J. Enzyme Inhibition, 1996, Vol. 10, pp. 81–91
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A CYSTEINE-RICH SERINE PROTEASE INHIBITOR (GUAMERIN II) FROM THE NON-BLOOD SUCKING LEECH *WHITMANIA EDENTULA*: BIOCHEMICAL CHARACTERIZATION AND AMINO ACID SEQUENCE ANALYSIS

DONG RYOUNG KIM,[1] SEOK JIN HONG,[1] KWON-SOO HA,[2]
CHEOL O JOE[1] and KE WON KANG[1,*]

[1]*Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305–701, Korea*

[2]*Biomolecule Analysis Group, Korea Basic Science Center, Taejon 305-701, Korea*

(Received 31 July 1995)

A cysteine-rich serine protease inhibitor (Guamerin II) was isolated from the non-blood sucking leech *Whitmania edentula*. The new inhibitor was identified as a low molecular weight (6,012 Da) polypeptide with some sequence similarities to antistasin, hirustasin and guamerin. The inhibitor contained 56 amino acid residues with 76.8% sequence similarity to guamerin, 48.2% to hirustasin and 28.6% to the first domain of antistasin. This new inhibitor was the first completely sequenced serine protease inhibitor from a non-blood sucking leech. Analysis of the inhibitor revealed that it was active against neutrophil elastase and chymotrypsin, but had no activity against a variety of other proteases. The P1 reactive site residue was identified as methionine and the residues surrounding the Pl site were hydrophobic amino acids. The primary structure of the inhibitor showed no similarity to well-known elastase inhibitors from leeches such as eglin.

KEY WORDS: Leech, protease inhibitor, *Whitmania edentula*, amino acid sequence, cysteine-rich polypeptide, Guamerin II

INTRODUCTION

Protease inhibitors have complex mechanisms for controlling the activity of their target proteases. A large number of protease inhibitors have been isolated from various organisms.[1] Leeches have been known to contain several types of protease inhibitors.[2] Most of them have canonical interactions through a binding loop with their corresponding protease. After binding, cleavage of the reactive site of the inhibitor is possible resulting in a change of its inhibitory activity.[3] Most of the inhibitors from leeches have shown some therapeutic potential for human diseases. These include hirudin,[4] eglin,[5] antistasin,[6,7] hirustasin,[8]

*Correspondence: Phone: 82–42–869–2612; Fax: 82–42–869–2610.

gelin,[9] and guamerin.[10] These are, in general, small and compact proteinaceous inhibitors. Among the hundreds of leech species inhabiting the world, the medicinal leech *Hirudo medicinalis* has been the most extensively studied for protease inhibitors. For example, hirudin is a thrombin inhibitor and eglin is an inhibitor of elastase, chymotrypsin and cathepsin G. Eglin has a similar activity spectrum to gelin, but has a different primary structure and its amino acid composition lacks cysteine residues. Guamerin, isolated from *Hirudo nipponia*, is the specific inhibitor of elastase with the lowest K_i value. Antistasin, isolated from the genus *Haementeria*, is a specific inhibitor of factor Xa and trypsin, while hirustasin, from *Hirudo medicinalis*, is an inhibitor of cathepsin G, chymotrypsin, kallikrein, trypsin, and plasmin. Despite the close sequence similarities and the characteristic spacing between the cysteines among gelin, guamerin, antistasin, and hirustasin, these serine protease inhibitors act on different target proteases.

The protease inhibitory activity in two species of non-blood sucking North American leeches has been reported.[11] In the present paper, we describe a new elastase and chymotrypsin inhibitor isolated from the non-blood sucking leech, *Whitmania edentula*. The inhibitor has a high sequence similarity to guamerin with an identical spacing of the cysteines, but there is an important difference in the N-terminal region of variable amino acid composition which has a glutamate deletion. This difference suggests that the specificity of these types of inhibitors is influenced by more than the reactive site.

