

Gene Expression and Secretion of the Anticoagulant Hirudin in *Saccharomyces cerevisiae*

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Hirudin, a 65-amino acid protein isolated from the salivary gland of the bloodsucking leech, *Hirudo medicinalis*, is a potent thrombin-specific inhibitor and blocks the thrombin-mediated conversion of fibrinogen to fibrin in clot formation. We have studied the gene expression and secretion of hirudin in yeast, *Saccharomyces cerevisiae*. A gene coding for hirudin was synthesized based on the amino acid sequence and cloned into a yeast expression vector YEG α -1 containing the α -mating factor pre-pro leader sequence and galactose-inducible promoter, *GAL10*. Recombinant *S. cerevisiae* was found to secrete biologically active hirudin into the extracellular medium. The secreted recombinant hirudin was recovered from the culture medium and purified with ultrafiltration and reverse phase high performance liquid chromatography. Approximately 1 mg of hirudin per liter was produced under suboptimal culture conditions and brought to about 90% purity in two steps of purification.

Hirudin is a protease inhibitor isolated from the salivary gland of the bloodsucking leech, *Hirudo medicinalis* (18). It is a potent thrombin-specific inhibitor with reported K_i values between 10^{-11} and 10^{-14} M (26), and a relatively low concentration of hirudin efficiently blocks thrombin-mediated conversion of fibrinogen to fibrin due to its high potency and specificity (28).

Amino acid sequences of several isoforms of hirudin have been published and designated as HV 1 (5), HV 2 (9), and HV 3 (6) according to their chronological appearance in the literature. Additional variants have also been described and a sequence has been predicted from cDNA (9). Hirudin is a 65-66 amino acid polypeptide that is comprised of a compact N-terminal domain containing three disulfide bonds and a C-terminal tail rich in acidic amino acid residues. Natural hirudin contains a sulfated tyrosine residue at position 63, but this post-translational modification is reported not to be essential for antithrombin activity, although "desulfato-" hirudin shows a 2-10 fold decrease in its thrombin binding affinity (3).

Structural studies on hirudin-thrombin complex re-

vealed a novel mechanism of thrombin inhibition which is unique compared with that of other serine proteases (4, 24). This mechanism involves noncovalent interaction with multiple sites on thrombin, including the catalytic site, the apolar binding site, and the anion-binding exosite. The interaction of hirudin with the basic specificity pocket of thrombin is not essential for its inhibitory activity. The C-terminal domain appears to bind the anion binding exosite on thrombin which is the non-catalytic site. This binding inhibits thrombin cleavage of fibrinogen by inducing a conformational change of thrombin, but does not inhibit the catalytic site for amidolytic activities.

In animal studies, hirudin has demonstrated efficacy in preventing various types of thrombotic diseases. In addition, hirudin exhibits low toxicity, little or no antigenicity, and a very short clearance from circulation (19). The apparent superiority of hirudin, which is a monocomponent with defined action, over a classical anticoagulant, heparin, which has many and varied activities in the prevention of thrombosis and the limited availability of natural hirudin, encouraged the development of recombinant DNA methods for its large-scale production. Natural and synthetic genes coding for HV 1 and HV 2 have been expressed in *E. coli*, but the yield of active

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hirudin was low (7, 9). Good levels of HV 2 production were achieved using secretion vector in yeast with mating factor α promoter or PGK promoter (16). The *Saccharomyces* galactose-inducible promoters GAL1, GAL7, and GAL10 have been exploited extensively for regulated and high-level production of foreign proteins in yeast (22). We report here the expression and secretion of recombinant hirudin variant 2, using GAL10 promoter, yeast mating factor α pre-pro leader sequence, and the chemically synthesized hirudin gene based on the amino acid sequence.

MATERIALS AND METHODS

Strains and Plasmids

Haploid *S. cerevisiae* 2805 (*Mat a pep4::HIS3 prb1- δ can1 GAL2 his3 δ ura3-52*) and 2806 (*Mat a pep4::HIS3 PRB1 his3 δ ura3-52 gal2*) were used for assays of β -galactosidase activity and for gene expression and secretion of hirudin. *E. coli* HB101 (*F⁻ hsdS20(r⁻m⁻) recA13 proA2 galK2*) or DH5 α (*F⁻ lacZ Δ M15 hsdR17(r⁻m⁻) gyrA36*) was used for cloning of genes and propagation of plasmids.

