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# Isolation and purification of novel hirudins from the leech *Hirudinaria manillensis* by high-performance liquid chromatography

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## ABSTRACT

The isolation and purification of novel hirudins from a crude extract of the leech *Hirudinaria manillensis* and their analytical characterization are reported. Initial purification by gel permeation chromatography on Sephadex G50 and anion-exchange chromatography on Q Sepharose fast-flow removed most contaminants and yielded a highly active extract. Two isohirudins (designated hirudin P6 and P18) were isolated and purified by successive reversed-phase high-performance liquid chromatography on silica-based stationary phases and anion-exchange chromatography on Mono Q. The final products were characterized by reversed-phase high-performance liquid chromatography, $^{252}$Cf plasma desorption time-of-flight mass spectrometry and capillary zone electrophoresis. The molecular masses determined by $^{252}$Cf plasma desorption mass spectrometry were 7416 dalton for hirudin P6 and 7199 dalton for hirudin P18.

## INTRODUCTION

Hirudin is the best characterized compound from the leech *Hirudo medicinalis*. In the late 1950s, the polypeptide was isolated and found to be a very specific thrombin inhibitor [1] with a high therapeutic potential [2]. The complete sequence of the 65 amino acid polypeptide was elucidated in 1984 [3]. Amino acid sequences of several isoforms have been published by different groups [4–6] and a sequence has been predicted from cDNA [7]. No glycosylation has been found so far, although hirudin is a secretory protein.

The chemical synthesis of a gene coding for hirudin and its expression in *Escherichia coli* or *Saccharomyces cerevisiae* was carried out by different groups [8,9]. The conformations of natural and recombinant hirudin in solution were determined by two-dimensional NMR spectroscopy [10,11]. Kinetic data for the

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inhibition of human alpha thrombin by hirudin have been presented [12,13], the most profound study [14] giving a $K_I$ value of 22 f*M*.

With the exception of hirudin from the leech species *Hirudo medicinalis* thrombin inhibitors from other leeches have not been reported to date. In this paper the isolation of hirudins from a crude extract of the leech *Hirudinaria manillensis* is described for the first time. Separation of hirudins from by-products was achieved by gel permeation chromatography and anion-exchange chromatography. Purification of isoforms was achieved with reversed phase high-performance liquid chromatography (RP-HPLC) and anion-exchange chromatography. The purified products were characterized by analytical RP-HPLC, $^{252}$Cf plasma desorption mass spectrometry (PD-MS) and capillary zone electrophoresis (CZE).

Inhibitory activities against human alpha thrombin, amino acid analysis and sequence data will be published elswhere [15].

## EXPERIMENTAL

### *Materials*

The preparation of crude lyophilized leech extract from whole leeches has been described [16] and was carried out in a similar way. Briefly, to homogenized frozen leeches, cold aqueous acetone (60% v/v) and sodium chloride were added. After acidification to pH 4.5 with trifluoroacetic acid, hirudin was precipitated with acetone. The precipitate was dissolved in aqueous $Na_2CO_3$ solution, filtered (Amicon UM 05) and lyophilized. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with chemicals from BioRad (Richmond, CA, U.S.A.), Molecular Weight Standard low was purchased from the same company. The gels were stained with Coomassie Brillant Blue from Serva (Heidelberg, F.R.G.). Isoelectric focusing (IEF) was done with Servalyt gel pH 3–10 and Protein Test mix 9 (Serva). Chromozym TH (TosGlyProArg-4-nitroanilide) from Boehringer (Mannheim, F.R.G.) and Test-Thrombin 6 IU from Behringwerke (Marburg, F.R.G.) were used for testing inhibitory activity during the purification process. All buffers were prepared with MilliQ water (Millipore-Waters, Milford, MA, U.S.A.), filtered through a 0.45-$\mu$m Nalgene unit (Sybron/Nalge, Rochester, NY, U.S.A.) and degassed. Sephadex G50 superfine, MonoQ and Q Sepharose fast-flow were used as stationary phases for gel permeation chromatography and anion-exchange chromatography (Pharmacia/LKB, Uppsala, Sweden). Nucleosil $C_{18}$ (Macherey & Nagel, Dueren, F.R.G.) and 219 TP phenylsilica (Vydac) were used as stationary phases for RP-HPLC. Hydrochloric acid 6 *M* and Amino Acid Standard H were from Pierce (Rockford, IL, U.S.A.). The hydrolysate was derivatized with (4-dimethylamino)azobenzenesulphonyl chloride (DABS-Cl) (Fluka, Buchs, Switzerland). All other chemicals were of highest purity available.

