AFFINITY CHROMATOGRAPHIC PURIFICATION OF RECOMBINANT HUMAN GROWTH HORMONE

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ABSTRACT

AFFINITY CHROMATOGRAPHIC PURIFICATION OF RECOMBINANT HUMAN GROWTH HORMONE

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The purpose of the study is to purify human growth hormone from the fermentation broth by affinity chromatography. For this purpose, human growth hormone specific oligonucleotide aptamers are selected among an aptamer library; selected oligonucleotides were synthesized and used as ligands. Effect of pH on ligand-human growth hormone complex formation was investigated and the highest complex formation was obtained at pH= 7.0. Human growth hormone is separated from the fermentation broth with 99.8% purity and 41% overall yield. The equilibrium data obtained was described by Langmuir type isotherm where saturation constant (q_0) and affinity constant (K) are calculated as 0.338 mg hGH/μmol aptamer and 0.059 mg hGH/ml, respectively. Further, equilibrium

data obtained using aptamer affinity column was described by Langmuir type isotherm where saturation constant (q_0) and affinity constant (K) are 0.027 mg hGH/µmol aptamer and 1.543 mg hGH/ml, respectively. It is possible that, selected aptamer can be used for purification of bulk amounts of recombinant human growth hormone by using aptamer affinity chromatography.

Keywords: Aptamer, human growth hormone, affinity chromatography, liquid-solid, column, separation, purification.

ÖZ

AFİNİTE KROMATOGRAFİ YÖNTEMİYLE REKOMBİNANT İNSAN BÜYÜME HORMONUNUN SAFLAŞTIRILMASI

Balcı, Oğuz
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Çalışmanın amacı insan büyüme hormonunun fermentasyon ortamından afinite kromatografi yöntemi ile ayrılmasıdır. Bunun için, insan büyüme hormonuna özgün oligonükleotit aptamer, bir aptamer bankası içerisinden seçilmiş ve bu seçilen aptamer sentezlenip ligand olarak kullanılmıştır. Ligand-insan büyüme hormonu complex oluşumuna pH'ın etkisi araştırılmış ve en yüksek complex oluşumunun pH=7.0 değerinde gerçekleştiği tespit edilmiştir. İnsan büyüme hormonu fermentasyon ortamından %99.8 saflık, %41 geri kazanım ile ayrılmıştır. Denge verileri Langmuir tipi izoterme uymuş; doygunluk sabiti (q_0) ve afinite denge sabiti (K) sırasıyla, 0.338 mg hGH/µmol aptamer ve 0.059 mg hGH/ml olarak hesaplanmıştır. Kolon deneyleriyle elde edilen denge

verileri de Langmuir tipi izotermelerine uymuştur; doygunluk sabiti (q_0) ve afinite denge sabiti (K) sırasıyla, 0.027 mg hGH/µmol aptamer ve 1.543 mg hGH/ml olarak hesaplanmıştır. Aptamer afinite kromatografi yöntemiyle, rekombinant insan büyüme hormonu yüksek saflıkla elde edilmiştir.

Anahtar kelimeler: Aptamer, insan büyüme hormonu, afinite kromatografi, sıvı-katı, kolon, ayırma, saflaştırma.

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Abbreviations

$(C^0_{Pro}-C_{Pro})/ C^0_{Pro}$	Solid phase (adsorbed) hGH-free fermentation broth protein concentration per total hGH-free fermentation broth protein concentration
$(C^0_{Apt}-C_{Apt})/ C^0_{Apt}$	Solid phase (adsorbed) aptamer concentration per total concentration
$(C^0_{hGH}-C_{hGH})/ C^0_{hGH}$	Solid phase (adsorped) hGH concentration per total hGH concentration
A_λ	Absorbance at wavelength λ
C	Liquid phase solute concentration at time t (mg/ml)
C^0	Initial liquid phase solute concentration (mg/ml)
C^0_{Apt}	Initial liquid phase aptamer concentration (mg/ml)
C^0_{hGH}	Initial liquid phase hGH concentration (mg/ml)
C^0_{Pro}	Initial liquid phase hGH-free fermentation broth protein concentration (mg/ml)
C_{Apt}	Liquid phase aptamer concentration (mg/ml)
C_{hGH}	Liquid phase hGH concentration (mg/ml)
C_{Pro}	Liquid phase hGH-free fermentation broth protein concentration (mg/ml)
DAAC	Differential aptamer affinity column
ddNTP	dideoxynucleotide triphosphate
DF	Dilution Factor
dNTP	deoxynucleotide triphosphate
dsDNA	Double stranded DNA