Plasmid YEp352 which is a multicopy number yeast-*E. coli* shuttle vector containing pBR322 sequence, yeast URA3 and yeast 2 μ replication origin (10) was used for the backbone of the general yeast expression and secretion vectors. To construct a promoter-*lacZ* fusion plasmid, pBM258 (*GAL1-GAL10* promoter) (14), pMA 56 (*ADH1* promoter) (1), pPH05 (*PHO5* promoter) (2), pLG669-Z (*CYC1* promoter) (8) and YEpIPT (*PGK* promoter) (11) were used as promoter sources and YEp353 (21) as *E. coli* β -galactosidase gene source. YEp70 α T (11) was used as PCR template to obtain mating factor α pre-pro leader sequence.

Media

For general cultivation of auxotrophic yeast strains, cells were grown at 30°C on YPD medium (2% peptone, 1% yeast extract and 2% glucose). All plasmid-bearing yeast cells were grown in minimal selective medium containing, per liter, 0.67 g of yeast nitrogen base without amino acid, 0.7 g of amino acid mixture lacking uracil and 20 g of glucose (MinGlu); 20 g of galactose (MinGal). Low inorganic phosphate (Pi) medium was prepared according to Rubin (23) and high-Pi medium was prepared by supplementing low-Pi medium with 0.2% KH₂PO₄.

Enzymes and Chemicals

Restriction endonuclease, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase and T4 polynucleotide kinase were obtained from KOSCO Biotech. [α -³²S] dATP α S was from Dupont. Calf-intestine alkaline phos-

phatase, RNase, human thrombin and Chromozym TH were from Boehringer Mannheim. ONPG (o-nitrophenyl- β -D-galactopyranoside) and standard hirudin from leech, *Hirudo medicinalis*, were purchased from Sigma Chemical Co. The GENECLEAN and MERMAID kits were obtained from Bio101 and were used according to the manufacturer's instructions.

Recombinant DNA Techniques

General DNA manipulations were performed as described by Maniatis (17). DNA fragments required for subcloning experiments were gel purified using GENECLEAN and MERMAID kit. Yeast cells were transformed using the lithium-acetate method (12).

Polymerase Chain Reactions (PCR)

PCR was done in 100 μ l volume with the following components: 10 μ l 10 \times Taq polymerase reaction buffer (Boehringer Mannheim), 10 μ l 10 \times dNTP mixture (2 mM), 1 μ l primer I (100 pmole) (dCAATGAATTCGAT-TAAAAGAATGAGATTTTC), 1 μ l primer II (100 pmole) (dTTTATCTAGAGATACCCCTTCTTCTTAGC), 5 μ l template DNA solution (20 ng), 72 μ l sterile dH₂O and 1 μ l Taq DNA polymerase (5 units) (Boehringer Mannheim). Amplification was done in a DNA thermal cycler (Ericomp Instrument) using the program set to denature DNA at 94°C for 1 min., anneal at 50°C for 2 min. and extend at 70°C for 3 min.. This sequence was repeated for 25 cycles.

DNA Synthesis

Oligonucleotides were synthesized using an Applied Biosystems model 391 DNA synthesizer and purified by polyacrylamide gel electrophoresis. All purified oligomers except the 5' end segment were phosphorylated, mixed in equimolar ratios, and ligated. The full-length synthetic gene was purified by MERMAID kit after agarose gel electrophoresis.

DNA Sequencing

DNA sequence of the synthetic gene encoding hirudin was determined by dideoxy chain-termination method (25) using fragments subcloned into pBluescript.

β -Galactosidase Activity Assay

β -Galactosidase activities were measured according to Miller (20). Single colonies of yeast transformants from MinGlu plate were grown at 30°C until an optical density at 600 nm of about 1.0 was reached. Cells permeabilized with chloroform and SDS were assayed for β -galactosidase activity using ONPG as substrate. The assay results were normalized with respect to culture cell density (OD₆₀₀/ml) and incubation time (min.).

