Purification and Biochemical Characterization of Recombinant Hirudin Produced by *Saccharomyces cerevisiae*

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ABSTRACT: Recombinant hirudin was produced by the yeast *Saccharomyces cerevisiae* using the α-pheromone prepro sequence to direct its secretion into the culture medium. The secreted hirudin was isolated to ≥95% purity as measured by 205-nm absorbance integration from a reverse-phase chromatogram. One major activity peak corresponding to the complete, correctly processed molecule and two minor activity peaks corresponding to C-terminally truncated forms were identified. The primary structure of the major peak, determined by N-terminal sequencing of tryptic peptides, was that predicted from the cDNA sequence, and the molecular mass analyzed by fast atom bombardment mass spectrometry (FAB-MS) was 6892.6 (calculated 6892.5). UV spectral analysis suggested that, in contrast to the natural molecule, recombinant hirudin produced by *S. cerevisiae* is not sulfated.

Hirudin is a low molecular weight polypeptide protease inhibitor synthesized in the peripharyngeal glands of the medicinal leech, *Hirudo medicinalis*. It is specific for thrombin, the ultimate regulatory enzyme of the blood coagulation cascade, and is extremely potent as an inhibitor; the K_i has been reported as approximately 10^{-11} M (Markwardt & Walsmann, 1958; Fenton et al., 1979; Dodt, 1984) and 10^{-14} M (Stone & Hofsteenge, 1986). Due to its high affinity for thrombin, relatively low inhibitor concentrations are sufficient to block blood coagulation.

The complete amino acid sequences of the natural hirudin preparations described to date display ≥85% sequence homology (Figure 1). We have designated them HV1 (Dodt et al., 1984), HV2 (Harvey et al., 1986), and HV3 (Dodt et al., 1986a) according to their chronological appearance in the literature. Recently seven additional variants have been described (Tripier, 1988).

Hirudin is a 65–66 amino acid polypeptide containing 6 cysteines located in the N-terminal half of the molecule. The C-terminal portion is rich in acidic residues and contains a stretch of homology (residues 40–48) with a thrombin cleavage site in prothrombin (V-T-V-E-V-I-P-R-S, residues 149–157, cleavage site 156–157) (Petersen et al., 1976). This observation led to the proposition that the prothrombin-like region may interact with the thrombin active site. Like fibrinogen, the principal substrate of thrombin, natural hirudin contains a sulfated tyrosine residue (position 63) (Petersen et al., 1976; Dodt et al., 1984). It is not clear what role this posttranslational modification plays with respect to the thrombin inhibitory activity of the molecule. Although a secreted protein, there is no indication that natural hirudin is glycosylated. Recently, the conformation of hirudin (HV1) in solution has been determined by proton NMR studies (Clore et al., 1987) and shows the structure to be composed of three domains: a central core, a protruding "finger" consisting of an antiparallel β-sheet, and an exposed loop. Despite the NMR studies that describe HV1 as having two β-sheets and no α-helices (Sukumaran et al., 1987), other structural information has recently been derived from circular dichroism studies where 27% α-helix has been measured for HV1 (Konno et al., 1988), which is consistent with the computational prediction of a "kinked" amphipathic α-helical structure for the C-terminus of that molecule (Krstenansky et al., 1987).

Hirudin prevents not only fibrinogen clotting but other thrombin-catalyzed reactions as well, for example, the activation of factors V, VIII, and XIII and platelet activation (Mürer, 1972; Walsmann & Markwardt, 1981; Hoffmann & Markwardt, 1984). Pharmacological studies have shown that natural leech hirudin is an effective prophylactic agent in animal models of venous and arterial thrombosis (Markwardt et al., 1982). Moreover, natural hirudin very efficiently prevents experimental disseminated intravascular coagulation (DIC) (Markwardt et al., 1977; Nowak & Markwardt, 1980; Ishikawa et al., 1980). Pilot studies with human volunteers showed that administration of 1000 ATU[1] of hirudin/kg of body weight produced a clear anticoagulant effect as monitored by the thrombin time with no influence on cardiac or respiratory function and no allergic or immune response (Markwardt et al., 1984). Furthermore, Vogel and Markwardt (1987) recently demonstrated that subcutaneous administration at 8-h intervals of 0.1 mg/kg hirudin to a patient with chronic DIC led to normalization of fibrinogen and platelet levels during the treatment.

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[1] Abbreviations: TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PTH, phenylthiohydantoin; DTT, dithiothreitol; 4-VP, 4-vinylpyridine; ATU, antithrombin units (NIH units); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Da, dalton; TCA, trichloroacetic acid; FAB-MS, fast atom bombardment mass spectroscopy; 4-PEC, *S*-(β-4-pyridylethyl)cysteine; $[MH]^+$, protonated molecular ion.

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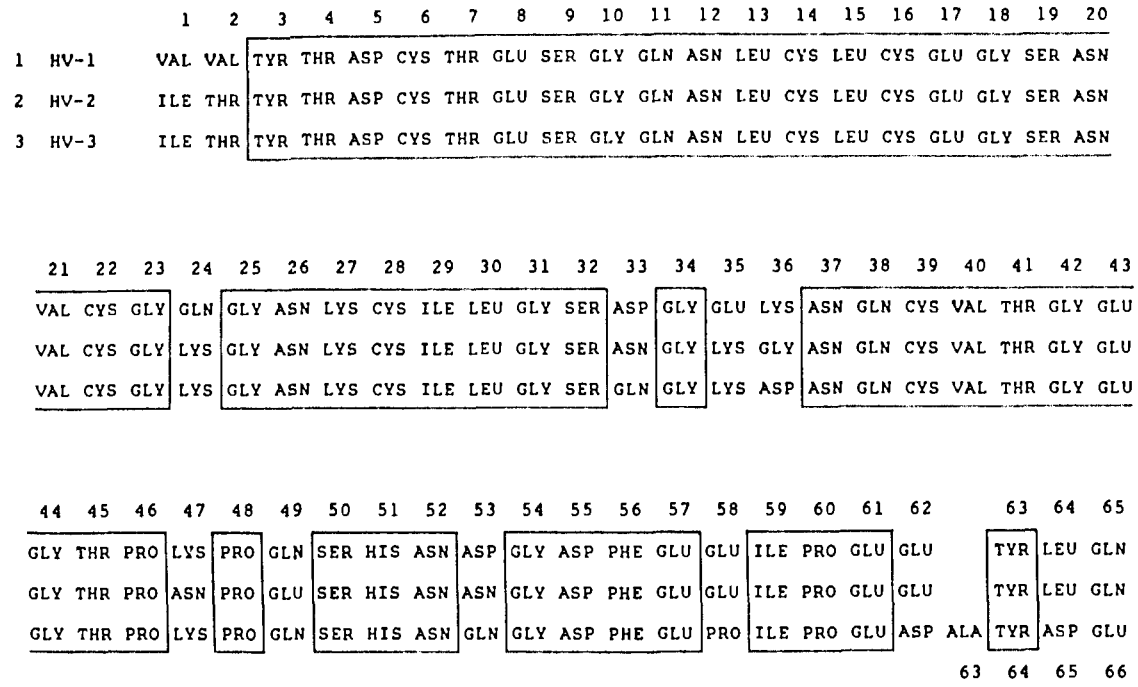

