Expression, secretion and processing of hirudin in *E. coli* using the alkaline phosphatase signal sequence

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Received 21 April 1986; revised version received 29 May 1986

A DNA fragment coding for the *E. coli* phoA signal peptide was synthesized and inserted into the expression vector pKK223-3. A single *Hin*dIII restriction site is located just at the end of the signal sequence. A gene coding for the proteinase inhibitor hirudin, which has previously been synthesized, was inserted into this *Hin*dIII site. The hybrid protein was expressed under control of the tac-promoter and secreted into the periplasm of *E. coli*. From the periplasmic fraction two processed proteins were isolated. One of these was identical with desulfatohirudin and also had similar biological properties.

Hirudin *Thrombin-specific inhibitor* *(Leech,* Hirudo medicinalis) *Gene expression* *Alkaline phosphatase* *Signal sequence* *Desulfatohirudin*

1. INTRODUCTION

Hirudin, the proteinase inhibitor protein originating from the salivary glands of the leech, *Hirudo medicinalis,* consists of a single polypeptide chain composed of 65 amino acid residues [1,2]. It is the most potent thrombin-specific inhibitor so far known. It forms tight complexes with thrombin, which exhibit equilibrium dissociation constants as low as 4×10^{-11} M. The potency and specificity of hirudin stimulated interest in its possible use as an antithrombotic drug in clinical studies [3].

Besides this pharmacological interest, hirudin-thrombin complexes may function as an excellent model to study protein-protein recognition. For this reason novel hirudin molecules with distinct biological activities should be generated. We have therefore considered the use of recombinant DNA for the production of hirudin and hirudin mutants derived by oligonucleotide directed site specific mutagenesis. A prerequisite for these studies is the expression of biologically active hirudin.

Abbreviation: IPTG, isopropyl-β-D-thiogalactopyranoside

However, in recent years the expression of foreign genes in *Escherichia coli* and production of biologically active products have proven to be difficult, especially for polypeptides [4,5]. This was also shown for hirudin expression in previously published data. Only a small amount of the inhibitor was found in the cytoplasm [6,7]. Therefore protein folding, disulfide bonding and protection against proteolytic degradation have to be guaranteed. This should be accomplished by co-translational transport of the nascent polypeptide chain through the cytoplasmic membrane using bacterial or eukaryotic signal sequences [8,9]. One of the periplasmic proteins of *E. coli* is the alkaline phosphatase (phoA) which carries in its precursor form a signal sequence of 21 amino acids [10]. Making use of this signal sequence we here present the expression and processing of hirudin into the periplasm of *E. coli*.

2. MATERIALS AND METHODS

Deoxyoligonucleotides SP1 and SP2 were synthesized manually on a solid silica gel support, deoxyoligonucleotides SP3 and SP4 were assem-

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00145793/86/$3.50

bled on a DNA synthesizer using the phosphoamidite method [11]. Purification of the oligonucleotides was performed by HPLC. Restriction enzymes (Pharmacia and Boehringer), calf intestinal alkaline phosphatase and T_4 DNA ligase (Boehringer) were used according to the suppliers recommendation. Small scale DNA preparations for rapid analysis were performed by a LiCl-boiling method [12]. DNA gene fragments required for cloning were separated and isolated from polyacrylamide gels [13]. The expression vector pKK223-3 was purchased from Pharmacia. Cultures of *E. coli* BMH71-18 (*(lac, pro), thi, supE/F', lacI^q, lacZ,* M15 *pro*$^+$) [14], harbouring plasmid pHIR21 (controls: *E. coli* BMH71-18/pKK223-3) were grown at 37°C in LB-broth containing 100 μg/ml ampicillin to A_{578} unit of 0.5. Induction of gene expression was achieved by bringing the cultures to 0.5 mM IPTG. The time course of hirudin expression was followed by a thrombin inhibition assay [15] in cell extracts, periplasmic and extracellular fractions. The enzyme activity was measured at 25°C using tos-gly-pro-arg-*p*-nitroanilide (Chromozym TH, Boehringer) as a substrate. The periplasmic fraction [9] of *E. coli* BMH71-18/pHIR21 was passed through a DEAE-Sephadex A-25 column, equilibrated with 0.1 M ammonium acetate/0.18 M sodium chloride, pH 6.0, and developed by a gradient from 0.18 to 0.3 M sodium chloride. Fractions with thrombin inhibitory activity (0.28 M NaCl) were pooled and purified further by HPLC. Amino acid sequence analysis of the proteins from the resultant peaks was performed on a gas phase sequencer (Applied Biosystems 470A) before and after oxidative cleavage [16] of disulfide bonds as well as from fragments after cleavage with endoproteinase Lys-C. C-terminal analyses were obtained by the carboxypeptidase Y method [17].

