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BIOCHEMICAL CHARACTERISATION OF A PANCREATIC ELASTASE INHIBITOR FROM THE LEECH *HIRUDINARIA MANILLENSIS*

ASGAR ELECTRICWALA,[1]* NILS A.E. VON SICARD,[2]
ROY T. SAWYER[2] and TONY ATKINSON[1]

[1]*Division of Biotechnology, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, England;* [2]*Biopharm (UK) Ltd., Bryngelen House, 2, Bryngwili Road, Hendy, Wales*

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The jawed leech, *Hirudinaria manillensis* is closely related to *Hirudo medicinalis*, both belonging to the same family Arhynchobdellida. From *Hirudo*, two potent peptide inhibitors, hirudin (a thrombin inhibitor) and eglin (an elastase/chymotrypsin inhibitor) have been characterised in detail. During our studies to isolate thrombin inhibitor from the leech *Hirudinaria* a potent inhibitor, analogous to eglin, was also detected. Results indicate that this inhibitor, which we have named 'GELIN', is significantly different from eglin. Gelin was isolated and purified to homogeneity by ion exchange chromatography and reverse phase HPLC. The isoelectric point of Gelin was estimated to be 4.55, in contrast to 6.45 for eglin. The molecular weight of Gelin was similar to eglin, as estimated by SDS-PAGE. Amino-terminal sequence analysis of the first 29 residues show no sequence homology with eglin or any other serine protease inhibitors. Circular dichroism studies showed that the secondary structure of Gelin has no helix, 58% beta sheets and 42% random structures compared to 19% helix, 56% beta sheets and 25% random structures in eglin. Like eglin, Gelin inhibits elastase, cathepsin G and chymotrypsin but has little or no activity towards plasmin, thrombin, pepsin and trypsin. These data suggest that the elastase inhibitors from these two species of leech are fundamentally different in structure, indicative of independent evolutionary origin.

KEY WORDS: Pancreatic elastase inhibitor, eglin, gelin, circular dichroism, leech.

INTRODUCTION

In diseases like emphysema, arthritis, gingivitis and several other inflammatory conditions, it has been suggested that tissue destruction is caused by the enzyme elastase released from human leukocytes.[1–3] Elastase is one of the few serine proteases which are capable of solubilising fibrous proteins like elastin and collagen. It is chiefly present in the pancreas and in the azurophil granules of neutrophil leukocytes. Under normal physiological conditions, the proteolytic activity is regulated by endogenous inhibitors present in plasma and other body fluids. However, in the diseased state, a variety of proteolytic enzymes are released upon degranulation of human neutrophils and such inhibitors are likely to be inactivated by oxidants.[2,3]

* Correspondence.

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In such circumstances leukocytes elastase may come into direct contact with the host substrates, resulting in lysis of elastin and collagen. Consequently, a potent inhibitor to elastase might prove to be a potentially useful therapeutic tool in combating such disseases.[4]

Previous studies have shown that leeches produce potent inhibitors to various proteases. The jawed leech *Hirudo medicinalis* which has been widely studied for hirudin,[5] a potent thrombin inhibitor, has been shown to possess also a potent inhibitor of the enzymes elastase and chymotrypsin. This inhibitor has been named eglin and has been well characterised[6] and cloned in *Escherichia coli.*[7] Goldstein *et al.*[8] have also reported the presence of an elastase inhibitor in two other non-blood sucking species of North American leeches but these have not been studied in detail.

In the present study, a potent inhibitor to pancreatic elastase, chymotrypsin and cathepsin G, which we have named "GELIN"**, has been isolated and purified from *Hirudinaria manillensis*, a species of leech more speciliased to feeding on mammals. The partial amino acid sequence and biochemical properties of this inhibitor has been compared with that of eglin.

