PROTEIN EXPRESSION AND PURIFICATION **6,** 813–820 (1995)

Expression of a Synthetic Gene Encoding the Anticoagulant-Antimetastatic Protein Ghilanten by the Methylotropic Yeast *Pichia pastoris*

Robert G. Brankamp, Koti Sreekrishna, Philip L. Smith, Dale T. Blankenship, and Alan D. Cardin[1]
Hoechst Marion Roussel, Department of Molecular Genetics, 2110 East Galbraith Road, Cincinnati, Ohio 45215

Received March 29, 1995, and in revised form October 14, 1995

Ghilantens are a family of cysteine-rich inhibitors of the clotting enzyme, factor Xa, that are produced in the salivary glands of the South American leech *Haementeria ghilianii*. In this study, a gene, designed from the amino acid sequence of a specific ghilanten isoform, was assembled from eight double-stranded oligonucleotide fragments. A yeast expression plasmid, pPIC9HG-2, was constructed by making an in-frame fusion of the ghilanten-coding sequences with the region encoding the pre-pro α-mating factor signal sequence for secretion. The expression of ghilanten in pPIC9HG-2 was under the control of the methanol-inducible, alcohol oxidase (*AOX1*) promoter. *Pichia pastoris* yeast strains KM 71 and SMD 1168 were transformed with linearized pPIC9HG-2 to target integration of the plasmid to the chromosomal 5′-*AOX1* locus via homologous recombination. Both strains yielded His$^+$ transformants that secreted a potent anticoagulant activity into the medium. Product yield was improved by using buffered media (pH 6.0) supplemented with either casamino acids or a mixture of yeast extract and peptone. The protease-deficient strain, SMD 1168, secreted about a twofold higher level of *r*-ghilanten than KM 71. Significant clonal variation in the expression of *r*-ghilanten was found among the His$^+$ transformants. A high producing clone was selected for production at the 2-liter shake flask and 10-liter bioreactor scales. *r*-Ghilanten was recovered from the fermentation broths in a single step by heparin Sepharose affinity chromatography. Protein sequence analysis of the amino terminus showed that the correct processing to yield mature ghilanten varied with the fermentation conditions.

Leeches produce in their saliva a variety of highly potent and selective inhibitors to specific enzymes of the blood clotting mechanism . *Hirudo medicinalis* secretes a 65 amino acid inhibitor of thrombin termed hirudin (1–4). Analysis of the crystal structure of the thrombin–hirudin complex has yielded structural information which has been valuable in the design of small, organic inhibitors of thrombin (5). Recent clinical trials (Gusto II and TIM1-9)[2] indicate that thrombin inhibition leads to bleeding complications in patients. Thus, selective inhibitors to other enzymes of the hemostatic mechanism are being sought as alternative approaches in the treatment of vascular-related diseases. In that regard, Factor Xa (FXa) may be a preferred target for the treatment of thrombotic disorders (6–8), restenosis following angioplasty (9), and the blood-borne dissemination of cancer (10–12). FXa is the key enzyme regulating thrombin production. Its catalytic activity is enhanced by membrane-associated Factor Va in the prothrombinase complex (13).

Recently, we described a five-step purification of an anticoagulant-antimetastatic protein, ghilanten, from salivary gland extracts of the South American leech *Haementeria ghilianii* (10). Elucidation of its complete amino acid sequence showed 119 residues arranged in a twofold internal repeated homology with each of its two domains stabilized by five intramolecular disulfide bonds. We showed that the amino-terminal domain blocks the active site of FXa (10) and the carboxyl-terminus has a heparin-binding region which possibly mediates interactions with heparan sulfate proteoglycans on the arterial wall (14). In addition, we also reported the 3-dimensional structure of human DES (1–45) FXa at 2.2 Å resolution (15). The purpose of the current study was to express ghilanten in sufficient quantities to allow its structural elucidation. Unlike the cysteine-rich cerabratulus toxins (16), which can be expressed in *Escherichia coli*, the expression of ghilanten in this bacteria results in an improperly folded

[1] To whom correspondence should be addressed. Fax: (513) 948-7345.

[2] SCRIP No. 1916, p. 25, April 22, 1994.

1046-5928/95 $12.00

and insoluble product. The present report describes the gene synthesis of a specific ghilanten isoform and its expression in the methylotropic yeast *Pichia pastoris*.