3. RESULTS

A DNA coding for the phoA signal peptide [10] was assembled from four chemically synthesized oligonucleotides SP1–SP4 (fig.1). The ligation mixture of the four phosphorylated oligonucleotides was separated on a 15% polyacrylamide gel yielding about 13% of the 67 bp ligation product (not shown). This DNA was ligated into the *Eco*RI and *Hin*dIII restriction sites of the polylinker on

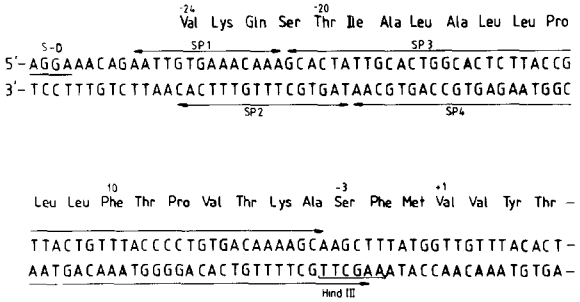

Fig.1. Amino acid and corresponding DNA sequence of the phoA signal peptide. The DNA sequence was assembled from the four oligonucleotides SP1–SP4. The resulting 67 bp ligation product was flanked by an *Eco*RI compatible 5'-terminus and a *Hin*dIII site at the 3'-terminus. This fragment was inserted into the corresponding restriction sites of pKK223-3 (cf. fig.2A). The first amino acid of native hirudin is numbered +1. Val^{-24}–Ala^{-4} is the phoA signal peptide. Additional amino acids from Ser^{-3} to Met^{-1} are caused by the design of the hirudin gene.

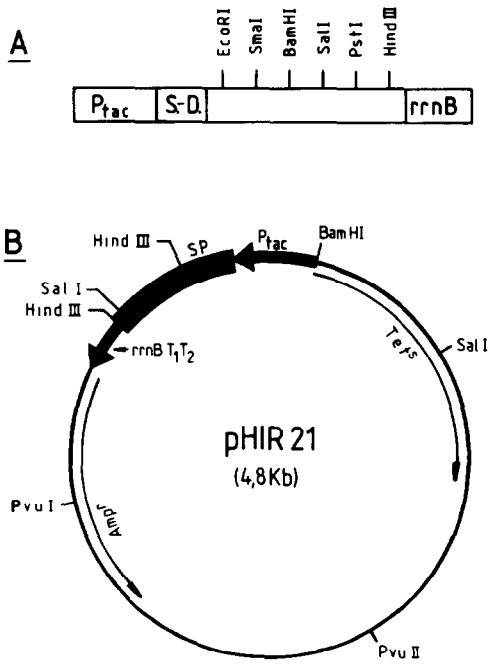

Fig.2. (A) Multiple cloning site of the expression vector pKK223-3. (B) The expression vector pHIR21 carrying the DNA sequence for the phoA signal peptide and the hirudin gene. The tac-promoter, direction of transcription and the terminator are indicated by filled arrows.

