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Susceptibility of mammalian deoxyribonucleases I (DNases I) to proteolysis by proteases and its relationships to tissue distribution: Biochemical and molecular analysis of equine DNase I

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Abstract

Equine (*Equus caballus*) deoxyribonuclease I (DNase I) was purified from the parotid gland, and its 1295-bp cDNA was cloned. The mature equine DNase I protein consisted of 260 amino acid residues. The enzymatic properties and structural aspects of the equine enzyme were closely similar to those of other mammalian DNases I. Mammalian DNases I are classified into three types — pancreatic, parotid and pancreatic–parotid-based on their tissue distribution; as equine DNase I showed the highest activity in the parotid gland, it was confirmed to be of the parotid-type. Comparison of the susceptibility of mammalian DNases I to proteolysis by proteases 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. However, site-directed mutagenesis analysis revealed that only a single amino acid substitution could not account for acquisition of proteolysis resistance in the mammalian DNase I family during the course of molecular evolution. These properties are compatible with adaptation of mammalian DNases I for maintaining their activity *in vivo*.

Keywords: cDNA cloning; Deoxyribonuclease I; *Equus caballus* (Horse); Mammal; Molecular evolution; Purification; Proteolysis

1. Introduction

Deoxyribonuclease I (DNase I, EC 3.1.21.1) has been considered to play a major role in digestion in the alimentary tract, because, in mammals, it is secreted by exocrine glands such as the pancreas and/or parotid, which are associated with the digestive system (Moore, 1981; Nadano et al., 1993; Napirei et al., 2004). DNase I was recently postulated to be responsible for the removal of DNA from nuclear antigens at sites of high cell turnover and necrosis, suggesting that maintenance of DNase I activity levels *in vivo* may be an essential factor for the prevention of systemic lupus erythematosus (Napirei et al., 2000; Yasutomo et al., 2001). Previously, we have shown that mammalian DNases I can be classified into three types – pancreatic, parotid and pancreatic–parotid (mixed) – according to their main source (Takeshita et al., 2000); in human, pig and dog (Kaneko et al., 2003), the pancreas exhibits the highest activity of DNase I; in rat and mouse, the parotid has the highest activity; and in ox and rabbit, the highest activity is detected in both the parotid and the pancreas. Furthermore, DNases I of the two latter types are more stable in low pH conditions than the pancreatic-type enzyme, perhaps reflecting the fact that those enzymes are secreted from the parotid and have to pass through the very acidic conditions present in the stomach. In this context, pancreatic-type DNase I is secreted into the duodenum as a component of pancreatic juice (Keller et al., 1958). Since several proteases such as trypsin and chymotrypsin are concomitantly

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present, it is postulated that DNase I proteins are not vulnerable to proteolysis by these proteases in order to fully maintain their digestion function activity. On the other hand, if parotid-type and mixed-type DNases I secreted into the oral cavity are able to pass through the stomach intact, then they need to be resistant to proteolysis by pepsin, in addition to being acid-stable. Therefore, low susceptibility of vertebrate DNases I to proteolysis by such proteases is indispensable in order for them to play their full role in digestion in the alimentary tract. However, it remains unknown whether these properties are shared by the mammalian DNase I family.

In this context, we have carried out comprehensive and comparative studies of vertebrate enzymes: mammalian (Yasuda et al., 1990; 1997; Takeshita et al., 1995; 1997; Mori et al., 2001; Kaneko et al., 2003), avian (Nakashima et al., 1999), reptilian (Takeshita et al., 2003), amphibian (Takeshita et al., 2001) and piscine (Yasuda et al., 2004a,b) DNases I have already been characterized. However, mammalian DNases I examined so far have been limited to those derived from Primate (human and chimpanzee), Lagomorpha (rabbit), Rodentia (rat and mouse), Carnivora (dog) and Artiodactyla (ox, pig and sheep), whereas the enzyme from the other typical class of mammals, Perissodactyla, has not been entirely investigated. Although the horse, *Equus caballus*, belonging to the Perissodactyla, is a common domestic animal, the biochemical and molecular properties of equine DNase I remain to be clarified. Therefore, comprehensive characterization of equine DNases I would allow us to elucidate the molecular evolutionary aspects of the mammalian DNase I family. In this study, we purified and characterized DNase I from the equine parotid gland, in addition to cloning its cDNA. Susceptibilities of vertebrate DNases I, including the equine enzyme, to proteolysis by some proteases were also examined.