MATERIALS AND METHODS

Materials

Hirudinaria manillensis leeches were obtained from Biopharm (UK) Ltd, Hendy, Wales, UK. Chromogenic substrates S-2251 and S-2238 were obtained from Kabi Diagnostica, Ealing. Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals, Scotland. Trifluoroacetic acid (TFA) was from Pierce Chemicals. Procine pancreatic elastase, (EC 3.4.21.11), chymotrypsin (EC 3.4.21.1) trypsin (EC 3.4.21.4), cathepsin G (EC 3.4.21.20) and chromogenic substrates, Benzoyl-arginine *p*-nitroanilide, *N*-succinyl Ala-Ala-Ala *p*-nitroanilide (SAAAP) were obtained from Sigma Chemical Co., Poole, Dorset. DEAE Sepharose, Q-Sepharose, Superdex 200, IEF and SDS-PAGE Phastgels were purchased from Pharmacia, Milton Keynes. Bovine thrombin (EC 3.4.21.5) was obtained from Diagnostic Reagents Ltd. Oxford. Low molecular weight calibration markers (Electran) for SDS-PAGE was obtained from BDH, Poole. Recombinant eglin *c* was a gift from Dr H. P. Schnebli of CIBA GEIGY, Basle, Switzerland. All other reagents were of analytical grade.

Assay

The elastase inhibitory activity of Gelin was assessed by measuring the inhibition of the release of *p*-nitroanilide group from the synthetic substrate, SAAAP, catalysed by pancreatic elastase. One inhibitory unit of activity is defined as the amount of Gelin necessary to inhibit the hydrolysis of 1 μmole of SAAAP/min at pH 8.3 and 25°C.

In brief, the assay consists of incubating Gelin with a known amount of pancreatic elastase in 0.1 M Tris/HCl buffer, pH 8.3 containing 1 M NaCl for 5 min at 25°C. The reaction is started by the addition of the chromogenic substrate and the absorbance at 405 nm monitored with time. A control reaction, in the absence of Gelin, is carried out under identical conditions. The activity of Gelin was

** GELIN is a registered trade name of EuroBiopharm BV.

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calculated from absorbance change per min and using molar extinction coefficient of 10,500 $M^{-1} cm^{-1}$.

Purification

(a) Crude extract
Hirudinaria manillensis leeches were dehydrated and extracted with four changes of ethanol. The extract was diluted with an equal volume of water and concentrated by ultrafiltration using a 10 kDa molecular mass cut off filter. The concentrated extract was adjusted to pH 5.5 with dilute HCl prior to chromatography.

(b) Chromatography on DEAE-Sepharose
The crude extract was filtered and loaded onto a column (10 × 8 cm) of DEAE-Sepharose Fast Flow, pre-equilibrated with 0.05 M Piperazine/HCl buffer, pH 5.5 at a flow rate of 10 ml/min. After washing, the bound material was eluted with a step gradient of NaCl in the equilibration buffer and each of the eluting peaks was collected as a separate fraction for measurement of anti-elastase and anti-chymotrypsin activity. The active peak, which eluted at 0.4 M NaCl, was desalted by dialysis overnight against distilled water.

(c) Chromatography on Q-Sepharose
The partially purified product was further purified by anion exchange chromatography on a column (10 × 5 cm) of Q-Sepharose, pre-equilibrated with 0.02 M Tris/HCl buffer, pH 7.5. The column was developed at a flow rate of 20 ml/min and the bound material was eluted with a linear gradient of 0–1 M NaCl in the equilibration buffer. The absorbance at 234 nm and the elastase inhibitory activity of the eluent was recorded (Figure 1). The active fractions were pooled and concentrated by ultrafiltration. This material was then gel filtered on Superdex 200 column (60 × 5 cm) equilibrated in 0.05 M Tris/HCl buffer, pH 7.5 containing 0.1 M NaCl. The active peak was pooled and lyophilised.