the expression vector pKK223-3 (fig.2A). The vector carries the tac-promoter and a Shine-Dalgarno sequence upstream of the cloning site and additionally ribosomal RNA transcription terminators (rrnB) downstream of this site. The *Eco*RI site was not reconstituted by the ligation procedure. A 229 bp DNA fragment coding for hirudin was derived from the vector pHIR1 [6] and ligated into the *Hin*dIII site of pKK223-3 carrying the phoA signal sequence. The vector pHIR21 showed the correct orientation of the hirudin gene fragment, as could be verified by restriction enzyme analysis with *Sal*I. pHIR21 directed a tac-promoter controlled synthesis of a fusion protein starting with the phoA signal peptide at the N-terminus and followed by hirudin (fig.2B).

Expression of the phoA signal peptide-hirudin fusion protein was induced in *E. coli* BMH71-18 harbouring the plasmid pHIR21 by bringing the cultures to 0.5 mM IPTG. Time course of the hirudin expression was followed by a thrombin inhibitory assay (fig.3). In the stationary phase (21 h after induction) hirudin activity reached a level of about 350 mIU/1 A_{578} unit cells in total cell extracts and periplasmic fractions. An appreciable amount of approx. 100 mIU/1 A_{578} unit cells was also found in culture supernatants. These hirudin levels were stable for another 10 h. The results obtained for hirudin from cell extracts and periplasm indicate that nearly all of the thrombin inhibitor was secreted into the periplasm. Secretion of hirudin into culture supernatants, however, is not yet understood.

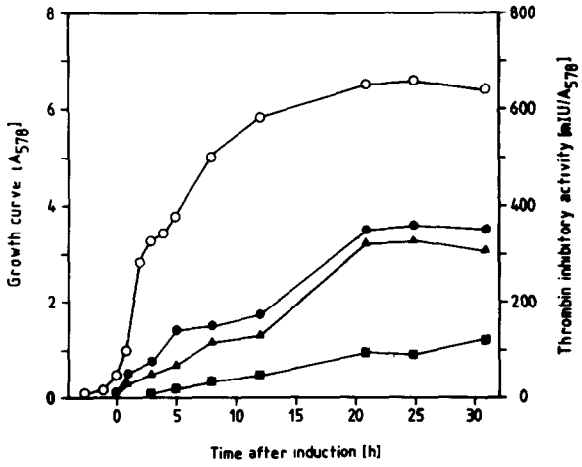

Fig.3. Thrombin inhibitory activity determinations of *E. coli* BMH71-18/pHIR21 after induction of gene expression by IPTG. (○—○) Growth curve. Thrombin inhibiting activities in: (●—●) cell extracts, (▲—▲) periplasmic fractions and (■—■) culture supernatants are given.

350 mIU/1 A_{578} unit cells were observed in the periplasmic fraction of *E. coli* BMH71-18/pHIR21 24 h after induction of gene expression. Proteins and peptides of this fraction were separated by ion-exchange chromatography on DEAE-Sephadex A-25 followed by HPLC (fig.4). This chromatography resulted in two protein peaks (I and II) which were analysed by amino acid sequence and thrombin inhibitory activity determinations. The data from sequencing studies confirmed the primary structure of proteins I and II (fig.5). Protein II indicates that the signal peptide was correctly processed by the leader peptidase at positions Ala^{-4}- Ser^{-3} during secretion into the periplasm. Surprisingly, peptide I showed the same amino acid sequence as determined for hirudin [2] except