*Instrumentation*

SDS-PAGE was performed in a Mini-Gel cell (BioRad); for IEF an LKB 2117 Multiphor electrophoresis system was used. Thrombin inhibition was monitored in 96-well microtiter plates using a microplate autoreader (Titerteck Multiskan, Flow Laboratories). Gel permeation chromatography and anion-exchange chromatography were performed on a Pharmacia Fast Protein Liquid Chromatography (FPLC) system consisting of two P-500 syringe pumps, a V-7 injector an LCC-500 controller, a UV-M monitor, a two-channel pen recorder REC-482 and a FRAC-100 fraction collector. RP-HPLC was done with a Waters system including 510 pumps, an automatic injector WISP 712, and a multiwavelength detector 490 linked to a Pharmacia 481 pen recorder. System control and data acquisition were done with a Professional 380 computer/controller (digital) using Waters software. Mass spectra were recorded on a $^{252}$Cf PD mass spectrometer Bioion 20 (Bio-Ion Nordic, Uppsala, Sweden). The CZE system was laboratory built and similar to those described in the literature [17]. The fused-silica capillary (Polymicro Technologies, Phoenix, AZ, U.S.A.) had a total length of 100 cm and an I.D. of 75 $\mu$m. A programmable FUG power supply HCN-35-35000 (FUG, Rosenheim, F.R.G.) and a Gilson M221 autosampler (Synmedic, Zürich, Switzerland) were interfaced with an HP series 300 computer system (Hewlett Packard, Palo Alto, CA, U.S.A.). The controlling and data acquisition software was written in Pascal. A 206 PHD absorbance detector equipped with a capillary flow cell (Linear Instruments, Reno, NV, U.S.A.) was used. The signal was displayed on a chart recorder (Philips, Netherlands) digitalized with an A/D converter (SRS 245, Stanford Research Systems, Sunnyvale, U.S.A.) and stored in the HP 300 computer.

*Purification of the crude extract*

*Gel permeation chromatography.* Crude extract (1 g) was dissolved in 0.1 *M* acetic acid (20 ml) and centrifuged. The clear brown supernatant was applied to a Sephadex G50 superfine column (Pharmacia, 100 cm × 26 mm I.D. 400-ml bed volume) preequilibrated with 0.1 *M* acetic acid and connected to an FPLC system. The elution was carried out with 0.1 *M* acetic acid at a flow-rate of 0.2 ml/min, and monitored at 280 nm. Fractions (6 ml) were collected and the activity was tested by their ability to inhibit the thrombin-mediated release of *p*-nitroaniline from Chromozym TH [5]. The active fractions were pooled and lyophilized.

*Anion-exchange chromatography.* The lyophilized active fractions from the gel permeation chromatography were dissolved in 2 ml of 0.02 *M* histidine · HCl pH 5.6 (eluent A) and centrifuged. The clear supernatant was applied to a Q Sepharose fast-flow anion-exchange column (Pharmacia, 40 cm × 16 mm I.D., 66-ml bed volume). Elution was carried out using a step gradient: 30 min at 0% B (B = 0.02 *M* histidine · HCl pH 5.6 + 1 *M* NaCl), 40 min at 15% B, 65 min at 100% B at a flow-rate of 2 ml/min. The active fractions were pooled and lyophilized.