MATERIALS AND METHODS

Materials

The proteases porcine pancreatic elastase (PPE), human leukocyte elastase (HLE), chymotrypsin, and trypsin were purchased from Sigma Chemical Company (St. Louis, MO). The other proteases were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Chromogenic substrates were bought from Sigma Chemical Company. Chromatographic materials were obtained from Pharmacia LKB. All other chemicals were from Sigma Chemical Company.

Leech Extracts

Leeches were kept in a culture system of a laboratory aquarium until used. Thirty live leeches (15 g) were dissected and homogenized in 100 ml of 25 mM Tris/ HCl buffer (pH 8.0). Then the suspension was centrifuged at 6,000 × g for 15 min and the supernatant was freeze-dried.

Acetone Precipitation

The extract was dissolved in 50 ml of 25 mM Tris/HCl buffer (pH 8.0) containing 250 mM NaCl. An equal volume of cold acetone was added and the mixture was left to stand for 6 h at −20°C. After centrifugation, the supernatant was mixed with cold acetone at a final volume of 70%, centrifuged and the pellet was solubilized in a 25 mM Tris/HCl buffer (pH 8.0) containing 250 mM NaCl. The solution was concentrated to a final volume of 2.0 ml by passing it through a 3,000 Da membrane.

Gel Filtration Chromatography

The concentrate from the ultrafiltration step was loaded onto a Sephadex G-75 column (2 cm × 60 cm) pre-equilibrated with a 25 mM Tris/HCl buffer (pH 8.0) and eluted with the same buffer.

Anion-Exchange Chromatography

The active pool from the gel filtration step was loaded onto a DEAE-Sepharose CL-6B column (0.7 cm × 3 cm) pre-equilibrated with a 25 mM Tris/HCl buffer (pH 8.0). The column was eluted sequentially with buffer solutions containing 35 mM, 70 mM, 140 mM, 280 mM, and 1,500 mM NaCl.

Reverse-Phase (RP) HPLC

The final purification of the inhibitor was carried out by passing the active fractions, isolated by the DEAE-Sepharose CL-6B anion-exchange chromatography step, through a Delta-Pak C_{18} column (3.9 mm × 300 mm; Millipore Corporation, Bedford, MA) using a RP-HPLC method. The gradient program was run with mixtures of solvent A (0.1% v/v TFA in water) and solvent B (0.1% v/v TFA in acetonitrile) as follows: 0–20% v/v solvent B for 10 min, 20–45% solvent B for 80 min and, finally, 45–60% solvent B for 20 min.

Amino Acid Composition and Sequence Analysis

The amino acid composition of the inhibitor was analyzed with the Pico-Tag method after hydrolysis in constantly boiling 3.0 N HCl at 110°C for 24 h. The inhibitor (about 50 μg), dissolved in 200 μl of 100 mM Tris/HCl (pH 8.0) containing 6 M guanidine-HCl, was mixed with 10 μl of β-mercaptoethanol and incubated at 50°C for 5 h. Sulfhydryl groups were exposed to 15 μl of 4-vinylpyridine at ambient room temperature in the dark for 2 h with constant stirring. The reaction was stopped by quickly cooling to −76°C. The modified inhibitor was then purified by RP-HPLC, and mixed with 1 μg of endoproteinase Arg-C in 100 μl of 100 mM Tris/HCl (pH 8.0) at 37°C for 24 h. Sequencing was performed in an Applied Biosystem 476A protein sequencer.[12]