MATERIALS AND METHODS

Automated Protein and DNA Sequence Analysis

Recombinant ghilanten was purified for sequence analysis on a 2.1 × 30-mm C-8 column (Aquapore RP-300) using a protein/peptide Model 130A Separation System (Perkin–Elmer/Applied Biosystems Inc. (PE/ABI), Foster City, CA). Proteins were loaded onto the column equilibrated in 0.1% trifluoroacetic acid (TFA) in H_2O (buffer A) and were then eluted with a linear gradient to 70% CH_3CN in 0.085% TFA (buffer B) over 150 min at a flow rate of 100 μl/min. Fractions containing ghilanten were resubjected to microbore reverse-phase HPLC using a linear gradient to 24% buffer B over 15 min and then to 35% buffer B over 75 min at a flow rate of 75 μl/min. Amino-terminal protein sequence was determined on a model 470A protein-peptide microsequencer (PE/ABI) with reagents, instructions, and standard programs supplied by the manufacturer. The phenylthiohydantoin-derivatized amino acids were identified at each cycle by a model 120 PTH analyzer (PE/ABI) directly on-line with the 470A. Sequence data were captured on a model 6000 Nelson analytical data system (Nelson Laboratories, Inc., Cupertino, CA). DNA sequence analysis of the gene was performed on a PE/ABI Model 373 automated DNA sequencer. Sequencing reactions on template DNA (1 μg) were performed with the Prism (PE/ABI) kit according to the procedures specified by the manufacturer. Sequence information was analyzed using the Inherit Data System (PE/ABI).

Design, Synthesis, and Assembly of a Synthetic Ghilanten Gene

The DNASTAR (DNASTAR Inc., Madison, WI) program was used to design a synthetic gene from the amino acid sequence of ghilanten isoform P5 (14) based on the mammalian preferred codons. The synthetic gene with *Eco*RI and *Hin*dIII restriction enzyme sites at the 5′ and 3′ termini, respectively, was assembled using 16 46-mer oligonucleotides (Fig. 1).

Oligonucleotides for gene construction and sequencing were synthesized on a PE/ABI Model 391 PCR-Mate or 394 DNA/RNA Synthesizer using standard β-cyanoethyl phosphoramidite chemistries as described by the manufacturer. After manual or automated cleavage of the oligonucleotides from the support, the oligos were deprotected by ammonialysis at 55°C for 12–15 h. Purification was performed either by OPC (Oligo Purification Cartridges, PE/ABI) using the manufacturers protocols or by high performance liquid chromatography (HPLC). HPLC of the DNA probes was performed on a binary Waters/Millipore (Milford, MA) system consisting of two Model 510 pumps, a Model 710B WISP, an automated gradient controller, a Model 440 fixed wavelength detector, and a Vydac (Hesperia, CA) 0.46 × 25 cm, 5 μm, C-18 reverse phase column. Elution was performed using a linear gradient from 93% of 50 mM triethylamine acetate, pH 6.95, to 25% acetonitrile in 50 min at a flow rate of 0.5 ml/min. Purity of the oligonucleotides was assessed by the chromatographic data and quantified by uv spectrophotometric measurements.

Oligonucleotides, with the exception of the two corresponding to the *Eco*RI and *Hin*dIII termini, were phosphorylated with T_4 polynucleotide kinase and equimolar amounts of the complementary oligonucleotides were annealed. The annealed DNA fragments were ligated using T_4 DNA ligase and the ligation product corresponding to the expected synthetic gene size (0.36 kb) was purified by preparative PAGE. The purified synthetic gene was cloned into the *Eco*RI and *Hin*dIII sites of pUC19 and the resulting plasmid is referred to as pUCHG-1.

Construction of pPIC9HG-2 Expression Vector for P. pastoris

The ghilanten coding sequence was excised from pUCHG-1 as a *Hin*dIII–*Eco* RI fragment, blunt ended using Klenow fragment of *E. coli* DNA polymerase and ligated into the *Sna*BI site of the *P. pastoris* secretion vector pPIC9 (18) for an in-frame fusion with the DNA sequence encoding the α-mating factor pre-pro secretion signal sequence of *Sacchromyeces cerevisiae*. Two constructs with the correct orientation were sequenced and one, designated as pPIC9HG-2 had the expected sequence. The sequence of the fusion junction, including the *KEX2* and diaminopeptidase (*DAP*) cleavage sites, as well as the start site of the mature ghilanten coding region are shown in Table 1.

Construction of P. pastoris Ghilanten Expression Strains

P. pastoris strains KM 71(*his4,aox1:ARG4*) (19) and SMD 1168 (*his4, pep4*) (M. A. Gleeson, personal communication) were transformed with *Sac*I-linearized pPIC9HG-2 using the spheroplast procedure (20). This method results in the stable integration of one or multiple copies of the linear DNA at the chromosomal alcohol oxidase (*AOX1*) locus (18,21,22). pPIC9 linearized with *Sac*I was used in the transformation as a vector control. The His^+ transformants were grown in BMGY medium and induced to secrete ghilanten in shake flask/tube cultures in four different induction medias (BMMY, BMMC, AMMY, and AMMC). All medias are described in Appendix I. Serial dilutions of the pool of His^+ SMD 1168 transformants were plated to obtain individual colonies. Ninety-six colonies were selected, grown in

TABLE 1

Amino Terminal Sequence Analysis of Recombinant Ghilanten Produced under Different Fermentation Conditions

2 liter shake flask: (BMMC medium)	$^{-4}$Y N S M E G P F G P . . .
10 liter bioreactor: 85% (Fermentor media) 15%	^{1}E G P F G P G (C)[a] E E A G . . .
	^{35}V Y (C)[a] S H G F Q R S R Y G

Predicted sequence from the open reading frame
(Expected cleavage sites for the *KEX2* and *DAP* like proteases are also indicated)

Ghilanten start site
v
. . .5′ GTA TCT CTC GAG AAA AGA GAG GCT GAA GCT TAC AAT TCA ATG GAG
V S L E K R E A E A $^{-4}$Y N S M E
^ ^ ^ ^
KEX2 *DAP* *DAP* Mature sequence

[a] Parentheses indicates a position in the sequence cycle in which the PTH amino acid was not identified and the residue inside the parenthesis indicates the amino acid predicted from the nucleotide sequence at that position. The percentage values indicate the approximate proportion of the particular sequence present in the mixture.

