

Primary structures of new 'iso-hirudins'

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Crude hirudin (12.7 U/ μ g), a complex mixture of polypeptides obtained from the leech, could be separated by microbore HPLC. A combination of amino acid analysis, N-terminal microsequencing and chemical as well as enzymatic fragmentation made the primary sequence of the new isohirudins Ia–IIIb' accessible. The biological activity determined in the thrombin inhibition test showed a comparable value for all of these compounds. The results presented address the question as to whether these isohirudins are true mutations from a family of genes or a family of leeches.

Hirudin; Microsequencing, N-terminal; HPLC, microbore; Cleavage, Asn-Gly; Trypsin digestion

1. INTRODUCTION

Hirudin is a polypeptide of 65 amino acids containing 3 disulfide linkages isolated from the head-parts of the leech *Hirudo medicinalis*. It is the most potent natural thrombin-inhibitor known [1] ($K_i \leq 10^{-10}$). The complete sequence including disulfide bridges has been published [2,3]. Here, hirudin was found to exhibit Val-Val as N-terminal amino acids. Previous workers [4,5] also found Ile-Thr as the N-terminus of hirudin. In recent years, the concept of hirudin as a 'family of isoinhibitors' [8] emerged. In this work 10 new hirudin sequences were determined with microanalytical methods, which support this concept.

2. MATERIALS AND METHODS

2.1. Materials

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Abbreviations: HPLC, high-performance liquid chromatography; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; TFA, trifluoroacetic acid; PTH-amino acid, phenylthiohydantoin amino acid

P. Walsmann and Dr F. Marquardt [9]. Trypsin (TPCK-treated) was obtained from Merck and Boehringer (sequencing grade).

2.1. HPLC analysis of hirudins

HPLC analysis was carried out on a microbore column (Applied Biosystems, Aquapore, 1×250 mm, pore size 300 Å, silica C8 reversed-phase derivatized, bead size 7 μ m) with an HPLC gradient system (flow rate, 50 μ l/min; column temperature, 39°C). UV monitoring was carried out at 205 nm with a Shimadzu detector. The mobile phase consisted of 5% acetonitrile (MeCN) in water (w/w) plus 0.1% trifluoroacetic acid (TFA) (A) and 45% MeCN in water (w/w) plus 0.1% TFA (B). After each run the column was washed with 90% MeCN plus 0.1% TFA.

For preparative purposes, larger quantities (70 μ g) of native hirudin mixture were injected and eluted with a shallow gradient (0.06% buffer B/min). Identical peaks were pooled and each fraction was submitted to final purification using a steeper gradient (0.4% buffer B/min).

2.2. Amino acid analysis

Aliquots of peptides (3.5–7 μ g) were dried in 4×20 mm silica test tubes and hydrolysed in the vapor phase under vacuum deoxygenation using constant-boiling HCl plus 10% (w/v) phenol for 22 h at 106°C. Analysis was carried out on a Beckman 6300 amino acid analyser using ninhydrin technology.

2.3. Sequence analysis

Sequence analysis was carried out on an Applied Biosystems 470A sequencer coupled on-line with a PTH amino acid analyser (Applied Biosystems 110A).

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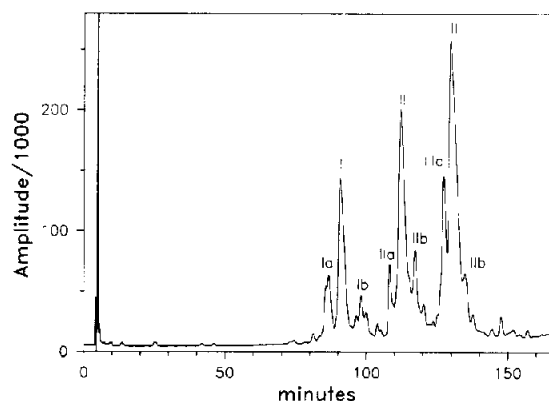


Fig.1. HPLC analysis of batch 404. 70 μ g native hirudin mixture eluted with a linear high-pressure gradient (0.06% buffer B/min) starting from 10% acetonitrile. Buffer A: 95% H_2O , 5% acetonitrile, 0.1% TFA; buffer B: 55% H_2O , 45% acetonitrile; UV monitoring at 205 nm; column temperature 39°C; column: Aquapore 300 Å, C8, 7 μ m, 1 $\times$ 250 mm.

digested (enzyme/substrate ratio 1:100) in 0.2 M *N*-methylmorpholine buffer (pH 8) at 25°C for 30 min. Subsequently, the mixture was analysed with microbore HPLC using a gradient of 1.7% buffer B/min. The structures of the tryptic fragments were determined by sequence analysis.