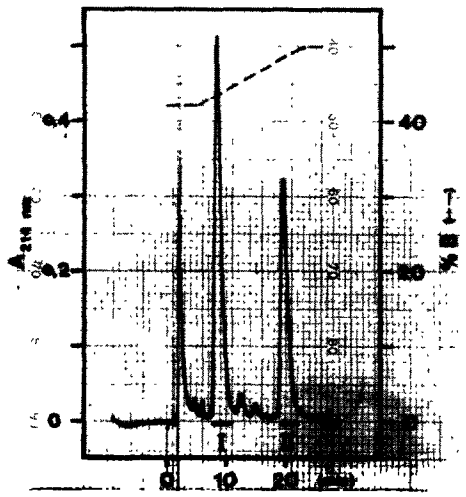

Fig.4. HPLC separation of thrombin inhibitory activity containing fractions derived from ion-exchange chromatography. Chromatographic conditions: column, Beckman ODS (0.46×25 cm); solvent A, 0.1% trifluoroacetic acid in water (v/v); solvent B, 0.1% trifluoroacetic acid in acetonitrile (v/v) mixed with solvent A in a ratio of 3:2 (v/v); flow, 1 ml/min; detector, 1.0 AUFS at 214 nm; temperature, 25°C; gradient, as indicated by dashed line. The retention times of I and II were 8.4 and 19.5 min, respectively.

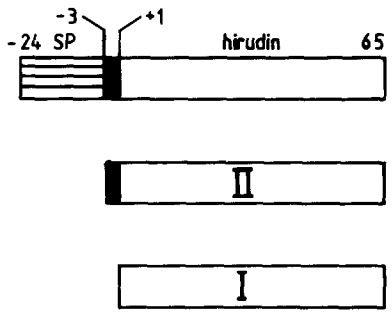

Fig.5. Schematic structure of the phoA signal peptide-hirudin fusion and processed hirudin forms from periplasmic fractions. The primary structures of protein peaks I and II isolated as in fig.4 were confirmed by amino acid sequence analyses. The first amino acid of mature hirudin is numbered +1.

that it is missing a sulfated tyrosine residue in position 63. This amino acid was identified as a tyrosine by C-terminal sequence analysis with carboxypeptidase Y (cf. [2]). The specific activity of protein I (desulfatohirudin) was determined as 550–570 IU/mg and is therefore comparable to 530–560 IU/mg for desulfatohirudin obtained by arylsulfatase treatment of native hirudin from leeches [18]. Thrombin inhibitory activity of protein II was 500-fold less than that for desulfatohirudin and therefore negligible under assay conditions. A recalculation of the data obtained for the specific activity of desulfatohirudin (protein II) and total thrombin inhibitory activity in periplasmic fractions 24 h after induction yielded 4 mg desulfatohirudin/l in *E. coli* BMH71-18/pHIR21 cultures.

4. DISCUSSION

Prior usage of the phoA system is documented in current patenting literature (Eur. Pat. Appl. EP 133,321). Using this system we have obtained 25- and 3500-times more hirudin activity than in prior reports (cf [6] and [7], respectively). As hirudin is a secretory protein from the salivary glands of the leech the expression of the synthetic gene fused to a leader sequence may mimic segregation under natural conditions. This indication is supported by the sequence of a cDNA clone of a naturally occurring hirudin form which presumably is translated as a precursor [7]. The amino acid sequence analyses of the hirudin forms I and II from the periplasm demonstrate that the precursor protein is exported across the cytoplasmic membrane via the signal peptide dependent process. Process of the gene product by a leader peptidase to hirudin form II took place as expected, whereas the origin of form I is still inexplicable. Thrombin inhibitory activity measured in the culture supernatants was also unexpected. Penetration of hirudin across the outer membrane may be due to its small molecular size and/or its hydrophilic nature [19]. The possibility that hirudin is set free by cell lysis is excluded by the data shown in fig.3 (hirudin can already be detected in supernatants 3 h after induction when cells are still in logarithmic growth).

Hirudin obtained from *E. coli* does not carry a sulfated tyrosine residue in position 63 as was shown for hirudin from leeches. This however is not a drawback as the removal of the sulfate group from hirudin molecules has only a very weak influence on the inhibition strength [20]. To obtain the sulfatohirudin an effort may be made in a eukaryotic expression system, but its success is doubtful as the specificity of arylsulfotransferases is not yet understood.

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