15 ml BMGY medium in 50-ml shake tubes, and then induced in 2 ml of BMMC. Culture supernatants were then evaluated for anticoagulant activity using the prothrombin clotting time assay described below.

Fermentation

Five liters of BSM containing 10% glucose were sterilized in a 10-liter Bioflo IV fermentor (New Brunswick Scientific, Edison, NJ) and allowed to cool to the set temperature of 31°C. The medium was adjusted to pH 6.0 with 50% NH_4OH and then 40 ml of biotin stock solution and 40 ml of PTM1 were added. The innoculum consisted of a yeast clone grown to an O.D. of 2–10 at 600 nm in a 2-liter shake flask containing 1 liter of MD. The fermentation was started with this innoculum and continued for 20 h. At this point, 250 ml each of 10% yeast extract and 20% peptone solutions were added to the medium and the fermentation was continued for an additional 20 h. Addition of yeast extract and peptone stimulated the cell growth and the cells grew to a density of 25 g dry cell weight per liter. Next, a fed-batch phase on methanol was initiated to induce the cells. The feed consisted of 980 ml of 100% MeOH, 10 ml of PTM1, and 10 ml of biotin stock solution which were filter-sterilized. The infusion was delivered at 0.2 ml/min by a Waters Model 510 HPLC pump over 3 days and the culture medium was sampled at 24-, 48-, and 72-h intervals for ghilanten activity.

Measurement of Clot Times

Prothrombin times were measured spectrophotometrically using an Electra 800 automatic coagulation timer (Medical Laboratory Automation Inc., Pleasantville, NY), using the standard programs and reagents recommended by the manufacturer. Briefly, various amounts of media (5–25 μl), containing *r*-ghilanten, were added to 100 μl of citrated sheep plasma (BioResources Inc., Irving, TX). After a 5-min incubation, clot times were determined with the addition of thromboplastin reagent (Baxter Healthcare Corp., Miami, FL).

Mass Spectrometry Analysis

Samples containing recombinant ghilanten were assayed on a Kratos Kompact III (Shimadzu Scientific Instruments, Inc. Columbia, MD) matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS). Molecular mass analysis was performed on 1- to 2-μl aliquots of the crude or purified proteins and cocrystallized with 1–2 μl of 45 mM sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid in a 2:3 v/v mixture of acetonitrile and 0.1% TFA).

RESULTS

Gene Design and Expression of Recombinant Ghilanten

Based on the amino acid sequence (14) we designed a synthetic gene (Fig. 1) which was then assembled and cloned into pUC19. This plasmid was digested with *Eco*RI/*Hin*dIII, blunt ended, and cloned into the *Sna*BI site of the yeast shuttle vector pPIC9 to yield the ghilanten expression plasmid pPIC9HG-2 (Fig. 2). This plasmid, with the expected cloning junction sequence, was selected from two independent *E. coli* clones.

pPIC9HG-2 was linearized with *Sac*I at the unique site in the 5′-*AOX1* region. The linearized DNA bearing the *HIS4* gene as the selectable marker was then introduced by homologous recombination into the 5′-*AOX1* chromosomal locus of *P. pastoris* strains KM 71 and SMD 1168. Transformants were selected based on the His^+ phenotype. Several hundred His^+ colonies were pooled and an aliquot was used to test for ghilanten expression. As shown in Table 2, both the KM 71 and

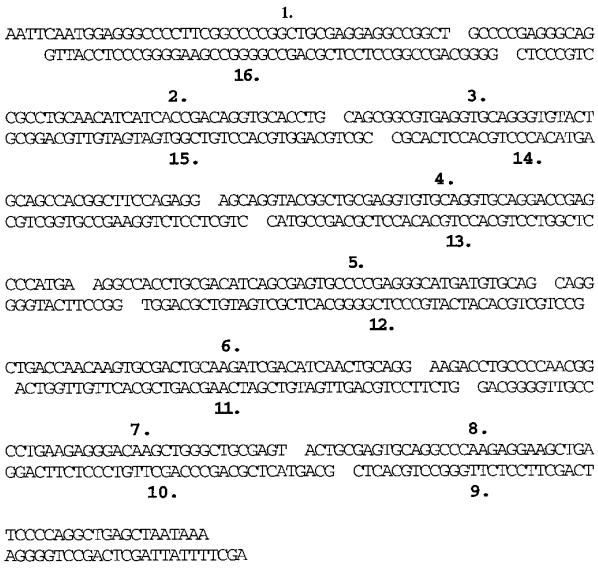

FIG. 1. The synthetic ghilanten gene was designed based on the amino acid sequence of ghilanten isoform P5 and assembled from 16 single-stranded oligonucleotides. The 5′ and 3′ termini of the synthetic gene correspond to *Eco*RI and *Hin*dIII restriction ends to facilitate directional cloning into plasmid pUC-19. The GenBank Accession No. is U20787.

