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Purification and characterization of hementin, a fibrinogenolytic protease from the leech *Haementeria ghilianii*

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SUMMARY

The fibrinogenolytic enzyme hementin, present in extracts of the posterior salivary glands of the giant leech *Haementeria ghilianii*, was isolated by ultrafiltration, high-performance ion-exchange chromatography and subsequent reversed-phase liquid chromatography. Approximately 100 μg (1 nmol) of hementin, present at less than 0.5% in the crude leech salivary extract, was brought to about 90% purity in three steps. Hementin migrated at an M_r of about 73 000 on non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and at 82 000 on reducing SDS-PAGE. The amino terminal sequence was determined to be TTLTEPEPDL. The amino terminal sequences of two inactive proteins that partially coeluted with hementin in the first chromatographic step were also determined.

INTRODUCTION

Extracts of the salivary glands of the leech *Haementeria ghilianii* contain proteolytic activity. The enzyme present in the anterior gland is more abundant than that in the posterior gland, so the extract of the anterior gland has been the principal object of investigation. Fibrin strip zymography of electrophoretic separations of extracts of posterior and anterior glands indicated that the enzymatic activity may originate from the same protein resident in both glands[1,2]. Hementin is a fibrinogenolytic enzyme acting as a blood anticoagulant with properties substantially different than those found in other leech species. Unlike hirudin[3], an inhibitor of thrombin, hementin acts directly on fibrinogen, proteolyzing it such that it is rendered incoagu-

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lable. Only a few, discrete bands are observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of hementin-treated fibrinogen, indicating that the enzyme has a high degree of specificity. Hementin was previously observed to be inactivated by metal-chelating and disulfide-reducing reagents, but the proteolytic activity was found to be unaffected by typical inhibitors of serine protease activity[4]. In a previous attempt to isolate hementin[4], chromatography on diethylaminoethylcellulose (DEAE), followed by ammonium sulfate (AS) precipitation and chromatography on carboxymethylcellulose (CMC), did not afford a homogeneous preparation, so non-denaturing polyacrylamide gel electrophoresis was used to prepare sufficient material for a determination of molecular weight (M_r), which was reported to be 120 000 daltons.

The extract of the posterior salivary gland of *Haementeria ghilianii* is a complex mixture, containing many proteins. In this work, the proteins associated with activity of hementin were isolated. The amino terminal sequences of three proteins that co-eluted in ion-exchange chromatography during hementin purification were determined by automated Edman sequencing.

EXPERIMENTAL

Materials

Ammonium hydrogencarbonate was purchased from Sigma (St. Louis, MO, U.S.A.). Calcium chloride dihydrate, sodium chloride, 25% glutaraldehyde, ammonia solution, HPLC-grade 2-propanol (iPA) and gradient-tested trifluoroacetic acid (TFA) were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Bromphenol blue and N,N,N′,N′-tetramethylethylenediamine were purchased from LKB Instruments (Gaithesburg, MD, U.S.A.). The silver stain kit, which used ammoniacal silver[5], was from ICN Biochemicals (Irvine, CA, U.S.A.). Utrapure 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Molecular weight markers (composed of a mixture of proteins of M_r equivalent to 14 300, 18 400, 25 700, 43 000, 68 000, 97 400 and 200 000 daltons), ultrapure glycine and ultrapure Tris were from Bethesda Research Labs. (Gaithesburg, MD, U.S.A.). Ultrapure acrylamide, ultrapure N,N′-methylenebisacrylamide and Coomassie Brilliant Blue R250 (CBB) were obtained from Schwarz/Mann Biotech (Cleveland, OH, U.S.A.). Sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (BME) were purchased from Pierce (Rockford, IL, U.S.A.). The fibrinogen used to test for hementin activity was of human origin (Grade L; Kabi Vitrum, Stockholm, Sweden). Solvent filters (0.2 μm) were from Rainin Instrument (Woburn, MA, U.S.A.). The preparation of the posterior gland extract has been described previously[4]. Briefly, dried posterior salivary glands, obtained from a breeding facility at the University of California (Berkeley, CA, U.S.A.). were homogenized with a cell disruptor in 20 m*M* HEPES buffer (pH 7.8) containing 10 m*M* calcium chloride. Cell debris was removed by centrifugation and the supernatant collected.

