The functional domain of hirudin, a thrombin-specific inhibitor

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Received 19 October 1983

Hirudin is a thrombin-specific inhibitor of M_r 8000 (65 amino acid residues). Native hirudin contains 3 disulfide linkages within the first 39 amino-terminal residues, and a highly acidic C-terminal segment which is freely accessible to enzyme digestion by both endo- and exo-peptidases. Removal of the acidic C-terminal amino acids of native hirudin by both chemical and enzymatic methods resulted in a concomitant loss of hirudin inhibition activity. It is concluded that this acidic C-terminal segment of hirudin is essential for hirudin–thrombin interaction. The implication of the hirudin–thrombin interaction for the enzymatic specificity of thrombin is also discussed.

Hirudin *Tyrosine sulfate* *Micro C-terminal sequencing* *Thrombin specificity*

1. INTRODUCTION

Hirudin is a thrombin-specific inhibitor and is the most potent thrombin inhibitor known [1–3]. It binds to α-thrombin (with K_i of 6.3×10^{-11} M as compared to 10^{-6} M of fibrinogen) and inactivates the enzyme irreversibly. This exceedingly high binding affinity, together with the observation that diisopropylphosphoryl-thrombin binds to hirudin with virtually the same K_i as native thrombin [1,3,4] has led to the speculation that interaction of hirudin and thrombin must comprise multiple binding sites [3–5].

The structure of hirudin [2] has some unique features (fig.1). (a) There are 3 disulfide linkages located within the first 39 N-terminal residues of native hirudin. (b) Hirudin contains a highly acidic C-terminal segment. There are 5 acidic amino acids, 4 Glu and one Tyr-SO_3H, within the last 9 C-terminal residues. (c) One of the likely reactive sites of hirudin comprises a Lys residue flanked by two prolines. Those distinct features imply that the domain structure of native hirudin may play an important role in the hirudin–thrombin interaction. We attempt here to characterize the functional role of the acidic C-terminal domain of native hirudin.

Note: Thrombin refers to α-thrombin unless otherwise indicated

Abbreviations: DABITC, dimethylaminoazobenzene isothiocyanate; DABS-Cl, dimethylaminoazobenzenesulfonyl chloride

2. MATERIALS AND METHODS

2.1. *Materials*

Hirudin was isolated from leech and was kindly provided by Dr U. Seemüller (University of Munich). The purity of hirudin was ascertained by SDS–gel electrophoresis and by quantitative N-terminal analysis [6]. DABITC and DABS-Cl were obtained from Fluka. Trypsin (TPCK treated) and carboxypeptidase Y were purchased from Worthington. Staphylococcal protease V8 was obtained from Miles. Bovine thrombin and the substrate Tos-Gly-Pro-Arg-pNA were obtained from Boehringer.

2.2. *Methods used in protein structure determination*

N-terminal amino acids of polypeptides were quantitatively analysed by the DABITC method [6]. Amino acid compositions of polypeptides were determined by the DABS-Cl precolumn derivatiza-

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00145793/83/$3.00

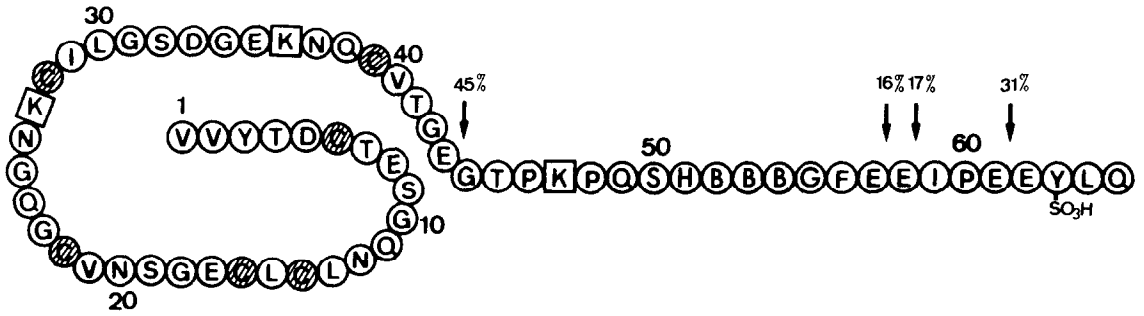

Fig.1. The primary structure of hirudin. Arrows indicate the Glu–X bonds of native hirudin cleaved by staphylococcal protease after 1 h digestion (also, see footnote to table 1). Cleavage positions were determined by 4 steps of automatic Edman degradation [25] and percentages of cleavage were based on the recovery of N-terminal Val as 100%. The sequence is taken from [2] and the precise sequence between residues 50–55 has not been established.

tion method [7,8]. C-terminal sequencing was performed by combination of the carboxypeptidase Y digestion [9] and DABS-Cl methods [7,8].