FIGURE 1: Primary structure and sequence homology of three hirudin preparations: HV1 (Dodt et al., 1984), HV2 (Harvey et al., 1986), and HV3 (Dodt et al., 1986).

Since it can be obtained only in trace amounts from leech extracts (~20 μg/leech head; Markwardt, 1970), hirudin has become an attractive target for genetic engineering. A number of products of pharmaceutical interest have been produced by recombinant DNA techniques, and the most widely used host has been *Escherichia coli*. Hirudin expression in *E. coli* has been reported (Bergmann et al., 1986; Dodt et al., 1986b; Fortkamp et al., 1986; Harvey et al., 1986). One of the drawbacks in producing recombinant proteins intracellularly in *E. coli* is that a large fraction of the peptide produced can contain an additional N-terminal methionine residue corresponding to the translation initiation codon. It was found that the portion of hirudin produced by *E. coli* that contains an extra methionine displayed greatly reduced specific activity compared to the correctly processed fraction (our unpublished results). In addition, low expression levels were obtained due to proteolysis of the hirudin within the *E. coli* cell. To circumvent these problems, we have produced hirudin using a *Saccharomyces cerevisiae* secretion vector based on the α-pheromone export signals (Loison et al., 1988). Here we describe the purification and characterization of recombinant hirudin (rHV2) from the yeast culture supernatants.

Materials and Methods

Materials

Reagents and Instrumentation. Media components were from the following suppliers: yeast nitrogen base without amino acids and casamino acids (DIFCO, Detroit, MI), glucose monohydrate (Roquette, Aniche, France), and ethanol (Merck, Darmstadt, FRG).

Chromozym TH, tosylglycylprolylarginine 4-nitroanilide acetate (No. 206849), was obtained from Boehringer Mannheim (Mannheim, FRG), natural hirudin (H4256) was from Sigma (St. Louis, MO), and bovine thrombin was from Roche (Basel, Switzerland). Protein (low molecular weight) standards for SDS–PAGE were from Bethesda Research Laboratories (Gaithersburg, MD) and myoglobin peptides from BDH (Poole, England). The Immobiline solutions for a pH gradient 3.8–4.8 were obtained from LKB (Sweden). Standard proteins from the low p*I* calibration kit (pH 2.5–6.5) (Pharmacia, Uppsala, Sweden), glucose oxidase p*I* = 4.15 and soybean trypsin inhibitor p*I* = 4.55, were used for the standard curve.

TPCK-trypsin for tryptic mapping was from Worthington (Malvern, PA) and 4-vinylpyridine from Aldrich (Milwaukee, WI). PTC amino acid standards for quantitative amino acid composition analysis were from Pierce (Rockford, IL). All sequencing reagents were obtained from Applied Biosystems (Foster City, CA). All solvents used were of HPLC grade. Acetonitrile used for protein purification and HCl (Ultrex grade) for amino acid hydrolysis were purchased from Baker Chemical Co. (Phillipsburg, NJ).

Batch fermentation cultures were performed by employing 20-L bioreactors from LSL-Biolafitte (St. Germain-en-Laye, France). Filtration of culture medium was accomplished by using either a Millipore HPCF 230 F1 system or a Romicon HF Lab5 filtration unit. Cell removal was achieved by using Millipore 0.45-μm membranes or Romicon cartridges with molecular weight cutoffs of 50000 or 100000 (HF5-43-PM50 or HF5-43-PM100).

All HPLC separations of proteins or peptides were performed on Hewlett-Packard HP1090 chromatographs equipped with 1040A diode array detectors. HPLC separations of amino acids were performed on a Beckman system (114M pumps, 421A controller) with a WISP autosampling device (Waters-Millipore, Milford, MA). Amino acid sequence analysis was accomplished by using an Applied Biosystems gas-phase sequencer (Model 470A) with phenylthiohydantoin amino acids identified by reverse-phase chromatography on an on-line PTH analyzer (Model 120).

Positive and negative FAB mass spectra were obtained by using a VG Analytical ZAB-SE double-focusing mass spectrometer (Manchester, England).

Methods

Yeast Expression Vector. Plasmid 1833 is an *E. coli*–yeast shuttle vector designed to direct the secretion of foreign proteins in yeast. It contains a fragment derived from pJDB207 (Beggs, 1981) that encompasses the 2-μm segment and the yeast *LEU2* gene, as well as the Ap^R gene and the origins of

replication of pBR322. The other DNA fragments inserted in this plasmid are the yeast *URA3* gene and the expression cassette for the hirudin gene, which corresponds to a fusion of HV2 mature coding sequence to the yeast MFα1 prepro sequences (Kurjan & Herskowitz, 1982). In the corresponding protein fusion, the Lys–Arg residues corresponding to amino acids 84–85 of the prepro region are immediately followed by the Ile–Thr of mature hirudin.

Yeast Fermentation Conditions. Batch cultures (14 L) of *S. cerevisiae* strain TGY1sp4-3 transformed with plasmid pTG1833 (Loison et al., 1986, 1988) were performed by using the following medium composition (L^{-1}): yeast nitrogen base without amino acids, 14 g; casamino acids, 10 g; glucose, 1 g; ethanol, 20 g.