(d) High pressure liquid chromatography
Partially purified sample was reconstituted and applied in aliquots to reverse phase microbore Aquapore C8 column, equlibrated in 0.1% TFA. The bound material was eluted with 0–40% linear gradient of 60% CH_3CN + 0.09% TFA over 10 min and 40–100% over 20 min. Each peak was collected as a separate fraction and checked for anti-elastase activity (Figure 2). The active peak was lyophilised and used for further biochemical and structural studies.

Molecular Weight and Isoelectric Focusing

The molecular weight and isoelectric point of purified Gelin were determined on a Pharmacia PhastSystem using a 20% homogenous SDS-PAGE Phastgel and an IEF gel of pH range 3–9, respectively. For IEF, Gelin was applied centrally on the gel. The appropriate reference markers were used for each experiment. The gels were developed and the resulting bands were visualised by silver staining, according to the manufacturer's instructions.

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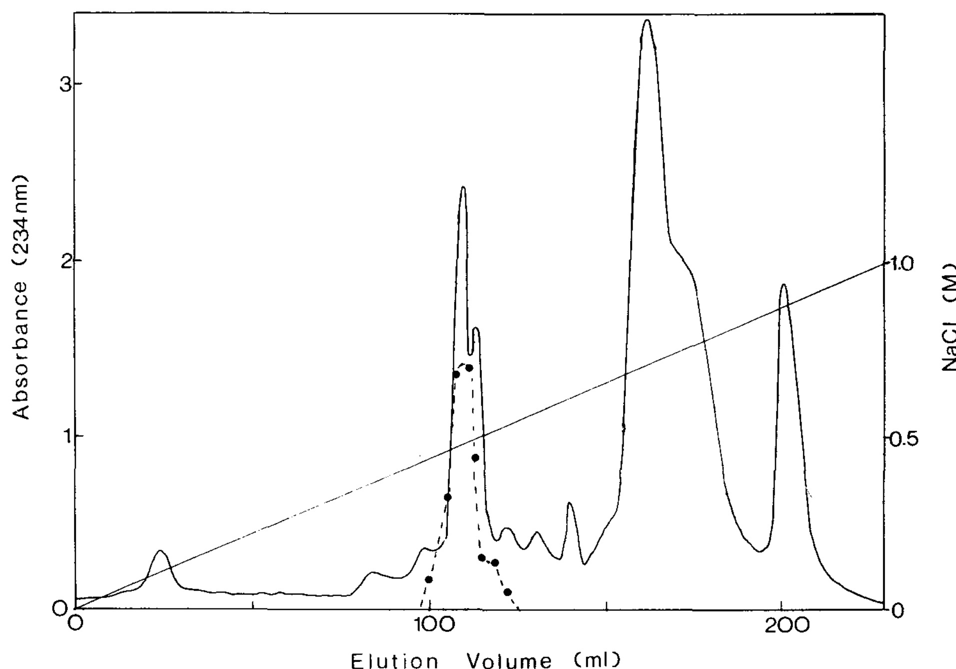

Figure 1 CHROMATOGRAPHY OF GELIN ON Q-SEPHAROSE. Partially purified Gelin was loaded onto the column and the bound material eluted with a linear NaCl gradient. Flow rate 20 ml/min, wavelength 234 nm. Fraction volume 4 ml. The dotted line indicates elastase-inhibitory activity.

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Amino Acid Composition

Purified Gelin was hydrolysed with gaseous ARISTAR HCl in vaccuo at 110°C for 24 h. The hydrolysed mixture was analysed for amino acid composition on an AminoChrome system using dabsyl chloride precolumn derivatization technique.[9]

Amino Acid Sequence

N-terminal amino acid sequence of purified Gelin was determined by Edman degradation on an Applied Biosystem gas sequencer (Model 477A) linked to an on-line PTH amino acid analyser (Model 120A).[10] For deterination of cysteine residues in the sequence, the sample was reduced with dithiothreitol and derivatised with 4-vinyl pyridine, before sequence analysis.