*Purification of isohirudins*

*$C_{18}$ RP-HPLC.* The lyophilized hirudin extract was dissolved in doubly distilled water at a concentration of 9 mg/200 μl. Aliquots of 200 μl were applied to a Nucleosil 7 μm 300 Å $C_{18}$ column (250 × 8 mm I.D.) and eluted with a linear gradient from 18% to 28% B in 40 min at a flow-rate of 5 ml/min; eluent A was 0.1% TFA, eluent B was acetonitrile + 0.08% TFA. Single peak fractions were manually collected and immediately dried in a vacuum centrifuge. The fractions of interest, *e.g.* P6 and P18, were pooled and rechromatographed under the same conditions.

*Phenylsilica RP-HPLC.* Fractions P6 and P18 were pooled again, dried in a vacuum centrifuge, and redissolved in doubly distilled water at a concentration of 700 μg/100 μl. Aliquots of 100 μl were applied to a 5 μm 300 A phenylsilica RP column (150 × 4.6 mm I.D.) and eluted at a flow-rate of 1 ml/min. Eluents and gradient were as before. The main peak fractions were collected and dried in the vacuum centrifuge.

*MonoQ anion-exchange chromatography.* The last purification step was carried out on an FPLC system with a MonoQ HR 5/5 column. Eluent A was 0.05 *M* ammonium formate (pH 5.1); eluent B was 0.05 *M* formic acid (pH 2). The flow-rate was 1.5 ml/min. The peak fraction to be purified was dissolved in 1 ml of A and applied to the column. Elution was done with a linear gradient from 0% to 100% B in 30 min. The main peak fractions were collected and lyophilized.

*Analytical characterization*

*SDS-PAGE.* Slab gels containing 12.5% acrylamide and 0.1% SDS were prepared according to the manufacturer's protocol. Samples were denatured and reduced by mixing equal parts of sample and sample buffer (pH 6.8) containing 0.01 *M* dithiothreitol (DTT), and heating the mixture for 5 min at 95°C. Staining was done with Coomassie Blue.

*IEF gel.* Servalyt gel pH 3–10 and Protein test mix 9 were used. Proteins were detected by Coomassie Blue staining.

*Quantitative amino acid analysis.* Approximately 1.5 μg of each isohirudin was hydrolysed with 6 *M* HCl for 24 h at 110°C and derivatized with DABS-Cl [18].

*$^{252}Cf$ PD MS.* Approximately 4 μg of peptide dissolved in 10 μl of doubly distilled water were used. The solution was placed on the sample holder (aluminium foil covered with a thin film of nitrocellulose). After 10 min material that had not been adsorbed was rinsed off the holder with water. The spectra were collected at + 19 kV accelerating potential. A 1 ns time resolution was employed. The spectra were calibrated on the $H^+$ and $Na^+$ peak centroids.

*CZE.* The samples were injected by using electro-osmotic flow to transfer a small volume into the capillary (up to 7 kV during 7 s). Electrophoresis was carried out at pH 8.3 in 20 m*M* Tris [tris(hydroxymethyl)aminomethane], 20 m*M* Tricine {N-[tris(hydroxymethyl)methyl]glycine}, and 0.2 m*M* DAB (1,4-diaminobutane) with 280 V/cm at 8 μA. The absorbance wavelength was 200 nm.

## RESULTS AND DISCUSSION

The characterization of crude extract by SDS-PAGE and IEF is shown in Fig. 1. The majority of the peptides exhibit masses below 16 kD, but bands up to 30 kD are visible. From the IEF gel it is apparent that most peptides have isoelectric points in the pH range 3.6–5.5.

Owing to differences in mass, charge and hydrophobicity, as outlined in Table I, isolation and purification of hirudin were carried out using a combination of chromatographic separations. The main purpose of the first two steps was to get an extract containing mainly hirudin. The separation of the two isohirudins (designated P6 and P18) and their purification by HPLC was achieved in purification steps 3 to 5. Our approach minimizes the number of steps by employing volatile buffers and an appropriate column order.