Instrumentation

Ultrafiltration was performed using an 8MC stirred cell (Amicon, Danvers, MA, U.S.A.) equipped with a YM-100 (Amicon) 100 000-dalton cutoff membrane. Some samples were concentrated using a Centricon 30 (Amicon) 30 000-dalton mi-

croconcentrator and a Model RC-5B Sorvall (DuPont, Wilmington, DE, U.S.A.) refrigerated centrifuge. The ion-exchange chromatograph consisted of Model 2150 pump, a Model 2152 controller and an injection valve from LKB Instruments. The ion-exchange system was equipped with a Mono Q high-performance anion-exchange column (Pharmacia, Piscataway, NJ, U.S.A.).

Reversed-phase liquid chromatography (RPLC) was performed on a Spectra-Physics (San Jose, CA, U.S.A.) 8700 system. This was equipped with a type 201-TP54 C_4 Vydac column (Separations Group, Hesperia, CA, U.S.A.). This column had a 5-μm particle size support with 300-Å pores, packed in a 15 cm × 4.6 mm I.D. stainless-steel column. Chromatographic detection was effected by measuring the absorbance at 220 or 280 nm, using a Model 116 detector (Gilson Medical Electronics, Middleton, WI, U.S.A.).

Densitometry of electrophoretic gels was performed on an LKB 2202 Ultrascan laser densitometer. Sequencing was performed on either an 890M-2 Sequencer (Beckman, Palo Alto, CA, U.S.A.) or on an Applied Biosystems (Foster City, CA, U.S.A.) 470A gas-phase sequencer. Separation and quantitation of the phenylthiohydantoin (PTH)-amino acids was performed using a previously described system[6]. Data were acquired and analyzed using a Beckman (Waldwick, NJ, U.S.A.) Computer Inquiry Systems Computer Automated Laboratory Systems (CALS) data system.