Elastase Inhibition Assay

Inhibition of PPE activity was monitored throughout the purification processes. PPE was dissolved in 100 mM Tris/HCl buffer (pH 8.0) containing an equimolar quantity of NaCl.The substrate solution consisted of 100 mM NaCl, 0.02% (w/v) sodium azide and 0.1% (w/v) azocasein in 100 mM Tris/HCl buffer (pH 8.0). In each reaction vial, 10 μl of PPE (about 1 unit) and 20–50 μl of the sample were mixed, and then incubated for 30 min. This step was followed by the addition of 500 μl of pre-warmed azocasein solution to each vial and an additional incubation for 1 h at 37°C. The reaction was stopped by the addition of 400 μl of 15% (w/v) TCA at 4°C. After centrifugation at 12,500 × g for 5 min, the absorbance was measured at 440 nm.[13] One elastase inhibitory unit (EIU) is defined as the inhibitory activity blocking one elastase unit from completely hydrolyzing 1 mg of elastin during a 20 min incubation in a pH 8.8 buffer at 37°C.[14]

TABLE 1
Summary of the purification of guamerin II.

	Protein (mg)	Total activity (EIU)	Specific activity (EIU/mg)	Yield (%)	Purification (fold)
Crude extract	1400	780	0.56	100	1
Acetone ppt.	42	600	14.3	77	25.5
Sephadex G-75	30	540	18.3	69	32.7
DEAE-Sepharose	2.4	480	200	62	357
RP-HPLC[a]	0.2	86	430	11	768

[a] Data are based only on peak D (see Figure 3).

Inhibitory Constants and Protease Specificity

Inhibitory constants for inhibition of PPE, HLE and chymotrypsin were analyzed with chromogenic substrates as previously described.[15] Synthetic peptide substrates, *N*-succinyl-$(Ala)_3$-*p*-nitroanilide for elastases and *N*-benzoyl-Tyr-*p*-nitroanilide for chymotrypsin, were used. The inhibitions of thrombin,[16] trypsin[17] and factor Xa[18] were measured with Chromozyme TH, *N*-benzoyl-Arg-*p*-nitroanilide and *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide, respectively. The inhibition against subtilisin was measured with azocasein as the substrate.

Miscellaneous Methods

The molecular weight of the inhibitor was measured by MALDI mass spectrometry[19] and 16.5% (w/v) Tricine/SDS-polyacrylamide gel electrophoresis.[20] Protein was measured by the method of Bradford *et al.*[21] The search for similar peptide sequences used the EMBL data base (Heidelberg).[22] The secondary structure was predicted by the HDSSOP program (version 1.0, 1991) supported by the EMBL data base.[23]

RESULTS

A serine protease inhibitor against elastase and chymotrypsin was purified from the leech *Whitmania edentula* by the sequential procedures of acetone precipitation, gel filtration chromatography, anion-exchange chromatography, and, finally, reverse-phase HPLC. The purification procedures are summarized in Table 1. The crude extract was fractionated by the addition of cold acetone at various concentrations. Most of the activity was concentrated between 50% (v/v) and 70% (v/v) cold acetone. Approximately 97% (w/w) of the inactive proteins were removed by acetone precipitation. Gel filtration was a very efficient method of fractionating the acetone precipitate (Figure 1). Most of the activity was bound to DEAE-Sepharose CL-6B at pH 8.0. Over 90% (unit/unit) of the activity bound to this gel was eluted with a 25 mM Tris/HCl buffer (pH 8.0) containing 140 mM NaCl (Figure 2). Elution at a low salt concentration implies that the inhibitor has a low isoelectric point. The remaining activity was eluted with a buffer containing 280 mM NaCl. The fractions eluted

