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Improved Purification and Characterization of Equine DNase I

Junko FUJIHARA[1], Tamami MORITANI[1], Miki TONGU[2], Takaya YAMADA[2], Mari TABUCHI[3] and Haruo TAKESHITA[1]

[1]*Department of Legal Medicine, Shimane University Faculty of Medicine, Izumo 693-8501, Japan*

[2]*Department of Experimental Animals, Center for Integrated Research in Science, Shimane University Faculty of Medicine, Izumo 693-8501, Japan*

[3]*Department of Chemistry and Research Information Center for Extremophile, Rikkyo University, Tokyo 171-8501, Japan*

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Deoxyribonuclease I (DNase I) from equine parotid gland was purified by reformation with a yield of 48 % to electrophoretic homogeneity using three-step conventional column chromatography. The purified enzyme showed a molecular mass of about 37kDa and maximum activity at pH 7.0. It required divalent cations Mg^{2+} and Ca^{2+} for its activity and was inhibited by EDTA and EGTA. The purified enzyme preparation was found to contain no other detectable nucleases. An antibody against the purified enzyme was found to be monospecific against the equine parotid gland and the pure antigen, and completely blocked the activity of the purified enzyme. Inhibition by globular actin (G-actin) was investigated in seven mammalian enzymes. Activity inhibition by G-actin was very strong in an equine enzyme. In G-actin-inhibited human DNases I, four amino acid residues, Tyr-65, Val-66, Val-67 and Ala-114, were involved in actin binding. In equine, Tyr-65 is substituted with Phe-65. Considering that the enzymatic activity of a rabbit enzyme with Phe-65 and Ala-67 was less efficiently inhibited by G-actin, the structural involvement of Phe-65 and Val-67may result in a strong DNase I and G-actin complex.

Key words: characterization, DNase I, actin inhibition, equine, purification

INTRODUCTION

Deoxyribonuclease I (DNase I, EC 3.1.21.1) is a divalent cation-dependent enzyme that cleaves double-stranded DNA to produce oligonucleotides with 5'-phospho and 3'-hydroxy-termini (1). DNase I is considered to play a major role in the digestion of dietary DNA because, in vertebrates, it is secreted by exocrine/endocrine glands (2). However, the presence of the enzyme in mammalian tissues other than the digestive organs suggested that it might have other function(s) *in vivo*; endogenous DNase I has been regarded as a candidate endonuclease responsible for internucleosomal DNA degradation during apoptosis (3). Furthermore, the relevance of DNase I to disease has recently been elucidated. DNase I has been postulated to be responsible for the removal of DNA from nuclear antigens at sites of high cell turnover and necrosis and, thus, for the prevention of systemic lupus erythematosus (4). Moreover, it was demonstrated that an abrupt elevation of serum DNase I activity occurs within ~3h of the onset of symptoms in patients with acute myocardial infarction (AMI) and that DNase I activity in serum then exhibits a marked time-dependent decline within 12 h, returning to basal levels within 24 h (5).

Recently, the structural gene of equine DNase I has been isolated and sequenced (6). On the basis of DNase I activity distribution in tissue, mammalian enzymes have been classified into three types (pancreatic, parotid, and pancreatic-parotid); enzymes of the pancreatic type are more sensitive to low pH than those of the other types (2). Equine DNase I has been classified as the parotid type (6). In this paper, we describe a novel purification procedure and the biochemical properties of the equine DNase I. In addition, we evaluated the effect of globular actin (G-actin) inhibition due to the differences in the amino acid residues involved in actin binding in equine and other mammalian enzymes.

Correspondence: H. Takeshita, M.D., Ph.D. Department of Legal Medicine, Shimane University Faculty of Medicine, 89-1 Enya, Izumo, Shimane 693-8501, Japan
Tel: +81-853-20-2156
Fax: +81-853-20-2155
E-mail: htakeshi@med.shimane-u.ac.jp

MATERIALS AND METHODS

Materials and biological samples

DEAE Sepharose CL-6B, Phenyl Sepharose CL-4B, and Superdex 75 were purchased from Amersham Biosciences (Tokyo). Rabbit muscle G-actin and salmon testis DNA were from Sigma (St. Louis, MO). DNase I was purified from human (7), rabbit (8), rat (9), mouse (10), porcine (11), and bovine (12) according to previously described methods. All other chemicals used were of reagent grade and commercially available. These mammalians were acquired, maintained, and used in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH, USA; revised 1985).

