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ISOLATION OF RECOMBINANT HIRUDIN BY PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The purification of recombinant hirudin variant 2-Lys^{47} (rHV2-Lys^{47}), produced by a genetically engineered yeast strain, is described. rHV2-Lys^{47} expressed and secreted into the culture medium was the starting material for the purification process of hirudin from the culture broth after cell harvesting by centrifugation. Initial purification of the product by preparative reversed-phase high-performance liquid chromatography (HPLC) using step-gradient elution, followed by precipitation of rHV2-Lys^{47} in the presence of acetone, removed most of the contaminants from the culture medium. The pure product was obtained by successive preparative anion-exchange and reversed-phase HPLC on silica based stationary phases. Characterization of the final product by analytical HPLC, isoelectric focusing gel electrophoresis, quantitative amino acid composition and sequence analysis did not reveal any contaminants. Liquid secondary ion mass spectrometry was used to confirm its primary structure. The isolated product was tested in an inhibition assay of human α-thrombin and proved to be fully active.

INTRODUCTION

Natural hirudin, as first described by Markwardt[1,2], is one of the most potent inhibitors of α-thrombin. It is produced in trace amounts as a mixture of closely related polypeptides of 64–66 amino acids by the leech *Hirudo medicinalis*[3–8]. The rather limited quantities that can be isolated from this natural source and the additional complications caused by its heterogeneity have prevented more extensive and systematic pharmacological evaluations of natural hirudin. However, from various animal studies, evidence has been obtained that natural hirudin functions as a powerful anticoagulant *in vivo*[9]. The first clinical trials have substantiated these results and no adverse side effects have been observed when therapeutic doses were used[10,11].

Only recently has it become possible to produce hirudin in genetically engineered microorganisms, such as *Escherichia coli*[7,12–14] and *Saccharomyces cerevisiae*[15]. This permits the production of recombinant hirudin in quantities sufficient for systematic pharmacological evaluations, provided that the polypeptide can be efficiently purified.

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Recombinant hirudin variant 2 (rHV2) has been expressed and secreted from *Saccharomyces cerevisiae* as previously described[15]. A variant of HV2 in which Asn^{47} has been replaced by a Lys residue (rHV2-Lys^{47}) has been shown to have improved inhibitory properties[16].

In this article we describe the isolation of rHV2-Lys^{47} from the culture medium of a recombinant yeast strain by preparative high-performance liquid chromatography (HPLC). The final product has been characterized by analytical reversed-phase and anion-exchange HPLC, determination of its amino acid sequence and amino acid composition, isoelectric focusing gel electrophoresis, liquid secondary ion mass spectrometry (LSIMS) and its specific inhibitory activity against human α-thrombin.

MATERIALS AND METHODS

Materials

Chromozym PL (tosylglycylprolyllysine-4-nitroanilide acetate) was obtained from Boehringer Mannheim (Mannheim, F.R.G.). Human α-thrombin was obtained from Sigma (St. Louis, MO, U.S.A.). The purity and integrity of α-thrombin was assessed to be higher than 95% by 10% polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) as previously described[17]. rHV2-Lys^{47} of the described quality is also available from Sigma. Immobiline solutions and protein standards for the range pH 3.8–4.8 were obtained from Pharmacia/LKB (Uppsala, Sweden). Amino acid standards for quantitative amino acid analysis were from Pierce (Rockford, IL, U.S.A.) and sequencing reagents from Applied Biosystems (Foster City, CA, U.S.A.). All solvents used were of HPLC grade from Farmitalia Carlo Erba (Milan, Italy). The HCl used for amino acid composition analysis was of Ultrex grade from Baker Chemical (Phillipsburgh, NJ, U.S.A.). Stationary phases used for analytical HPLC separations were either from Macherey & Nagel (Nucleosil C_8; Düren, F.R.G.) obtained through Société Française Chromato Colonne (SFCC) (Neuilly-Plaisance, France) or from Pharmacia/LKB (Mono Q). Stationary phases used for preparative HPLC were obtained from either SFCC [silica derivatized with an adsorbed and cross-linked coating of poly(vinylimidazole) and poly(vinylpyrrolidone) that was partially quaternized (PVDI-silica) for anion-exchange chromatography] or from Separations Technology (ST/C_8 reversed-phase support; Wakefield, RI, U.S.A.).