SMD 1168 His$^+$ pools yielded anticoagulant activity relative to the corresponding pPIC9 transformed controls. The protease-deficient strain SMD 1168 yielded approximately a twofold higher level of ghilanten activity as compared to KM 71. With the SMD1168 strain, induction with BMMY or BMMC produced more ghilanten than did AMMY or AMMC. Anticoagulant activity with KM71 strain was consistently higher in BMMY

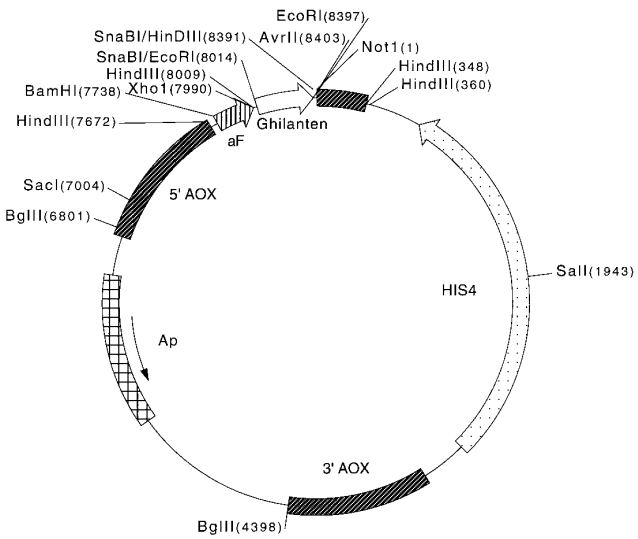

FIG. 2. After confirming the DNA sequence in pUC-19, the gene was excised by a double restriction digest (*Eco*RI/*Hin*dIII), blunt ended with Klenow, and cloned into the *Sna*BI site of plasmid pPIC9 to yield the expression vector pPIC9HG-2.

TABLE 2

Effect of Strain and Media Conditions on Ghilanten Secretion

	Prothrombin time in seconds			
	Strain KM71		Strain SMD1168	
Media	Transformed	Control	Transformed	Control
BMMY	53.5	23.8	97.5	33.2
BMMC	25.0	17.5	61.7	20.5
AMMY	33.5	21.8	54.2	36.0
AMMC	23.5	17.2	32.1	20.9

Note. Media (20 μl) collected after 48 h of induction were assayed. The A_{600} of the culture was approximately 60.0 units.

than in BMMC, whereas, with the SMD1168 strain, both BMMY and BMMC produced comparable amounts of ghilanten. The BMMY media components interfered with visualization of the product by SDS–PAGE. Therefore, BMMC was chosen for further study. Both SDS–PAGE and MALDI-TOF mass spectral analyses confirmed the presence of ghilanten in BMMC broth from the induced SMD1168 cells transformed with the ghilanten expression plasmid pPIC9HG2.

Purification and Analysis of Recombinant Ghilanten

The purification of recombinant ghilanten from BMMC media (SMD 1168) by heparin Sepharose chromatography is shown in Fig. 3. The peak anticoagulant activity eluted at 0.75 M NaCl as determined by conductivity measurements. The inset shows the SDS–PAGE of the ghilanten-containing fractions. The electrophoretic mobilities of recombinant (Fig. 3, inset) and native ghilanten (data not shown) are similar and run near the lysozyme standard (see Fig. 3 legend). The purified protein from the 2-liter shake flask fermentation had the unique amino-terminal sequence $^{-4}$Tyr-Asn-Ser-Met-^{1}Glu-Gly-Pro-Phe-Gly-Pro-Gly showing the correct processing of the α-mating factor pre-pro leader sequence as well as the Glu-Ala-Glu-Ala spacer predicted from the open reading frame (Table 1). The additional four residues of the amino terminus predict a mass of 13,775 amu vs. 13,601 amu observed by MALDI-TOF MS (Fig. 4). The additional residues had no detectable effect on either the anticoagulant activity or heparin-binding properties. Based on the processing pattern, we conclude that both the *KEX2*-like protease activity and the *DAP* activity are present in the protease-deficient strain.