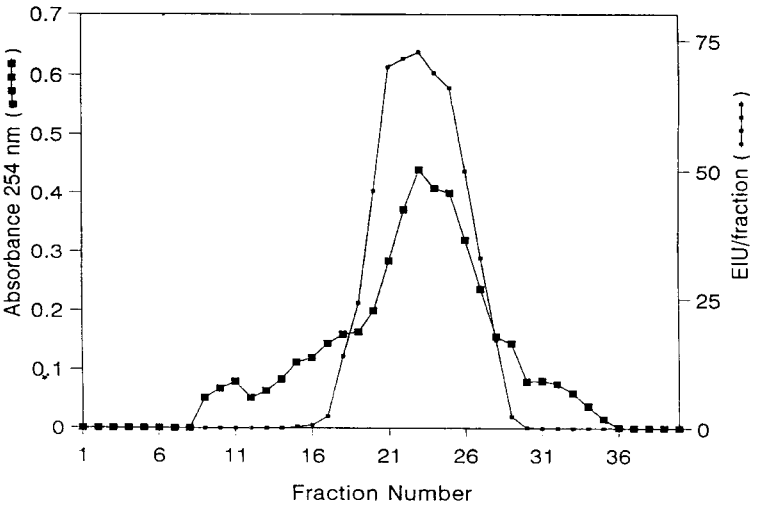

FIGURE 1 Gel filtration chromatography of elastase inhibitors on a Sephadex G-75 column. The concentrate from the acetone precipitation step was applied to the column. The peak of the inhibitory activity against porcine pancreatic elastase was found in fractions 17–29.

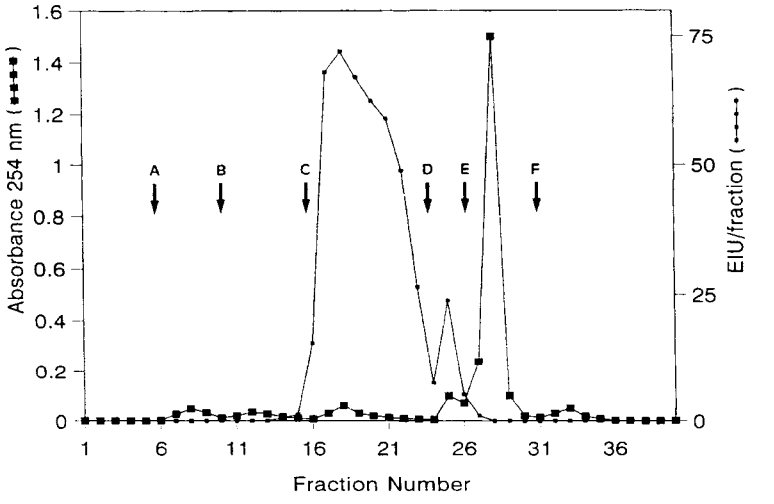

FIGURE 2 Anion-exchange chromatography of elastase inhibitors on a DEAE-Sepharose CL-6B column. The active pool from the Sephadex G-75 column was loaded onto the column, and the column was washed and eluted with a step gradient: A, 35 mM; B, 70 mM; C, 140 mM; D, 280 mM; and E, 1,500 mM NaCl. The final eluant (F) contained 0.2 N NaOH. Fractions 15–23 were concentrated and the buffer was changed for HPLC.

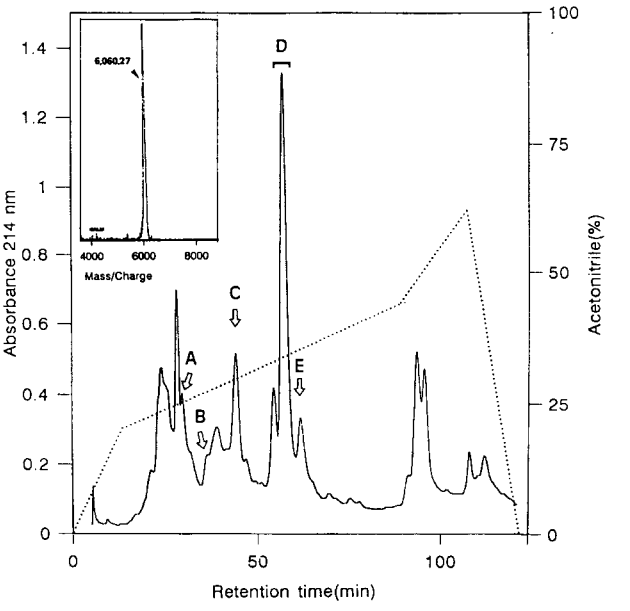

FIGURE 3 High-performance liquid chromatography. A Delta-Pak C_{18} (Millipore) column was used. Solvent A = 0.1% (v/v) TFA in water; solvent B = 0.1% TFA in acetonitrile. The gradient was 0–20% solvent B for 10 min, 20–45% solvent for 80 min and 45–60% solvent B for 20 min. The flow rate was 0.5 ml/min. The peaks of elastase inhibitory activities were denoted by the symbols A, B, C, D, and E based on the order of elution. Peak D was used for following analyses. The insert shows the molecular weight of peak D from MALDI mass spectroscopy.