Purification of DNase I from equine parotid gland

All the procedures described below were carried out at 0-4℃. Parotid gland samples (approximately 0.3g) were minced separately and homogenized in a 5-10 mL Tris/HCl buffer, pH 7.5 (buffer I), containing 1 M ammonium sulfate. After centrifugation (10,000× g, 25 min), the supernatant was applied to a Phenyl Sepharose CL-4B column (1.6×15 cm) pre-equilibrated with the same buffer, and the adsorbed materials were eluted with a 400 mL linear reverse ammonium sulfate concentration gradient (1.0-0 M) in buffer I. The active fractions were collected, dialyzed against buffer I, and applied to a DEAE Sepharose CL-6B column (1×15 cm) pre-equilibrated with buffer I, and the adsorbed materials were eluted with a 100 mL linear NaCl concentration gradient (0-1.0 M) in buffer I. The active fractions eluted over the NaCl concentration range of 250-300 mM were concentrated using polyethylene glycol 6,000 and then subjected to gel filtration using the ÄKTA *FPLC* system (Amersham Pharmacia Biotech) equipped with a Superdex 75 column (1.6×60 cm) with buffer I containing 150 mM NaCl as the eluent. The active fractions were collected and used as the purified enzymes for the subsequent experiments. A specific rabbit antibody against purified DNase I from equine was prepared as described previously (7).

Analytical Method

The activity of DNases I was determined by the test-tube method (13, 14) or the single radial enzyme diffusion (SRED) method (15). One unit of DNase I activity was defined as an increase of 1.0 unit in the absorbance at 260 nm. The enzymological properties of the enzyme and the inhibitory effects of specific antibodies on its activity were examined as described previously (13, 14). The purified enzyme was analyzed by 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresed proteins were subjected to the staining with 0.2 % (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad), or western blot analysis using a rabbit anti-equine DNase I antiserum after the electrophoresed protein had been transferred on to a Durapore membrane (Millipore, Bedford, MA) by electroblotting according to previously described methods (8, 9). The actin-binding assay was performed as described previously (16). Briefly, an actin solution in a reaction mixture containing double-stranded DNA was incubated with the prepared DNase I, and the absorbance was measured as described above. Control reactions were performed by incubating samples in a reaction buffer without an actin solution. The remaining DNase I activity was determined by comparisons with controls.

RESULTS AND DISCUSSION

Purification and characterization of DNase I from equine parotid gland

In this study, we describe a three-step chromatographic procedure for the purification of equine DNase I to apparent homogeneity. A typical equine parotid gland DNase I purification procedure is summarized in Table 1; this resulted in a 37-fold purification with a 48 % yield. When the purified equine DNase I was subjected to SDS-PAGE followed by protein staining, immunostaining, and activity staining, only a single band was detected (Fig.1). The purified DNase I had a molecular mass of approximately 37 kDa, as determined by both SDS-PAGE and gel filtration on a Superdex 75 column. This purification method appeared to function well with only three-step conventional column chromatography and resulted in higher quality of the purified protein than current methods (6). The pH activity profile of the enzyme was bell-shaped and exhibited an optimal pH of 7.0 (data not shown). The addition of 20 mM EDTA (final concentraton of 1 mM) to the reaction mixture completely abolished the activity of the enzyme.

Table 1. Summary of the purification of DNase I from equine parotid gland[a]

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude	44	560	12.7	1	100
Phenyl Sepharose CL-4B	12	364	30.3	2	65
DEAE Sepharose CL-6B	1.2	318	265	21	57
Superdex 75	0.56	266	475	37	48

[a]Enzyme activity was measured in 0.1 M Tris-HCl, pH 7.5, containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ as described in the text (13, 14).

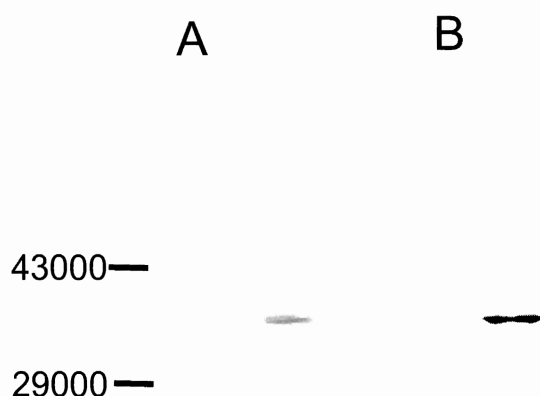

Fig. 1. SDS-PAGE patterns of purified equine DNase I detected by Coomassie Blue staining (A) and immunostaining (B). Samples were treated with SDS-PAGE sample buffer containing 2% w/v SDS and 25 mM dithiothreitol at 100 ℃ for 3 min, followed by electrophoresis with 12.5 % acrylamide gel.