2.3. *Determination of the inhibition activity of native hirudin and modified hirudins*

The activities of native hirudin and modified hirudins were measured by their ability to inhibit thrombin from releasing colored *p*-nitroaniline from the chromogenic substrate Tos-Gly-Pro-Arg-pNA. Twenty microliters of thrombin solution (5 IU/ml, in 0.25 M phosphate, pH 6.5) was diluted with 890 μl triethanolamine buffer (0.1 ml/l, pH 8.4) and mixed with 5 ng hirudin (in 50 μl triethanolamine buffer). The mixture was incubated at 25°C for 5 min, then added with 125 μl Tos-Gly-Pro-Arg-pNA solution (1.5 mM). The molar ratio of hirudin : thrombin was 1 : 2. The increase of absorbancy was monitored at 405 nm within a 5 min period.

3. RESULTS

3.1. *The acidic C-terminal segment of native hirudin is accessible to enzyme digestion by both endo- and exo-peptidases*

Although its 3-dimensional structure is not known, the unique primary structure of hirudin [2] provides clues for one to suggest that native hirudin may contain an N-terminal compact domain (held by 3 disulfide linkages) and a C-terminal hydrophilic (acidic) domain which is exposed on the surface of the hirudin molecule. This two-domain model is supported by the following

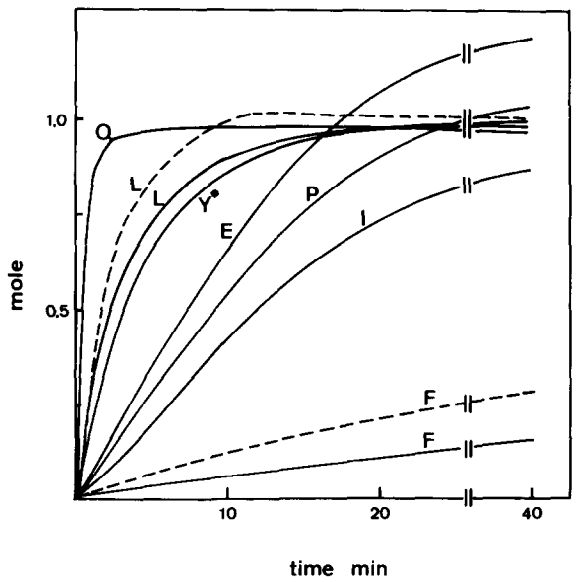

Fig.2. Release of C-terminal amino acids of native hirudin by carboxypeptidase Y digestion (solid curves). One nmol native hirudin was digested with 1 μg carboxypeptidase Y in 50 μl acetate buffer (pH 5.4) at room temperature. Aliquots (10 μl) were removed at time intervals (3, 10, 20 and 40 min), freeze-dried and derivatized with DABS-Cl. Five percent of each derivatized sample (10 pmol) was applied for HPLC analysis according to the method described in fig.3. The yield of Tyr-SO_3H (Y*) was corrected for 12% desulfated derivative. The Glu curve represents only 50% of the Glu yield. The release rates of Leu and Phe (dashed line) from a reduced carboxymethylated hirudin were selected for comparison. The release rates of C-terminal amino acids fit correctly to the reported hirudin sequence.

experiments. (a) Native hirudin is not digested by aminopeptidase M, which indicates that the N-terminal amino acid is buried within the N-terminal compact domain. (b) The C-terminal amino acids of native hirudin were released by carboxypeptidase Y at a rate which is comparable to that of reduced carboxymethylated hirudin (fig.2), clear evidence that the C-terminal segment of native hirudin must be freely accessible to enzyme digestion on the surface of the molecule. (c) When native hirudin was digested by staphylococcal protease [10] (an enzyme that specifically cleaves Glu–X bonds), cleavage only occurred at Glu–X bonds located in the C-terminal region (fig.1). There was no detectable cleavage with the three Glu–X bonds which are located within the N-terminal compact domain. (d) While oxidized and reduced carboxymethylated hirudins were readily digested by trypsin at Lys–Cys and Lys–Asn bonds (both located within the N-terminal com-