at 140 mM NaCl were pooled and used for the following analysis. The final step of the purification by RP-HPLC revealed five peaks of inhibitory activity (Figure 3). The highest elastase inhibitory activity per mg protein was found in peak C of the HPLC chromatogram, but this peak of activity was contaminated by inactive polypeptides. The polypeptides that appeared in peak D were the second-most active fraction and were homogeneous; they had approximately 11% of the total activity in the crude extract. The homogeneity was confirmed by a single band on 16.5% (w/v) Tricine/SDS-polyacrylamide gel electrophoresis (data not shown). A single sharp peak from MALDI mass spectrometry (see the insert in Figure 3) showed an apparent molecular weight of 6,060 Da. The actual molecular weight calculated from the sequence data of the automated Edman degradation was 6,012 Da.

The amino acid composition determined by Pico-Tag analysis was virtually identical with the final sequence data (Table 2). It was found that the inhibitor has a high aspartate/asparagine and glutamate/glutamine content, but no tryptophan or leucine residues. The amino acid sequence was determined after reduction and S-pyridylethylation by β-mercaptoethanol and 4-vinylpyridine. The direct sequence of the first forty residues from the N-terminus was assigned without any difficulty and the rest of the sequence was determined from the endoproteinase Arg-C digestion based on the Pico-Tag data. The full sequence of 56 amino acids was constructed from the direct sequence and the two peptide fragments from the endoproteinase Arg-C digestion.

TABLE 2
Amino acid composition of guamerin II.

Amino acid	Guamerin II Pico-Tag (Sequence)	Guamerin[b]	Antistasin[b]	Hirustasin[b]	Gelin[c]
Ala	5.29 (5)	5	3	4	7.8
Arg	1.05 (1)	1	12	2	1.97
Asp/Asn	10.17 (10)	8	9	4	14.9
Cys	N.D.[a] (10)	10	20	10	N.D.
Gly	4.77 (4)	4	11	6	9.1
Glu/Gln	7.43 (6)	7	11	6/7[e]	6.2
His	1.05 (1)	1	2	1	0
Ile	3.05 (3)	2	6	1	2.66
Leu	0.91 (0)	2	6	2	4.76
Lys	2.27 (2)	2	9	6	5.76
Met	0.93 (1)	1	4(3)[d]	0	1.28
Phe	2.10 (2)	2	3	0	2.41
Pro	3.50 (3)	3	9	1	6.25
Ser	1.59 (1)	1	6	3	4.4
Thr	3.38 (3)	4	5	3	2.55
Trp	N.D. (0)	0	0	0	N.D.
Tyr	0.15 (1)	1	2	2	0.98
Val	3.42 (3)	3	1(2)[d]	3	11.92
Total	56	57	119	54/55	N.D.

[a]Not determined. [b]The quantity of each residue was obtained from the amino acid sequence. [c]The quantity of each residue was calculated from the analysis of the amino acid composition based on a molecular weight of 8,100 Da. [d]The value in the parenthesis is the number of isoforms of antistasin differing only at position 35 (Met/Val variation). [e]An isoform has an additional glutamate at the C-terminus of hirustasin.

The inhibitory constants (K_i) against PPE and HLE were 8.1 nM and 9.3 nM, respectively. Specificity tests revealed that it was also active against chymotrypsin (48.7 nM), but essentially inactive against other proteases (Table 3).