hGH	Human growth hormone
K	Affinite constant (mg hGH/ml)
L	Path Length (cm)
OD_λ	Optic density at wavelength λ
PCR	Polymerase chain reaction
Q	Volumetric liquid flow rate (cm^3/sec)
q	Solid phase solute concentration (mg solute/μmol aptamer)
q_0	Saturation constant (mg solute/μmol aptamer)
rhGH	Recombinant human growth hormone
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	Single strand DNA
t	Incubation time (sec)
T_m	Melting temperature (ºC)
y	Liquid phase solute concentration (mg/ml)

Greek Letters

ε	Molar extinction coefficient
λ	Wavelength (nm)

CHAPTER 1

INTRODUCTION

This thesis focuses on separation and purification of a therapeutic human protein, i.e., human growth hormone, a protein synthesized naturally in human metabolism. Proteins are linearly synthesized from the 20 amino acids (monomers) through an intracellular reaction mechanism based on sequential transfer of information from the genes in the DNA to RNA and then to protein (Çalık et al., 2002).

Proteins are found in all living systems ranging from bacteria and viruses through the unicellular and simple eukaryotes to higher animals such as humans. Proteins can have vastly different functional roles, including signaling, catalysis, protection, regulation, and mechanical support in our cells and bodies (Lodish et al., 2004). In bodily disorders and for the treatment of disease, proteins are frequently either supplemented or targeted; in modern medicine, almost all drug targets are proteins (Drews et al., 2000).

Most of the biotechnology products are proteins which must be prepared in large quantities in purified form (Khodabandeh et al., 2003). Generally, combinations of different methods have to be applied for purifications of proteins. Multi-step purification of proteins by conventional procedures is frequently laborious and incomplete, and the yields are often low (Cuatrecasas et al., 1968). The ultimate objective in production of a recombinant protein after achieving the production in the bioreactor system is separation and purification

from the bioreactor product mixture (Çelik et al., 2007), by simplifying the separation and purification process which may be achieved by elimination of the time consuming and expensive steps such as chromatography (Khodabandeh et al, 2003).

Human growth hormone is a well known pituitary derived protein with a wide range of biological functions such as protein synthesis, cell proliferation and metabolism (Tritos and Mantzoros, 1998). Recombinant human growth hormone was subsequently approved for human therapy in the USA in 1986, and at present there are several preparations of recombinant human growth hormone available including Humatrope (Eli Lilly, USA), Saizen and Serostim (Serono Laboratories, USA), Genotonorm (Pharmacia, USA), and Norditropin (Novo Nordisk, USA) for their therapeutic applications such as treatment of dwarfism, bone fractures, skin burns, bleeding ulcers and AIDS (Roehr, 2003).

Separation of globular proteins from liquid mixture by aptamer based affinity chromatography is a novel approach for separation and purification. Aptamers are nucleic acids selected for binding target molecules of interest with high affinity and selectivity (Cao et al, 2005). For selection of aptamers against a specific target, first an aptamer library of oligonucleotides usually containing 10^{14} to 10^{15} random sequences is synthesized. This library is then incubated with the target molecule of interest under certain conditions (Cao et al, 2005). The sequences that chemically interact with and bind to the target molecules are isolated and amplified by polymerase chain reaction (PCR) for the next round of incubation (Cao et al, 2005). This process is repeated until a sequence that binds to the target with the highest affinity and selectivity is determined (Cao et al, 2005).