Antithrombin Activity Assay

Determination of thrombin inhibitory activity in culture supernatants was done using chromogenic thrombin substrate, Chromozym TH. The amidolytic cleavage of Ch-

romozym TH by thrombin was measured as the rate of increase in absorbance at 405 nm with microtiter plate reader (Biorad). Antithrombin activity was expressed in ATU: one unit neutralized 1 NIH unit of thrombin (Lot J, Sigma Chemical Co.) at 37°C. Commercial hirudin from leeches (Sigma Chemical Co.) was used as a reference standard.

Amino Acid Analysis and N-terminal Amino Acid Sequencing

The amino acid composition of purified proteins was analyzed by Beckman Amino Acid Analyzer (Model 6300) after 24 h of hydrolysis at 110°C. N-terminal amino acid sequencing was done by manual Edman degradation and the derivatized amino acid residues were identified by HPLC (Beckman System Gold).

RESULTS AND DISCUSSION

Promoter Selection

Promoter fragments of various yeast genes were isolated from a number of yeast expression vectors in order to screen for the strong promoters. Promoters tested in this study were three constitutive expression promoters (*CYC1*, *ADH1* and *PGK*) and two inducible expression promoters (*GAL1-GAL10* and *PHO5*). Each of these promoters was fused into a reporter gene, *lacZ* and the promoter strength of these constructs was scored by measuring the β -galactosidase activity. A series of β -galactosidase expression plasmids were constructed as

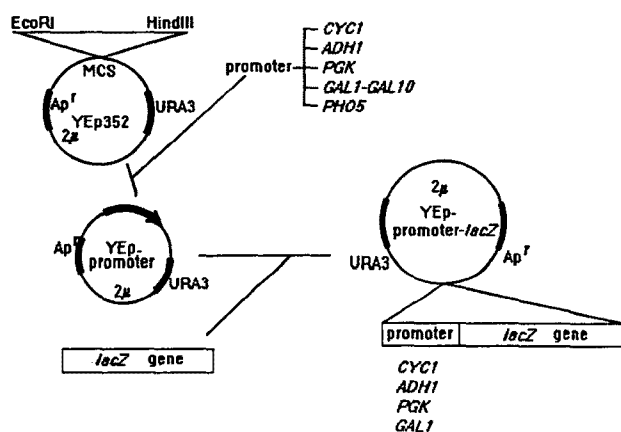


Fig. 1. Construction of promoter-*lacZ* in-frame fusion plasmids, YEp-promoter-*lacZ*.

Promoter fragments were originated from pLG669-Z (*CYC1*), pMA56 (*ADH1*), YEpIPT (*PGK*), pBM258 (*GAL1-GAL10*) and pPHO5 (*PHO5*). *E. coli lacZ* gene from YEp353 was modified by addition of a sequence (dGAATTCATG) to include *EcoRI* restriction site and translational initiation codon.

shown in Fig. 1. In order to construct various yeast promoter and *E. coli lacZ* in-frame fusion plasmids, each promoter fragment was inserted into the multiple cloning site of YEp352 (YEp-promoter). For the facilitation of in-frame gene fusion of promoter and *lacZ*, gene *lacZ* gene lacking initiation codon and the first seven codons obtained from YEp353 was modified by addition of the sequence (dGAATTCATG) containing *EcoRI* restriction site and translational initiation codon, and subcloned downstream to YEp-promoter. These plasmids (YEp-promoter-*lacZ*) were transformed to yeast *S. cerevisiae* 2805 strain and single colonies of transformant were grown in MinGlu, MinGal, high Pi or low Pi medium (Table 1). The result showed that all promoters employed directed the expression of β -galactosidase gene according to the original expression mode of each promoter. The *lacZ* expression levels of each promoter, however, differed to a considerable extent. Inducible promoters directed more β -galactosidase gene expression than constitutive expression promoters did. Among the promoters tested, *GAL10* and *GAL1-GAL10* divergent promoters showed maximum activities with a minimal difference between the two. This result suggests that galactose-inducible *GAL10* or *GAL1-GAL10* divergent promoter is suitable for expression vector to produce foreign proteins in yeast *S. cerevisiae*.