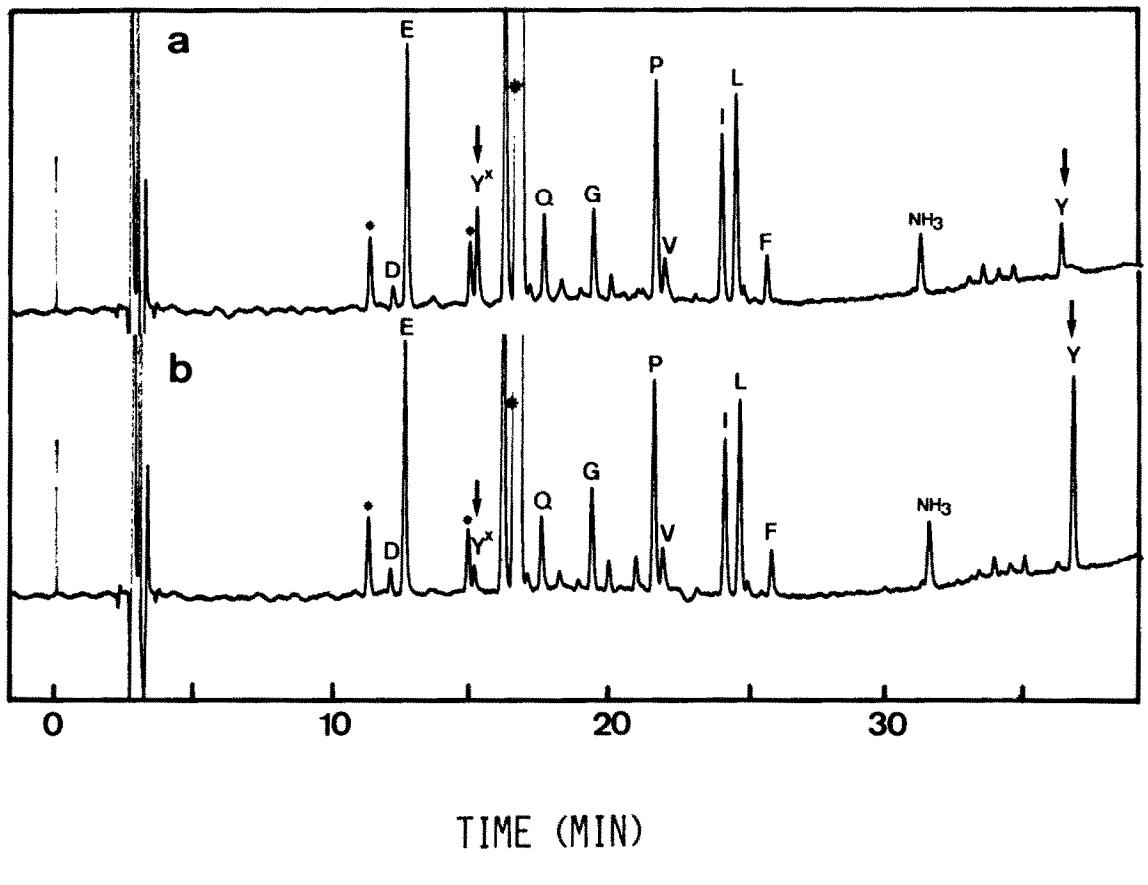

Fig.3. Structure characterization of C-terminal modified hirudins by analysis of amino acids released from hirudin after carboxypeptidase Y digestion. (a) 200 pmol native hirudin were digested with 0.2 μg carboxypeptidase Y at room temperature for 20 min. The sample was freeze-dried and derivatized with DABS-Cl, and 5% of the sample (10 pmol) was injected for amino acid analysis. (b) 200 pmol native hirudin were incubated with 20 μl of 30% trifluoroacetic acid at 70°C for 15 min, freeze-dried and then processed as described in (a). The arrows indicate the conversion of Tyr-SO_3H to Tyr (desulfation) after acid treatment. DABS-amino acids are symbolized by the one-letter code of their corresponding amino acids. The appearance of Gly was not expected from the sequence of hirudin and is likely to be a contaminant. Chromatographical conditions were: solvent A, 12 mM phosphate (pH 6.5); solvent B, acetonitrile containing 4% dimethylformamide. Gradient, 12–37% B/0–22 min, 37–60% B/22–35 min, kept at 60% B from 35–40 min, then 60–12% B/40–45 min. Column, Merck Lichrosorb RP-18 (5 μm). Column temperature, 50°C. Detector, 436 nm, 0.01 absorbance full scale.