Aptamers have been used for purification of proteins because aptamers have several clear advantages. First, their production is easy and cheap. Because oligonucleotides have robust structures, aptamers can withstand harsher experimental conditions and can be stored and reused without causing much degradation. Aptamers can also be easily labeled or modified in controllable ways

for different molecular recognition applications. Finally, they can be easily immobilized onto solid surfaces without much expected change in their binding affinities to proteins (Cao et al, 2005).

Purification of recombinant human growth hormone has been performed using combinations of several purification methods such as gel filtration chromatography, ion-exchange chromatography, hydrophobic-interaction chromatography, and immunoaffinity chromatography. Nevertheless, in the literature there is no work reporting neither on aptamer synthesis for hGH, nor separation of hGH using an aptamer based separation and purification process.

In the thesis, a single step separation and purification process for recombinant human growth hormone from the bioreactor product medium by developing an aptamer-affinity chromatography is presented. For this purpose, an aptamer library is synthesized and human growth hormone specific aptamer is selected among the aptamer library and immobilized onto the micro-particles for separation of human growth hormone from the bioreactor reaction medium. Finally, batch equilibrium and batch affinity column experiments are performed to calculate saturation constant (q_0) and affinity constant (K).

CHAPTER 2

LITERATURE SURVEY

2.1 Protein separation and purification methods

Advancement in recombinant DNA technology has made possible the expression of proteins in host cells, such as *Escherichia coli* (Wallis and Wallis, 1990; Violand *et al.,* 1994), *Bacillus subtilis* (Westers et al., 2004) and *Pichia pastoris* (Çelik et al., 2007). After the intracellular synthesis (e.g. by r-*E.coli*) or the synthesis and secretion, i.e., extracellular production, of the target protein, an efficient downstream process for separation from other proteins, nucleic acids, carbohydrates, lipids, excreted amino and organic acids, and other molecules in the fermentation broth, is needed (Çalık et al., 2008).

Proteins in solution show profound changes in solubility as a function of pH, ionic strength, temperature and electrostatic properties of the solvent (Khodabandeh et al, 2003). Ion exchange chromatography (Brostedt and Roos, 1989; Çalık et al., 2002), affinity chromatography (Wingfield et al., 1987; Cuatrecasas et al, 1968), hydrophobic interaction chromatography (Oliveira et al, 1999), metal-chelate affinity chromatography (Mukhija et al, 1995), gel filtration (Bonnerjea, 1986), and isoelectric precipitation (Bailey and Ollis, 1986) are some of the separation methods performed. In Table 2.1 separation processes and physicochemical basis for the processes are summarized (Grandison and Lewis, 1996).

Table 2. 1 Physicochemical basis for the development of separation process

Physicochemical basis	Separation process
Charge	Ion exchange chromatography
	Electrodialysis
	Aqueous two-phase partitioning
	Reverse miscelle extraction
Hydrophobicity	Hydrophobic interaction chromatography
	Reversed phase chromatography
	Precipitation
	Aqueous two-phase partitioning
Specific binding	Affinity chromatography
Size	Gel filtration
	Ultrafiltration
	Dialysis
Electric mobility	Electrophoresis
Isoelectric point	Chromatofocusing
	Isoelectric focusing
Sedimentation rate	Centrifugation
Surface activity	Adsorption
	Foam fractionation
Solubility	Solid-liquid extraction
	Supercritical fluid extraction

2.2 Affinity chromatography

In the late 1960s, Cuatrecasas et al., (1968) demonstrated that enzymes could be purified using a specific competitor inhibitor immobilized on solid matrices covalently attached. Affinity chromatography, or more precisely

biospecific interaction chromatography, comprises a number of highly selective purification techniques in which chemical molecules are bonded to a solid support and undergo specific and reversible interactions with a biomolecule to be purified. In the process the biomolecule forms a stable, reversible association complex with the support-bound biospecific ligands. The resulting association constant K_a varies between $10^3 M^{-1}$ and $10^{15} M^{-1}$ depending on the biospecificity (Street et al., 1994).