Temperature was regulated at 30 ± 0.1 °C, and the pH was adjusted to 5.5 ± 0.1 with 4 N NaOH and 2 N H_2SO_4. Aeration was maintained at 1 VVM and agitation speed varied so that the dissolved oxygen concentration (measured by an Ingold polarographic oxygen electrode) remained above 20% saturation. At an absorbance A_{600nm} of ~20 (after 54 h of growth), cultures were harvested and the clarified medium concentrated by using either the Millipore or Romicon filtration apparatus.

Purification of rHV2. Clarified concentrated culture supernatants containing rHV2 were loaded on a semipreparative Brownlee Aquapore RP300 (C-8) 7-μm (0.7 × 250 cm) reverse-phase column, equilibrated in solvent A (0.1% TFA/water). Elution with solvent B (0.1% TFA/acetonitrile) was performed at 4 mL/min in a two-step gradient: 0–15% solvent B in 15 min and 15–30% in 30 min. Column fractions were screened with the chromogenic assay. Active fractions were dried under vacuum (Speed Vac, Savant Instruments) to remove acetonitrile and TFA, resuspended in 20 mM Tris-HCl buffer, pH 7.5, and were further purified by anion-exchange chromatography on an HR10/10 Mono Q column equilibrated in the same buffer. Elution was performed over 60 min with a linear 0–0.25 M NaCl gradient, followed by a 0.25–0.5 M NaCl wash of the column.

The major activity peak was rechromatographed on the Aquapore RP300 (C-8) reverse-phase column with a three-solvent system: solvent A, 10 mM HFBA/water; solvent B, 10 mM HFBA/acetonitrile; solvent C, 10 mM HFBA/1-propanol for altered selectivity (Roitsch & Barnes, 1985). Separation was achieved by simultaneous gradients of 14% B/h and 25% C/h. A final purification step on Aquapore RP300 was accomplished by using a shallow 0.1% TFA/acetonitrile gradient (15% or 20%/h). Purified hirudin was dried under vacuum and stored at −20 °C until subsequent analysis.

Antithrombin Activity (Chromogenic) Assay. Inhibition of bovine thrombin amidolytic activity with the synthetic substrate Chromozym TH is measured at 410 nm. The units of antithrombin activity are calculated from a standard curve constructed by using commercially available natural hirudin. One antithrombin unit (ATU) neutralizes 1 National Institutes of Health (NIH) unit of thrombin at 37 °C, and 1 NIH unit clots a standard fibrinogen solution in 15 s at 37 °C. If thrombin inhibition exceeds 40%, the test sample is diluted in assay buffer until the value is lower than 40% inhibition.

Hirudin standard (50 antithrombin units/mL in water) is diluted 10-fold in assay buffer (0.1 M Tris-HCl, pH 8.5, 0.15 M NaCl, 0.1% PEG 6000) to use amounts from 10 to 60 μL (0.05–0.3 ATU) for the standard curve. Stock substrate, Chromozym TH, is made 10 mM in water. Immediately prior to the assay 1 mg/mL stock bovine thrombin (63 units/mg) in 0.25 M sodium phosphate, pH 6.5, and 0.1% PEG 6000 is diluted 10-fold in the assay buffer.

rHV2 (generally 10-μL samples in the appropriate dilution or hirudin standard, 10–60 μL) is incubated for 30 s at room temperature with 60 μL of 0.1 mg/mL bovine thrombin in assay buffer. The volume of the reaction mixture is 990 μL. Ten microliters of 10 mM Chromozym TH is added to the incubation and mixed, and ΔOD 410 nm/min is recorded. A standard curve of ΔOD 410 nm/min vs units of hirudin can thus be constructed.

All reagents and measurements are at room temperature except for the thrombin, which is at 4 °C until the incubation. Measurements were made by using a spectrophotometer equipped with a multicell transport for six or seven cuvettes.

Clotting Assay. Inhibition of coagulation was determined as described by Markwardt (1970). Specific activity measurements were made by using bovine thrombin standardized with reference to the first international human thrombin standard (National Institute of Biological Standards and Control, London).

Protein Quantitation. Amino acid analysis (see below) was used as the ultimate method of pure hirudin quantitation. Determinations of protein at earlier stages of purification were as follows.

(*a*) *Bradford Assay.* Crude estimations of total protein have been made by using the Bio-Rad protein assay following the literature description of that procedure (Bradford, 1976).

(*b*) *Absorbance at 205 nm.* With the peptide backbone monitored at 205 nm, amounts of protein in HPLC effluents have been estimated from integrated recorder traces using an average extinction coefficient

$$\epsilon_{0.1\%}^{205nm,1cm} = 30$$

(Roitsch & Barnes, 1985).

(*c*) *Absorbance at 280 nm.* Protein quantitation based on the characteristic absorbance of aromatic amino acid residues and disulfide bridges at 280 nm has been accomplished from integrated recorder traces using the extinction coefficient

$$\epsilon_{0.1\%}^{280nm,1cm} = 0.45 \pm 0.02$$

determined as described in a subsequent section.

Reduction, Alkylation, and Amino Acid Analysis. Quantitative amino acid composition analysis was accomplished following total acid hydrolysis (6 N HCl, 105 °C, 24 h) of duplicate protein samples (500 pmol each) and precolumn phenyl isothiocyanate derivatization of $^1/_{10}$ the hydrolyzed sample, using the PICO-TAG system of Waters Associates (Milford, MA) (Bidlingmeyer et al., 1984). Cysteine content was established by reduction and alkylation with 4-VP prior to acid hydrolysis forming the stable derivative *S*-(β-4-pyridylethyl)cysteine (4-PEC) (Tarr, 1986).