Instrumentation

Preparative HPLC was performed on a ST/1200-XP HPLC system controlled by a microcomputer (Separations Technology). Analytical HPLC separations were carried out on an HP-1090 chromatograph equipped with an HP-1040A diode-array detector and a microcomputer (Hewlett-Packard, Waldbronn, F.R.G.). Specific activity determinations were performed with a 8451A diode-array spectrophotometer (Hewlett-Packard). Amino acid composition analysis was performed on a 420A derivatizer, connected to a 130A on-line HPLC analyzer equipped with a 920A microcomputer (Applied Biosystems) after total hydrolysis in a Pico Tag workstation (Waters Assoc., Milford, MA, U.S.A.). For protein sequencing a 477A protein sequencer was employed connected to a 120A on-line phenylthiohydantoin (PTH)-amino acid HPLC analyzer, equipped with a 920A microcomputer (Applied Biosystems).

LSIMS was performed on a VG Analytical ZAB-SE double-focusing mass spectrometer (Manchester, U.K.), using an ionization current of *ca.* 1 μA from 30-keV Cs^+.

Isolation procedure

Recombinant hirudin (rHV2-Lys^{47}) was expressed in yeast and secreted into the culture medium as previously described[15]. After cell harvesting by centrifugation, the supernatant was clarified by microfiltration at 0.1 μm through a hollow-fibre cross-flow filtration system (Amicon, Danvers, MA, U.S.A.). The filtered supernatant was directly loaded onto a reversed-phase C_8 column (50 cm × 4 in.; Separations Technology ST/C_8, 50 μm) and subsequently eluted using water–2-propanol (70:30) at a flow-rate of 300 ml/min. After removing the 2-propanol by rotaevaporation, rHV2-Lys^{47} was precipitated at 4°C with 80% (v/v) aqueous acetone in 20 m*M* sodium phosphate buffer (pH 7.5) in the presence of 150 m*M* NaCl. The precipitated material was pelleted by centrifugation and resolubilized in 20 m*M* sodium phosphate buffer (pH 6.4).

This solution was loaded onto an anion-exchange column (PVDI-silica, 15–20 μm, 25 cm × 2 in.), and rHV2-Lys^{47} subsequently eluted using a gradient from 0–1 *M* NaCl in 20 m*M* sodium phosphate (pH 6.4) in 65 min at a flow-rate of 80 ml/min.

Reversed-phase HPLC on a C_8 stationary phase (Nucleosil, 10 μm, 30 cm × 5 cm) was employed as the final purification step. An 80-min gradient of 0–50% 2-propanol in 10 m*M* sodium acetate (pH 5) at a flow-rate of 70 ml/min was used for elution. rHV2-Lys^{47} was pooled according to the results of analytical reversed-phase and anion-exchange HPLC. Fractions showing homogeneity in both analytical systems were pooled.

Desalting of the final material was achieved by reversed-phase HPLC on a C_8 stationary phase (Nucleosil, 10 μm, 30 cm × 5 cm) with 30% aqueous 2-propanol for elution at a flow-rate of 90 ml/min. After rotaevaporation of 2-propanol under vacuum, the final product was lyophilized from water.

Analytical characterization

Analytical reversed-phase HPLC was performed on Nucleosil-C_8 (3 μm, 10 cm × 0.46 cm) at a flow-rate of 1 ml/min with a gradient from 15% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) to 30% acetonitrile in 0.1% aqueous TFA in 15 min. For the analysis of crude samples, a 10-min wash with 15% acetonitrile in 0.1% aqueous TFA preceded the gradient. Detection was performed by UV absorption at 205 nm (for attenuation see individual figures).

Analytical anion-exchange HPLC was done on Mono Q (10 μm, 5 cm × 0.5 cm) at a flow-rate of 1 ml/min with a gradient from 20 m*M* Tris–HCl (pH 7.5) to 0.3 *M* NaCl in 20 m*M* Tris–HCl (pH 7.5) over 20 min (detection by UV absorbance at 280 nm with 0.05 a.u.f.s.).

Protein sequence analysis from the N-terminus was carried out by automated Edman degradation with identification of the individual PTH-amino acids by on-line HPLC analysis.

Complete amino acid composition analysis was performed by acid hydrolysis, followed by derivatization of the amino acids with phenyl isothiocyanate (PITC) and subsequent analysis by HPLC[18].