Circular Dichroism

Circular dichroism (CD) studies were performed using a Jasco J600 recording spectropolarimeter. The instrument was calibrated with a solution of ammonium d-10-camphorsulphonic acid (0.6 w/v).[11] CD spectra of Gelin and eglin were obtained under identical conditions between 190 and 250 nm using 0.02 cm pathlength cell in 0.1% TFA, pH 2.1, at room temperature. The data were then analysed by the computer programme CONTIN to estimate the secondary structure content of the protein.[12]

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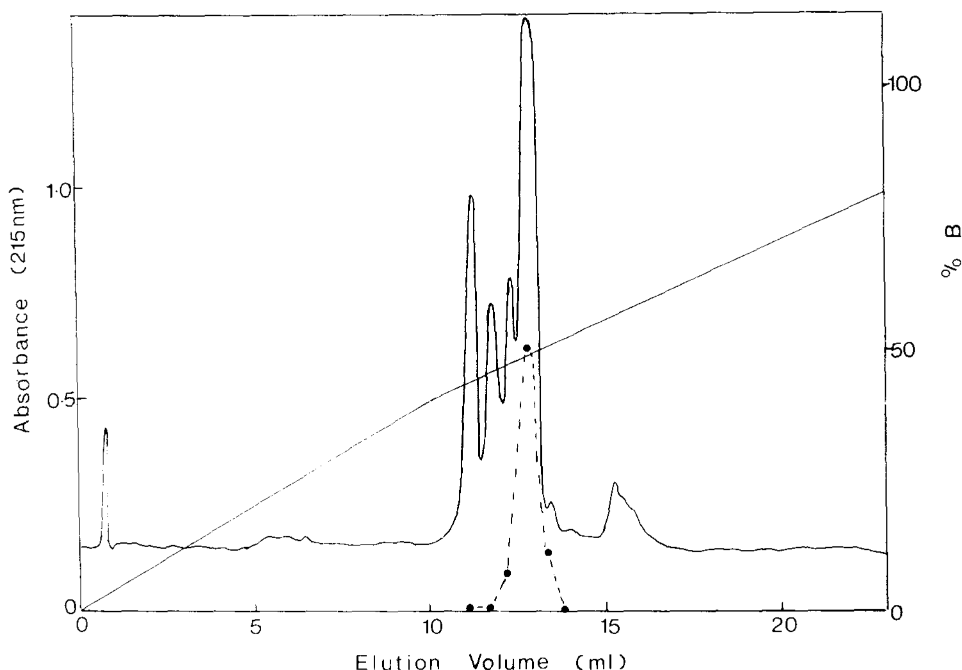

Figure 2 PURIFICATION OF GELIN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY. Column: Aquapore C8; solvent A 0.1% (v/v) TFA in water; solvent B 0.09% (v/v) TFA+60% (v/v) acetonitrile in water; gradient 0–40% B over 10 min and 40–100% B over 20 min. Flow rate 0.25 ml/min, wavelength 215 nm. The dotted line indicates elastase inhibitory activity.

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Specificity

The inhibitory activity of Gelin towards different serine proteases was compared using established chromogenic assay methods.[13] In brief, a fixed amount of enzyme was incubated with different concentrations of Gelin in the appropriate buffer at 37°C for 5 min. The reaction was started by the addition of the substrate and the increase in absorbance was monitored at 405 nm. The initial rate of the control assay without the inhibitor was taken as 100% activity for each enzyme. From the data the inhibition constant, K_i, was calculated.