Buffer preparation

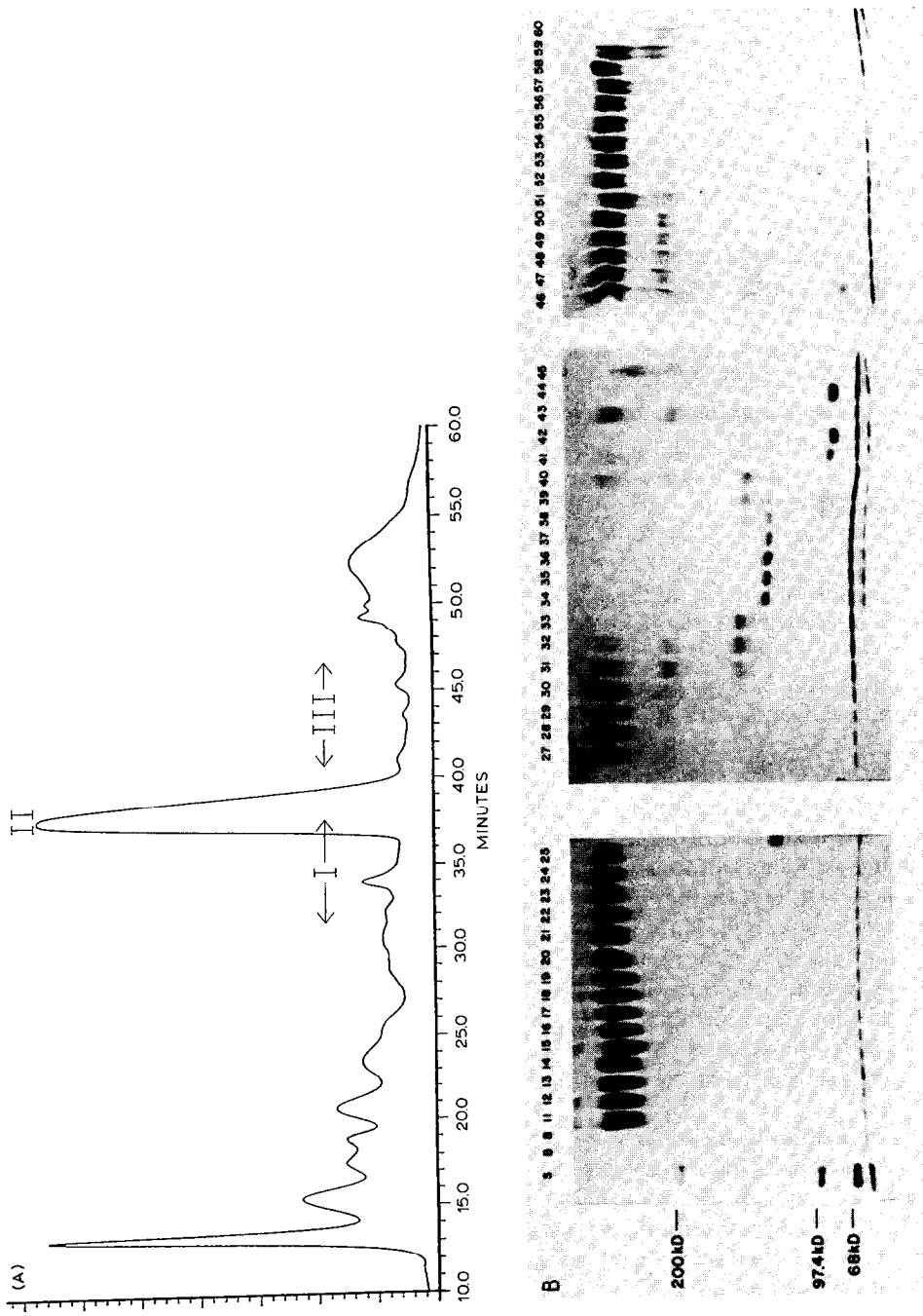
Buffers for all separation steps prior to RPLC contained 20 m*M* ammonium hydrogencarbonate adjusted to pH 8.0 ± 0.1 with ammonia solution. The ion-exchange starting buffer additionally contained 100 μM calcium chloride, and the elution buffer (IEC-B) additionally contained both 100 μM calcium chloride and 500 m*M* sodium chloride. The buffer used for transfer and concentration following ion-exchange chromatography was 250 m*M* in sodium chloride. All buffers and chromatographic eluents were filtered through 0.2-μm filters.

Ion-exchange chromatography (IEC)

A 600-μl portion of crude posterior gland extract was combined with 2.0 ml of 20 m*M* ammonium hydrogencarbonate buffer and ultrafiltered at 4°C and constant volume with 9 ml of the same buffer, using a YM-100 ultrafiltration membrane. The retentate was chromatographed by high-performance anion-exchange chromatography at 1 ml/min. The elution program was 0% IEC-B (5 min), to 8% (1 min), to 25% (19 min), to 40% (5 min), to 65% (15 min), to 100% (10 min). Detection was at 280 nm. Fractions of 1 ml were collected and assayed for fibrinogenolytic activity as described below. Portions of many of the fractions obtained by high-performance IEC were subjected to SDS-PAGE. Certain proteolytically active fractions were pooled and washed at 4°C using the Centricon 30 ultrafiltration apparatus.