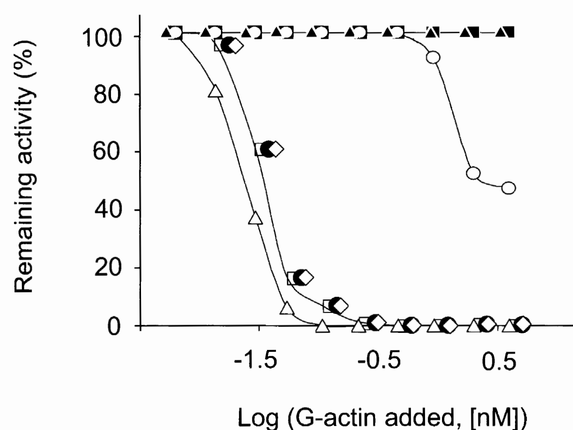

Fig. 2. Inhibitory effects of G-actin on mammalian enzyme activities of DNases I. Aliquots equivalent to approximately 0.3 units of each enzyme activity were incubated with various concentrations of G-actin, as indicated in the figure; residual enzyme activities were then measured by test tube method (13, 14). Residual activity of enzyme in the presence of each concentration of G-actin was determined at least in triplicate. The precision of assays was estimated to be within 10%. Equine (△), human (□), bovine (◇), mouse (●), rabbit (○), rat (▲), and porcine (■) DNases I.

DNase I activity appeared in the presence of Mn^{2+}, Mg^{2+}, and Ca^{2+} but was not detected in the absence of divalent cations when examined at pH 7.0 using 10 mM of each cation in the chloride form. An antibody against the purified enzyme was found to be monospecific against the equine parotid gland and the pure antigen, and completely blocked the activity of the purified enzyme. Contamination with other endonucleases was examined in the final pure enzyme preparation by the SRED method, but no DNase II, secretory RNase, or nonsecretory RNase activity was detected.

Inhibitory effects of G-actin on the enzyme activities of mammalian DNases I

Actin, a component of microfilaments, is present in monomeric form (G-actin; globular actin) or polymeric form (F-actin; fibrous actin). G-actin is known to bind preferencially to DNase I, and potently inhibit its enzymatic activity (17). The inhibitory effects of G-actin on the enzyme activities of mammalian DNases I were examined (Fig. 2). Porcine and rat DNase I were not inhibited by G-actin, and the enzymatic activity of rabbit DNase I was less efficiently inhibited by G-actin. Human, bovine, and mouse DNase I were inhibited to the same extent, but the equine DNase I was very strongly inhibited to the magnitude of 10. In human and bovine, main-chain interactions result from the parallel ß-strands formed by Tyr-65, Val-66 and Val-67 of DNase I and Gly-42, Val-43 and Met-44 of G-actin (18-20). The DNase I side-chains forming the core of the interface are hydrophobic and include Tyr-65, Val-67, and Ala-114; interactions peripheral to this central hydrophobic region are polar in nature and involve His-44, Asp-53, and Glu-69 (20). It has been suggested that four amino acid residues (Tyr-65, Val-66, Val-67 and Ala-114) are mainly responsible for actin binding in

human and bovine DNase I (17, 19). Figure 3 shows the amino acid sequences of actin binding site for equine (6), human (20), bovine (12), mouse (21), rabbit (8), rat (22), and porcine (11). Four amino acid residues, Tyr-65, Val-66, Val-67 and Ala-114, were critically involved in actin binding in G-actin-inhibited DNases I. In equine DNase I, Tyr-65 was substituted with Phe-65. Considering that the enzymatic activity of rabbit DNase I with Phe-65 and Ala-67 was less efficiently inhibited by G-actin, the structural involvement of Phe-65 and Val-67 may result in a strong DNase I and G-actin complex. Recently, we found that actin inhibition and folding of vertebrate DNase I are affected by mutations at residues 67 and 114 (16), and suggested that Val-67 may be essential for actin binding, that Phe-114 may be related to the folding of DNase I in reptiles and amphibians, and that Ala-114 may be indispensable for actin binding in mammals.

This study indicates that further comparative examinations and mutational analysis of mammalian DNase I about the residue 65 are needed to understand the mechanism of the actin inhibition of DNase I.

	60 73	111 118
Equine	PNTYHFVVSEPLGR	REPAIVKF
Human	PDTYHYVVSEPLGR	REPAIVRF
Bovine	PNTYHYVVSEPLGR	REPAVVKF
Mouse	PDTYRYVVSEPLGR	REPAIVKF
Rabbit	ADTYRFVASEPLGR	REPAVVRF
Rat	PDNYRYIISEPLGR	REPAIVKF
Porcine	PNNYHHVVSEPLGR	REPSVVKF

Fig. 3. Comparison of amino acid residues in the two actin-binding domains between equine (6), human (20), bovine (12), mouse (22), rabbit (8), rat (21) and porcine (11) DNases I. The amino acid redisues of 65-67 and 114 were gray-shaded. GenBank accession numbers for these amino acid sequences are as follows: equine (AB162819), human (M55983), bovine (AB048832), mouse (U00478), rabbit (D83038), rat (X56060), and porcine (AB048832).

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