RESULTS

The elastase inhibitor from the leech *Hirudinaria manillensis* was purified by a combination of ion exchange and gel filtration chromatography and reverse phase HPLC. The crude extract was coloured and after initial purification on DEAE-Sepharose, followed by Q-Sepharose, a highly purified colourless material was obtained. Figure 1 shows the elution profile during chromatography on Q-Sepharose. A final step of reverse phase HPLC was required to purify the inhibitor to near homogeneity (Figure 2). The purity of the sample was confirmed by a single band on SDS-polyacrylamide and isoelectric focusing gels and by a single amino acid

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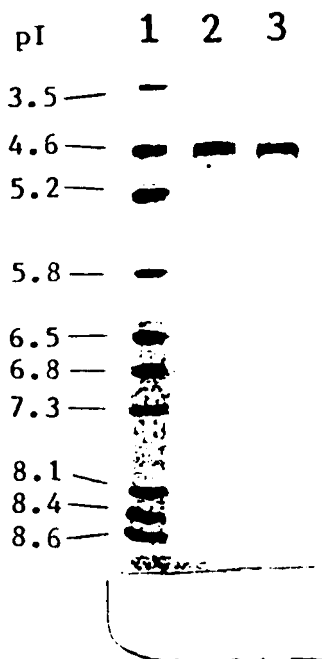

Figure 3 ISOELECTRIC FOCUSING OF GELIN. The pI of Gelin was determined on a Phastgel IEF 3–9. The sample was applied centrally and visualised by silver staining. Lane 1: pI calibration markers; Lanes 2 & 3: Gelin.

residue at the *N*-terminus during amino acid sequence analysis (see below). The active inhibitor was lyophilised in aliquots and used for further studies.

The isoelectric point of purified Gelin was determined on an IEF Phastgel with a pI range 3–9 in relation to its mobility with those of the reference proteins present in the broad pI calibration markers, under identical conditions (Figure 3). The results showed that Gelin has a mobility very similar to that of the reference protein, soybean trypsin inhibitor, with a pI value of 4.55 ± 0.05. This value for Gelin is significantly different from the published pI values of 6.45 and 6.6 for eglin *c* and eglin *b* respectively.[14]

The molecular weight of Gelin was estimated by SDS-gel electrophoresis on a 20% homogenous gel using low molecular weight calibration markers in the range 2.5 to 16.9 kDa, under reducing conditions. For comparison, eglin was also subjected to electrophoresis under identical conditions. The results show that Gelin has similar mobility to eglin with an apparent molecular mass of about 6.5 kDa (Figure 4). It has been noted by previous investigators that the molcular mass of eglin, estimated by SDS-PAGE, results in a lower value than that calculated from amino acid sequence; the latter was reported to be 8.1 kDa.[14] In view of the similar mobility of Gelin and eglin, and in the absence of the complete primary structure of Gelin, it is assumed that Gelin has a similar molecular mass of about 8.1 kDa.

Amino acid composition of Gelin was determined after 24 h acid hydrolysis and the values calculated are listed in Table 1 in comparison with that of eglin *c*. The

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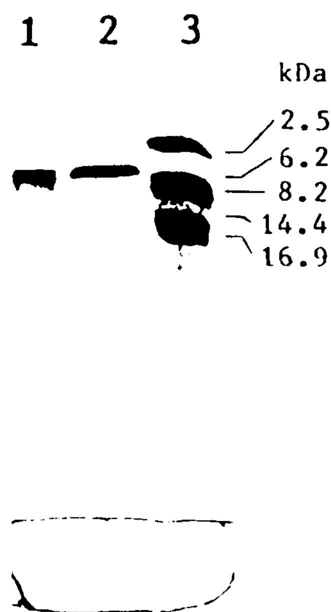

Figure 4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF GELIN. The molecular weight of Gelin was determined on a Phastgel 20% homogenous gel. The bands were visualised by silver staining. Lane 1: Gelin; Lane 2: eglin; Lane 3: molecular weight markers.