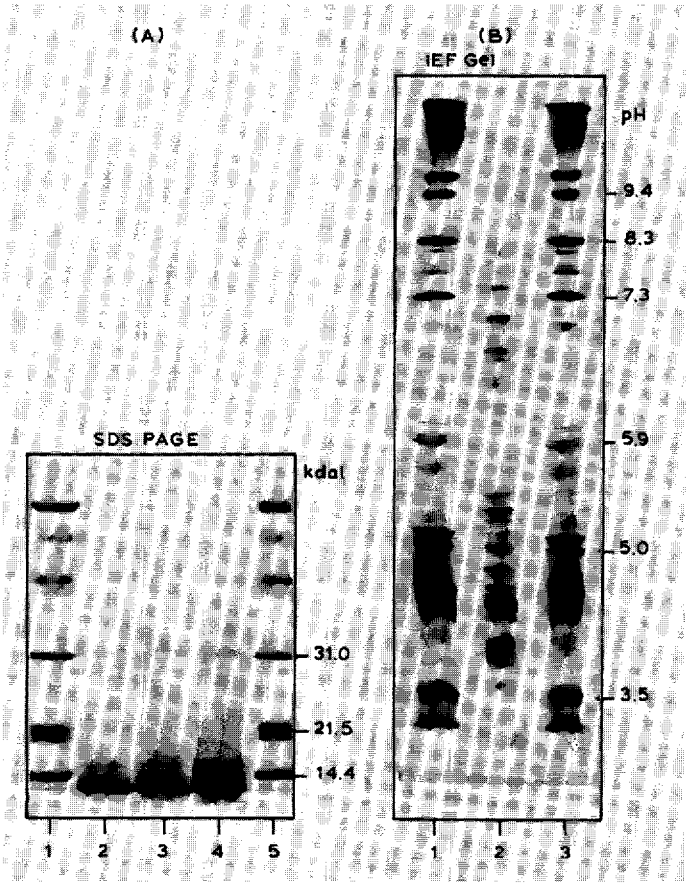


Fig. 1. (A) 12% SDS-PAGE of crude extract: lane 2, 18.7 μg; lane 3, 37.5 μg; lane 4, 75 μg; lanes 1 and 5, molecular mass standards. (B) IEF gel of crude extract with Servalyt gel pH 3–10. Lane 2, 262 μg crude extract; lanes 1 and 3, p*I* standards (see Experimental).

TABLE I

PURIFICATION AND YIELDS OF HIRUDIN P6 AND P18

Protein amounts were determined [a]by weight, [b]peak areas at 214 nm, [c]quantitative amino acid analyses.

| Purification step | Total amount | Yield (%) |
|---|---|---|
| *Isolation of hirudins* | | |
| 0 Starting material | 4300 mg[a] | 100 |
| 1 Gel permeation (Sephadex G50 sf) | 1075 mg[a] | 25 |
| 2 Anion exchange (Q Sepharose fast-flow) | 129 mg[b] | 3 |
| *Purification of isohirudins* | | |
| 3 $C_{18}$ RP-HPLC | 2.0 mg P6[b] | 0.047 |
| | 0.50 mg P18 | 0.012 |
| 4 Phenyl RP-HPLC | 1.3 mg P6[b] | 0.03 |
| | 0.28 mg P18 | 0.007 |
| 5 Anion exchange (MonoQ) | 0.328 mg P6[c] | 0.008 |
| | 0.128 mg P18 | 0.003 |

Gel permeation chromatography was chosen (Fig. 2) as a first step in the purification of the crude extract. Thus, the active fractions were separated not only from lower and higher molecular mass impurities but also from a strongly coloured compound and possible proteolytic enzymes. For the next purification step with Q Sepharose fast-flow, a buffer of pH 5.6 (histidine · HCl) was used. At this pH a large amount of unwanted peptides was eluted in the flowthrough (see Fig. 3). A step gradient from 0.15 *M* NaCl to 1 *M* NaCl was employed for the elution of hirudin to ensure that the two isoforms were eluted at once. The active