LSIMS was performed as described[19].

For isoelectric focusing the manufacturer's specifications were followed. An Immobiline system in the range pH 3.8–4.8 was modified as described[20].

The specific activity was determined by inhibition of human α-thrombin as described[16]. Assays were performed in polystyrene cuvettes at 37°C in 5 m*M* 1,4-piperazinediethanesulphonic acid (PIPES)–NaOH (pH 7.9), 0.18 *M* NaCl, 0.1% poly (ethylene glycol) (average molecular weight: 6000 daltons) at a final volume of 1 ml. Increasing amounts of rHV2-Lys^{47} were mixed with 4.3 n*M* α-thrombin [determined by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB) as previously described[22]] and thermally equilibrated for 3 min. The reaction was started by adding 20 μl of chromozym PL (20 mg in 3 ml water) to a final concentration of 0.2 m*M* and free thrombin was determined by *p*-nitroaniline release at 405 nm. Generally, 9–12 data points were determined and the velocity of substrate hydrolysis was analyzed by non-linear regression[16,21]. The amount of rHV2-Lys^{47} necessary for complete thrombin inhibition was deduced from the x-axis intercept. The concentration of the rHV2-Lys^{47} solution was determined independently by quantitative total amino acid analysis.

RESULTS

Isolation of rHV2-Lys^{47} from the culture medium of a genetically engineered yeast strain was achieved using a series of preparative HPLC steps. After separation of cells from the medium by centrifugation, the supernatant was clarified by cross-flow microfiltration with a 0.1-μm cut-off. The amount of rHV2-Lys^{47} present in this starting material and in the individual pools throughout the purification process was determined by analytical reversed-phase HPLC and integration of the absorbance peak at 205 nm. Purified rHV2-Lys^{47} which had been quantified by total amino acid analysis was used as a standard to calibrate the HPLC system.

The isolation of rHV2-Lys^{47} consisted of a number of individual purification steps, serving two main purposes: (1) the removal of contaminants from the culture medium unrelated to rHV2-Lys^{47} such as nutrients and other additives; (2) the separation of the product from closely similar protein contaminants. The initial purification step which is not listed here was the secretion of the molecule into the culture medium taking place at the level of fermentation. This resulted in a considerable enrichment of rHV2-Lys^{47} and facilitated the subsequent purification process. This is illustrated in Fig. 1 showing the reversed-phase HPLC analysis of the microfiltered culture supernatant. It was found that only the late-eluted peak in the chromatogram corresponds to rHV2-Lys^{47}. However, a closer look at the region of interest in the chromatogram showed that next to the major product peak there were a number of closely similar contaminants which had to be removed during the purification procedure. Analysis of these contaminants after the purification process showed that some of them represented either C-terminal degradation products or partially deamidated forms of rHV2-Lys^{47}.

The initial reversed-phase purification served as a rapid way of concentrating rHV2-Lys^{47} and of removing the bulk of the contaminants (Table I). A pigmented compound that was isolated together with the product as seen by monitoring the analytical reversed-phase HPLC at 450 nm (Fig. 2) was efficiently removed in the

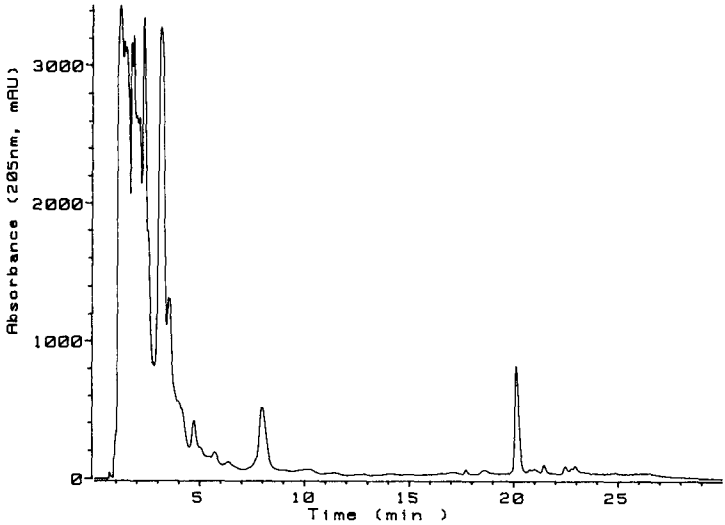

Fig. 1. Reversed-phase HPLC analysis of the culture supernatant after filtration at 0.1-μm cut-off. Experimental conditions: 10 cm × 0.46 cm column (Nucleosil C_8, particle diameter 3 μm); flow-rate, 1 ml/min; gradient, 15% acetonitrile in 0.1% aqueous TFA to 30% acetonitrile in 0.1% aqueous TFA in 15 min after a 10-min wash with 15% acetonitrile in 0.1% aqueous TFA; detection, UV absorption at 205 nm in milliabsorbance units (mAU).