Reversed-phase liquid chromatography

Portions of the enzymatically-active pooled IEC fractions were injected onto the RPLC column. Elution buffers were 0.1% aqueous TFA (buffer A) and 0.1% TFA–iPA (buffer B). Elution was accomplished by a programmed linear gradient at a flow-rate of 0.75 ml/min. The starting condition was 95% A, which was held for 2 min after injection, and the gradient proceeded to 70% B at 55 min. Detection was at 220 nm. Fractions were taken at 1-min intervals.


(A)
II
I
III
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
60.0
MINUTES
B
200kD
97.4kD
68kD

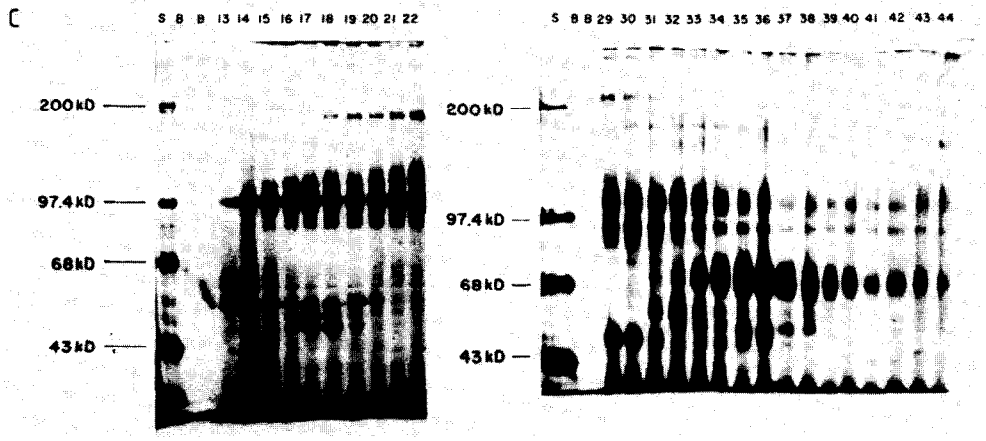


Fig. 1. *High-performance anion-exchange chromatography of crude hementin.* Leech salivary gland extract was ultrafiltered through a YM-100 membrane and the retentate applied on a Mono Q column. Elution was effected at a flow-rate of 1 ml/min with buffers containing 20 m*M* ammonium hydrogen-carbonate and 100 μM calcium chloride, pH 8.0; the elution buffer contained 0.5 *M* sodium chloride. Numerals along the horizontal axes are the chromatographic retention time in minutes. (A) Elution profile at 280 nm of retentate on IEC. (B) SDS-PAGE pattern (5% acrylamide, CBB stain) of fibrinogen incubated at 37°C with aliquots of column fractions from (A) (retentate). (C) Non-reducing SDS-PAGE (10% acrylamide, silver stain) of aliquots of column fractions from (A) (retentate). The symbols S and B in (B) and (C) indicate standards and blanks, respectively. Along the vertical axes of (B) and (C) are marked the electrophoretic migration positions of the protein standards in kilodaltons. Fractions 33–38 are designated peak I, the peak at 38 min is designated peak II and fractions 41–45 are designated peak III.

Activity assay

The column fractions were tested for fibrinogenolytic activity by incubation at 37°C with fibrinogen (1 mg/ml) in 150 m*M* HEPES (pH 7.9) containing 10 m*M* calcium chloride. To test the IEC fractions, 5 μl of sample were combined with 180 μl of fibrinogen solution. To test the RPLC fractions, 20 μl of sample were combined with 280 μl of fibrinogen solution. Aliquots of the digestion mixtures were taken at appropriate intervals, quenched with non-reducing electrophoresis sample loading buffer and electrophoresed by non-reducing SDS-PAGE.