pact domain), native hirudin was not accessible to trypsin digestion at all. It is clear that both Lys–Cys and Lys–Asn bonds were digested by trypsin only when the 3 disulfide linkages were disrupted. The other trypsin-susceptible site, Lys–Pro located near the C-terminal domain. However, it is known that Lys–Pro is not readily digested by trypsin.

3.2. *Removal of the C-terminal acidic amino acids of native hirudin resulted in a concomitant loss of hirudin inhibition activity*

Three types of hirudins modified at the C-terminal region were prepared in order to characterize the functional role of the acidic C-terminal domain. (a) Hirudin with the Tyr-SO_3H at position 63 desulfated. (b) Hirudin with the last 7 C-terminal amino acids deleted by carboxypeptidase Y digestion. (c) Hirudin with the last 22 C-terminal amino acids deleted by staphylococcal protease digestion. The structures of the desulfated and carboxypeptidase Y digested hirudins were determined by amino acids analysis (fig.3). The structures of the staphylococcal protease digested hirudins were determined by automatic Edman degradation (fig.1). Table 1 summarizes the structures and surviving activities of those modified hirudins.

In preparing the enzymatically modified hirudins, an unorthodox approach has been adopted due to the scarcity of hirudin. For instance, both carboxypeptidase Y and staphylococcal protease modified hirudins were assayed in the presence of those enzymes and the released amino acids or peptides. However, control experiments were carried out to show that both carboxypeptidase Y and staphylococcal protease do not exhibit any inhibition activity under the conditions described (table 1). From table 1, several conclusions can be drawn. (a) The native hirudin used in this study was only 88% sulfated at residue 63. (b) Desulfation of native hirudin was accompanied by a linear decrease of hirudin inhibition activity and the Tyr-SO_3H residue probably accounts for less than 30% of the total hirudin inhibition activity. It is, however, arguable that denaturation of native hirudin caused by acid treatment may also account for the loss of inhibition activities. (c) Removal of the last 7 C-terminal amino acids (including two Glu and one Tyr-SO_3H) from native hirudin resulted in a loss of at least 90% of the hirudin inhibition activity. (d) Removal of the last 22 C-