Construction of Secretion Vector YEG α -1

The construction strategy of yeast expression and secretion vector YEG α -1 is summarized in Fig. 2. To introduce secretion signal of yeast mating factor α gene to downstream of *GAL10* promoter, YEp352-GAL containing galactose-inducible *GAL1-GAL10* promoter was modified by gene fusion of 3' end of *GAL10* promoter and yeast mating factor α pre-pro leader sequence (ppL) (Fig. 2). Mating factor α ppL of YEp70 α T was modified by polymerase chain reactions with primer I and primer

Table 1. Comparison of β -galactosidase activities under the control of various yeast promoters in *S. cerevisiae* 2805 strain

Promoters	Media & carbon sources				
	YPD	MinGlu	MinGlu		MinGal
			High-Pi	Low-Pi	
host control	2.3	—	—	—	—
<i>CYC1</i>	254.0	284.7	—	—	1032.2
<i>ADH1</i>	354.3	359.3	—	—	213.2
<i>PGK</i>	260.4	266.0	—	—	513.5
<i>GAL1</i>	2.3	2.5	—	—	634.2
<i>GAL10</i>	2.7	2.4	—	—	1248.6
<i>GAL1-GAL10</i>	2.0	2.2	—	—	1300.0
<i>PHO5</i>	—	—	39.2	456.8	—

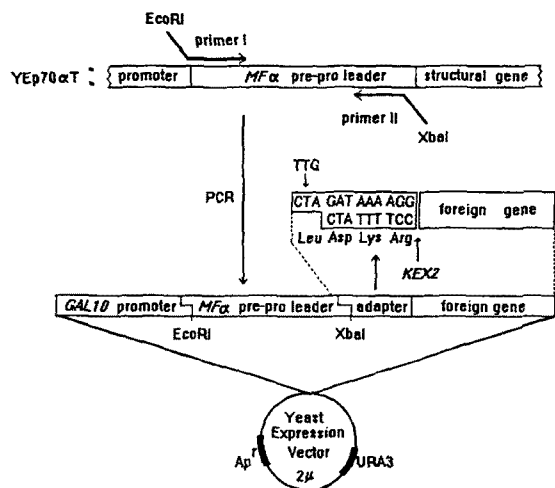


Fig. 2. Construction of yeast expression and secretion vector YEG α -1.

PCR products of primer I and primer II with YEp70 α T as template contain *EcoRI* and *XbaI* restriction sites at each end and sequences coding for 82 amino acid of the mating factor α pre-pro leader peptide. Adapter (dCTGGA-TAAAAGA) coding for a peptide (Leu-Asp-Lys-Arg) was needed for the expression of fusion protein composed of secretion signal peptide and foreign protein to be processed correctly by *KEX2* protease.

II for the purpose of eliminating the Glu-Ala-Glu-Ala dipeptidyl aminopeptidase processing site located downstream from the *KEX2* protease processing site (15) and to introduce appropriate restriction sites. The product of PCR contained unique *EcoRI* restriction site at 5' end of the fragment and a portion of the yeast mating factor α ppL (82 aa) modified at codon 82 to create *XbaI* site 3 aa upstream from the *KEX2* processing site. The resulting *EcoRI/XbaI*-digested 268 bp fragment of modified mating factor α ppL was inserted into YEp352-GAL and designated as YEG α -1.

Expression and Secretion of Recombinant Hirudin

The synthetic gene segment encoding hirudin was constructed from 10 overlapping synthetic oligonucleotides, as shown in Fig. 3. The nucleotide sequence of the hirudin gene was optimized by using codons preferred in *S. cerevisiae*, and additional restriction sites flanking the structural gene were added to allow only one orientation after ligation into the vector. To obtain correctly processed hirudin which has Ile at N-terminus, the yeast endoprotease processing site (Leu-Asp-Lys-Arg) was introduced to the 5' end of the synthetic hirudin gene. The *XbaI/SalI*-digested fragment was inserted into YEG α -1 and the resulting plasmid YEG α -HIR5 (Fig. 4) was used to transform the yeast host strain.



Fig. 3. Nucleotide and amino acid sequence of synthetic hirudin gene.