Electrophoretic methods

Non-reducing sample buffer was prepared by combining 20 ml of glycerol, 8 ml of 1 *M* Tris–HCl (pH 6.8), 10 ml of 10% aqueous SDS, 0.5 ml of 0.4% aqueous bromphenol blue and 71 ml of water. Reducing sample buffer was prepared by combining non-reducing sample buffer with BME in the volume ratio 95:5. Samples were electrophoresed using the Laemmli system[7]. Up to twenty samples were simultaneously electrophoresed on 12.5 × 14 × 0.15 cm slab gels with 2.5-cm stacking gels. Activity assays were performed on 5% acrylamide gels using CBB staining. Individual IEC fractions were assayed on 7.5% acrylamide gels (non-reducing) with glutaraldehyde-enhanced silver staining[5]. A pool of IEC fractions 33–38 was assayed on 10% acrylamide (reducing and non-reducing) with CBB staining and silver stain overlay. RPLC fractions and the final preparation of hementin were assayed on 10% acrylamide gels with silver staining. Gels were scanned by laser densitometry, with the output being directed to the CALS system for analysis.

RESULTS

Ion-exchange chromatography

The retentate from ultrafiltration contained approximately 87% of the original hementin. It was injected onto a Mono Q IEC column and eluted with an ascending salt gradient. Fig. 1A shows the elution profile (280 nm) of the retentate. The small peak at about 34 min contained hementin. This peak, designated I, was calculated from the absorbance to comprise about 3.8% of the crude extract.

Fig. 1B shows the non-reducing SDS-PAGE of samples of fibrinogen treated with aliquots of fractions obtained from IEC of the retentate as shown in Fig. 1A. Fig. 1C shows the non-reducing SDS-PAGE of fractions 13–22 and 29–44. To summarize the results shown in Fig. 1B and C, fibrinogenolytic activity elutes from the Mono Q column in seemingly distinct fractions at 31–38, 40–45, and 59–60 min. The activity in the first two of these fractions appears to be correlated with the appearance of a band at slightly above 68 000 daltons in non-reducing SDS-PAGE.

Fractions 33–38 (peak I) were pooled and concentrated to a volume of 2.0 ml on a Centricon 30. Fractions 41–45 (peak III) were similarly pooled. The principal electrophoretic band of peak I migrated at 76 000 daltons on non-reducing SDS-PAGE and 82 000 daltons on reducing SDS-PAGE. Neglecting material at the dye front, the principal band was found to be about 64% (non-reducing gel) and 57% (reducing gel) of the densitometric area. No single contaminant band represented more than about 5% of the densitometric area. Silver stain overlay, while revealing numerous trace contaminants, gave no evidence of the existence of inhomogeneity of

the principal band. Based on comparison of the CBB band areas with those of a proprietary sample of similar molecular weight, the concentration of the 76 000-dalton (non-reducing SDS-PAGE) band in peak I was calculated to be 64 μg/ml. Although RPLC subsequently demonstrated that the CBB band was inhomogeneous, the estimate of concentration was useful for the purpose of evaluating sequence data.

Reversed-phase liquid chromatography

Approximately 400 μl of peak I were further purified by RPLC. Aliquots of each fraction were assayed for composition by SDS-PAGE and for fibrinogenolytic activity. The results are shown in Fig. 2B–D. Fig. 2B shows the RPLC elution profile, Fig. 2C shows the SDS-PAGE of column fractions (10% acrylamide, silver stain) and Fig. 2D shows SDS-PAGE of 2-h digests of fibrinogen. The material that elutes in a sharp peak at 34–35 min, peak IA, is inactive. By comparison of the three panels, it can be seen that the fibrinogenolytic activity is contained largely in fraction 38, designated peak IB, characterized by an M_r of 73 000 daltons on non-reducing SDS-PAGE. The component in fractions 40–41, peak IC, is inactive, but has an M_r identical with that of IB.

RPLC of pooled peak III was also performed, and results very similar to those shown for peak I were obtained. The chromatographic trace is shown in Fig. 2A. The proteolytic activity appeared in a single peak at the same retention time as was observed for peak IB. Based on RPLC peak areas, it was calculated that only 10% of the total hementin eluted by IEC is contained in peak III.