Tryptic Peptide Mapping and Complete Amino Acid Sequence Analysis. A dried rHV2 sample was resuspended at a 1–2 mg/mL concentration in 0.1 M *N*-ethylmorpholine and 0.1 mM $CaCl_2$, pH 8.3, containing TPCK-trypsin at a 1:20 enzyme:substrate ratio. The reaction was allowed to proceed for 4 h at 37 °C and stopped by adjustment of the solution to 1% TFA. The peptides were separated by reverse-phase HPLC. After a 5-fold dilution in 0.1% TFA/water, the sample was injected on a Vydac 218TP54 (C18, 5 μm, 4.6 × 250 mm, The Separations Group, Hesperia, CA) column equilibrated at 0.2 mL/min in 0.1% TFA/5% acetonitrile/H_2O. The column was eluted by a linear gradient from 0 to 40% 0.1% TFA/acetonitrile over 60 min at the same flow rate. Peptides were detected simultaneously at 205, 254, and 280 nm, collected, and directly used for further analysis. Since 4-PEC

strongly absorbs at 254 nm, this facilitates rapid identification of cysteine-containing peptides and clear distinction of the N-terminal peptide, which contains four cysteines, and the C-terminal peptide, which contains only one.

N-Terminal amino acid microsequence analysis (automated Edman degradation) of the tryptic peptides was accomplished by using gas-phase sequencing with TFA/acetonitrile conversion of the amino acids into their PTH derivatives.

SDS–PAGE. Gradient Laemmli (1970) SDS–polyacrylamide gels from 10 to 25% acrylamide were run in the presence of 0.7 M β-mercaptoethanol. Samples (±4-VP treated or standards) were heated 5 min at 96 °C with sample buffer containing 0.7 M β-mercaptoethanol.

Immediately following the run, a gel was fixed 30 min with 50% TCA/water, stained for 10 min with 0.1% Coomassie Blue R250 in methanol/water/acetic acid (100:100:20, v/v/v), and destained overnight in water containing 5% methanol and 7.5% acetic acid.

Isoelectric Focusing. An Immobiline gel of pH gradient 3.8–4.8 was prefocused according to LKB Application Note 324. Samples of 5, 10, and 40 μg of highly purified rHV2 were loaded (4 mg/mL).

Fast Atom Bombardment Mass Spectrometry. rHV2 (~500 μg, ≥95% pure protein) prepared for FAB mass spectral analysis was eluted from a 7-μm reverse-phase (C-8) Aquapore RP 300 HPLC column (7 × 250 mm) into a glass tube and dried under vacuum to remove residual TFA and acetonitrile. The sample was neither subjected to reducing conditions nor derivatized.

The FAB source had an accelerating voltage of 8 keV. The fast xenon atom beam was operated with an emission current of 0.1 mA at 8 keV. Mass spectra were recorded with the VG 11-250 data acquisition system, and calibration was performed with CsI. Usually three scans were accumulated to provide a good signal. rHV2 was dissolved in 5% acetic acid/water, and then 1 μL of this solution, corresponding to approximately 20 μg of rHV2, was added to a 1-thioglycerol matrix.

The mass spectrometer was tuned at a resolution of 1000 to obtain the highest sensitivity. Chemical mass measurements were performed in the wide scan mode with ±0.5 Da accuracy as described (Van Dorsselaer et al., 1989).

The protonated peak of rHV2 was easily seen on the oscilloscope. In scanning up to 15000 Da, no signal was visible higher than 7000. Thus, for purity criteria, only the gap between mass 4000 and 8500 was studied.

Wavelength Scan. UV wavelength scans (200–350 nm) were made of hirudin eluting from the analytical Aquapore RP300 (C-8) reverse-phase support with an acetonitrile gradient containing 0.1% TFA. By use of a UV–vis diode array detector, wavelength scans at the upslope, apex, and downslope of a symmetrical peak were made and superimposed to check for peak homogeneity.

Extinction Coefficient at 280 nm. For the determination of the extinction coefficient at 280 nm, a symmetrical reverse-phase HPLC peak of ≥95% pure rHV2, recorded at both 205 and 280 nm, was integrated and quantified initially by using $\epsilon_{0.1\%}^{205nm,1cm} = 30$. The amount of protein was confirmed by amino acid analysis. Since the protein quantity was known by two different methods, the extinction coefficient at 280 nm was calculated by surface area integration of the tracing at 280 nm.

Results

Purification of Recombinant Hirudin. Hirudin was synthesized in *S. cerevisiae* by using a vector in which a cDNA coding for HV2 was inserted in frame with the MFα1 prepro sequence (Loison et al., 1988). It has been demonstrated that the yeast prepro α-factor leader sequence can be employed to direct secretion of heterologous proteins into the culture medium (Bitter et al., 1984; Emr et al., 1983; Brake et al., 1984; Singh et al., 1984). Secretion as opposed to intracellular production provides a useful initial purification compared to extraction from total yeast protein.

Table I: Purification (Average of Five Purifications)

step	protein amount[a] (mg)	act. (10^6 ATU)	spec act. (units/mg)	yield (%)
crude supernatant	ND	3.18	–	100
0.45-μm filtration	ND	2.24	–	70
concentration	858 (1)	2	2330	63
Aquapore RP300, TFA/CH_3CN	ND	1.19		37
Mono Q	36.5 (2)	0.44	12060	14
Aquapore RP300, HFBA/CH_3CN/1-propanol	17 (2)	0.21	12530	6.6
Aquapore RP300, TFA/CH_3CN	15 (3)	0.20	13500	6.3

[a] Protein amount was calculated by Bio-Rad assay (1), by integration of the active peak on the chromatogram at A_{280nm} (2) or A_{205nm}, and by amino acid analysis (3). ND, not determined.

rHV2 was concentrated and further purified by four HPLC steps as outlined in Table I. Semipreparative reverse-phase chromatography on Aquapore RP 300 C-8 isolated one broad peak of activity that was still highly colored (Figure 2A). The tailing of this peak was not recovered. Fractions with highest activity were then rechromatographed on Mono Q, which separated two active areas: one major and one minor peak (Figure 2B). The major activity peak was recovered and subsequently purified.

Reverse-phase chromatography of the major activity peak using a three-solvent system allowed the separation of some minor UV-absorbing contaminants (Figure 2C). A final reverse-phase separation was performed by using a shallow TFA–acetonitrile gradient to optimize the removal of remaining low-level contaminants with retention properties similar to those of rHV2 (Figure 2D).

Material prepared in this way was suitable for HPLC analysis, microsequencing, and amino acid composition analysis. Material to be analyzed by FAB-MS was rechromatographed on Aquapore RP300 (TFA/acetonitrile) and collected into glass vials.

