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The chymotrypsin-like activity of human prostate-specific antigen, γ-seminoprotein

Kazuko Akiyama, Takanori Nakamura*, Sadaaki Iwanaga* and Mitsuwo Hara

Department of Legal Medicine, Kurume University School of Medicine, Kurume, Fukuoka 830 and **Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812, Japan*

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γ-Seminoprotein (γ-Sm) is a human prostate-specific antigen and a serine protease judging from the complete amino acid sequence which shows extensive homology with the kallikrein family. The enzymatic activity of γ-Sm was defined as a chymotypsin-like activity using reduced and *S*-3-(trimethylated amino)propylated lysozyme and insulin-oxidized A and B chains as substrates. The -Leu↓Ser- peptide bond of lysozyme was rapidly hydrolyzed by γ-Sm. γ-Sm also hydrolyzed the -Phe↓Glu- of lysozyme and the -Leu↓Cys(SO_3H)- of insulin B chain. Insulin A chain and arginyl- or lysyl-linkage of these proteins were not hydrolyzed by γ-Sm at all.

Prostate-specific antigen; Seminal plasma protease; Chymotrypsin-like enzyme; γ-Seminoprotein

1. INTRODUCTION

γ-Seminoprotein, a major seminal-specific antigen, is a glycoprotein isolated from human seminal plasma [1]. The complete amino acid sequence (237 residues) of γ-Sm [2,3] has been recently determined using protein chemical methods. Sequence comparison of γ-Sm with serine proteases shows a high degree of homology, especially with the kallikrein family. Although the biological function of γ-Sm has not been established, the utility of γ-Sm has been shown in forensic medicine to identify seminal stain [4] as well as in the diagnosis and monitoring of prostate cancer [5].

During these studies, Lundwall and Lilja [6] have also presented the amino acid sequence of a human prostate-specific antigen (PSA) deduced from a cDNA clone which is identical to that of γ-Sm [3]. The primary structure of the prostate-specific antigen (PA) presented by Watt et al. [7] is also very similar to those of γ-Sm and PSA. Of these antigens, PA displays low but detectable activity towards not only chymotryptic but also tryptic substrates [7], and PSA displays no detectable activity against *N*-benzoyl-tyrosine ethyl ester but against Arg/Lys containing substrates [8]. The substrate specificity of prostate-specific antigen has been so obscure and there are no clear experimental data which can define the cleavage sites of protein substrates by the prostate-specific antigen. Here we describe the cleavage sites of reduced and *S*-3-(trimethylated amino)propylated lysozyme and insulin-oxidized B chain by γ-Sm.

Correspondence address: K. Akiyama, Department of Legal Medicine, Kurume University School of Medicine, Kurume, Fukuoka 830, Japan

Abbreviations: γ-Sm, γ-seminoprotein; RSA-lysozyme, reduced and *S*-3-(trimethylated amino)propylated lysozyme; pNA, *p*-nitroanilide

2. MATERIALS AND METHODS

γ-Sm prepared from human seminal plasma [3] was passed through a benzamidine-Sepharose 6B

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column (Sigma, 2 ml) with 0.1 M ammonium bicarbonate containing 1 M guanidine-HCl, pH 8.0. The unbound fraction was used as γ-Sm in this experiment. RSA-lysozyme [9] and insulin-oxidized A and B chains (Sigma) were used as protein substrates. The substrate (1 mg) dissolved in 300 μl of 0.05 M Tris-HCl buffer, pH 8.0, was incubated with γ-Sm (10 μg) at 37°C. After 60 min, the digest was subjected to reverse-phase high performance liquid chromatography (HPLC) to separate peptides by a Beckman HPLC system (see legend to fig.1A). For an examination of the time course of digestion, an aliquot of the digest of RSA-lysozyme was removed after 10, 60 and 120 min incubations and followed by SDS-polyacrylamide gel electrophoresis (PAGE) [10]. The rate of hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA (1 mM) and Suc-Ala-Ala-Pro-Leu-pNA (1 mM) (Bachem, Feinchemikalien AG) was measured spectrophotometrically at 405 nm after incubation with γ-Sm (10 μg) in 250 μl of 0.1 M Tris-HCl buffer, pH 8.0, at 37°C for 60 min followed by adding 800 μl of 0.6 M acetic acid.