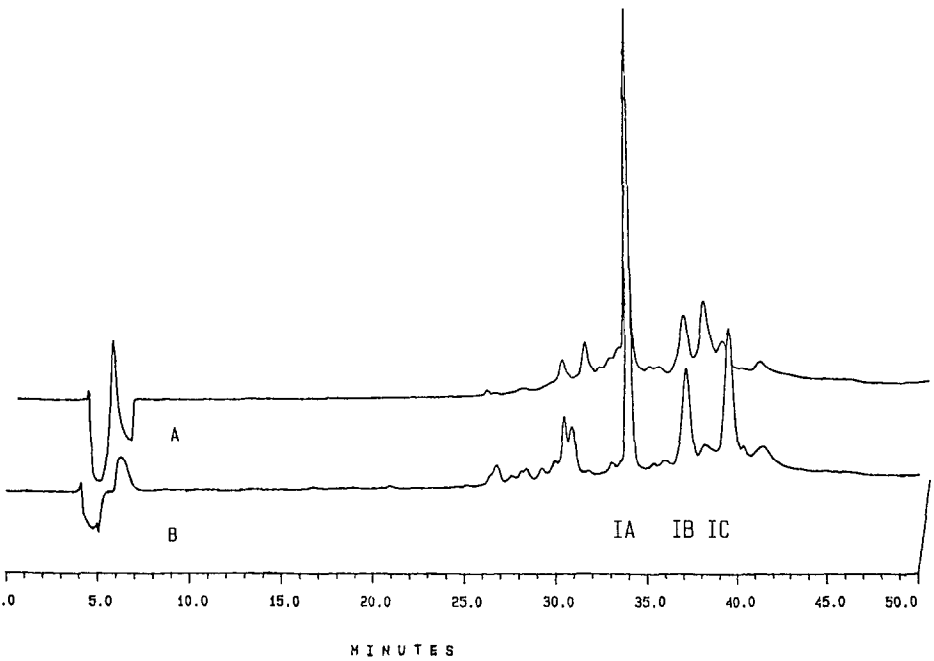


Fig. 2.

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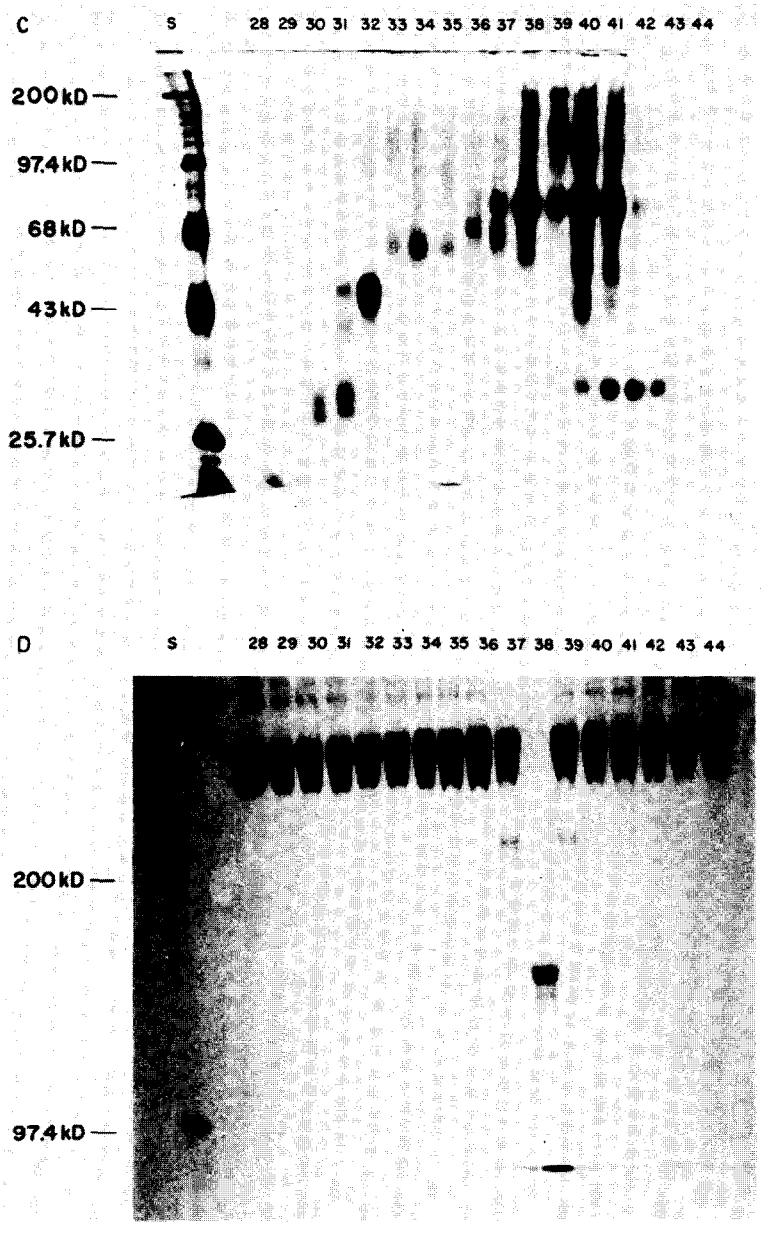