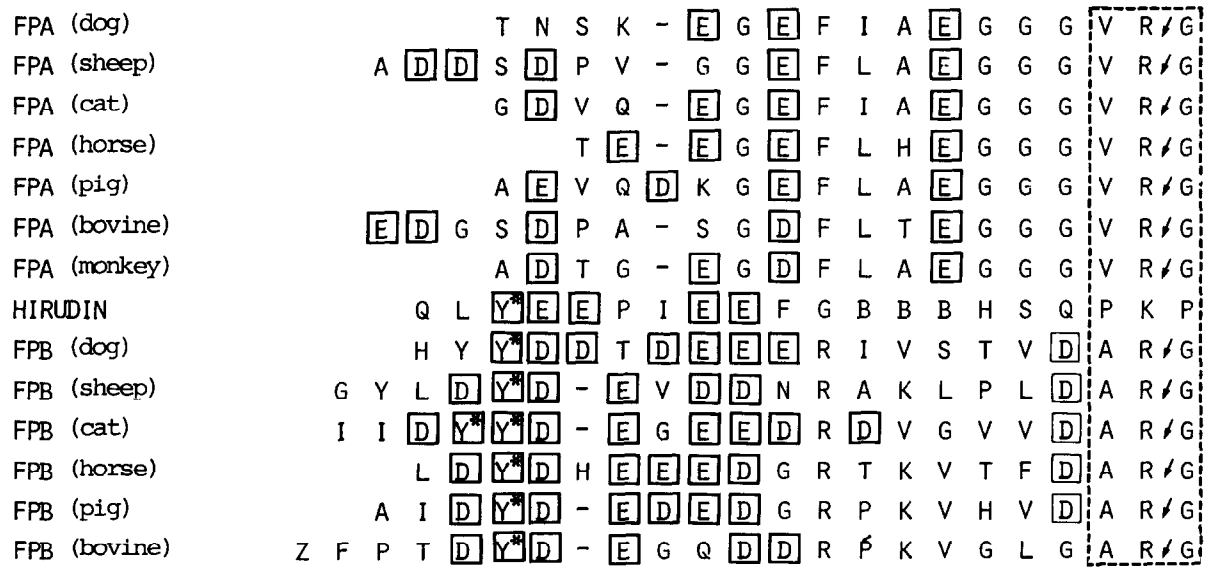

Fig.4. The structure similarity of hirudin C-terminal segment and fibrinopeptides A (FPA), fibrinopeptides B (FPB). Acidic amino acids, Asp (D), Glu (E) and Tyr-SO_3H (Y*) are boxed. The sequence of hirudin is aligned from the C-terminal direction toward the N-terminal direction.

Table 1

The structure and surviving activity of modified hirudin

Treatment of hirudin[a]	Structure[a]	Surviving[b] activity (%)
Native hirudin	12% desulfated	100
30% Trifluoroacetic acid, 50°C, 25 min	46% desulfated	89
30% Trifluoroacetic acid, 60°C, 15 min	60% desulfated	87
30% Trifluoroacetic acid, 70°C, 15 min	93% desulfated	73
30% Trifluoroacetic acid, 80°C, 15 min	100% desulfated	45
Carboxypeptidase Y, 20 min	At least 95% of hirudin with the last 5 C-terminal amino acids removed, see fig.2	8
Carboxypeptidase Y, 40 min	See fig.2	7
Staphylococcal protease, 20 min	Not determined	42
Staphylococcal protease, 1 h	45% of the C-terminal 22 amino acids deleted	27
Staphylococcal protease, 2 h	74% of the C-terminal 22 amino acids deleted	16
Staphylococcal protease, 4 h	Not determined	3

[a] Three types of modified hirudin were prepared. (a) Four samples of 1.5 nmol native hirudin were incubated with 20 μl of 30% aqueous trifluoroacetic acid under varied conditions. The samples were freeze-dried, redissolved in 30 μl water. One-third of the sample (0.5 nmol) was removed for C-terminal analysis (see fig.3) to determine the extent of desulfation. The remaining samples were used for bioassay. (b) Two samples of native hirudin (1.5 nmol each) were digested with 1.5 μg carboxypeptidase Y in 30 μl acetate buffer (pH 5.4) at room temperature for 20 and 40 min. Ten μl (0.5 nmol) of each sample was removed for the analysis of the released C-terminal amino acids and the remaining samples were freeze-dried and used for bioassay. A blank sample (with only 1.5 μg carboxypeptidase Y) was carried out for control experiment. (c) Four samples of 2 nmol each of native hirudin were incubated with 2 μg staphylococcal protease in 40 μl of 0.1 M ammonium bicarbonate (pH 8.0) at 37°C for 20 min, 1 h, 2 h and 4 h. Fifty percent of each sample was removed for 4 step N-terminal sequencing to determine the cleaving point and the remaining samples were used for bioassay. A blank sample (containing only 2 μg staphylococcal protease) was carried out as a control experiment

[b] The inhibition activity of native hirudin (12% desulfated) was taken as 100%

terminal amino acids causes nearly quantitative abolishment of hirudin inhibition activity.

The above results clearly indicate that the acidic C-terminal segment of hirudin is essential for hirudin activity. However, efficient binding of hirudin to thrombin requires the intactness of the hirudin molecule, as reduced carboxymethylated and oxidized hirudins [2], or fragmented C-terminal segments of hirudin do not exhibit appreciable inhibition activities (less than 3% of the native one).

4. DISCUSSION

α-Thrombin has been classified as a 'weak serine proteinase' with a preferential arginine specificity [4,5,11]. It is also known that the high-affinity protein-binding or recognition site which is independent of its catalytic site accounts for the high enzymatic specificity of thrombin toward protein substrate [4,5]. However, the nature of this recognition site has not yet been unequivocally defined.