DISCUSSION

A number of protease inhibitors have been isolated from leeches and characterized. They can be grouped as follows: (i) thrombin specific inhibitors such as hirudin; (ii) relatively high molecular weight inhibitors such as bdellin B-3;[24] (iii) low molecular weight cysteine-rich inhibitors such as antistasin, and (iv) other low molecular weight inhibitors without cysteine residues such as eglin.[2,5] In the course of screening for leech-derived protease inhibitors from native Korean species, a low molecular weight elastase inhibitor with very low K_i values containing 10 cysteine residues was detected in non-blood sucking species.

TABLE 3
Comparison of inhibitory constants.

Enzyme	Guamerin II (K_i)	Guamerin[a] (K_i)	Antistasin[b] (IC_{50})	Hirustasin[c] (K_i)	Gelin[d]	Eglin[e]
PPE	8.1 nM	N.D.	–	–	36 nM	+
HLE	9.3 nM	0.81 fM	–	–	N.D.	+
Trypsin	–[f]	–	5 nM	7 nM	N.D.	
Chymotrypsin	48.7 nM	–	–	6.4 nM	1 nM	+
Factor Xa	–	–	1 nM	–	N.D.	
Thrombin	–	–	–	–	N.D.	
Subtilisin	–	–	–	–	N.D.	+
Cathepsin G	N.D.[g]	N.D.	N.D.	2.9 nM	1.9 nM	+
Tissuekallikrein	N.D.	N.D.	N.D.	3 nM	N.D.	
Plasmin	N.D.	N.D.	N.D.	38 nM	N.D.	

[a]Guamerin data are from Jung *et al.* [10] [b]Antistasin data are from Tuszynski *et al.*[6] and Nutt *et al.*[7] [c]Hirustasin data are from Söllner *et al.*[8] [d]Gelin data are from Electricwala *et al.*[9] [e]Eglin data are from Seemüller *et al.* The + marks mean 0.1–0.01 nM for the K_i values. [f]No inhibition against the protease. [g]Not determined.

Eglin from the leech *Hirudo medicinalis* might have a therapeutic value for treating such diseases as the tissue destruction which is caused by HLE. Although the biochemical characterization, molecular cloning[25] and X-ray crystallography[26] of eglin showed a compact molecule without disulfide bonds, clinical investigations and animal model studies have not yet been published. A recently identified elastase inhibitor from *Hirudinaria manillenis*, gelin, also showed a similar specificity spectrum as eglin.[9] However, the partial amino sequence of gelin showed little similarity to eglin. Although their apparent molecular weights on SDS-polyacrylamide gel electrophoresis were similar, their amino acid compositions were quite different. It may be that different inhibitors of the same enzyme have evolved different mechanisms for inhibition during the evolution of leeches.

We have found at least five different polypeptides with activities against elastases in whole body extracts from a non-blood sucking leech. These are the first proteinaceous inhibitors isolated from the genus *Whitmania*. One of the isolated and sequenced peptides showed a close sequence similarity with guamerin and had inhibitory activities against chymotrypsin in addition to elastase.

The new inhibitor from the non-blood sucking leech had a similar primary structure and spacing of the cysteine residues to antistasin, hirustasin and guamerin. The new inhibitor was very similar to guamerin, an elastase specific inhibitor, which was identified by Jung *et al.*[10] It was, therefore, named guamerin II. The sequence similarity with guamerin is 76.8%, 48.2% with hirustasin, and 28.6% and 32.1% with the antistasin domain 1 and domain 2, respectively. There was virtually no sequence similarity with eglin but it showed a sequence similarity with the N-terminal region of gelin which has been only partially sequenced at present.[9] Gelin is known to have inhibitory activities against elastase and chymotrypsin. When guamerin II is aligned with the potentially homologous inhibitors on the basis of their cysteines, the positions of the ten cysteine residues and the P1 reactive site