2.2.1 Preparation of affinity packing

Each affinity packing or adsorbent contains two structural elements; the solid support and the ligand.

2.2.1.1 The solid support

The support in the affinity chromatography should have several properties. The particles must be rigid and must exhibit good mechanical and pressure stability. Particles must have a hydrophobic non-ionic inner surface with reactive functional groups. And, particles must have high chemical stability to withstand buffer solutions. (Street et al., 1994)

There are different supports that have been used in affinity chromatography some of which are, among the inorganics: porous glass, porous silica, and alumina; among the natural polymers: agarose, cellulose, and dextran; among the synthetic polymers: poly(hydroxy-methylacrylamide), poly(acrylamide), and poly(hydroxylmethacrylates) (Street et al., 1994).

2.2.1.2 Activation of the solid support

Specific chemical reactions are required to prepare an affinity packing, and are accomplished by several consecutive steps. First step is the “activation step” that includes the pretreatment of the support and the subsequent reaction with a chemical of defined functionality, whereby it is covalently bound to the support. The linked group consists of an anchor group to the support, a spacer,

and a reactive terminating group. The second step is the "coupling step" in which the activated support is subjected to reaction with a "biomimetic chemical or biochemical molecule" under gentle conditions to couple the ligands. Finally in the third step, in order to avoid non-specific interactions, the residual activating groups or residues from the ligand coupling procedure have to be removed by means of appropriate reactions or treatment (Street et al., 1994).

2.2.1.3 The ligand

The bonded biochemicals or biomimeting chemicals functioning as ligands are grouped as:

i) chemical ligands or chemical biomimetics: e.g., benzamidine, triazine dyes, metal chelates); and

ii) biological ligands or biochemical mimetics: e.g., sugars, e.g. lectin, hormones, nucleic acids, enzymes, antibodies (Street et al., 1994).

Depending on the ligand immobilized, affinity adsorbents are classified as:

a) high specificity: capable of binding typically one biomolecule, and

b) moderate specificity: the group-specific ligands, capable of binding many similar biomolecules.

A ligand suitable for process affinity separation should have the following properties:

1. It forms a reversible complex with the molecule to be purified with a dissociation constant value between 10^{-4} and 10^{-8} M;
2. It possesses functional group(s) not involved in the affinity interaction, via which it can be immobilized;
3. It remains stable during the chemical procedure of immobilization;
4. Its effective cost should represent only a fraction of the value of the total product that can be obtained by using a fixed amount of the ligand.

There exists several affinity ligands some of which are dye ligands, antibodies, aptamers, antigens, protein A, protein G, substrate analogues, enzyme

inhibitors, complimentary nucleotide base-pair sequence, receptors, amino acids, poly-(amino acids), lectins, concanavalin A, immobilized metal ions.

2.2.2 Affinity chromatography separation steps

Affinity chromatography separations usually involve three stages:

i) adsorption,

ii) wash, and

iii) elution.

In affinity chromatography, the solute to be purified is passed through a column containing a cross-linked polymer or gel to which a specific competitive inhibitor of the solute has been covalently attached (Cuatrecasas et al, 1968). All proteins without substantial affinity for the bound inhibitor will pass directly through the column, whereas one that recognizes the inhibitor will be retarded in proportion to its affinity constant (Cuatrecasas et al, 1968). Elution of the bound solute is readily achieved by changing such parameters as salt concentration or pH, or by addition of a competitive inhibitor in solution (Cuatrecasas et al, 1968). These stages may be carried in a finite batch system, a fixed bed, a periodic countercurrent bed, a continuous countercurrent bed, a fluidized bed, or a magnetically stabilized fluidized bed (Street et al., 1994).