An aliquot (100 μl) from fractions of the reverse-phase HPLC was hydrolyzed in 5.7 M HCl at 110°C for 20 h. The hydrolysates were analyzed by the phenylthiocarbamyl method on a Pico Tag system (Waters, Millipore Corp., Milford, MA), in conformity with the operator's manual. Approx. 5 nmol each of the fragments derived from the 60 min-digest of RSA-lysozyme was analyzed on their NH_2-terminal sequences by manual Edman degradation according to the film method [11]. The PTH amino acids were identified by HPLC on a Zorbax CN column (4.6 × 250 mm) with a ternary gradient system [12].

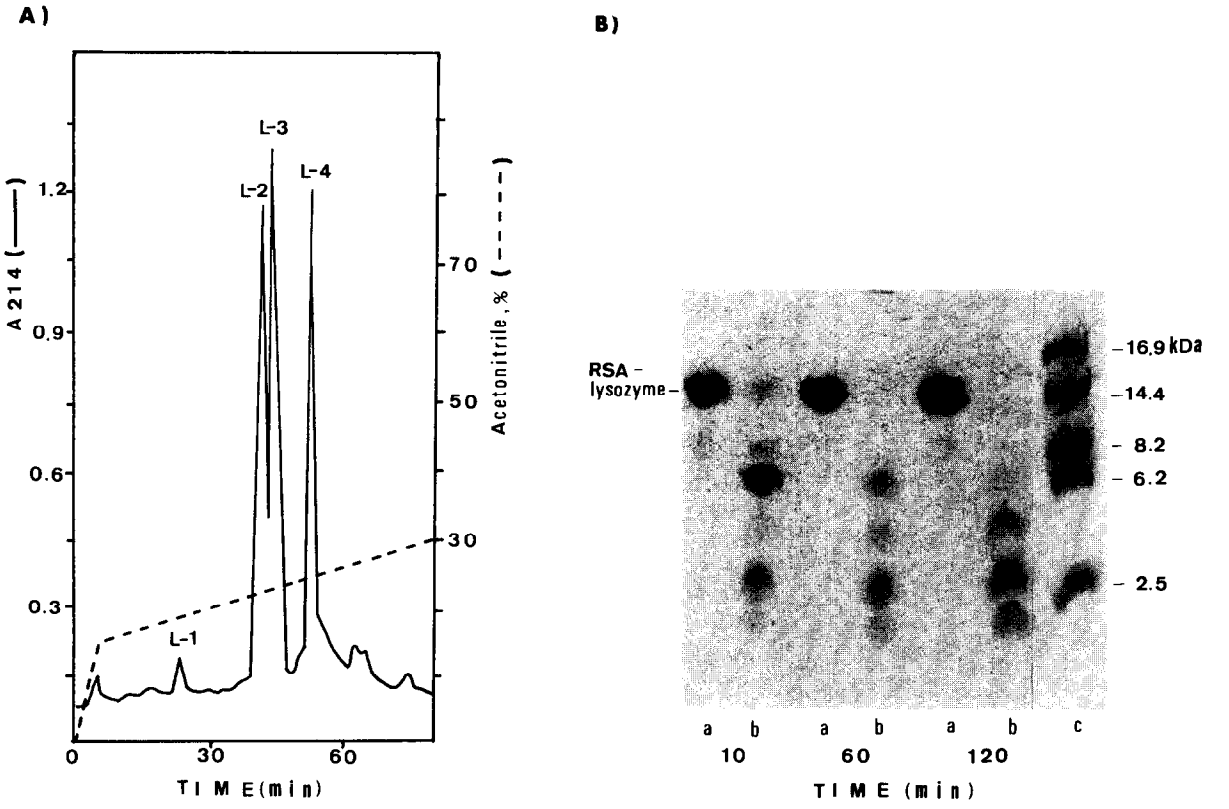

Fig.1. Analyses of the digests of RSA-lysozyme with γ-Sm. (A) Reverse-phase HPLC of the digest (60 min) on a Cosmosile 5C18P column (10 × 250 mm) at a flow rate of 0.5 ml/min. Elution was performed with an 80 min gradient from 0 to 30% solvent B; solvent A, 0.1% (v/v) trifluoroacetic acid in water; solvent B, 0.1% (v/v) trifluoroacetic acid in acetonitrile. (B) SDS-PAGE (12% polyacrylamide gel with 8 M urea) of the digests at 10, 60 and 120 min. (a) Control; (b) digests; (c) molecular mass markers (Fluka AG).