Table 1 Comparison of the amino acid composition of Gelin and eglin. The values for Gelin are calculated based on a molecular weight of 8100 daltons after 24 h hydrolysis

	Gelin		Eglin *c**
Amino acid	moles/mole		moles/mole
Asp (+Asn)	14.9	(15)	7
Glu (+Gln)	6.2	(6)	7
Ser	4.4	(4)	3
Thr	2.55	(3)	5
Gly	9.1	(9)	5
Ala	7.8	(8)	1
Arg	1.97	(2)	4
Pro	6.25	(6)	6
Val	11.92	(12)	11
Met	1.28	(1)	0
Ile	2.66	(3)	0
Leu	4.76	(5)	5
Phe	2.41	(2)	5
Cys	N.D.		0
Lys	5.76	(6)	2
His	0	(0)	3
Tyr	0.98	(1)	6
Trp	N.D.		0

*Values obtained from ref. 14. N.D. = not determined.

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Table 2 Comparison of the *N*-terminal sequence of Gelin and eglin

	1				5					10					15
Gelin	Val	Asp	Glu	Lys	Ala	Glu	Val	Thr	Asp	Gly	Leu	Cys	Gly	Asp	Trp
Eglin*	Thr	Glu	Phe	Gly	Ser	Asn	Leu	Lys	Ser	Phe	Pro	Asn	Val	Val	Gly
	16				20					25					
Gelin	Thr	Cys	Ser	Gly	Ala	Gln	Val	X	Gln	Asn	Asp	Ala	Ala	Val	
Eglin*	Lys	Thr	Val	Asp	Asn	Ala	Arg	Glu	Tyr	Phe	Thr	Leu	His	Tyr	

*Data from ref. 14. X = not determined.

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data indicate that the two inhibitors differ significantly; notably in the absence of His and the presence of Cys, Met and Ile in Gelin. In the present analysis, the total number of cystine or cysteine was not determined but the limited amino terminal sequence data (see below) indicates the presence of cysteine residue in Gelin at positions 12 and 17, which is in marked contrast to its absence in eglin. This indicates that the tertiary structure of Gelin, and especially the conformation of the primary binding loop, may be stabilised by intramolecular disulphide linkage(s).

The *N*-terminal amino acid sequence of Gelin was determined by automated gas phase sequencing and this resulted in a single sequence up to residue 29, beyond which the decreased signal to noise ratio prevented further assignment of residues with accuracy. Comparison of this partial sequence with that of eglin shows no sequence homology (Table 2). There was also no similarity in the structure of Gelin with that of the chymotrypsin inhibitor I from potatoes[15] or other serpins. The results indicate that the primary structure of Gelin and eglin are fundamentally different, at least in the amino terminal part of the molecule. However, the data does not preclude the possibility that the three dimensional elastase binding domain of the two inhibitors may be similar and probably located at the *C*-terminal region of the molecule.

The secondary structure was Gelin was examined by circular dichroism and compared with that of eglin, under identical conditions. The CD spectra of these two inhibitors, as shown in Figure 5, are markedly different. The secondary structure content calculated from these data shows that Gelin is composed of 58% beta sheet and 42% non-ordered structure and no alpha helix, as compared to 56% beta sheet, 25% non-ordered structure and 19% alpha helix in eglin. These data suggest that the overall secondary structure of Gelin is significantly different from that of eglin.

Comparison of these physico-chemical properties show that Gelin is a significantly different molecule from eglin. However, Gelin appears to be functionally similar to eglin in terms of its inhibition characteristic toward various serine proteases. Like eglin, Gelin is a potent inhibitor of pancreatic elastase, chymotrypsin and cathepsin G, with K_i values of 36 nM, 1 nM and 1.9 nM respectively. It has little or no activity towards trypsin, plasmin, pepsin and thrombin. Also, it is relatively thermostable and survives boiling at 100°C for up to 1 h, with negligible loss of inhibitory activity (data not shown).