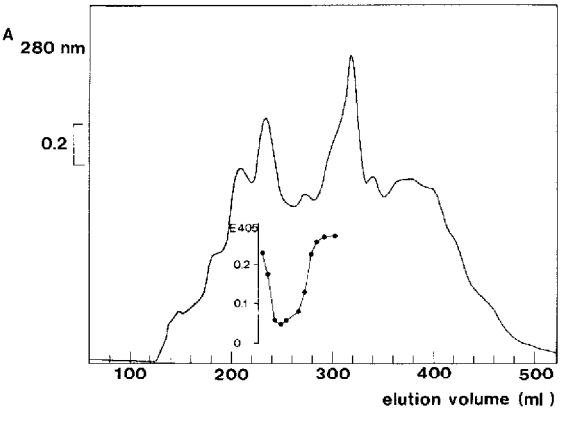


Fig. 2. Gel permeation chromatography of 1 g of crude extract with Sephadex G50 superfine. Solvent, 0.1 *M* acetic acid; flow-rate, 0.2 ml/min. Inset: thrombin inhibition, see Experimental. Hirudin-containing fractions exhibiting $E_{405} < 0.2$ were pooled.

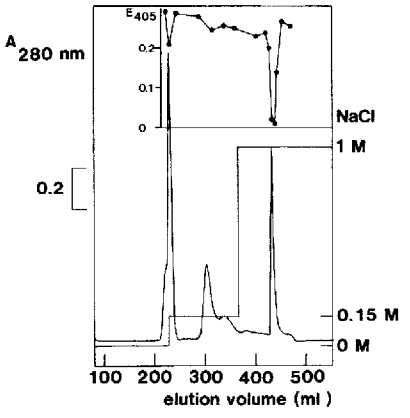


Fig. 3. Q Sepharose fast-flow anion-exchange chromatography of 180 mg of extract from gel permeation chromatography dissolved in 2 ml of 0.02 *M* histidine·HCl pH 5.6 (eluent A). Eluent was carried out using a segmented gradient: 30 min of eluent A, 40 min of 15% B (eluent B 0.02 *M* histidine · HCl pH 5.6 + 1 *M* NaCl), 65 min of 100% B. The flow-rate was 2 ml/min. Inset: human alpha thrombin inhibition, see Experimental. Hirudin-containing fractions exhibiting $E_{405} < 0.2$ were pooled.

fractions were submitted to $C_{18}$ RP-HPLC without desalting. The hirudins were eluted with a shallow linear gradient, and peak fractions were collected and assayed for thrombin inhibition. In order to save time and solvent, the fractions P6 and P18 were pooled for further purification. Fig. 4A shows the HPLC analysis of the pooled fractions on an RP $C_{18}$ column. Peak P18 exhibits a slight shoulder. Using a phenylsilica column, this shoulder was resolved into two distinct peaks (Fig. 4B). The subsequent purification step 4 was therefore done by changing the stationary phase from $C_{18}$ to phenylsilica and employing the same elution conditions (18% acetonitrile + 0.1% TFA to 28% acetonitrile + 0.1% TFA in 40 min). Trifluoroacetate salts of peptides and salts in general interfere in MS analysis. Therefore, ion-exchange chromatography with formate buffers (data not shown) was chosen as final purification step, although the capacity of the MonoQ column used is higher than the capacity of the RP columns employed in preceding steps. The purification yields are listed in Table I. The recovery of antithrombin activity was not determined because of considerable differences in activities between P6 and P18 [15].

The peptide homogeneity of hirudin P6 and P18 as estimated from peak areas at 214 nm from an analytical HPLC run on a phenyl column was greater than 95% for P6 and 100% for P18. These estimates were later corroborated by sequencing data. Analysis by CZE revealed another type of heterogeneity: as will be reported elswhere [15], P6 and P18 are glycosylated to various degrees at one site. This carbohydrate heterogeneity is indicated in the electropherogram in Fig. 5. The main peak of both P6 and P18 exhibits a distinct shoulder, which cannot be assigned to the 5% peptide inhomogeneity.