The sequence was constructed from ten overlapping synthetic oligonucleotides that ranged in length of 35 to 54 nucleotides. Synthetic oligomers (S1-S10) are bracketed. *XbaI* and *SalI* restriction sites at each end and the adapter sequence (Fig. 2) were co-synthesized with hirudin gene. Marked nucleotides are those which have been changed from the originally reported cDNA sequence (5) to represent preferred codons in *S. cerevisiae*. Three consecutive translational stop codons are underlined.

Yeast cells transformed with expression/secretion vectors with (YEG α -HIR5) or without (YEG α -1) hirudin gene were grown in different culture media for 48 h, and the antithrombin activities of the cleared broth were determined by chromogenic substrate assay. Cells harboring YEG α -HIR5 grown in a minimal medium (1 xUra⁻) containing galactose (MinGal) were found to secrete biologically active hirudin into the medium as shown in Fig. 5, where a series of serial dilution of the culture broth showed antithrombin activities. Control cells containing YEG α -1 which were lacking in the hirudin gene insert did not exhibit any detectable antithrombin activity. The broth from the culture of cells harboring hirudin gene grown on glucose medium (MinGlu) showed no antithrombin activity (data not shown), as expected, since the *GAL10* promoter was repressed by glucose and induced by galactose. The cells first grown in glucose me-

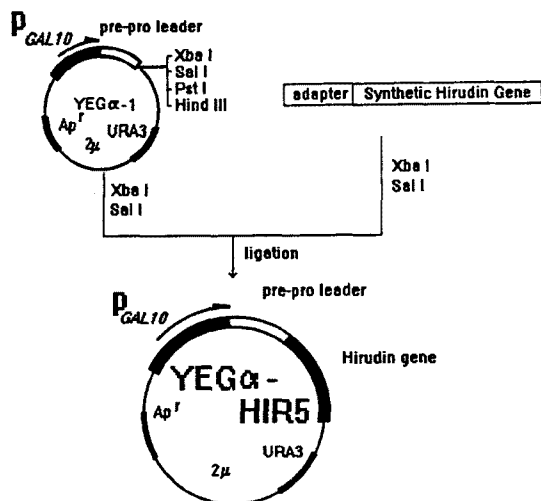


Fig. 4. Construction of YEG α -HIR5 for gene expression and secretion of hirudin.

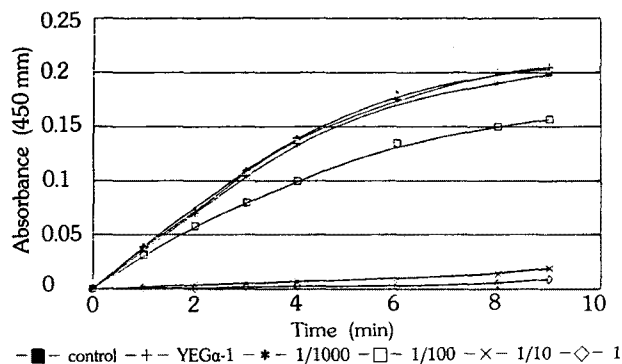


Fig. 5. Antithrombin activities of extracellular medium from cultures of cells containing YEG α -1 or YEG α -HIR5.

Antithrombin activities were assayed using chromogenic thrombin substrate (Chromozym TH). Control (—■—) with only thrombin and substrate in the assay mixture showed a rapid increase in the absorbance at 405 nm as did the control without hirudin gene (YEG α -1, —+—). Inhibitory activities of a series of diluted culture medium from cells containing YEG α -HIR5 are shown.

dium (MinGlu) prior to transfer to the galactose medium (MinGal) yielded a considerably lower antithrombin activity (ca. 1/10) compared to those grown directly on galactose medium from the beginning. This observation is to the contrary of the general practise for a galactose-inducible expression system where the cell culture is grown to a high cell density prior to the addition of galactose (16).