DISCUSSION

The leech *Hirudo medicinalis* has been widely studied for the thrombin specific inhibitor, hirudin. From this species, a potent elastase/chymotrypsin inhibitor has

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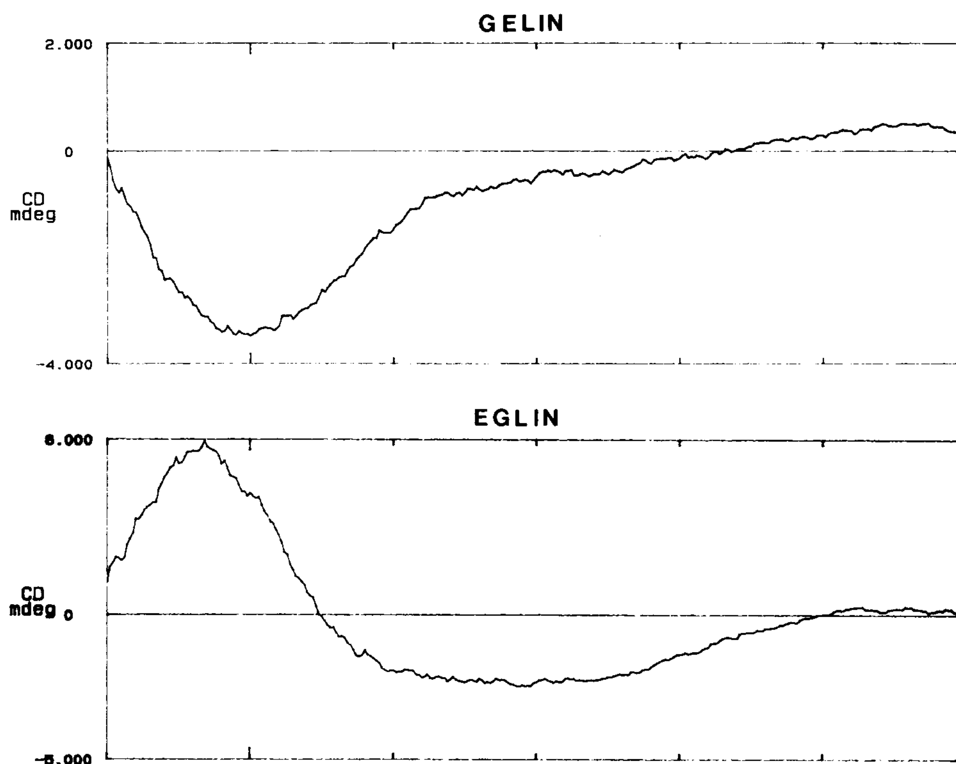

Figure 5 COMPARISON OF CIRCULAR DICHROISM SPECTRA OF GELIN AND EGLIN. Solutions of 0.45 mg/ml of Gelin and eglin in 0.1% TFA were used to obtain CD spectra over the range 190 to 250 nm. Time constant 4 s, scan speed 10 nm/min, 0.02 cm path length.

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also been isolated and characterised.[6,14] We chose to study these two inhibitors in another closely related, but evolutionary advanced species of leech *Hirudinaria manillensis*. Both these species belong to the same class Hirudinea and the same order Arhynchobdellida but have different geographical distribution; *Hirudo* is widespread in Europe and feeds on frogs and mammals, while *hirudinaria* is prevalent in the Asian subcontinent and is parasitic on mammals, especially water buffaloes.[16]

In a previous study, we have purified and characterized the thrombin inhibitor from this leech.[17] In the present study, the inhibitor to pancreatic elastase/chymotrypsin was isolated from this leech and its biochemical and structural properties compared with eglin. The present data clearly shows that the elastase inhibitor from these two species of leech, *Hirudo* and *Hirudinaria* are functionally similar but are fundamentally different in their physico-chemical properties. The limited amino acid sequence data suggest that the two elastase inhibitors may not have evolved from a common ancestral gene. This is in contrast to the thrombin inhibitors of these two leech species where there is about 70% structural homology between the two molecules.[18] The

Note added proof: Limited studies indicate that Gelin also inhibit human neutrophil elastase.

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fundamental difference between the elastase inhibitors from these two related leech species is entirely unexpected. The biological and evolutionary significance of the difference is unclear at present.

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