Fig. 2. Reversed-phase liquid chromatography of hementin. Fractionation was effected by applying peaks I and III of IEC-purified hementin on a C_4 column at 0.75 ml/min. The gradient was from 95% to 30% of 0.1% aqueous TFA with 0.1% TFA–iPA as the organic constituent of the mobile phase. Numerals on the horizontal axis indicate chromatographic retention times in minutes. The peak at 34–35 min is designated IA, that at 38 min is IB and that at 40–41 min is IC. (A) RPLC of IEC peak III measured at 220 nm. (B) RPLC of IEC peak I measured at 220 nm. (C) Non-reducing SDS-PAGE (10% acrylamide, silver stain) of aliquots of fractions from (B). (D) Non-reducing SDS-PAGE (5% acrylamide, CBB stain) of fibrinogen incubated at 37°C with aliquots of column fractions from (B). The symbols S and B in (C) and (D) indicate standards and blanks, respectively. On the vertical axes (C) and (D) are indicated the electrophoretic migration positions of the protein standards in kilodaltons.

Amino terminal sequencing

RPLC peak IA was found to have the sequence EVYTNYASFL[a]. Peak IB contained hementin and its sequence was TTLTEPEPDL (Table I). Of the residues other than hementin observed in sequencing this fraction, many were assigned as derived from peak IA, leaving little ambiguity in the assignment of the sequence. If one were to calculate the purity on a weight percent basis, ignoring the trace residues shown in the third row of Table I, the purity of the material in peak IB would be about 90%. Peak IC was found to have the sequence S(D)TGEEGA(K)RDV. This sequence is clearly unrelated to that observed for hementin.

DISCUSSION

The fibrinogenolytic acitivity of the posterior salivary gland of *Haementeria ghilianii* was determined to arise from a single protease migrating at an apparent M_r of 82 000 daltons on reducing SDS-PAGE, and having an amino terminal sequence TTLTEPEPDL. The principal residual impurity, proved to be inactive, was a polypeptide of M_r < 18 400 daltons, and having an amino terminal sequence EVYTNYASFL. Two minor impurities of M_r 62 000 and 67 000 daltons were also noted, but were not sufficiently abundant to sequence. Densitometric analysis indicated that these trace impurities were approximately equally divided between RPLC fractions 37 and 38, yet the fibrinogenolytic activity is clearly centered in fraction 38. Therefore, these can be excluded as sources of fibrinogenolytic activity. Several other minor impurities appear as a streak at molecular weights greater than hementin. All of these bands are far more abundant in fraction 39, so these can also be excluded as sources of fibrinogenolytic activity.