The purification was achieved in five steps, giving an overall average yield from five 20-L fermentations of 6% for the major product (Table I). The low yield can be attributed to the number of steps required for the isolation of the major activity. Activity losses were incurred during the volume reduction, the removal of visible-absorbing compounds, and the removal of minor activity peaks.

Biochemical/Biophysical Properties. (a) Molecular Mass Determination. SDS–PAGE analysis of rHV2 on a gradient gel (10–25%) using the Laemmli (1970) system reveals a molecular mass of 8300 when cystines have been reduced with DTT and blocked with 4-VP (Figure 3). It should be noted that the addition of six *S*-(β-4-pyridylethyl) groups on the cysteine residues increases the molecular mass of rHV2 from ~6900 to 7500. Thus, the 8300 molecular mass observed is within 10% of that expected.

The average molecular mass of oxidized rHV2 (i.e., containing three disulfide bridges) calculated, allowing for the presence of natural isotopes of carbon, nitrogen, and oxygen, is 6892.5. Two separate FAB mass spectral measurements of rHV2 gave protonated chemical $[MH]^+$ mass values of

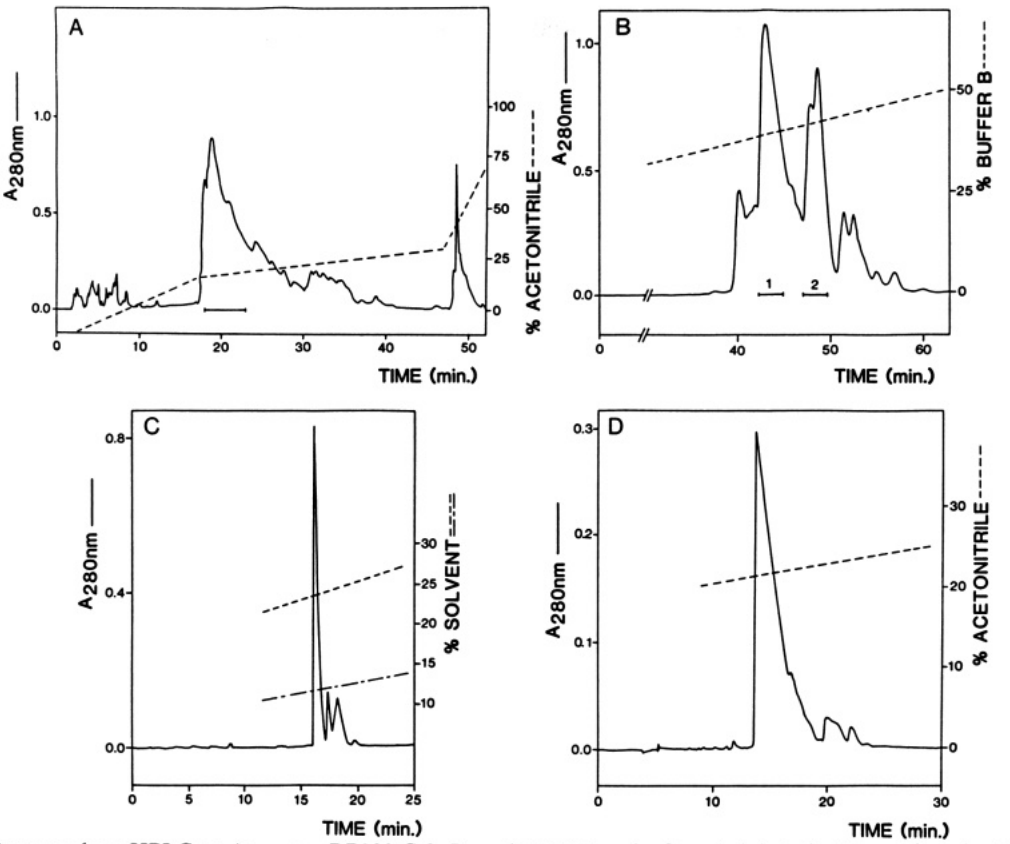

FIGURE 2: (A) Reverse-phase HPLC on Aquapore RP300 C-8, 7 μm (7 × 250 mm). Sample injected: 26 mg of crude rHV2. Solvent A: 0.1% TFA/water. Solvent B: 0.1% TFA/acetonitrile. Flow rate: 4 mL/min. Horizontal bar shows the active area pooled for further processing. (B) Anion-exchange chromatography on the Mono Q HR 10/10 column. Sample injected: 9 mg of rHV2 from first reverse-phase pool. Buffer A: 20 mM Tris-HCl, pH 7.5. Buffer B: 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl. Flow rate: 4 mL/min. Horizontal bars show the major (1) and a minor (2) active peak. (C) Reverse-phase HPLC on Aquapore RP300 C-8, 7 μm (7 × 250 mm). Sample injected: 1.65 mg of rHV2 from major Mono Q pool. Solvent A: 10 mM HFBA/water. Solvent B: 10 mM HFBA/acetonitrile. Solvent C: 10 mM HFBA/1-propanol. Flow rate: 2 mL/min. (---) Acetonitrile gradient. (– – –) 1-Propanol gradient. (D) Reverse-phase HPLC on Aquapore RP300 C-8, 7 μm (7 × 250 mm). Sample injected: 4 mg of rHV2 from second reverse-phase pool. Solvent A: 0.1% TFA/water. Solvent B: 0.1% TFA/acetonitrile. Flow rate: 2 mL/min.

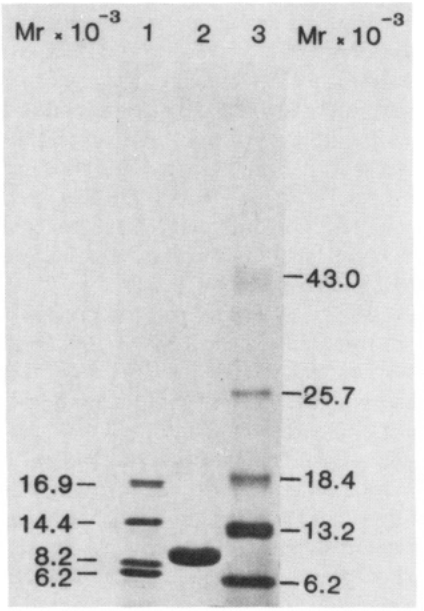

FIGURE 3: 10–25% polyacrylamide gradient–SDS gel with Coomassie Blue staining: (Lane 2) pure rHV2, reduced and 4-VP blocked (15 μg); (lanes 1 and 3) molecular weight standards from BDH and BRL, respectively (5 μg/band).