The acidic C-terminal segment of hirudin, in addition to its demonstrated function of interacting with thrombin, displays an intriguing structural similarity to both fibrinopeptides A and fibrinopeptides B (fig.4) [12]. They all have similar length and they are all very acidic. One post-translationally modified amino acid, Tyr-SO_3H, which was found in hirudin is also present in the fibrinopeptides B of a large number of species [13]. If one aligns the last 20 C-terminal amino acids of hirudin (from the C-terminal toward the N-terminal direction) with fibrinopeptides A and B, the homology becomes more evident. There are clusters of acidic amino acids located at regions about 9–17 amino acids apart from the thrombin

active site binding regions. The most interesting feature of the hirudin C-terminal segment is perhaps the region which aligns with the thrombin cleaving points of fibrinogen Aα and Bβ chains. While Aα and Bβ chains possess Val-Arg-Gly and Ala-Arg-Gly structures, the potential reactive site of hirudin has a unique structure of Pro-Lys-Pro. The preceding proline serves to enhance the affinity of the hirudin reactive site to the active site of thrombin [3,5], and the succeeding proline presides to resist the cleavage of the hirudin reactive site by the active site of thrombin.

Although there is no precedent for comparing the amino acid sequence from reversed directions, this intriguing sequence homology (fig.4) at least raises the plausibility that thrombin may recognize its substrate through those homologous acidic regions. There exists in thrombin a basic recognition site which is independent of but adjacent to its catalytic site and is comprised of clusters of Arg and Lys. With large protein substrates, only those Arg–X (or Lys–X) bonds which have acidic regions at approximate distances apart from the cleavage site can be more effectively recognized and cleaved by thrombin. In the hirudin–thrombin complex, the acidic C-terminal segment of hirudin binds to the recognition site of thrombin, while Pro-Lys-Pro fits into the active site of thrombin.

This proposed mechanism is well supported by mounting documented evidence. (a) In the B-chain of α-thrombin, there exists a highly conserved Arg/Lys cluster [3]. Cleavage at this basic segment converts highly clotting α-thrombin to β- and γ-thrombins [14,15]. Hirudin complexing with α-thrombin prevents enzymatic conversion to β- and γ-thrombins [3]. This implies that hirudin binding masks the basic recognition site which is responsible for the high clotting activity (or high protein binding affinity) of α-thrombin. (b) Acetylated thrombin [4,16] lost most of its clotting activity whereas it retained substantial esterase activity. Nor does acetylated thrombin bind to hirudin. It is likely that acetylation blocks the lysine ϵ-groups which are located in the recognition site of thrombin. (c) Kinetic studies [17] on the varied size of synthetic Aα peptides have shown that the Asp residue at the P_{10} position of fibrinopeptide A plays an important role in the fibrinopeptide A–thrombin interaction. (d) Though thrombin is a serine proteinase [4], it is not inhibited by proteinase inhibitors of plant origin [3] or by pancreatic inhibitors which inhibit trypsin and plasmin. On the other hand, hirudin is only thrombin specific [1,2] and it does not inhibit trypsin or plasmin. All these facts suggest that thrombin and hirudin must interact via a unique structure that is independent of the active site of serine proteinase. (e) Most well characterized thrombin-susceptible Arg–X bonds [5,18] are about 8–15 amino acids apart from an acidic amino acid region. The significant difference of thrombin cleavage rates between human and ovine growth hormones [19] is probably due to the higher acidity and more optimal distance of the acidic region presented in ovine growth hormone:

Human growth hormone [20,21]
G I E T L M G R L E D G S P R↙ T
(residues 120–135)

Ovine growth hormone [22]
E E G A L M R E L E D V T P R↙ A
(residues 119–134)

(f) In several genetically abnormal fibrinogens, the C-terminal Arg of fibrinopeptide A is replaced by His. This His–Gly bond has been reported to be cleaved by thrombin [23,24]. This occurrence is likely due to the strong interaction of the acidic fibrinopeptide A with the recognition site of thrombin which brings the relatively weak basic side chain of His into the Arg binding pocket and thus results in His–Gly cleavage.

ACKNOWLEDGEMENTS

The author thanks Dr U. Seemüller for providing the hirudin sample and Mr J. Dodt for assistance in performing the bioassay of modified hirudins.

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