2.2.2.1 Adsorption and adsorption isotherms

Two types of adsorption may occur (Smith, 1983):

a) Physical adsorption: is nonspecific and the atomic or molecular forces attracting the molecules in the fluid to the solid surface are relatively weak, and the heat evolved during the exothermic adsorption process is of the same order of magnitude as the heat of condensation ($-\Delta H = 0.5$ to 5 kcal/mol).

b) Chemical adsorption or chemisorption: is specific and involves forces much stronger than in physical adsorption. The adsorbed molecules are held to the active sites or immobilized ligands by valence forces of the same type as those occurring between atoms in molecules.

Analysis of an adsorption process is based on identifying an equilibrium relationship between bound and free solute and performing a solute material balance. The equilibrium relationship between the solute concentration in the liquid phase and that on the adsorbent's surface at a given condition is called an isotherm (Raja, 2006). From the point of view of bioseparations, three types of isotherms:

1. Linear adsorption,
2. Freundlich adsorption, and
3. Langmuir adsorption

are important. Most isotherms are linear when the solute concentration is very low. The linear isotherm is given by:

$$q = Ky \tag{2.1}$$

where q is the amount of solute adsorbed per amount of adsorbent, y is the solute concentration in solution, and K is linear equilibrium constant.

The adsorption of antibiotics, steroids and hormones generally follow Freundlich type isotherm:

$$q = Ky^n \tag{2.2}$$

where the constants n and K must be determined experimentally. These constants are best determined by means of log-log plot of q versus y.

The Langmuir isotherm is applicable when there is a strong specific interaction between the solute and the adsorbent. Ion exchange and affinity type adsorptions generally follow Langmuir isotherm:

$$q = yq_0 / (K+y) \tag{2.3}$$

in which q_0 and K are constants which must be determined experimentally. The constants are best determined by means of a plot of q^{-1} versus y^{-1}. The intercept on such a plot is q_0^{-1} and the slope is K/q_0.

$$q^{-1} = Ky^{-1}q_0^{-1} + q_0^{-1} \tag{2.4}$$

2.3 The use of nucleic acids as ligands in affinity chromatography

Aptamers are short oligonucleotides (<100 bases=nucleotides), nucleic acids, selected for binding target molecules of interest with high affinity and selectivity (Cao et al, 2005). The word "Aptamer" is derived from the Greek word "aptus", which means, "to fit" and describes early selection results with a lock and key mode of interaction between the nucleic acid and the target (Kulg et al., 1994). Aptamers present the same high specificity and affinity for their targets as antibodies. In aptamer-affinity chromatographic purification, the oligonucleotides of greater than 20 bases in length, which is flanked by known primer sequences (Tuerk and Gold, 1990; Klug and Famulok, 1994; Bruno, 1997), are used as ligands.

The idea of using single-stranded nucleic acids (aptamers) as affinity molecules for proteins, first described in 1990 (Tuerk and Gold, 1990; Ellington and Szostak 1990) and has shown modest progress. The first nucleic acid selection to utilize a protein target that does not biologically bind to RNA or DNA was the generation of a ssDNA aptamer against thrombin (Bock et al., 1992).

Since 1990 (Ellington et al., 1990; Tuerk et al., 1990), aptamers have been created for a wide variety of targets such as small molecules, amino acids, peptides, and proteins, with affinities for their targets typically in the low nanomolar (nM) to picomolar (pM) range (Table 2.2).