6893.5 and 6893.6 (Figure 4), which correspond to an average chemical mass of 6892.6 with an estimated error of ≤0.5 Da as described (Van Dorsselaer et al., 1989). These data are consistent with a completely oxidized molecule on the basis

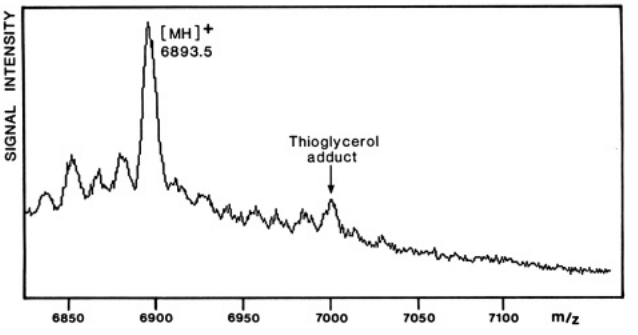

FIGURE 4: Accurate chemical mass measurement of the protonated rHV2 by FAB-MS. Calibration was done with cesium iodide (CsI) clusters of 6887.96 and 7147.77 Da. A thioglycerol (M_r 108) adduct was clearly visible at m/z $[MH]^+ + 107$.

of the calculated mass (6892.5) predicted from the cDNA sequence.

(*b*) *pI Determination.* The p*I* determined by computer calculation (DNASTAR, Inc., Madison, WI) is 4.12. The measured isoelectric point of rHV2 was 4.17–4.18 as compared to the standards that were migrated in an adjacent lane by using an Immobiline matrix and a narrow gradient range of 4.8–3.8 (data not shown).

(*c*) *Amino Acid Composition and Primary Amino Acid Sequence Analysis.* The total amino acid composition of rHV2 corresponds well with that predicted by the cDNA sequence (Table II). Discrimination between acid residues (Asp and Glu) and their amidated derivatives (Asn and Gln) was directly obtained by amino acid sequence analysis. Edman degradation

Table II: Amino Acid Composition Analysis of Recombinant HV2 (Average of 10 Analyses)

amino acid	composition	
	actual	expected
D + N	10.00	10
E + Q	11.26	11
S	3.88	4
G	10.37	10
H	0.92	1
R	0	0
T	4.86	5
A	0	0
P	3.44	3
Y	1.96	2
V	1.86	2
M	0	0
C	*a*	6
I	2.87	3
L	4.02	4
F	0.99	1
K	2.95	3

[a] Cysteine residues cannot be accurately determined by this method. Reduction and blocking with 4-VP prior to sequence analysis showed all six cysteines derivatized.

of 4-PEC rHV2 was performed on the complete molecule up to residue 40 and on the three easily resolved reverse-phase tryptic peptides (data not shown). The sequence determined was identical with that expected through glutamine-65 (Figure 1).

(*d*) *Reverse-Phase Retention Properties and Extinction Coefficient Determination.* The retention time of rHV2 in 0.1% TFA/acetonitrile on a Brownlee Aquapore RP300 column (C-8) as described under Materials and Methods is 27.23 ± 0.15 min (Figure 5A). The extinction coefficient of rHV2 at 280 nm was determined from these reverse-phase conditions to be $\epsilon_{0.1\%}^{280nm,1cm} = 0.45 \pm 0.02$. This low value for a 280-nm extinction coefficient is due primarily to the lack of tryptophan in this molecule and thus is predominantly due to the two tyrosine residues and, to a lesser extent, the three cystine bridges and the single phenylalanine present.

(*e*) *Purity Analysis.* Integration of the peak surface area at 205 nm after the final reverse-phase chromatography gave a purity ≥95%.

Amino acid analysis was a further quality check for accuracy of compositon as well as definitive quantification. The amino acid composition of the highly purified molecule corresponds well, within a 10% range, with that predicted by the cDNA sequence (Table II) as well as with the single unambiguous amino acid sequence detected by Edman degradation.

The retention time of rHV2 is characteristic of the complete and correctly matured 65 amino acid rHV2 molecule as determined by complete amino acid sequence analysis. In addition, UV wavelength scans from 240 to 320 nm at three positions of the peak are superimposable, indicating peak homogeneity (Figure 5B). One or two other active protein peaks, always minor, could be purified from batch cultivations performed by using several different media. These forms are easily separated by anion-exchange chromatography on the Mono Q column and can be further purified on Aquapore RP300 (C-8) using a TFA/acetonitrile gradient. They display characteristic retention times easily distinguishable from the major form on reverse-phase HPLC. Amino acid composition and/or sequence analysis showed that a more hydrophobic peak corresponds to rHV2 minus Gln^{65} (RT = 28.1 ± 0.1 min), and a more hydrophilic one is rHV2 minus Leu^{64}–Gln^{65} (RT = 24.6 ± 0.1 min). This difference in retention time was exploited in the purification scheme to optimize the isolation

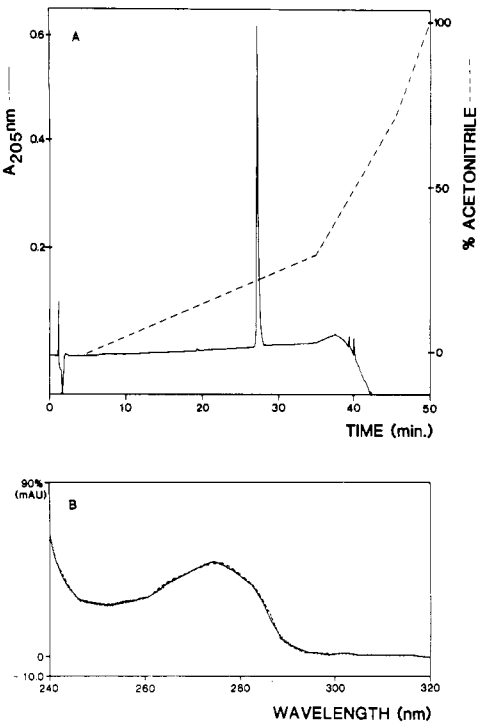

FIGURE 5: Analytical reverse-phase HPLC on Aquapore RP300 C-8, 10 μm (4.6 × 100 mm). Solvent A: 0.1% TFA/water. Solvent B: 0.1% TFA/acetonitrile. Flow rate: 1 mL/min. Sample injected: 8.5 μg of rHV2 (A). UV wavelength scans at the upslope (—), apex (···), and downslope (- - -) of the peak were normalized at 240 nm (B).

of the major active component.