subsequent precipitation of rHV2-Lys[47] with 80% (v/v) aqueous acetone at 4°C (Fig. 3). Precipitation was quantitative after 15 min and did not affect the activity of the molecule.

The pellet was isolated by centrifugation and dissolved readily in the initial buffer for the subsequent anion-exchange purification step. Anion-exchange HPLC was done on a silica matrix of particle diameter 15–20 μm and average pore diameter 30 nm derivatized with an adsorbed and cross-linked coating of poly(vinylimidazole) and poly(vinylpyrrolidone) (PVDI) that was subsequently quaternized[23]. The proteins were adsorbed in 20 m*M* sodium phosphate buffer at pH 6.4 and subsequently eluted with a gradient of increasing sodium chloride concentration. Fractions were pooled according to the results of analytical reversed-phase HPLC, giving a purity of 90% by integration at 205 nm (Fig. 4). The two major contaminants at about 8.5 and 12 min respectively corresponded to C-terminally degraded forms of rHV2-Lys[47]

TABLE I

PURIFICATION YIELDS OF RECOMBINANT HIRUDIN

Purification step	*Concentration (mg/l)*	*Total amount (mg)*	*Yield (%)*
Starting material	90	1930	100
1st Reversed-phase HPLC	677	1460	76
Acetone precipitation	619	1238	64
Anion-exchange HPLC	307	1093	57
2nd Reversed-phase HPLC	1510	801	42
Final product	–	750	39

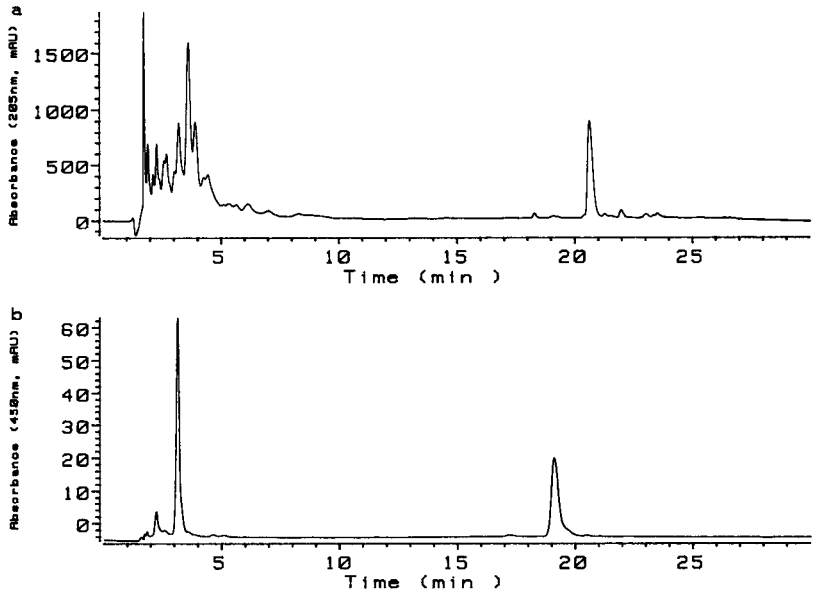

Fig. 2. Reversed-phase HPLC analysis after the first purification step by adsorption to a C_8 reversed-phase stationary phase. Experimental conditions as in Fig. 1 with detection at (a) 205 and (b) 450 nm.

with 63 and 64 amino acids as determined by quantitative total amino acid analysis and LSIMS (results not shown).