2.5. Hydroxylamine cleavage

Purified hirudin (fraction III) was dissolved in 200 μ l cleavage [11] buffer containing 6 M guanidine and 2 M hydroxylamine (pH 9) and incubated at 45°C for 4 h. After quenching the cleavage reaction with 200 μ l acetic acid, the peptides were isolated by HPLC using a gradient of 0.4% buffer B/min and submitted to sequence analysis.

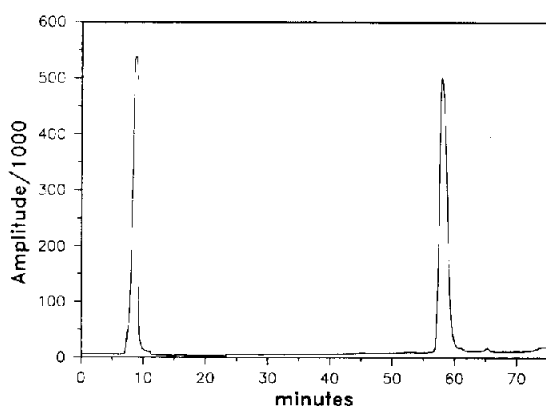


Fig.2. Rechromatogram of hirudin III; eluted with a linear high-pressure gradient (0.4% buffer B/min) starting from 10% acetonitrile; 15 μ g purified hirudin III was obtained.

3. RESULTS

3.1. Microbore-HPLC analysis of hirudin Ia-IIIb.

70 μ g of the native hirudin mixture was separated into 9 purified compounds (chromatogram, fig.1). Three main peaks (I-III) and their satellite peaks ('a' and 'b') were obtained. All these peaks exhibited specific thrombin inhibition activity. The rechromatogram of hirudin III is shown in fig.2 as an example.

3.2. Amino acid composition of hirudin I-III

The amino acid composition was determined for the three purified main peaks I-III. They are similar in composition to published hirudins [2-8].

3.3. N-terminal sequence analysis of hirudin Ia-IIIb

The complete sequence of each purified peak was determined with a sequencer-load of 1 nmol. In the few cases where interpretation of the last degradation steps was inconclusive, trypsin diges-

Fig.3 Amino acid sequences of hirudin Ia-IIIb

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tion was performed in order to isolate the C-terminal peptide (fig.3). Some degradation steps gave rise to the identification of two amino acids, which amounted to up to 30%. This indicated that

the starting material was not homogeneous and consisted of a mixture of two proteins (designated for the minor component by a prime). Nevertheless, quantitative interpretation of the sequence data allowed us to characterise the minor component of the mixture.

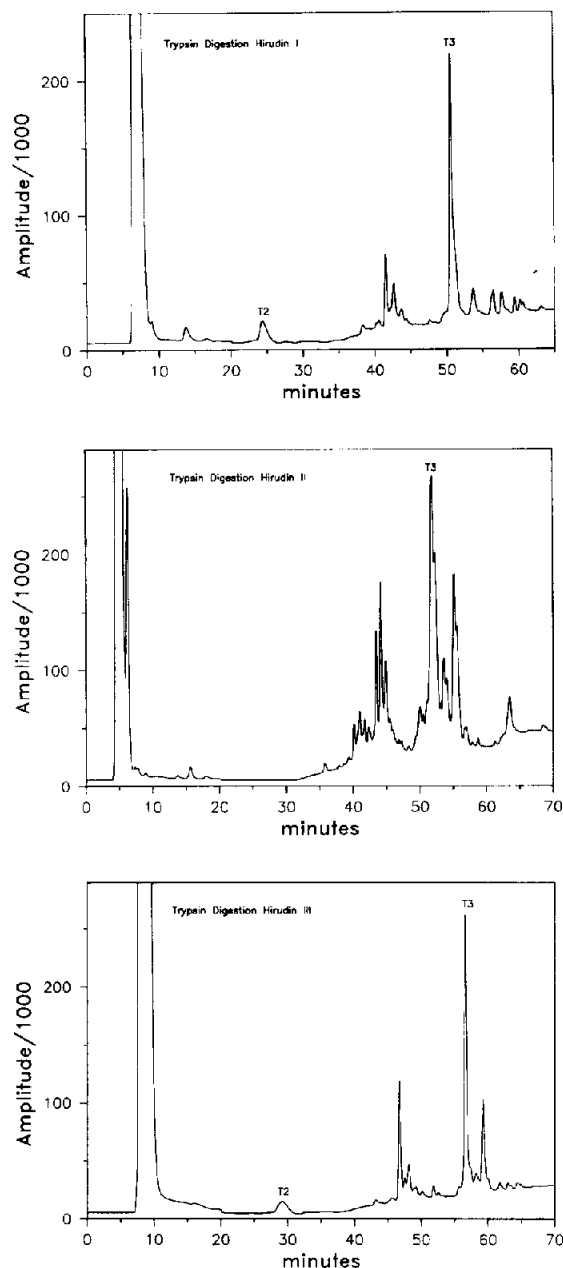
The sequence analysis of hirudin Ia and I terminated in every case at step 17 (fig.3), irrespective of whether native or oxidized hirudin was analysed. Furthermore, amino acid analysis and trypsin digestion (HPLC mapping) proved that the starting material consisted of full-sized hirudin. No explanation could be found for this unusual behaviour. No determination of Tyr *O*-sulfate at position 63 was achieved.