Fermentation

We analyzed 96 individual colonies in order to isolate a high-producing ghilanten clone of SMD 1168. As shown in Fig. 5, a high degree of clonal variation in *r*-

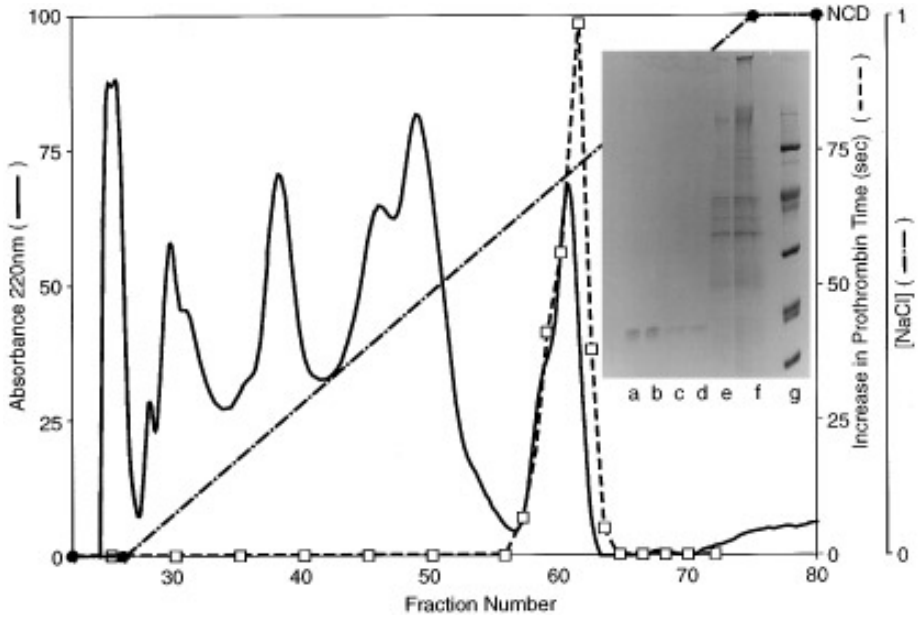

FIG. 3. Purification of recombinant ghilanten by heparin Sepharose chromatography. Protein elution was monitored at 220 nm. Elution involved a 15-min wash with 10 mM Hepes, pH 7.4, followed by a linear gradient of NaCl to 1 M, in 10 mM Hepes, pH 7.4, over 60 min, with a flow rate of 1 ml/min; 1-ml fractions were collected. Inhibitor activity was assayed as the increase in sheep plasma prothrombin time using 20 μl of the fractions. *Inset:* SDS–polyacrylamide gel analysis of heparin Sepharose fractions. Lanes a and b represent the equivalent of 100 μl of fractions 60 and 61 (concentrated by TCA precipitation); lanes c and d represent 15 μl of fractions 60 and 61; lanes e and f represent 15 μl of the His^+ pool and 288 h post methanol induction; lane g, protein molecular weight markers: (from top to bottom) phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400), aprotinin (6,500).

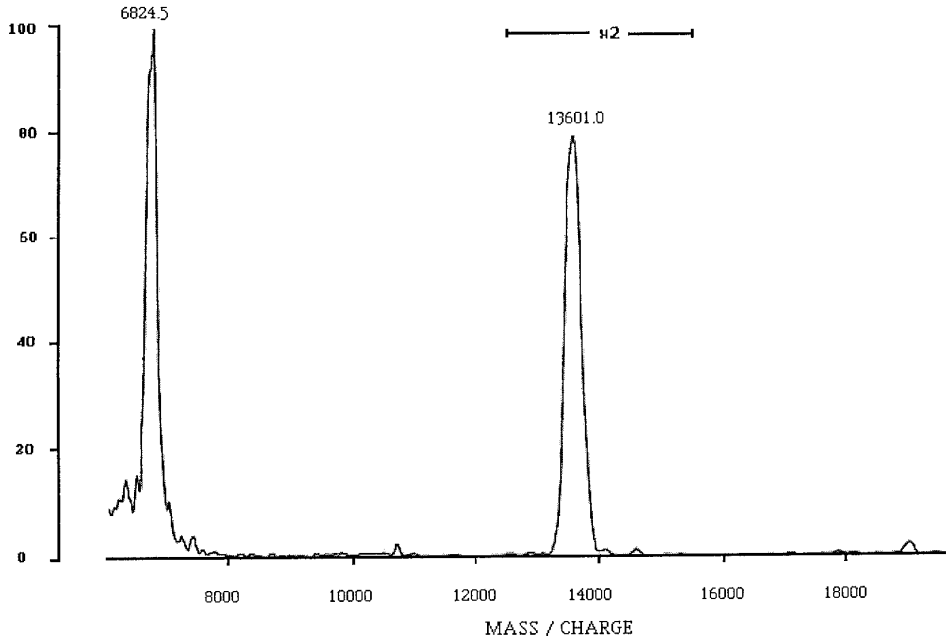

FIG. 4. Matrix-assisted time of flight mass spectral (MALDI-TOF MS) analysis of recombinant ghilanten.

