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Novel method of expression and purification of hirudin based on pBAD TOPO, pTYB12 vectors and gene synthesis

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Abstract

To express recombinant hirudins in *Escherichia coli* cells, a fragment of chemically synthesized DNA was used, containing codons for the individual amino acids preferred by the host cells. Gene synthesis was based on the design of two DNA fragments, so-called mega primers H1 and H2 with a complementary fragment, and their incubation with *Taq* polymerase. The gene obtained in this fashion was multiplied using the PCR, and then expressed in *E. coli* cells with the use of TOPO vectors pBAD and pTYB12. Using this method, hirudins were obtained in the amount of 17 mg/l *E. coli* strain, with the activity of 17 antithrombin units (ATU)/mg protein. The method can be considered as an easy and inexpensive route to small protein synthesis.

Keywords: Hirudin; pBAD; pTYB12; Direct thrombin inhibitor

The therapeutic properties of medicinal leech (*Hirudo medicinalis*) were already known in ancient times. Treatments with their help were popular until the beginning of the 20th century. In the 1830s, European hospitals made use of about 5–6 million leeches, whereas American hospitals imported close to 30 million leeches from Germany. During this time, leeches were used with the aim of decreasing blood volume in patients—as a blood-letting agent of sorts, in the treatment of varicose veins and hemorrhoids, as well as inflammation of the epididymis and the eye.

In 1984, the blood-thinning agent (anticoagulant) hirudin was isolated for the first time from leech salivary glands [1]. Today, hirudin is the most specific and active of known thrombin inhibitors not influencing on other peptidases [2]. Hirudin is a protein built of 65 amino acid residues containing N-terminal, 48-peptide rigid fragment stabilized by three disulfide bridges, as well as a C-terminal chain, which assumes random conformation in solution [3–5]. Hirudin molecule contains numerous acidic amino acid residues (including the modified amino acid tyrosine-*O*-sulfate), but it does not contain methionine, tryptophan, alanine or arginine [6]. The recombinant hirudin, not having the sulfur group on the tyrosine residue, exhibit similar activity and anti-thrombin properties as wild protein [7]. The synthetic N-terminal hirudin fragment containing 47 amino acids residues (hirudin$^{1-47}$) was shown to exclusively block the active center of thrombin, competitively inhibiting the hydrolysis of short substrates ($K_i = 4.2 \times 10^{-7}$ M) and fibrinogens ($K_i = 4.6 \times 10^{-7}$ M) [8]. The high activity and specificity of this inhibitor is the result of the mutual fit. Crystallographic studies demonstrated that about half of the 65 amino acid residues in hirudin are found at a distance smaller than 4 Å from the thrombin surface. Hirudin interacts both with the active center, as well as with thrombin fibrinogen-recognition exosite (FRE).[1] The N-terminal hirudin fragment blocks the catalytic center of thrombin. The Ile^1 residue occupies the S_2 pocket of the enzyme, while the amino group creates a hydrogen bond with the Ser^{195} in

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[1] *Abbreviations used:* FRE, fibrinogen-recognition; ATU, antithrombin units.

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catalytic center of the enzyme. The aromatic ring of the Tyr^3 residue occupies the hydrophobic S3 pocket in the enzyme [9].

The C-terminal hirudin fragment interacts with thrombin FRE, which contains numerous basic amino acid residues. Both of these active hirudin fragments are connected by a hexapeptide connector (hirudin$^{49–54}$). Hirudin$^{55–65}$, which specifically blocks FRE, is therefore a very specific thrombin inhibitor. Unfortunately, its low activity and quick degradation in the presence of proteases makes it difficult to use in therapy. Currently, this protein is undergoing third phase of clinical trials [10].

Reported studies indicate that hirudin is a specific anticoagulant that is well tolerated, non-toxic and stable. Moreover, it is an exceptionally weak antigen [11]. Recent reports on hirudin-related research [12–15] have demonstrated the growing interest in the development of hirudin application. Therefore in this report we present rapid, easy and efficient method of hirudin expression based on synthetic gene and pBAD TOPO and pTYB12 vectors.