A yield of about 1 mg hirudin/L culture medium was obtained under the suboptimal culture condition employed in the present study. This yield is expected to be

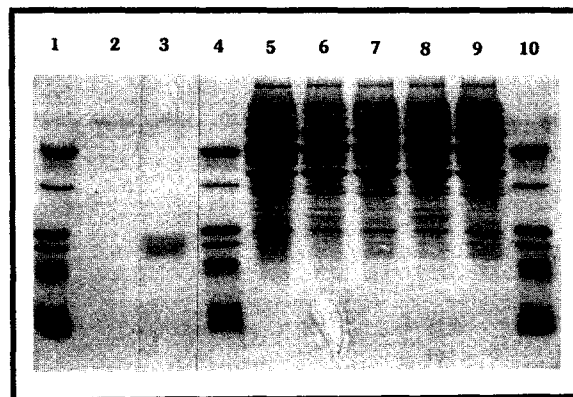


Fig. 6. SDS-PAGE analysis of extracellular medium and cell free extracts of cultures containing YEG α -1 or YEG α -HIR5 grown on different medium.

Lanes 1, 4, and 10; standard molecular weight markers (43.0, 29.0, 18.4, 14.3, 6.2, and 3.0 kDas) Lanes 2 and 3; extracellular medium (2: YEG α -1, MinGal medium; 3: YEG α -HIR5, MinGal medium) Lanes 5-9; cell free extracts (5: host cell only, grown on YPD medium; 6: cells with YEG α -1, grown on MinGlu medium; 7: cells with YEG α -1, grown on MinGal medium; 8: cells with YEG α -HIR5, grown on MinGlu medium; 9: cells with YEG α -HIR5, grown on MinGal medium)

improved by optimization of the culture medium and conditions.

Purification and Characterization of Recombinant Hirudin

The protein profiles of the broth from the cultures of cells containing either vector only or vector plus hirudin gene grown on galactose-minimal media (MinGal) were analyzed by SDS-PAGE after the broth was passed through ultrafiltration membrane (YM30, Amicon) (Fig. 6). The protein profile of the extracellular medium revealed a relatively simple pattern already at this stage. In the case of cells containing hirudin gene, a major protein band was located at the MW 12,000~13,000 region which corresponded to the reported molecular weight of non-alkylated hirudin (16), whereas control cells lacking hirudin gene showed no protein band on that molecular weight region. SDS-PAGE analysis of the cell free extracts revealed no prominent protein bands migrating to the same position as the major protein band in the extracellular medium.

To determine whether these major protein bands in the medium exhibit thrombin inhibitory activity, the concentrated broth was subjected to HPLC-purification, and the peak showing antithrombin activity was analyzed by SDS-PAGE. The broth which had been passed through YM30 membrane was concentrated by another passage through YM03 membrane, and the concentra-

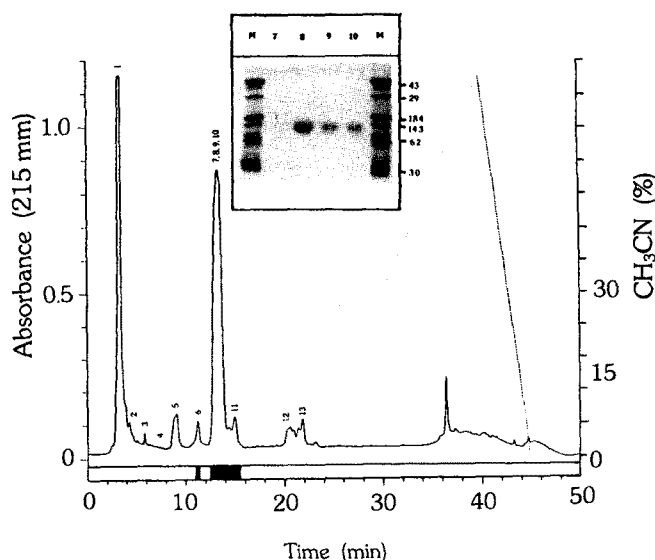


Fig. 7. HPLC chromatogram of concentrated extracellular medium from culture of cells containing YEG-HIR5 grown on MinGal medium.

