopus laevis
Rana catesbeiana
Bufo vulgaris japonicas
Gallus gallus domesticus
Cyprinus carpio
Oreochromis mossambica
Anguilla japonica
Heterodontus japonicus
Triakis scyllia
Agkistrodon blomhoffii
Elaphe quadrivirgata
Elaphe climacophora