2. Materials and methods

2.1. Materials and biological samples

Various tissue samples from a 3-year-old female Thoroughbred horse (*E. caballus*), weighing about 450 kg, were obtained from the Laboratory of Racing Chemistry, Tochigi, Japan. LipofectaminPlus, fetal calf serum, 3′-rapid amplification of cDNA ends (RACE) system, the 5′ GeneRacer kit and trypsin (0.25% solution) were obtained from Invitrogen Corp. (Carlsbad, CA, USA); Phenyl Sepharose CL-4B, Mono Q 5/50 GL and Superdex 75, and Con A-agarose were from Amersham Pharmacia Biotech (Uppsala, Sweden), and Seikagaku Kogyo (Tokyo, Japan), respectively; chymotrypsin (Type I-S), pepsin (3200–4500 units/mg protein) and G-actin were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Analytical methods

DNase I activity was assayed by single radial enzyme diffusion (SRED) using a reaction mixture (50 mM MES buffer, pH 6.5) containing 10 mM $MgCl_2$ and 1 mM $CaCl_2$ (Nadano et al., 1993), and one unit of the activity was defined as reported previously (Yasuda et al., 1990). The enzymological and chemical properties of the enzymes were examined as described previously (Yasuda et al., 1990; Takeshita et al., 1995; 2001). Proteins were determined with a protein assay kit using BSA as a standard. SDS/PAGE was performed in 12.5% (w/v) gels according to the method of Laemmli (1970). Activity staining for DNase I was performed by the DNA-cast PAGE method (Nakajima et al., 1998). Digestion of the purified DNase I by the recombinant peptide: *N*-glycosidase F (PNGaseF; Toyobo Co. Ltd., Osaka, Japan) prior to electrophoresis was performed as described previously (Yasuda et al., 1993).

Each vertebrate DNase I in medium secreted from COS-7 cells transfected with the corresponding expression vector as described below was used for analysis of proteolysis by proteases; each DNase I (0.2 unit) was incubated at 37 °C with 1 μg/mL chymotrypsin in PBS, 10 μg/mL trypsin in PBS, or 1.5 μg/mL pepsin in 50 mM sodium acetate buffer, pH 3.0, for various periods. Control reactions were performed by incubating the enzyme without each protease. Then, the activities were assayed by the SRED method and remaining activities were determined by comparison with those of the controls.

2.3. Purification of equine DNase I from parotid glands

All procedures were carried out at 0–4 °C. Parotid gland (approximately 0.5 g) obtained from a horse was minced and homogenized in 25 mM Tris/HCl, pH 7.5 (buffer I), containing 1.0 M ammonium sulfate and 1 mM PMSF. After centrifugation, the supernatant (crude extract) was applied to a Phenyl Sepharose CL-4B column (1.6×15 cm) pre-equilibrated with the same buffer. The adsorbed materials were eluted with a 300-mL linear reverse gradient of 1–0 M ammonium sulfate in buffer I. DNase I-active fractions were collected, dialyzed against buffer I, then run on an ÄKTA *FPLC* system equipped with a Mono Q 5/50 GL column (0.46×10 cm) pre-equilibrated with the same buffer. The adsorbed materials were eluted with a 100-mL linear gradient of 0–2 M NaCl in the same buffer. The DNase I-active fractions were collected and dialyzed against buffer I, containing 1 mM $CaCl_2$, 10 mM $MgCl_2$ and 150 mM NaCl. The dialysates were applied to a Con A-agarose column (1.6×5 cm) pre-equilibrated with the same buffer. The adsorbed materials were eluted with 300 mM methyl-α-D-mannopyranoside in the same buffer. The active fractions were collected, concentrated, and subjected to gel filtration with the ÄKTA *FPLC* system equipped with a Superdex 75 column (1.6×60 cm) pre-equilibrated with the same buffer containing 400 mM NaCl. The active fractions were collected and used as the purified enzyme for subsequent experiments. A specific antiserum against equine DNase I was prepared by the methods described previously (Yasuda et al., 1990).

2.4. Construction of the cDNA encoding horse DNase I

Total RNA was isolated from the horse parotid gland using a RNeasy Mini kit (QIAGEN Inc., Chatsworth, CA). First, a portion of the DNase I cDNA was amplified by reverse transcriptase (RT)-PCR using two degenerate sense and antisense