The pattern of degradation products observed on treatment of fibrinogen with purified hementin was identical with that obtained from treatment with crude extract, suggesting that the extract contains only a single protease. The fibrinogenolytic activity exhibited by IEC fractions 59–60 is believed to be due to the effects of non-specific adsorption to the Mono Q column. The digestion pattern in fractions 41–45 is consistent with extensive degradation of fibrinogen by hementin. On collecting peak III and subjecting the pool to RPLC, only about 10% of the total hementin (relative to that in peak I) was recovered; no other protease was discovered and the hementin specific activity was qualitatively consistent with the mass.

In the original attempt to isolate hementin[4], the AS and CMC steps increased the purity by a combined factor of only three. The purification scheme relied on DEAE and on non-denaturing electrophoresis to achieve the greatest increases in purity. Hementin did not bind to CMC and, therefore, probably has few exposed basic residues. The acidic residues were the principal determinants of both chromatographic (DEAE) and non-denaturing electrophoretic mobility, so these techniques were not mechanistically independent. The five-step procedure apparently did not resolve hementin from a 120 000-dalton protein. Resolution of hementin from components of M_r near 120 000 daltons was barely achieved in this work with the the high-performance techniques available today (Fig. 2C).

RPLC has become increasingly popular as a method for the purification of

[a] Throughout this article, the single-letter code for amino acids is used.

TABLE I

AMINO TERMINAL SEQUENCING OF PEAK IB

Sequence	*Cycle*									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Principal sequence of hementin residues (pmol)[a]	T(9.9)	T(6.1)	*L*(9.4)	T(5.9)	*E*(7.0)	*P*(7.5)	E(6.0)	*P*(6.1)	D(4.6)	L(5.4)
Minor sequence residues (pmol)	E(5.5)	V(2.3)	Y(2.6)		N(2.7)	Y(2.3)	A(3.9)			
Other amino acid residues[b]	L,W			P	A	E	F			

[a] Residues in italics were confirmed on the Beckman 890M-2 sequence.

[b] Residues other than those derived from hementin or peak IA were present in amounts below the limits of determination.

enzymes with retention of activity, although it is known that reversible or irreversible unfolding can occur during chromatography[8–10], generating multiple or distorted peaks[10]. Therefore, it was a matter of concern that RPLC separated two components of identical molecular weight, one active and one inactive. Edman sequencing established the sequence S(D)TGEEGA(K)RDV for the inactive component, dispelling the possibility that the two might have the same primary sequence. The active and inactive species represent completely unrelated species that, by chance, co-migrate in both IEC and SDS-PAGE.

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REFERENCES

1 A. Z. Budzynski, S. A. Olexa and R. T. Sawyer, *Proc. Soc. Exp. Biol. Med.*, 169 (1981) 259–265.
2 A. Z. Budzynski, S. A. Olexa, B. S. Brizuela, R. T. Sawyer and G. S. Stent, *Proc. Soc. Exp. Biol. Med.*, 168 (1981) 266–275.
3 F. Markwardt, *Methods Enzymol.*, 19 (1970) 924–932.
4 S. M. Malinconico, J. B. Katz and A. Z. Budzynski, *J. Lab Clin. Med.*, 103 (1984) 44–58.
5 B. R. Oakley, D. R. Kirsch and N. R. Morris, *Anal. Biochem.*, 105 (1980) 361–363.
6 D. Hawke, P. M. Yuan and J. E. Shively, *Anal Biochem.*, 120 (1982) 302–311.
7 U. K. Laemmli, *J. Mol. Biol.*, 80 (1973) 575–585.
8 M. P. Strickler and M. J. Gemski, in W. S. Hancock, (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, pp. 349–355.
9 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227–243.
10 X. M. Lu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 (1986) 19–29.