3.4. Trypsin digestion of hirudin I-III

About 1 nmol oxidized hirudin was digested with trypsin. The mixture was analysed via microbore HPLC (figs 4-6) and the carboxy-termini T2 and T3 (fig.7) were isolated. In the trypsin digestion of hirudin II, the short fragment T2 (Gly-Asn-Lys) could not be isolated. Fragment T1 could not be isolated as a homogeneous fraction, but as several peaks caused by secondary cleavage. The isolation yield of each fragment amounted to up to 400 pmol. The structure of the isolated fragments was determined by N-terminal sequence analysis (fig.7).

3.5. Asn-Gly bond cleavage with hydroxylamine

Amidation of aspartic acid at position 33 leads to an Asn³³-Gly³⁴ bond, which can be cleaved specifically with hydroxylamine [11]. Although the cleavage yield of this procedure, which was not optimised for hirudin, was poor, it was a valuable method for chemically determined proof of the mutation from Asp³³ to Asn³³. 2 nmol purified hirudin III were subjected to hydroxylamine cleavage and the reaction mixture was analysed on micro-bore HPLC (fig.8). Fraction F1 was isolated and submitted to sequence analysis. The results agreed with a structure cleaved at position 33. Due to the Cys-bridge conformation the two fragments remained chemically linked via the disulfide bonds (Cys¹⁶-Cys²⁸ and Cys²²-Cys³⁹). Thus, N-terminal sequence analysis of the cleaved product exhibited two amino-termini, one resulting from the regular N-terminus, the other from the Asn-Gly cleavage site (table 1).



Figs 4-6. Trypsin digestion of hirudin I-III. 7 μ g (1 nmol) purified hirudin (I-III) was digested with trypsin and injected and eluted with a linear high-pressure gradient (1.7% buffer B/min) starting from 0% acetonitrile.

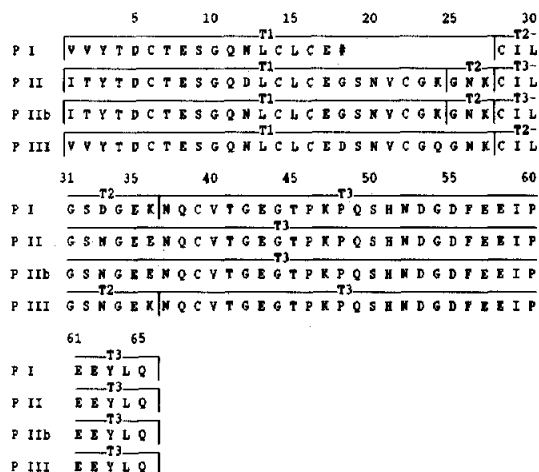


Fig.7. Hirudin fragments obtained by trypsin digestion.

4. DISCUSSION

The hirudin sequences Ia-IIIb' are not identical to those of the hirudins published previously [2-8] (see fig.9). Their overall homology is in the range of 66%. No change occurs at the N-termini Val-Val (hirudin Ia-I and IIIa-IIIb') or Ile-Thr (hirudin IIa-IIb). The same is true for the Cys residues and for their number and location. We did not determine the Cys bridges but argue, based on their activity, for an identical geometry as in [3]. Striking identity is also observed for the C-terminus starting at position Val⁴⁰ for all our variants. Based on [12,14,15], this part is considered to be essential for binding at the anionic binding site of thrombin [13]. All hirudins except as described in [7] have a Lys⁴⁷ (fig.9) at the P1 position (nomenclature, see [16]) according to Dodt et al. [15]. In contrast to [7,8], no Ala⁶² or Ala⁶⁴ was found at the C-terminus for Ia-IIIb'. A combination of Ile-Thr hirudins with a Lys²⁴ and Val-Val hirudins with a Gln²⁴ or Glu²⁴ seemed to be favoured.

