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Human growth hormone-specific aptamer identification using improved oligonucleotide ligand evolution method

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ABSTRACT

LETEG is a method developed and used for the separation and purification of proteins employing a single-step ligand (aptamers) evolution in which aptamers are eluted with an increasing temperature gradient. Using recombinant human growth hormone (rhGH) as the test purification target, and after avoiding cross reactions of aptamers with *Bacillus subtilis* extracellular proteins by negative SELEX, the effects of time and pH on aptamer binding to rhGH were investigated. The highest binding efficiency of aptamers on rhGH-immobilized microparticles was obtained at pH 7.0. The aptamers that interacted with rhGH were eluted by a multi-stage step-up temperature gradient in $\Delta T = 10\ ^{\circ}C$ increments within the range $T = 55–95\ ^{\circ}C$; and the strongest affinity binding was disrupted at $T = 85\ ^{\circ}C$ where $C_{Apt} = 0.16\ \mu M$ was eluted. The equilibrium binding data obtained was described by a Langmuir-type isotherm; where the affinity constant was $K_D = 218$ nM rhGH. RhGH was separated from the fermentation broth with 99.8% purity, indicating that the method developed is properly applicable even for an anionic protein.

Introduction

The separation and purification of proteins, particularly hormones and enzymes, is achieved by using well-designed downstream processes based on uniquely defined molecular interactions with the target biomolecule. Affinity purification is a highly selective purification approach in which biomolecules of interest in a mobile phase form a stable and reversible association with the specific ligands bound to a stationary phase. Protein-specific affinity interactions can be achieved *in vitro* by mimicking the protein–nucleic acid recognition interaction that plays an essential role in all mechanisms of gene expression and control. As these synthetic protein–nucleic acid interactions do not exist naturally, the choice of the single-stranded DNA or RNA to use is based on (i) the number of residues of amino acids or nucleotide bases involved in the binding interface; (ii) the count of ionic bonds and polar interactions, namely hydrogen bonds, formed in the binding action; (iii) the extent of the binding interface area. Aptamers are DNA or RNA molecules selected *in vitro* from random pools and chosen for their ability to bind to certain molecules such as nucleic acids, proteins, small organic molecules, and even entire organisms [1–4]. They are identified by a widely used method called *systematic evolution of ligands by exponential enrichment* (SELEX)[1] [4–6]. In a DNA SELEX process, a DNA library composed of synthetic random sequences flanked by specifically designed primer sequences is incubated with the target molecule. The target-bound oligonucleotides are eluted and then amplified by PCR, forming a new pool of oligonucleotide pool through separation of the relevant ssDNA, which can then be used for a binding reaction with the target molecule in the next SELEX round. The iterative cycles of selection and enrichment are repeated for 6–20 SELEX rounds [1,7,8].

Human growth hormone (hGH) is an anionic, non-glycosylated four helix-bundle protein also known as somatotropin, composed of 191 amino acid residues with a total molar mass of 22 kDa. Recombinant hGH (rhGH) has been used to treat hypo-pituitary dwarfism, injuries, bone fractures, bleeding ulcers, and burns [9]. Recently it is reported to have considerable therapeutic value for

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[1] *Abbreviations used:* SELEX, systematic evolution of ligands by exponential enrichment; hGH, human growth hormone; HIV, human immunodeficiency virus; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPCE, high-performance capillary electrophoresis.

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Nomenclature

hGH	human growth hormone
rhGH	recombinant human growth hormone
C_{Apt}	liquid phase aptamer concentration (mg/ml)
C^0_{Apt}	initial liquid phase aptamer concentration (mg/ml)
C_{rhGH}	liquid phase rhGH concentration (mg/ml)
C^0_{rhGH}	initial liquid phase rhGH concentration (mg/ml)
C^*_{rhGH}	liquid-phase equilibrium rhGH concentration (mg/ml)
C_{Pro}	extracellular rhGH-free *Bacillus* fermentation broth protein concentration (mg/ml)
C^0_{Pro}	initial extracellular rhGH-free *Bacillus* fermentation broth protein concentration (mg/ml)
K_D	affinity constant (nM rhGH/ml)
m_{rhGH}	mass of rhGH (mg)
m_{mp}	mass of microparticles (mg)
mp	microparticle
m_p	mass of *Bacillus* extracellular proteins (mg)
n_{Apt}	moles of aptamer (nmol)
q_{Apt}	solid phase aptamer concentration (mg aptamer/mg-mp)
q_{rhGH}	solid phase rhGH concentration (mg rhGH/mg-mp)
q^*_{rhGH}	solid phase equilibrium rhGH concentration (mg rhGH/mg-mp; nmol rhGH/μmol aptamer)
q_P	solid phase rhGH-free *Bacillus* fermentation broth protein concentration (mg protein/mg-mp)
q_0	saturation constant (mg rhGH/mg-mp; nmol rhGH/μmol aptamer)
t	residence time
V	reactor volume (μl)
V_R	reaction volume (μl)

girls with Turner's syndrome, children with chronic renal failure, and adults with growth hormone deficiency or human immunodeficiency virus (HIV) syndrome [10]. Although aptamers having molecular recognition properties similar to antibodies [11] have been used for targeting molecules varying from clinically important proteins [12,13] and peptides [14] to small molecules such as dyes [15], amino acids [16] and drugs [17,18], there is no report in the literature concerning the use of aptamer sequences specific for rhGH binding, nor their use in the process of separating and purifying rhGH.