Final purification was achieved by reversed-phase HPLC on a silica-based C_8

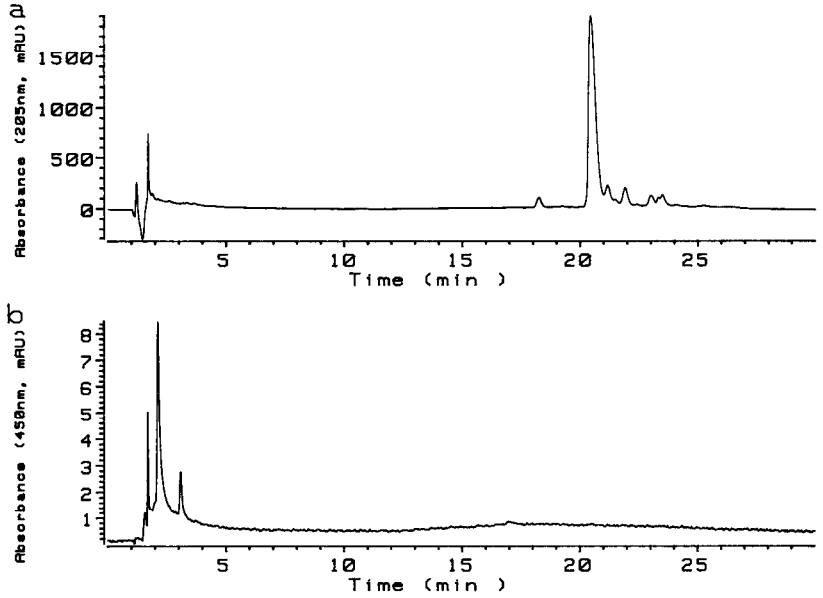

Fig. 3. Reversed-phase HPLC analysis after acetone precipitation of rHV2-Lys[47]. Experimental conditions as in Fig. 1 with detection at (a) 205 and (b) 450 nm.

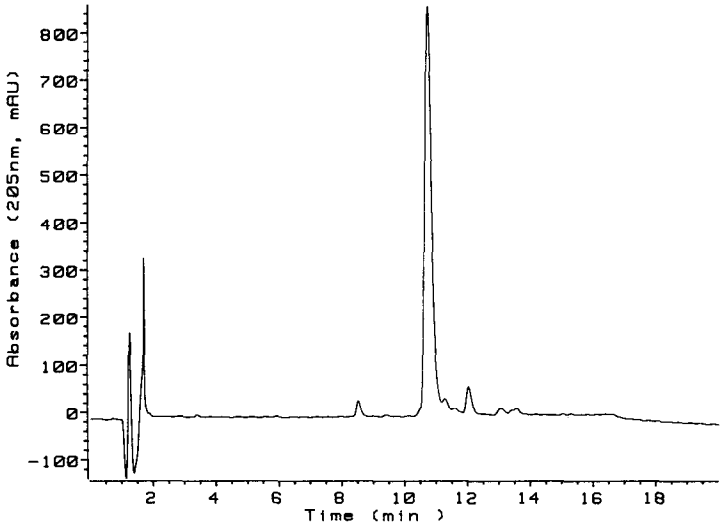

Fig. 4. Reversed-phase HPLC analysis of the pool obtained after preparative anion-exchange HPLC. Experimental conditions as in Fig. 1 but without the 10-min wash.

stationary phase with particle diameter 10 μm and average pore diameter 12 nm. rHV2-Lys^{47} was eluted using a gradient of increasing 2-propanol concentration in 10 m*M* sodium acetate at pH 5. Fractions which did not show any contaminating peaks by either analytical reversed-phase HPLC at pH 2 or by anion-exchange HPLC at pH 7.5 were pooled. After rotaevaporation of 2-propanol and desalting by reversed-phase HPLC, the final product was lyophilized from water and stored at -20°C. Analysis of the material after several months of storage, by reversed-phase or anion-exchange HPLC, did not reveal any degradation (Figs. 5 and 6).

rHV2-Lys^{47} isolated by the procedure described was further characterized by the following analytical methods (results not shown): (1) quantitative amino acid analysis after total acid hydrolysis; (2) complete amino acid sequence determination by automated Edman degradation; (3) assignment of disulphide bridges between residues 6–14, 16–28 and 22–39 as in the natural molecule by sequence analysis of peptides obtained after digestion with thermolysin. These criteria confirmed the primary structure and purity of the isolated molecule.