Hirudin IIa is the only naturally occurring compound which has been determined to have an Ile²⁹-Lys²⁹ exchange.

A remarkable mutation occurs in hirudin IIIa, III and IIIb where Asp is replaced by Gly in position 18, a β -turn region (17-20) according to [17]. Thus, it is interesting to speculate on the implications of this exchange for this antiparallel β -pleated sheet (15-16 and 21-22). Amidation and deamidation

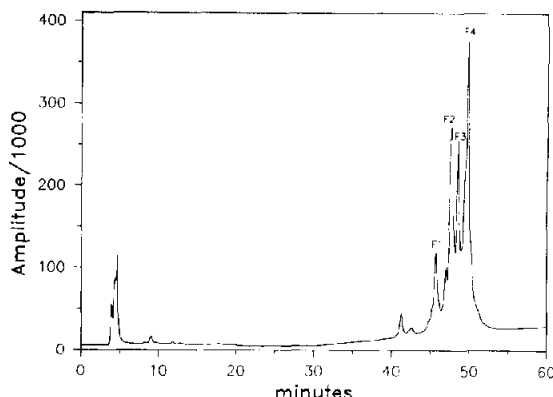


Fig.8. Hydroxylamine digestion of hirudin III. Reaction mixture was directly injected and eluted with a linear high-pressure gradient (0.4% buffer B/min) starting from 10% acetonitrile.

tion of side chains known in the literature [7] (fig.9) are also observed here (Ia-IIIb'). Hirudin IIIa' is especially noteworthy where Glu^{11,24,38} and Asp¹² are deamidated. The cDNA sequence reported by Harvey et al. [7] supports the true mutation and not an artefact due to the work-up procedure. In contrast to the Ile-Thr hirudins known, compounds IIa-IIb do not have a Lys^{35,36}. The lack of this lysine could be proven by trypsinolysis.

In conclusion, it can be said that these new hirudin sequences Ia-IIIb' are almost equivalent in biological activity towards thrombin but distinctly different in primary sequence. The character and position of the mutation seem not to be essential for the inhibitory activity. At the DNA level, all mutants can be explained by simple point mutations of a possible ancestral gene.

Thus, this work supports the hypothesis of a family of hirudins, the isohirudins. The family history can be investigated by cDNA studies of leeches.

From an analytical point of view, modern microbore HPLC and microsequencing in com-

Table 1
N-terminal sequence analysis of fraction F1

Degradation step	Amino acids (yields)
1	Val (17 pmol), Gly (41 pmol)
2	Val (17 pmol), Glu (50 pmol)
3	Tyr (24 pmol), Lys (37 pmol)

	10										20										30									
1	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
2	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
3	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
4	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
5	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
6	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
7	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
8	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
9	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
10	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
11	*	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
IIa	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
II	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
II'	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
IIb	I	T	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
IIIa	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
IIIa'	V	V	Y	T	D	C	T	E	S	G	E	D	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
III	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
IIIb	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L
IIIb'	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L	C	E	G	S	N	V	C	G	Q	G	N	K	C	I	L

	40										50										60										65							
1	G	S	D	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
2	G	S	Q	G	*	K	D	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	D	A	Y	D	E
3	G	S	N	G	*	K	G	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
4	G	S	D	G	*	Q	K	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
5	G	S	D	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
6	G	S	D	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
7	G	S	D	G	*	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
8	G	S	D	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
9	G	S	D	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
10	G	S	D	G	*	K	D	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	A	Q	
11	G	S	D	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
IIa	G	S	D	G	E	E	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
II	G	S	N	G	E	E	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
II'	G	S	N	G	E	E	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
IIb	G	S	N	G	E	E	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
IIIa	G	S	N	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
IIIa'	G	S	D	G	E	K	*	N	E	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
III	G	S	N	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
IIIb	G	S	N	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	
IIIb'	G	S	N	G	E	K	*	N	Q	C	V	T	G	E	G	T	P	K	P	Q	S	H	N	D	G	D	F	E	E	I	P	E	E	*	Y	L	Q	

Fig.9. Sequence of naturally occurring hirudins: (1-2) Dodt et al. [2,6], (3-4) Harvey et al. [7], (5-11) Tripier [8], (12-20) IIa-IIIb'. Only complete structures are listed.

ination with site-specific chemical and enzymatic cleavage have made these studies possible.

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