To select aptamers having high affinity to the target molecule, the present work reports for the first time the use of a single-step ligand (oligonucleotide) aptamer evolution by temperature gradient (LETEG) method, in which aptamers having high specificity to the target protein are separated from the low specificity aptamers by elution through an increasing temperature gradient. RhGH was chosen as the model recombinant therapeutic protein to be purified from the fermentation medium by the proposed method. Thus, to separate and purify rhGH from the fermentation broth of recombinant *Bacillus subtilis*, an aptamer specific for rhGH was selected by the proposed LETEG method, and binding efficiencies of the identified aptamer to rhGH and binding equilibrium characteristics were determined.

Materials and methods

Aptamer library and PCR primers

The aptamer library consists of the sequence TGAGGGAATCGGGTTT-*N21*-TAACAATAAGCTCGCA, in which sequence N21 is a random region of 21 nucleotides producing sufficient diversity in the random sequence such that it represents 4^{21} sequences less those representing palindromic and symmetric sequences. The forward primer TGAGGGAATCGGGTTT and reverse primer TGCGAGCTTATTGTTA were purchased as HPLC-purified sequences and obtained at the 0.2 μmol scale (Invitrogen, CA, USA).

Protein and aptamer immobilization onto microparticles

Microparticles composed of poly-glycidylmethacrylate with a 5 μm diameter and NH_2-modified surface functionality were purchased from Micromod Partikeltechnologie GmbH, Warnemuende, Germany. For covalent binding of proteins onto microparticles (mp), NH_2-modified surface functionality was activated with gluteraldehyde. Two microliters from 100 μg/μl microparticle solution, 1 μl from 25% (v/v) gluteraldehyde solution and 50 μl distilled water is mixed in a microcentrifuge tube; after 30 min of incubation at room temperature, microparticle were centrifuged 1 min at 13000 rpm and supernatant was removed. Thereafter, microparticles were washed three times with distilled water.

For protein and rhGH immobilization, the microparticles were resuspended either in 0.1 μg *Bacillus* proteins/μl or 0.1 μg rhGH/μl, in a total reaction volume of 200 μl, and kept at pH 7.0 for 30 min at 25 °C. After centrifugation, the microparticles carrying either *Bacillus* proteins or rhGH were incubated in 50 μl of 1 M glycine for 30 min for capping any uncoupled reactive aldehyde. Covalent binding of aptamer onto microparticles was done similarly to that of the protein immobilization method, microparticles are activated with glutaraldehyde leading to surface reactive aldehyde groups. 5′NH_2 modified aptamers (5′-NH_2TGAGGGAATCGGGTTTTTACGCTAGCAGACGCAGCATAACAATAAGCTCGCA-3′) were immobilized onto the microparticles via their NH_2 groups. After aptamer immobilization, glycine treatment was performed for capping uncoupled reactive aldehyde groups. The initial aptamer concentration used was C^0_{Apt} = 500 μM.

Aptamer selection for rhGH

Aptamers having affinity to *Bacillus* proteins were eliminated by negative SELEX [19] using the rhGH-free *B. subtilis* fermentation broth. Immobilized rhGH-free *Bacillus* extracellular proteins with q_P = 40 μg protein/mg-mp were incubated with 500 μM of the aptamer library in a 200 μl H_2O. Aptamers remaining from the negative SELEX operation were then mixed with rhGH-immobilized microparticles (q_{hGH} = 30 μg rhGH/mg-mp) in a 200 μl reaction volume. The aptamers interacting with rhGH were eluted using a multi-stage step-up temperature gradient with ΔT = 10 °C increments within the T = 55–95 °C range. In the gradient elution process, after each elution step (t = 5 min), the microparticles were centrifuged at 13,000 rpm for 1 min and resuspended in 50 μl distilled water for the next elution step.

PCR amplification, cloning, and sequencing

The eluted single-stranded DNA aptamers were amplified by PCR (Techne, UK) using the forward and reverse primers. Purified PCR products (PCR Purification Kit, Fermentas Inc., Vilnius, Lithuania) were then cloned into pGEM-T vector (Invitrogen Corporation, California, USA) and then sequenced (Amersham Biosciences, Buckinghamshire, UK).