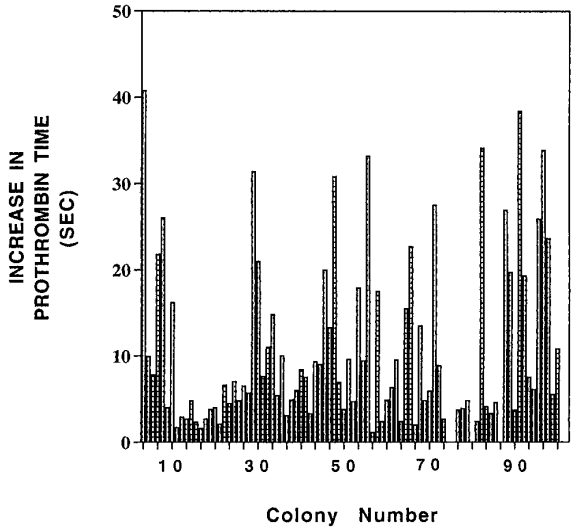

FIG. 5. Clonal variation of ghilanten expression in the SMD1168 strain of *P. pastoris.*

ghilanten expression was observed in shake tube studies. Several clones gave significantly higher levels of product than the His^+ pool. One of these, clone 72, was grown in a 2-liter shake flask culture in BMGY medium and then induced in BMMC. As shown in Fig. 6, maximum activity occurred within 64 h postinduction, and SDS–PAGE analysis indicated *r*-ghilanten comprised the major protein present in the broth (data not shown). The amount secreted into the medium was estimated to be approximately 10 mg/liter, with the cell density being approximately 260 g/liter wet weight. A pilot 10-liter bioreactor fermentation with clone 72 was performed in which cells were grown and induced in fermentor medium supplemented with yeast extract and peptone. Maximum expression occurred at 48 h postinduction. Amino terminal amino acid sequence of *r*-ghilanten purified from the bioreactor run showed $^{+1}$Glu-Gly-Pro-Phe . . . , indicating the complete processing to the mature form (Table 1).

DISCUSSION

The salivary glands of *H. ghilianii* are known to secrete powerful anticoagulants including a fibrinogenolytic enzyme termed hementin (25–28). Earlier, we reported the isolation of a family of FXa inhibitors from the salivary glands of the leech, *H. ghilianii,* and termed them ghilantens (10). We determined the primary sequence of the most potent isoform. The protein has 119 amino acids with two internal repeated homologies. These domains are compactly folded into two Kunitz protease inhibitor-type structures (residues 1–54 and 55–108), which are stabilized by five intramolecular disulfide bonds. Residues 93–119 are rich in basic amino acids and account for the heparin-binding properties of the inhibitor (14). Based on the protein sequence information, we designed a synthetic gene for expression in the methylotropic yeast, *P. pastoris.*

The synthetic gene was cloned into the expression vector designated pPIC9HG-2. The plasmid was then introduced into two different strains of *P. pastoris* by homologous recombination with the 5′-*AOX1* promoter locus of the endogenous alcohol oxidase gene to obtain stable transformants. Upon induction, secretion and proper folding of the cysteine-rich inhibitor occurred. A higher level of secretion was seen with the SMD1168 (Pep4 protease deficient) as compared to the KM71 (Pep4 protease normal). It is presumable that the higher level of secretion with SMD1168 is due to reduced proteolysis of the product. However, we cannot unequivocally draw this conclusion, because the strains also differ in their growth rates on methanol (KM71 has a methanol-slow phenotype, whereas SMD1168 grows normally on methanol). Purification of ghilanten from leech salivary gland extracts is a five-step process (10). Methanol induction of the SMD 1168 strain in defined BMMC media allowed a one-step purification by heparin Sepharose chromatography. At the shake tube and 2-liter shake flask scales in BMMC, the α-mating factor pre-pro signal sequence was efficiently processed yielding an active product with four additional amino terminal residues. At the 10-liter bioreactor scale, with growth and induction in fermentation medium, approximately 85% of the product represented the mature form lacking these four extraneous

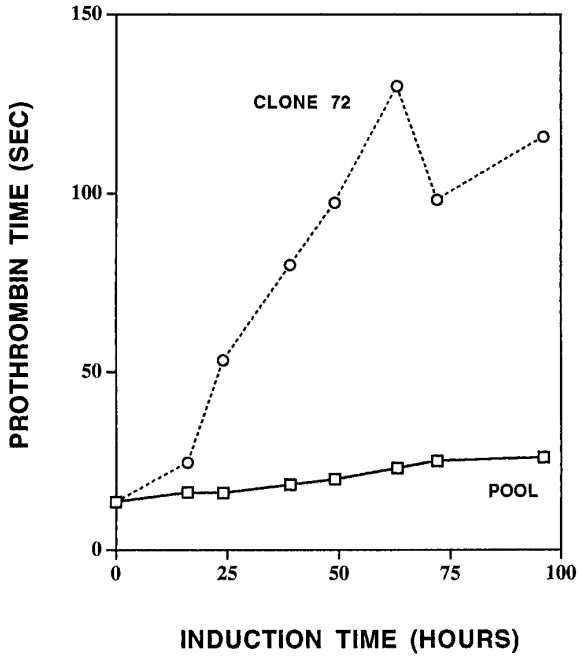

FIG. 6. The His^+ pool and clone 72 were induced to secrete *r*-ghilanten in BMMC medium. Clot inhibitor activity was measured as a function of time after BMMC induction.

residues (Table 1). Approximately 15% of the material was cleaved at ^{34}Arg-^{35}Val, i.e., the inhibitor reactive site (14), indicating specific cleavage by an endogenous serine protease. Efficient liberation of the amino terminal Glu at position 1 of the sequence indicates a lack of the pyroglutamase activity in the processing pathway of *r*-ghilanten in SMD1168. In leech salivary glands, this activity cyclizes and blocks the amino terminus of native ghilanten. A significant clonal variation in the expression of ghilanten was observed and we do not know the molecular basis for this variation. One possible explanation for this is the gene dosage, as has been noted in numerous other instances in the literature (21). However, more recently, novel *P. pastoris* transformants of tick anticoagulant protein (TAP) were identified, which contain a single copy of the expression cassette, yet they secrete TAP in excess of grams per liter (29).