The sample collected from a symmetrical reverse-phase HPLC peak was submitted to mass analysis on the range m/z 4000–8500. A single peak was observed on this wide mass range (data not shown). Though it is possible that suppression effects could hide contaminant peptides (Naylor et al., 1986), it has been shown that closely related hirudin molecules having similar or identical reverse-phase retention times have little if any suppression effects when two or three are mixed in approximately equivalent amounts (Van Dorsselaer et al., 1989). Our results show that no other peptide which would necessarily have similar hydrophobicity to rHV2 exhibited a detectable mass in the range m/z 4000–8500.

The presence of the single band on isoelectric focusing is another strong criterion of homogeneity. No contaminating species were observed even when the gel was overloaded with 40 μg of rHV2 (data not shown).

The antithrombin assay using a chromogenic substrate gives a specific activity value of ~13 500 ATU/mg for recombinant HV2 with the protein quantitation carried out by amino acid analysis. The value is similar to that found for rHV2 without an N-terminal methionine produced in *E. coli* and that found in natural isolates (our unpublished results). The antithrombin units determined by chromogenic assay are within 15% of those determined by standard clotting assay (F. Markwardt, personal communication). By use of the fibrinogen assay, the specific activity of rHV2 was determined to be 15 100 ATU/mg. This compares well with the activity of the natural hirudin preparation (16 300 ATU/mg) measured simultaneously.

Discussion

Recombinant HV2 secreted from *S. cerevisiae* has been purified to ≥95% purity from 20-L scale fermentation cultures, providing a new reproducible source of the biologically active, correctly processed thrombin inhibitor for evaluation of its antithrombotic properties. Active minor protein peaks can be

separated from the major activity by using a Mono Q matrix. These active peaks, which also have different retention properties on reverse-phase, have been shown by amino acid composition and sequence analysis to be missing one or two amino acids from the C-terminus. That these forms were present prior to the purification procedure was illustrated by chromatography of the crude supernatant on an analytical HPLC anion-exchange column. Indeed, proteolytic activity, which could be detected in some fermentation supernatants, clearly correlated with the presence of truncated forms (our unpublished results).

Craig and Wondrack (1986) reported the presence of carboxypeptidase and aminopeptidase activity during the production of recombinant growth hormone releasing factor using a similar expression system. The presence of C-terminally degraded forms has also been reported by Vlasuk et al. (1986) for secretion of recombinant human atrial natriuretic peptide from this expression system. These authors suggested that this processing may be due to an enzyme normally involved in the maturation of α-factor. However, this specific enzyme, carboxypeptidase yscα, has been shown to have a strong specificity toward basic residues such as Arg and Lys (Achstetter & Wolf, 1985). It seems therefore doubtful that this enzyme should cleave the Gln and Leu residues at the C-terminus of hirudin. More likely, these cleavages are due to one or more of the numerous other carboxypeptidases [for a review see Achstetter and Wolf (1985)] as proposed for human epidermal growth factor produced in yeast (George-Nascimento et al., 1988). Whether they occur intracellularly during secretion or extracellularly remains unclear. Indeed, carboxypeptidases can be present in the yeast culture medium by a possible perturbation of the secretion machinery or after low-level cell lysis.

The molecular weight of 8300 was determined in this work by SDS–PAGE analysis only after reduction under N_2 and alkylation using 4-VP. SDS–PAGE under stringent reducing conditions (10 mM DTT or 0.7 M β-mercaptoethanol) has reproducibly given molecular weights of approximately 13 000 for rHV2 (Loison et al., 1988). Molecular weight determination of rHV2 using HPLC gel filtration supports (either Superose 12 or TSKG2000) under dissociating conditions has yielded multimer values (data not shown). Molecular weight determinations of natural hirudins have been reported to be from 9.1 to 16.0 kDa by use of a wide range of physical techniques [see summary table, Bagdy et al. (1976)].

To address the monomer/multimer question, the underivatized recombinant molecule was analyzed by FAB-MS. While the FAB-MS molecular mass of rHV2 taken from reverse-phase conditions is clearly a monomer, this does not eliminate the possibility of multimerization in either physiological conditions or under various concentration equilibria. Further mass spectroscopy studies have been done on rHV2 and variants of this molecule obtained by site-directed mutagenesis confirming monomer molecular masses (Van Dorsselaer et al., 1989). These data suggest that the apparent multimer molecular weights are not due to covalent interactions.

Purity of the major final product has been assessed primarily by UV absorbance of a gradient-eluted reverse-phase HPLC column. While SDS–PAGE analysis is probably the most common method used in purity determination, the detection of underivatized hirudin by Coomassie Blue R250 after fixing in 50% TCA is ~10–20 times less than for standard proteins. In addition, attempts to silver stain hirudin using the method described by Bürk (1983) have reproducibly yielded negative stains. These difficulties in staining hirudin in SDS gels make this routine technique inadequate as a criterion of purity. UV monitoring of reverse-phase HPLC is a much more sensitive criterion of purity than gel analysis, as many of the potential contaminants from fermentation supernatants are UV-absorbing but either do not electrophorese or do not fix and stain on SDS–PAGE. Integration of a 205-nm absorbance trace of the final rHV2 peak gradient-eluted from reverse-phase HPLC showed high product purity. UV wavelength scans of rHV2 taken at three positions on a symmetrical reverse-phase peak gradient-eluted from reverse-phase HPLC are superimposable, a finding that is indicative of peak homogeneity.