Isoelectric focusing gel electrophoresis on Immobiline gels was subsequently employed in the range pH 3.8–4.8 to verify the homogeneity of the purified rHV2-Lys^{47} with respect to its isoelectric point (p*I*) (Fig. 7). No contaminating bands were detected even when overloading the gel with 50 μg rHV2-Lys^{47} and the measured p*I* of 4.30 corresponded exactly with the calculated value (DNASTAR, Madison, WI, U.S.A.). The resolution of the gel system was determined to be ± 0.01 pH units. In an independent experiment it was shown that forms of rHV2-Lys^{47} which were deamidated at individual amino acid residues were easily discriminated from the correct molecule in this gel system.

Further structural information on the isolated molecule was obtained by LSIMS as described[19]. A wide scan gave an average mass of 6907.0 $\pm$ 1 dalton for

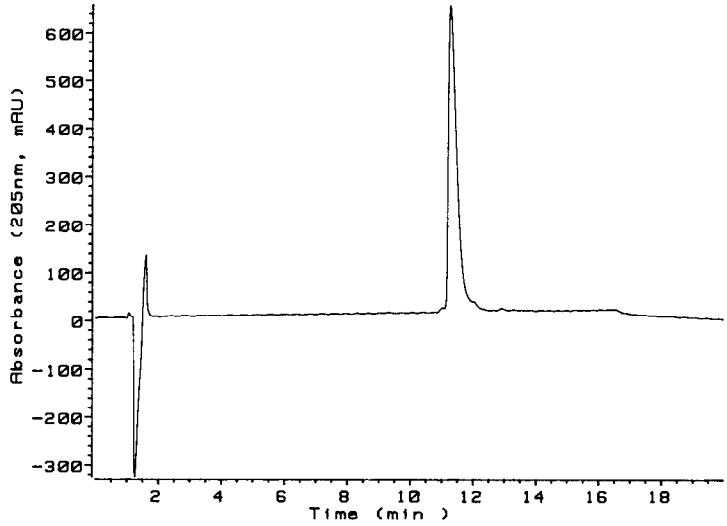

Fig. 5. Reversed-phase HPLC analysis of the product obtained after the final preparative reversed-phase purification step. Experimental conditions as in Fig. 1 but without the 10-min wash.

the protonated molecular ion (Fig. 8). Better accuracy of the measured average mass was obtained by using voltage scanning over only 300 mass units (narrow scan). This resulted in an average mass of 6907.5 daltons for the protonated molecular ion with an accuracy of ±0.5 dalton (data not shown). This value is identical to the calculated value of 6907.5 daltons.

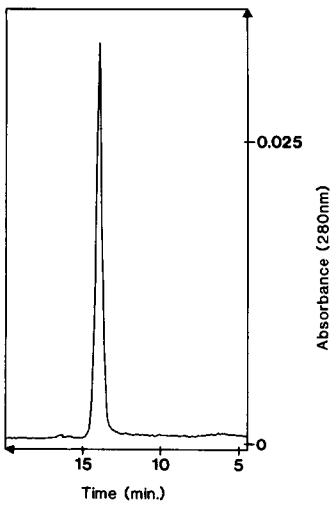

Fig. 6. Analysis of the final product by anion-exchange HPLC on Mono Q. Experimental conditions: 5 cm × 0.5 cm column (Mono Q, particle diameter 10 μm); flow-rate, 1 ml/min; gradient, 20 m*M* Tris–HCl pH 7.5 to 20 m*M* Tris–HCl pH 7.5 + 0.3 *M* NaCl in 20 min; detection, UV absorbance at 280 m.

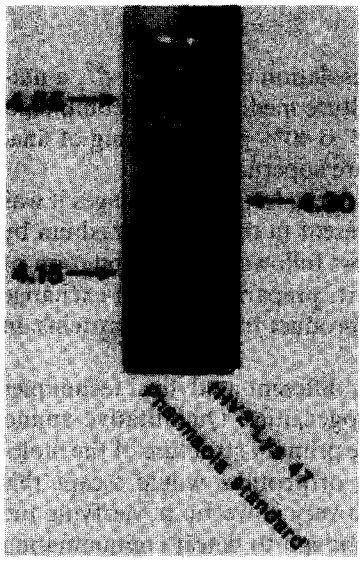

Fig. 7. Isoelectric focusing gel electrophoresis of the final product with an Immobiline system in the range pH 3.8–4.8. Left lane: p*I* standards (Pharmacia). Right lane: rHV2-Lys^{47} (purified).