APPENDIX I

YEAST GROWTH AND INDUCTION MEDIA

BMGY: 1% (v/v)glycerol, 2% (w/v) peptone, 1% (w/v) yeast extract, 1.34% (w/v) yeast nitrogen base without amino acids , 0.00004% (w/v) biotin, and 10% (v/v) potassium phosphate buffer (1 M, pH 6.0).

BMMY: same as BMGY, with the exception that 1% (V/V) methanol was used instead of glycerol.

AMGY and **AMMY:** Same as BMGY and BMMY with the exception that these media were adjusted to pH 2.9 with HCl.

BMMC and **AMMC:** Same as BMMY and AMMY with the exception that yeast extract and peptone were replaced by 2% (w/v) casamino acids.

PTM1 trace salts

Composition is for 1 liter final volume in water:

Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	6.0 g
Manganese sulfate ($MnSO_4 \cdot H_2O$)	3.0 g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	65.0 g
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	20.0 g
Sulfuric acid (H_2SO_4)	5.0 ml
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.5 g
Boric acid (H_3BO_3)	0.02 g
Sodium Molybdate ($NaMoO_4 \cdot 2H_2O$)	0.2 g
Potassium Iodide (KI)	0.1 g

BSM medium composition:

Composition is for 1 liter final volume in water:

Phosphoric acid, H_3PO_4 (85%)	26.0 ml
Calcium sulfate, $CaSO_4 \cdot 2H_2O$	0.9 g
Potassium sulfate, K_2SO_4	18.0 g
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	14.0 g
Potassium hydroxide, KOH	4.0 g

Biotin stock solution: 20 mg of *d*-biotin (Sigma Chem., St. Louis, MO) dissolved in 100 ml of water and filter sterilized.

MD: (per liter)20 g glucose, 2 ml of biotin stock solution, 13.2 g of yeast nitrogen base without amino acids, but with ammonium sulfate (Difco)

ACKNOWLEDGMENTS

We thank Ms. Jewel Rison for the preparation of this report and Drs. Maurizio Denaro, Mike Palfreyman, Arun Subramaniam, Lori Birkenberger, and Cynthia Gates for their careful review and helpful suggestions.

REFERENCES

1. Dodt, J. Seemuller, U., Maschler, R., and Fritz, H. (1985) The complete covalent structure of hirudin: Localization of disulfide bonds. *Biol. Chem. Hoppe Seyler* **366,** 379–385.
2. Mao, S. J., Yates, M. T., Blankenship, D. T. and Cardin, A. D. (1987) Rapid purification and revised amino-terminal sequence of hirudin: A specific thrombin inhibitor of the bloodsucking leech. *Anal. Biochem.* **162,** 514–518.
3. Markwadt, F. (1970) Hirudin as an inhibitor of thrombin. *Methods Enzymol.* **19,** 924–932.
4. Stone, S. R., and Hofsteenge, J. (1986) Kinetics of the inhibition of thrombin by hirudin. *Biochemistry* **25,** 4622–4628.
5. Rydel, T. J., Tulinsky, T., Bode, W., and Huber, R. (1991) Refined structure of the hirudin-thrombin complex. *J. Mol. Biol.* **221,** 583–601.
6. Sitko, G. R., Ramjit, D. R., Stabilito, I. I., Lehman, D., Lynch, J. J., and Vlasuk, G. P. (1992) Conjunctive enhancement of enzymatic thrombolysis and prevention of thrombotic reocclusion with the selective factor Xa inhibitor, tick anticoagulant peptide. *Circulation* **85,** 805–815.
7. Schaffer, L. W., Davidson, J. T., Vlasuk, G. P., and Siegl, P. K. S. (1991) Antithrombotic efficacy of recombinant tick anticoagulant peptide. *Circulation* **84,** 1741–1748.
8. Lynch, J. J., Sitko, G. R., Mellott, M. J., Nutt, E. M., Lehman, E. D., Friedman, P. A., Dunwiddie, C. T., and Vlasuk, G. P. (1994) Maintenance of canine coronary artery patency following, thrombolysis with front loaded plus low dose maintenance conjunctive therapy. A comparison of factor Xa versus thrombin inhibition. *Cardiovasc. Res.* **28,** 78–85.
9. Ragosta, M., Gimple, L. W., Gertz, S. D., Dunwiddie, C. T., Vlasuk, G. P., Haber, H. L., Powers, E. R., Roberts, W. C., and Sarembock, I. J. (1994) Specific factor Xa inhibition reduces restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. *Circulation* **89,** 1262–1271.
10. Brankamp, R. G., Blankenship, D. T., Sunkara, P. S., and Cardin, A. D. (1990) Ghilantens: Anticoagulant- antimetastatic proteins from the South American leech, *Haementeria ghilianii. J. Lab. Clin. Med.* **115,** 89–97.
11. Zacharshi, L. R., Dunwiddie, C., Huh, E. M., Hunt, J., and Memoli, V. A. (1991) Cellular localization of activated Factor X by Xa-specific probes. *Thromb. Haemostasis* **65,** 545–548.
12. Gasic, G. J., Viner, E. D., Budzynski, A. Z., and Gasic, G. P. (1983) Inhibition of lung tumor colonization by leech salivary gland extracts from *Haementeria ghilianii. Cancer Res.* **43,** 1633–1636.
13. Suttie, J. W., and Jackson, C. M. (1977) Prothrombin structure, activation and biosynthesis. *Physiol. Rev.* **57,** 1–70.