To complement the quantitative purity analysis of reverse-phase HPLC, the homogeneity of rHV2 was further examined by FAB-MS in the mass range 4000–8500, where no major impurity was found. Use of FAB-MS as a supplementary qualitative analysis technique following reverse-phase HPLC provides additional evidence of sample identity and homogeneity. FAB-MS has been proposed for routine batch analysis and identification of recombinant molecules like insulin, m/z 5803.65, and biosynthetically designed growth hormone releasing hormone, m/z 5076.72 (Grotjahn & Steinert, 1987), which are both smaller than hirudin.

FAB-MS was used to measure the molecular mass of rHV2, which is one of the highest accurate (±0.2 Da) molecular mass determinations made to date. Similar accuracy has been published only for eglin C, measured m/z 8090.78 (calculated 8091.03; Richter et al., 1985). The molecular mass for Paim I has been determined to be 7422.0 (calculated 7420.2), the difference being explained by partial disulfide bonding (Hirayama et al., 1987). The mass of thioredoxin has been determined to be 11 748.0 (calculated 11 750.2), but the accuracy for such a high mass is poorer (Johnson & Biemann, 1987).

It is certain that mass spectroscopy will be used even more frequently in the future as its sensitivity, accuracy, and speed are assets for routine testing of high-purity proteins and peptides with pharmaceutical applications. FAB-MS batch analysis can only be employed on pure protein and in complementation with many other high-resolution techniques to detect and/or confirm structural differences in oxidation, reduction, amidation, and insertion/deletion of amino acids.

The UV absorption spectra of rHV2 from both *E. coli* and yeast (λ_{max} = 280 nm) compared to that of natural hirudins (λ_{max} = 270 nm) show a hypsochromic shift in this absorption maximum as well as an enhanced absorbance in the 255–290-nm region. The hypsochromic λ_{max} shift of recombinant hirudins compared to the natural molecules can be correlated with the difference in the tyrosine sulfate UV absorption spectrum (λ_{max} = 260.3 nm) (Bauerle, 1983) compared to that of underivatized tyrosine (λ_{max} = 274 nm). Our results are consistent with the lack of tyrosine sulfation in the recombinant products. Most importantly, the sample used for chemical mass determination has been taken from a symmetrical reverse-phase peak, and thus it cannot be a mixture of the sulfated and nonsulfated forms that are separable on this gradient (our unpublished observations). The experimental positive ion chemical mass determination is consistent with rHV2 not being sulfated. In addition, no signal was observed in the negative ion mode usually used for detection of sulfated peptides (Gibson et al., 1987) in the mass range expected for a sulfated form of rHV2.

Despite this probable lack of sulfation, the specific activity is comparable to that of any natural variant isolated. The removal of the sulfate group in natural hirudin has only a slight or no effect on thrombin inhibition as described by Dodt

(1984). However, desulfated natural products exhibit a 10-fold increase in K_i (Stone & Hofsteenge, 1986; Dodt et al., 1988).

As thrombin inhibitory activity of hirudin requires complete disulfide bridging of the molecule (Bagdy et al., 1976; Chang, 1983), the fact that the rHV2 specific activity measured is comparable to that of the natural molecule is indicative of correct disulfide bond formation. Indeed, the disulfide bridge assignment has been made for a recombinant HV2 variant (P. Lepage, personal communication) and has been shown to be as previously described for natural hirudin (Dodt et al., 1985). Accurate disulfide bridging of proteins secreted from yeast under control of a prepro α-factor export signal has also been demonstrated for α-interferons (Zsebo et al., 1986) and epidermal growth factor (George-Nascimento et al., 1988).

Initial animal studies with recombinant hirudins (including site-directed mutants of rHV2) have demonstrated in vivo antithrombotic efficacy, especially in venous thrombosis and disseminated microthrombosis models (Courtney et al., 1989). These preliminary studies are strong support for the future potential of recombinant hirudin as an antithrombotic pharmaceutical.

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Mass Spectrometry Analyses of Recombinant Hirudins (7 kDa)

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ABSTRACT: The use of liquid secondary ion mass spectrometry (LSIMS) in the characterization of related recombinant 7-kDa peptides illustrates the adequacy of average mass measurement by scanning at low resolution. The difficulty in using the high-resolution technique in the case of poor LSIMS sensitive peptides is discussed, as well as the fact that it does not give, for these molecular weights, any real advantage. The average (or chemical) molecular weights of three recombinant hirudin molecules, hirudin variant 2 (rHV2, 6892.4 Da), hirudin variant 2-Lys47 (rHV2-Lys47, 6906.5 Da), and hirudin variant 2-Arg47 (rHV2-Arg47, 6934.5 Da), ≤10 μg each, have been measured with an accuracy ≤0.3 Da in the narrow-scan mode and ≤0.5 Da (from the protonated molecular ion) in the wide-scan mode within 10–15 min; this allows easy distinction of the three 65 amino acid proteins, which differ by a single amino acid. These three molecules could also be distinguished from one another in a mixture. Mass spectrometry and limited sequence characterization of several minor, similarly isolated peptides identified them to be N-terminal additions and/or C-terminal deletions of rHV2-Lys47. LSIMS analysis is consistent with there being no covalent dimer of rHV2-Lys47 as a narrow scan of the 7-kDa molecular ion cluster at high resolution shows it not to be a doubly charged ion.

Natural hirudin is a potent polypeptide (65–66 amino acids) thrombin inhibitor isolated from the salivary glands of the medicinal leech, *Hirudo medicinalis* [for a review, see Markwardt (1985)]. Pharmacological studies in animal models have suggested the potential of natural hirudin as a pharmaceutical agent in both venous and arterial thrombosis (Markwardt et al., 1982).

As hirudin is found only in low quantities in leeches, production through recombinant DNA technology has been undertaken (Bergmann et al., 1986; Dodt et al., 1986; Fortkamp et al., 1986; Harvey et al., 1986). Three recombinant hirudin variants, with either asparagine (rHV2), lysine (rHV2-Lys47), or arginine (rHV2-Arg47) at position 47, have been produced by using a *Saccharomyces cerevisiae* host and a secretion vector based on the α-pheromone export signals (Loison et al., 1988; Riehl-Bellon et al., 1989). All three of these recom-

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