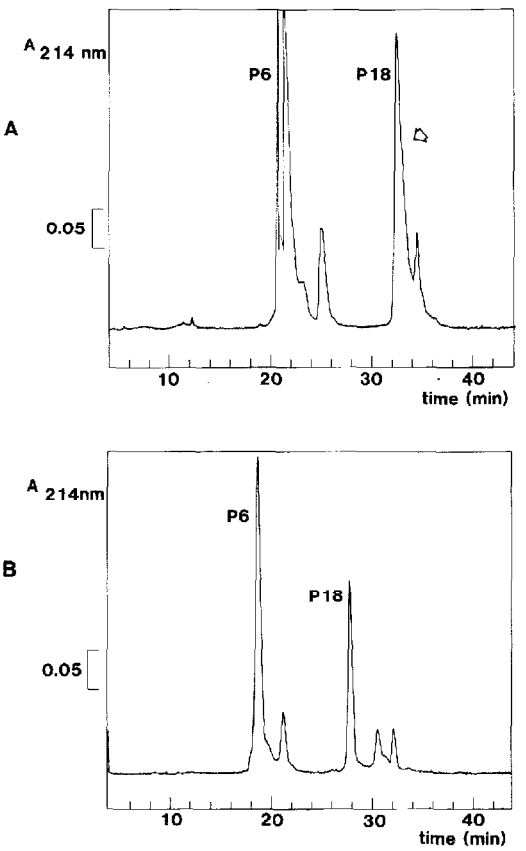


Fig. 4. Effect of change of solid phase shown for RP-HPLC analysis of peak fractions P6 and P18 after purification step 3. Solvent A, 0.1% TFA–water; solvent B, 0.08% TFA–acetonitrile; linear gradient from 18% to 28% B in 40 min; flow-rate, 1 ml/min. (A) RP-HPLC on Nucleosil $C_{18}$, 5 $\mu$m (150 × 4.6 mm I.D.). Peak P18 exhibits a slight shoulder (arrow). (B) RP-HPLC on Vydac 218 TP phenylsilica, 5 $\mu$m (150 × 4.6 mm I.D.). The shoulder from (A) is completely resolved.

Structural information on the isolated hirudins was obtained by $^{252}$Cf PD MS and gave an average mass of 7415.9 dalton for hirudin P6 and 7198.6 dalton for hirudin P18.

Further characterization was done by quantitative amino acid analysis, complete amino acid sequence determination and analysis of the carbohydrate moiety (results published elsewhere [15]).

Isolation of hirudin from crude leech extract has been described by different workers [3–5]. Our approach minimizes the number of steps for obtaining a fraction containing mostly hirudins by using volatile buffers and an appropriate column order. For further isolation of thrombin inhibitors, however, the use of thrombin affinity column is recommended (our unpublished results).