Materials and methods

Gene synthesis amplification and cloning

Primers H1: 5′GTCGTCTACACTGATTGTACTGAATCTGGTCAAAACTTGTGTTTGTGTGAAGGTTCTAACGTTTGTGGTCAAGGTAACAAGTGTATTTTGGGTTCTGACGGTGAAAAGAACCA3′ and H2: 5′TTGCAAGTACTCCTCTGGAATCTCCTCAAAATCACCATCGTTATGAGATTGTGGCTTTGGAGTACCTTCACCAGTAACACATTGGTTCTTTTCACCGTCAGAACCCAAAATAC3′ (Sigma) were used to build the gene coding hirudin. A reaction mixture containing 8 μl of each primer, H1 and H2, (10 μM), 10 μl dNTPs (2 mM), 1 μl *Taq* polymerase (Fermentas), 5 μl of 10×concentrated buffer (Fermentas) and 18 μl H_2O was incubated for 30 min. at 72 °C. Primers R2: 5′TTGCAAGTACTCCTCTGGAATCTCCTC3′ and F2: 5′GTCGTCTACACTGATTGTACTGAATCTGG3′ were used to amplify the gene coding hirudin. The thermal profile of the PCR was: initial denaturation at 93 °C, 120 s, denaturation at 93 °C, 30 s, addition of primers at 60 °C, 60 s, elongation at 72 °C for 100 s, final extension at 72 °C, 300 s. Primers HIR1: 5′TTTGGATCCCAGGTTGTTGTACAGAATTTGCAAGTACTCCTCTGGAATCTCC3′ and HIR2: 5′ TTATTCCGGATCCTCAGTCGTCTACACTGATTGTACTGAATC3′ enabled the cloning of the gene sequence using restriction endonuclease *Bam*H1 (Fermentas) and a specific recognition site for this enzyme in the appropriate vector. The thermal profile of the PCR was: initial denaturation at 93 °C, 120 s, denaturation at 93 °C, 30 s, addition of primers at 65 °C, 60 s, elongation at 72 °C, 100 s, final extension at 72 °C, 200 s. The pBAD TOPO plasmid (Invitrogen) was used as the vector into which the obtained gene was cloned, becoming a source of the hirudin coding sequence.

Expression and purification

The pTYB12 plasmid (New England BioLabs) acted as an expression vector. The *Escherichia coli* TOP10F' strain (Invitrogen) was used to prepare recombinant plasmids pBAD TOPO with the hirudin gene, and *E. coli* ER 2566 (New England BioLabs) was used to express fusion protein. *E. coli* ER 2566 strain was transformed, using recombinant plasmids enabling the expression of the gene under the control of the T7 promoter. The production was carried out in the LB medium with the addition of ampicillin (final concentration 100 μg/ml). After reaching OD_{600} 0.6 by the culture, the overexpression of hirudin was induced by the addition of IPTG, to a final concentration of 0.5 mM. The cultivation was continued at of 15 °C for 24 h. The bacterial cells were suspended in 10 ml of buffer I (10 mM Tris—0.605 g, 250 mM NaCl—7.312 g, H_2O distilled to 500 ml) and subjected to sonification in SONIPREP 150 device in four 30 s cycles, with one-min. interval after each cycle. Next obtained lysate was applied onto a chromatographic column containing chitin (10 ml of chitin beads for 20 ml cell lysate). Native proteins from *E. coli* were rinsed out from the column with buffer I. After "immobilisation" of recombinant protein, the column was filled with buffer II (20 mM Tris—0.242 g, 50 mM NaCl—0.272 g, 50 mM DTT—0.770 g, H_2O distilled to 100 ml) and the autohydrolysis reaction of the product was continued for 72 h at 4 °C. The concentration of the obtained protein fraction was determined according to the Bradford. IPTG, arabinose, agarose and all reagents for protein purification were purchased from Sigma.

Disulfide bond reshuffling

The disulfide bond reshuffling of hirudin was performed according to the method of Chang [16]. Protein sample was dialyzed in 0.1 M $NaHCO_3$ buffer (pH 8.3) in the absence of any extra salt to minimize dimmer formation. The reshuffling process was process was carried out at 4 °C overnight in microcentrifuge tube by adding cysteine and cystine to final concentration 8 and 4 mM, respectively. The efficiency of the reshuffling process was assayed using non-reducing SDS–PAGE.

Antithrombin activity assay

The following reagents were used for the determination of protein activity: bovine α-thrombin at 2500 NIH/ml (kindly supplied by the Medical Academy of Gdańsk), 1 NIH unit clots a standard fibrinogen solution in 15 s at 37 °C; Chromozyme-TH (Boehringer Mannheim) Tos-Gly-Pro-Arg-p-NA; buffer A: 0.1 M triethanolamine, 0.2 M NaCl (pH 8.4); buffer B: 0.05 M Tris, 0.1 M NaCl (pH 7.9). To determine the activity of the recombinant protein, a series of dilutions of recombinant hirudin (5–400 ng/ml) were prepared and mixed together with a solu-