The biological activity of rHV2-Lys^{47}, as prepared by the purification procedure described, was evaluated by determining its specific inhibitory activity against human α-thrombin, as described[16]. Briefly, increasing quantities of rHV2-Lys^{47} were added to a fixed amount of human α-thrombin, and the residual proteolytic activity of free thrombin was determined in a spectrophotometric assay, using a chromogenic substrate. This result verified that the rHV2-Lys^{47} preparation obtained was fully active as an α-thrombin inhibitor.

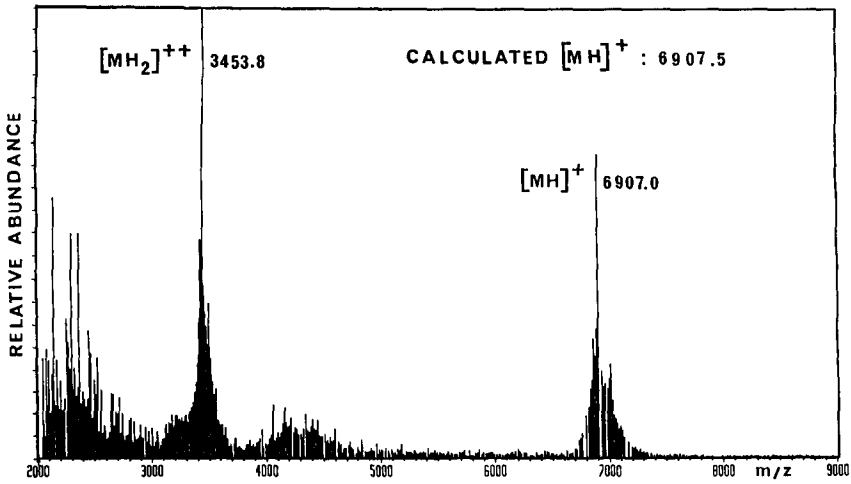

Fig. 8. Determination of the average molecular mass of rHV2-Lys^{47} by wide scan LSIMS. The spectrum shows the mono- and the diprotonated molecular ion.

DISCUSSION

The purification process described allowed the isolation of rHV2-Lys^{47}, a naturally occurring hirudin variant, starting from the culture medium of a recombinant yeast strain. The overall process yield ranged from 35 to 40% giving 750 mg of final product from *ca.* 2 g of starting material in the culture supernatant.

The purification process consisted of two major parts. In the initial stages it was necessary to remove the bulk of the contaminants present in the culture medium by adsorption of rHV2-Lys^{47} to a reversed-phase support followed by precipitation of the protein with aqueous acetone. In the second part, preparative anion-exchange and reversed-phase HPLC were used to separate the product from other proteins, in particular from some degraded forms.

Characterization of the isolated rHV2-Lys^{47} by different analytical techniques did not reveal any contaminants or structural heterogeneities. Quantitative amino acid analysis and amino acid sequencing confirmed the primary structure of the molecule. However, contaminants which are not proteins or peptides would escape this kind of analysis. In addition, these analyses, although very powerful in verifying the primary structure of the molecule, are not sensitive enough to detect contaminating proteins below the level of *ca.* 5%. They were therefore complemented by reversed-phase and anion-exchange HPLC analyses which had been optimized to separate either C-terminal degradation products or deamidated forms of rHV2-Lys^{47} which had been identified as possible contaminants. The sensitivity of these analytical methods was better than 1% with regard to these contaminants. All these techniques did not reveal any contaminants and the isolated rHV2-Lys^{47} had a minimum purity of 99% based on these criteria. Isoelectric focusing gel electrophoresis was subsequently used to measure the p*I* of rHV2-Lys^{47} which corresponded exactly to the calculated value.

LSIMS was employed as an additional criterion for assessing the structural integrity of the isolated rHV2-Lys^{47}. While this method is not able to detect contaminants below a level of *ca.* 10%, it has a high mass resolution and is thus able to detect structural changes in the molecule which result in a difference of 1 or 2 daltons, such ad deamidations of individual amino acids or reduced disulphide bridges.The isolated rHV2-Lys^{47} was homogeneous based on this criterion.

In conclusion it was shown that rHV2-Lys^{47} isolated by the procedure described was homogeneous with regard to its primary structure and pure to at least 99%. It was fully active as an inhibitor of human α-thrombin *in vitro*.

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