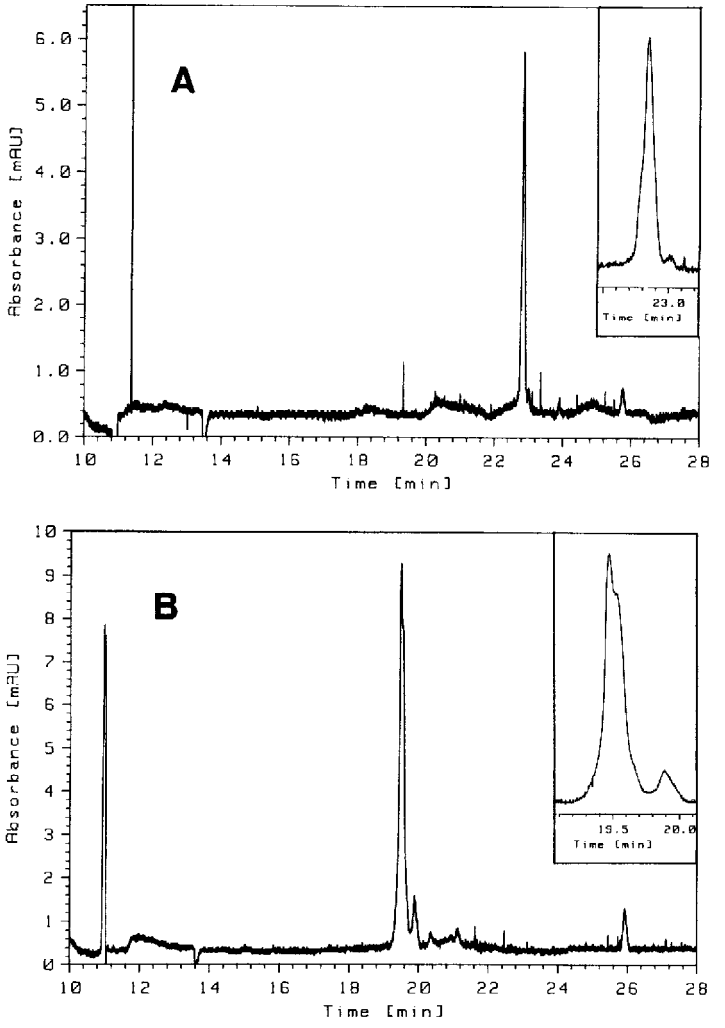


Fig. 5. (A) Electropherogram of hirudin P18 (0.1 $\mu$g/$\mu$l); electrolyte buffer, pH 8.3: 20 m*M* Tris–20 m*M* Tricine–0.2 m*M* DAB; injection, 7 kV for 7 s; length of capillary, 100 cm; voltage for electrophoresis, 280.0 V/cm at 8 $\mu$A; detection wavelength, 200 nm; inset, expanded view of main peak. (B) Electropherogram of hirudin P6 (0.4 $\mu$g/$\mu$l); injection, 5 kV for 5 s; other conditions as for P18.

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REFERENCES

1 F. Markwardt, *Hoppe Seyler's Z. Physiol. Chem.*, 308 (1957) 147.
2 P. Walsmann and F. Markwardt, *Pharmazie*, 36 (1981) 653.
3 J. Dodt, H. P. Mueller, U. Seemueller and J. Y. Chang, *FEBS Lett.*, 165 (1984) 180.
4 D. Tripier, *Folia Haematol.*, 115 (1988) 30.
5 S. J. T. Mao, M. T. Yates, D. T. Blankenship, A. D. Cardin, J. L. Krstenansky, W. Lovenberg and R. L. Jackson, *Anal. Biochem.*, 161 (1987) 514.
6 J. Dodt, W. Machleidt, U. Seemueller, R. Maschler and H. Fritz, *Biol. Chem. Hoppe-Seyler*, 367 (1986) 803.
7 R. P. Harvey, E. Degryse, L. Stefani, F. Schamber, J. P. Cazenave, M. Courtney, P. Tolstoshef and J.-P. Lecocq, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 1084.
8 G. Loisen, A. Findeli, S. Bernard, M. Nguyen-Juilleret, M. Marquet, N. Riehl-Bellon, D. Carvallo, L. Guerra-Santos, S. W. Brown, M. Courtney, C. Roitsch and Y. Lemoine, *Biotechnology*, 6 (1988) 72.
9 C. Bergmann, J. Dodt, S. Koehler, E. Fink, H. G. Gassen, *Biol. Chem. Hoppe Seyler*, 367 (1986) 731.
10 H. Haruyama and K. Wuethrich, *Biochemistry*, 28 (1989) 4301.
11 G. M. Clore, D. K. Sukumaran, M. Nilges, J. Zarbock and A. Gronenborn, *EMBO J.*, 6 (1987) 529.
12 J. W. Fenton II, *Ann. N.Y. Acad. Sci.*, 370 (1981) 468.
13 J. Dodt, S. Koehler and A. Baici, *FEBS Lett.*, 229 (1988) 87.
14 S. R. Stone and J. Hofsteenge, *Biochemistry*, 25 (1986) 4622.
15 V. Steiner, F. Raschdorf, R. Knecht, E. Gassmann, S. R. Stone, J. Y. Chang and F. Maschler, in preparation.
16 D. Bagdy, E. Barabas and L. Graf, *Thromb. Res.*, 2 (1973) 229.
17 H. Luedi, E. Gassmann, W. Maerki and H. Grossenbacher, *Anal. Chim. Acta*, 213 (1988) 215.
18 R. Knecht and J. Y. Chang, *Anal. Chem.*, 58 (1985) 2375.