3. RESULTS

The time course of hydrolysis of RSA-lysozyme by γ-Sm is shown by the patterns of SDS-PAGE of the digests (fig.1B). The peptide fragments of the 60 min-digest were separated by reverse-phase HPLC (fig.1A). The amino acid compositions and NH_2-terminal sequences of these fragments are shown in tables 1 and 2. Comparing these data with the sequence of lysozyme [13], the position of each fragment at the parent sequence was deduced. The fragments L-3 and L-4 corresponded respectively to the COOH-terminal portion (Ser-85–Leu-129) and to the NH_2-terminal portion (Lys-1–Leu-84) of lysozyme. Although the amino acid composition of L-2 corresponded to that of the NH_2-terminal to Leu-84, this fragment was found to be composed of two fragments (Lys-1–Phe-34 and Glu-35–Leu-84) by the NH_2-terminal sequence analysis (table 2). L-1 was assigned as the NH_2-terminal fragment from Lys-1 to Tyr-23.

On the other hand, insulin-oxidized B chain digested by γ-Sm was separated into four fragments (I-1, I-2, I-3 and I-4) by reverse-phase HPLC (not shown). The yields of these fragments were 14% for I-1 and I-4 and 2% for I-2 and I-3 which were deduced to be generated by a cleavage of -Leu-6↓Cys[SO_3H]-7-, and of -Tyr-16↓Leu-17-, respectively, based on their amino acid compositions (not shown). The HPLC chromatogram of the digested insulin-oxidized A chain showed the same pattern as that of the undigested sample. The rate of hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Leu-pNA by γ-Sm was calculated to be 2.10 and 1.05 μM/min per mg, respectively. However, these values were about 4000-times and 1000-times less than those of α-chymotrypsin, respectively.

Table 1

Amino acid compositions of four fragments obtained from the digest of RSA-lysozyme with γ-Sm

Amino acid	Residues per molecule				RSA-lysozyme
	L-1	L-2	L-3	L-4	
Asp	2.2(2)	15.5(14)	7.0(7)	15.8(14)	(21)
Thr		5.1(5)	2.0(2)	5.3(5)	(7)
Ser		5.7(6)	3.2(4)	5.9(6)	(10)
Glu	1.4(1)	4.2(4)	1.2(1)	4.2(4)	(5)
Pro		2.0(2)		2.0(2)	(2)
Gly	4.1(3)	8.3(8)	3.6(4)	8.1(8)	(12)
Ala	3.2(3)	7.7(7)	4.6(5)	7.4(7)	(12)
Cys/2	(1)	(5)	(3)	(5)	(8)
Val	1.2(1)	2.4(2)	2.8(4)	2.2(2)	(6)
Met	0.6(1)	0.5(1)	0.4(1)	0.5(1)	(2)
Ile		3.0(3)	2.1(3)	3.2(3)	(6)
Leu	2.0(2)	6.8(7)	1.4(1)	6.6(7)	(8)
Tyr	1.8(2)	2.7(3)	0.2	2.5(3)	(3)
Phe	1.0(1)	2.7(3)	0.2	2.6(3)	(3)
Lys	1.9(2)	3.3(3)	2.4(3)	3.1(3)	(6)
His	1.2(1)	1.0(1)		0.9(1)	(1)
Trp		(3)	(3)	(3)	(6)
Arg	4.2(3)	8.2(7)	3.9(4)	7.8(7)	(11)
Total	(23)	(84)	(45)	(84)	(129)
Position	1–23	1–34, 35–84	85–129	1–84	
Yield (%)	2.5	32.5	40.0	16.2	