Concentrated medium was chromatographed on C8 reverse phase HPLC column with 15~30% acetonitrile gradient over 30 min. Fractions exhibiting antithrombin activities (shown as black area beneath the peaks) were analyzed by SDS-PAGE, which is shown in the inset. Letter M denotes standard protein markers whose molecular weights are shown in kDas.

ted medium was chromatographed on C8 reverse phase HPLC column with 15~30% acetonitrile gradient over 30 min. (Fig. 7). Three isolated peaks corresponding to the fraction number 6, 7~9, and 10, respectively, were found to have antithrombin activity. When the fractions 7, 8, 9 and 10 of the major protein peak eluting at 22% acetonitrile were analyzed by SDS-PAGE, only a single protein band was detected in each fraction, and these protein bands gave the same mobility on the gel as the major protein bands found in the crude culture medium. The major peak with antithrombin activity from the HPLC column was rechromatographed on the same column under the same conditions and was shown to be about 90% pure (Fig. 8). The major protein peak from the second HPLC column was subjected to the analysis of amino acid composition and sequencing of N-terminal amino acid to confirm the identity of the recombinant protein. Amino acid composition of the purified recombinant protein shown in Table 2 was in good agreement with the theoretical values predicted from the DNA sequence of the hirudin gene. The N-terminal amino acid sequence of the recombinant protein was determined to be Ile-Thr-Tyr-Thr-Asp, which matched exactly to the predicted sequence, indicating that the

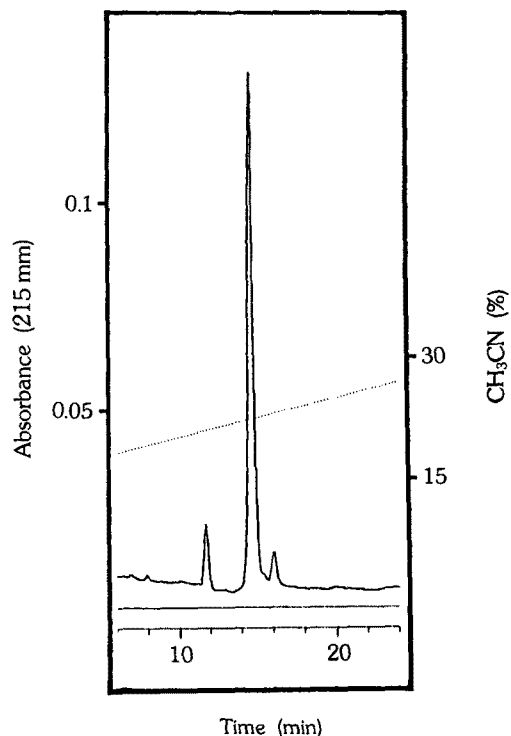


Fig. 8. HPLC chromatogram of the major activity peak rechromatographed on the second reverse phase column.

The chromatography condition was the same as described in Fig. 7.

Table 2. Amino acid composition of the secreted recombinant hirudin

Amino acid	Composition	
	Actual	Expected
Asp (Asn)	9.98	10
Thr	4.85	5
Ser	3.69	4
Glu(Gln)	10.89	11
Pro	3.00	3
Gly	9.94	10
Ala	0	0
Cys	6.34	6
Val	1.29	2
Met	0	0
Ile	2.81	3
Leu	4.00	4
Tyr	1.51	2
Phe	0.80	1
His	1.31	1
Lys	3.27	3
Arg	0	0
Trp	0	0

correct processing occurred at the Lys-Arg cleavage site of yeast mating factor α ppL to which the hirudin gene was directly fused. Two minor activity peaks from HPLC column could be the partially processed forms, although the N-terminal or C-terminal amino acid sequences of these peptides were not determined.

The specific antithrombin activity of the purified recombinant hirudin was assessed to be 13,000 ATU/mg protein based on the protein amount calculated from amino acid composition analysis. The protein concentration of hirudin varied considerably with the methods of determination. The obtained value of 13,000 ATU/mg protein was in general agreement with the reported values of specific activities of natural and recombinant hirudin variants which ranged from 12,000 to 15,000 ATU/mg protein (13).

In order to increase the expression level of hirudin gene while retaining the regulatable character of GAL promoter and secretion by the aid of yeast mating factor α pre-pro leader sequence, efforts are currently being made to incorporate GAL4 gene into the host chromosome whose product has been reported to increase the expression level of galactose-inducible promoters to a considerable degree.

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