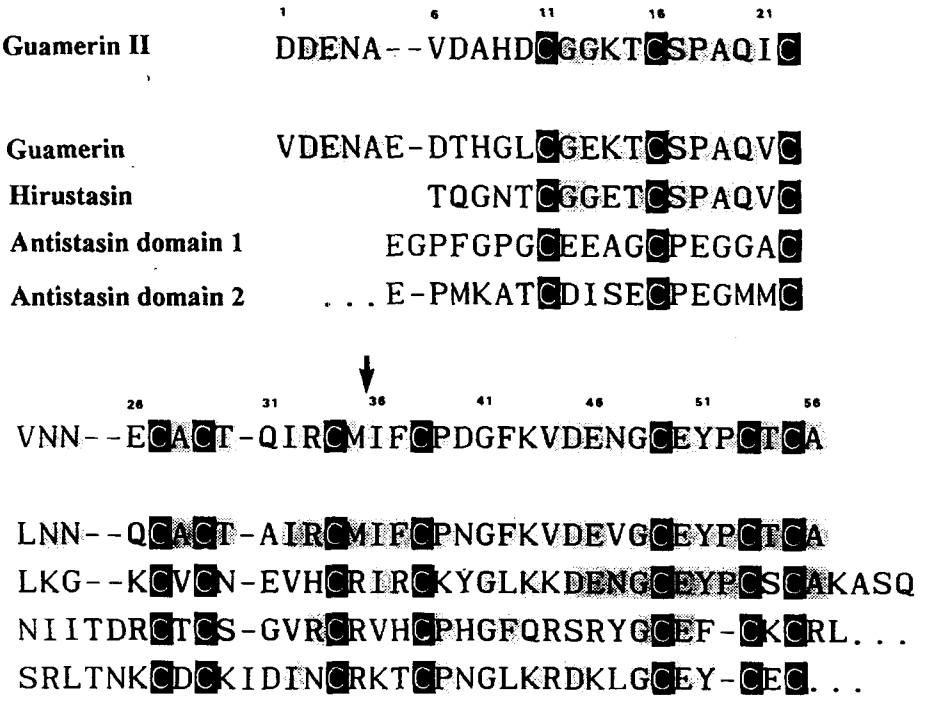

FIGURE 4 Sequence similarity of protease inhibitors. The primary amino acid sequence of guamerin II is compared with cysteine-rich low molecular weight protease inhibitors which were derived from leeches. The arrow indicates the scissile peptide bond of the reactive P1–P1′ site of the inhibitors. The identical residues with guamerin II are shaded.

match best with guamerin (Figure 4). The new inhibitor has methionine at the reactive site as guamerin but antistasin and hirustasin have arginines at these sites. The N-terminus and the sequences up to the first cysteines of these inhibitors are very interesting. A glutamate residue in the N-terminal region is missing from hirustasin and this newly isolated inhibitor. The sequences up to the first cysteine residues are the most variable including the pentapeptide (^{6}VDAHD^{10}). The sequence similarities suggest that these inhibitors evolved from a common ancestral protease inhibitor.

Guamerin II shows differences in affinity and/or specificity compared to antistasin, hirustasin, gelin, and guamerin despite their similar primary structures (Table 3). Guamerin II has a lower inhibitory constant (8.1 nM) for PPE than gelin (36 nM) but has higher inhibitory constants against elastases than guamerin. Guamerin II has inhibitory activity against chymotrypsin in addition to elastase despite its sequence similarity to guamerin, and does not inhibit trypsin unlike antistasin and hirustasin. Although chymotrypsin is inhibited by guamerin II, the K_i of 48.7 nM is much higher than the 1.0 nM for gelin that was isolated from *Hirudinaria manillensis*. These leech-derived and low molecular polypeptides show complex inhibitory spectra but similar amino acid sequences.