Values in parentheses are taken from the sequence data [13]

Table 2

NH_2-terminal sequences of four fragments obtained from the digest of RSA-lysozyme with γ-Sm

Cycle no.	L-1	L-2	L-3	L-4
1	Lys(N.Q.)[a]	Glu(1.26)	Ser(N.Q.)	Lys(0.51)
2	Val(1.10)	X[b]	Ser(N.Q.)	Val(1.19)
3	Phe(1.38)	Asn(0.88)	Asp(0.24)	Phe(0.45)
4	Gly(0.40)	Phe(1.76)	Ile(0.24)	Gly(1.09)
5	Arg(0.25)	Asn(0.61)	X	Arg(0.65)

[a] Not quantitated
[b] Not identified

Approx. 5 nmol each of the samples was applied to the sequence analysis. Values in parentheses are approximate yields (nmol) of phenyl-thiohydantoin derivatives calculated from peak areas on HPLC

4. DISCUSSION

Based on the primary structure, γ-Sm is a serine protease showing the conservation of residues (His-41, Asp-96 and Ser-189) in the vicinity of the active site of serine proteases [3] and a homology (62%) with human pancreatic kallikrein [14]. Comparing residue 183 at the bottom of the substrate binding pocket of γ-Sm with that of pancreatic kallikrein, this position is occupied in γ-Sm by a Ser residue and by an Asp residue in kallikrein. However, PA and PSA which seem likely to be the same enzyme as γ-Sm have been reported to express trypsin- and chymotrypsin-like specificities toward insulin A and B chains and recombinant interleukin 2 [7,8].

In this experiment, γ-Sm was further purified through a benzamidine-Sepharose column to exclude contamination, if present, responsible for trypsin-like activity. The cleavage sites of RSA-

Table 3

Cleavage sites of RSA-lysozyme and insulin-oxidized B chain by γ-Sm

	P4 P3 P2 P1 P1′P2′P3′ P4′	Rate of hydrolysis[a]
RSA-lysozyme	-Ser-Ala-Leu-Leu↓Ser-Ser-Asp-Ile-	rapid
	-Ala-Ala-Lys-Phe↓Glu-Ser-Asn-Phe-	rapid
	-Tyr-Arg-Gly-Tyr↓Ser-Leu-Gly-Asn-	very slow
Insulin-oxidized B chain	-Asn-Gln-His-Leu↓Cys[b]-Gly-Ser-His-	rapid
	-Glu-Ala-Leu-Tyr↓Leu-Val-Cys[b]-Gly-	very slow

[a] Based on the yield of the fragments generated after digestion for 60 min
[b] $Cys(SO_3H)$

lysozyme and insulin-oxidized B chain by γ-Sm are listed with their subsites in table 3. The Leu-84↓Ser-85 bond of RSA-lysozyme was found to be the most susceptible linkage. The Phe-34↓Glu-35 of RSA-lysozyme and Leu-6↓Cys(SO_3H)-7 of insulin-oxidized B chain were also hydrolyzed by γ-Sm. The Tyr-23↓Ser-24 of RSA-lysozyme and the Tyr-16↓Leu-17 of insulin-oxidized B chain were hydrolyzed slightly after 1 h incubation. Except for these bonds, neither the Leu-X (X = Ala, Val, Tyr, Gly, Gln, Asp or Cys-alkylated), the Phe-Y (Y = Phe, Tyr, Val, Asn or Gly), the Tyr-Z (Z = Arg, Gln, Gly, Thr or Cys(SO_3H)), lysyl nor arginyl bonds present in RSA-lysozyme, insulin-oxidized A chain or B chain were hydrolyzed by γ-Sm. Therefore, the data presented here indicate that the enzymatic activity of γ-Sm is defined as a chymotrypsin-like activity with strict substrate specificity. Leucyl, phenylalanyl and tyrosyl bonds followed by a suitable amino acid as the subsite of P1′ are concerned with the substrate specificity of γ-Sm (table 3). Also, the subsites of P3, P2 and P2′ may have some role in the specificity of γ-Sm, though any definite amino acid cannot be stated at present. Investigation of the physiological substrate for γ-Sm will be followed.

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