14. Blankenship, D. T., Brankamp, R. G., Manley, G. D., and Cardin, A. D. (1990) Amino acid sequence of ghilanten: Anticoagulant-antimetastatic principle of the South American leech, *Haementeria ghilianii. Biochem. Biophys. Res. Commun.* **166,** 1384–1389.
15. Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenship, D. T., Cardin, A. D., and Kisiel, W. (1993) Structure of human Des(1-45) factor Xa at 2.2 A resolution. *J. Mol. Biol.* **232,** 947–966.
16. Howell, M. L., and Blumenthal, R. M. (1989) Cloning and expression of a synthetic gene for *Cerabratulus lacteus* neurotoxin B-IV. *J. Biol. Chem.* **264,** 15268–15273.
17. Holmes, and Quigley (1981) A rapid boiling method for the plasmids preparation of bacteria. *Anal. Biochem.* **114,** 193–197.
18. Clare, J. J., Romanos, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., Sreekrishna, K., and Henwood, C. A. (1991) Production of mouse epidermal growth factor in yeast: High-level secretion using *pichia pastoris* strains containing multiple gene copies. *Gene* **105,** 205–212.
19. Cregg, J. M., and Madden, K. R. (1988) Development of the methylotrophic yeast, *pichia pastoris* as a host for the production of foreign proteins. *Dev. Ind. Microbiol.* **29,** 33–41.
20. Cregg, J. M., Barringer, K. J., Hessler, A. Y., and Madden, K. R. (1985) *Pichia pastoris* as a host system for transformations. *Mol. Cell Biol.* **5,** 3376–3385.
21. Sreekrishna, K. (1993) Strategies for optimizing protein expression and secretion in the methylotrophic yeast *pichia pastoris* Ind. Microorganisms: Basic and applied molecular genetics (Baltz, R. H., Hegeman, G. D., and Skatrud, P. L. Eds.), pp. 119–126.
22. Sreekrishna, K., McCombie, W. R., Potenz, R., Parker, R. A., Mazzaferro, P. K., Maine, G. M., Lopez, J. L., DiVelbiss, D. K., Holden, K. A., Barr, R. D., and Fuke, M. (1988) Clonal variation in the expression of human tumor necrosis factor in the methylotrophic yeast *pichia pastoris.* Fifth Annual Biotech U.S.A. Indus. Conf. and Exhibition, San Francisco, CA.
23. Barr, K. A., Hopkins, S. A., and Sreekrishna, K. (1992) Protocol for efficient secretion of HSA developed from *pichia pastoris. Pharm. Eng.* **12,** 48–51.
24. Budzynski, A. Z., Olexa, S. A., Brizuela, B. S., Sawyer, R. T., and Stent, G. S. (1981) Anticoagulant and fibrinolytic properties of salivary proteins from the leech *Haementeria ghilianii Proc. Soc. Exp. Biol. Med.* **168,** 266–275.
25. Budzynski, A. Z., Olexa, S. A., and Sawyer, R. T. (1981) Composition of salivary gland extracts from the leech *Haementeria ghilianii. Proc. Soc. Exp. Biol. Med.* **168,** 259–265.
26. Malinconico, S. M., Katz, J. B., and Budzynski, A. Z. (1984) Hementin: Anticoagulant protease from the salivary glands of the leech *Haementeria ghilianii. J. Lab. Clin. Med.* **103,** 44–58.
27. Malinconico, S. M., Katz, J. B., and Budzynski, A. Z. (1984) Fibrinogen degrading action of hementin, a fibrinogenolytic anticoagulant from the salivary glands of the leech *Haementeria ghilianii. J. Lab Clin. Med.* **104,** 842–854.
28. Oakley, B. R., Kirsch, D. R., and Morris, R. (1980) A simplified ultrasensitive silver strain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105,** 361–363.
29. Laroche, Y., Storme, V., De Meutter, J., Messens, J., and Lauwereys, M. (1994) High-level secretion and very efficient isotopic labeling of tick anticoagulant peptide (TAP) expressed in the methylotropic yeast, *Pichia pastoris. Biotechnology* **12,** 1119–1124.