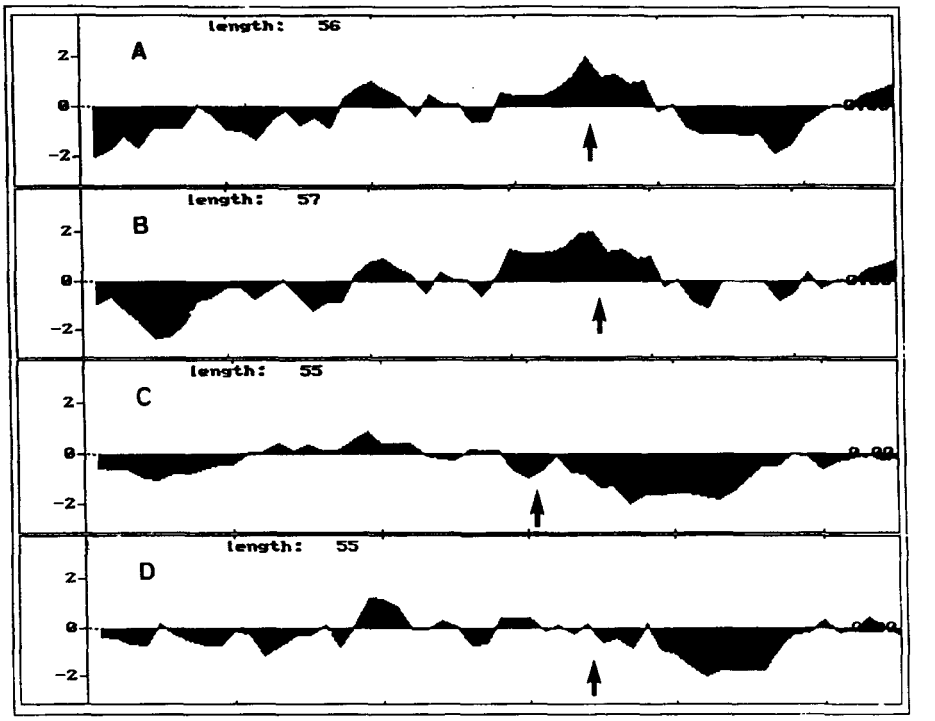

FIGURE 5 Hydrophobicity plots of guamerin II and other protease inhibitors. The Y-axis is the index of hydrophobicity based on a mean value of 7.0 residues for each inhibitor: A, guamerin II; B, guamerin; C, hirustasin; D, antistasin domain 1. The arrows indicate the scissile peptide bond.

The inhibitory properties might, however, be partially explained by several determinants. These are the hydrophobicity of the binding core,[27] the P1 reactive site residue[28] and the variable residues at the N-terminal region.[29] Hydrophobicity plots show hydrophobic cores around the reactive site in guamerin II and guamerin, and neutral or hydrophilic cores for the other inhibitors (Figure 5). The predicted secondary structures show β-sheets at the regions of the reactive site of these inhibitors.[23] This implies that the interactions of these inhibitors with their proteases are partially dependent on the loops of the β-sheet and that the differences of hydrophobicity in the loops affect the strength of the interactions or resulting inhibition of the proteases. The residue of the P1 reactive site has been known to be important for determining the specificity of the inhibitor. After the binding of the inhibitor to the protease, the inhibitor may or may not be cleaved between the P1 and P1′ residue depending on its properties and amino acids composition. Although the methionines of guamerin II and guamerin may determine their specificities toward elastase, and the arginines of antistatin and hirustasin toward trypsin, inhibitory activities against other proteases may not be sufficiently explained by the reactive sites alone.

Further studies are needed on the structure and functions of guamerin II, including N-terminal variability and other structural elements after the cloning of its gene. Because of the relatively elastase specific property of guamerin II, it might be a promising candidate for novel therapeutic applications for emphysema and other elastase-related diseases.[30]

Acknowledgements

This work was supported by grants from the Ministry of Science and Technology and from the Korea Science and Engineering Foundation. We thank Dr. M.K Song for helpful discussions.

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