Table 2. 2 Published studies about selected aptamers against different targets

Target	Template	Source
Thrombin	ssDNA	(Bock et al, 1992)
IgE	ssDNA	(Wiegand et al, 1996)
Hiv-1 RT	ssDNA	(Schneider et al, 1995)
Thrombin	RNA	(Latham et al, 1994)
Elastase	RNA	(Lin et al, 1995)
ATP	ssDNA	(Sassanfar et al, 1993)
Cyanocobalamin	RNA	(Lorsch et al, 1994)
Valine	RNA	(Majerfeld et al, 1994)
NAD	RNA	(Lauhon et al, 1995)
Tryptophan	RNA	(Famulok et al, 1992)
Guanosine	RNA	(Connell et al, 1994)
Arginine	RNA	(Geiger et al, 1996)
Dopamine	RNA	(Mannironi et al, 1997)
Streptomycin	RNA	(Wallace et al, 1998)

2.3.1 Selection of target specific aptamers among an aptamer library

Selection of target specific aptamers among an aptamer library is typically performed by first synthesizing a random oligonucleotide of greater than 20 bases in length, which is flanked by known primer sequences (Tuerk and Gold, 1990; Klug and Famulok, 1994; Bruno, 1997). Synthesis of the random region is achieved by mixing equimolar amounts of all four nucleotides at each locus in the sequence. Thus, the diversity of the random sequence is maximally 4^n minus the frequency of palindromes and symmetric sequences, where n is the length of the sequence (Klug and Famulok, 1994; Lorsch and Szostak, 1996). Therefore, the aptamer selection process begins with a library of synthesized oligonucleotides

usually containing 10^{14} to 10^{15} random sequences. Synthesized ssDNA aptamer library can directly be used to select a DNA aptamer or in vitro transcribed leading to an RNA library for selecting an RNA aptamer (Tuerk et al., 1995). In applications, which may require a stable aptamer it may be preferable use ssDNA because due to the 2' OH group present in RNA it is inherently less stable than ssDNA of the same basic sequence (Moran et al., 1994).

The library is then incubated with the target molecule of interest under certain conditions. The temperature of the binding reaction is an important consideration however many successful selections have been carried-out with binding taking place at room temperature (Rusconi et al., 2000). The sequences that interact with and bind to the target molecules are isolated; numerous methods of isolation of the sequences that bind to the target molecules have been used and include separation on nitrocellulose membranes (Jellinek et al., 1993), separation via use of a solid support matrix (Yang et al., 1998), and separation based on differences in electrophoretic gel mobility (Conrad et al., 1996). The binding nucleic acid molecules are then subjected to Reverse Transcription - Polymerase Chain Reaction (RT-PCR) in the case of RNA (Tuerk et al., 1995) or simply PCR in the case of ssDNA (Erlich et al., 1989) for the next round of incubation. This process is repeated until a sequence that binds to the target with the highest affinity and selectivity is determined. When the pool of nucleic acids has been enriched for binding species, individual molecules are cloned (Validis et al., 1998), sequenced (Graham et al., 2001), and characterized (Patel et al., 2000).

Figure 2.1 gives an illustration of systematic evolution of aptamers (ligands) by exponential enrichment process (Sampson, 2003). As seen in the figure, DNA library is synthesized with random sequence flanked by known primer sequences. By in vitro transcription, RNA library is synthesized and the library is incubated with the target. Selected sequences are reverse-transcribed to get double strand DNA and double strand DNA is enriched with PCR. As explained, selection and enrichment steps are repeated for several times. At the beginning of each selection and enrichment step, the target molecule has to

interact with single strand nucleic acid. For that reason, double strand nucleic acid has to be converted to single strand nucleic acid after PCR. That is performed with either in vitro transcription or ssDNA selection methods from dsDNA. When ssDNA selection methods from dsDNA are used, there is no need for DNA/RNA conversions (Sampson, 2003). Figure 2.2 (Howard et al, 2006) shows systematic evolution of aptamers (ligands) by exponential enrichment process with both ssDNA and RNA pools. As seen in the figure, starting nucleic acid pool (random ssDNA pool) is either transcribed to random RNA pool or directly used. If ssDNA pool is used, ssDNA has to be selected after dsDNA synthesis with PCR. If RNA pool is used, reverse transcription, PCR, and transcription has to be applied respectively.

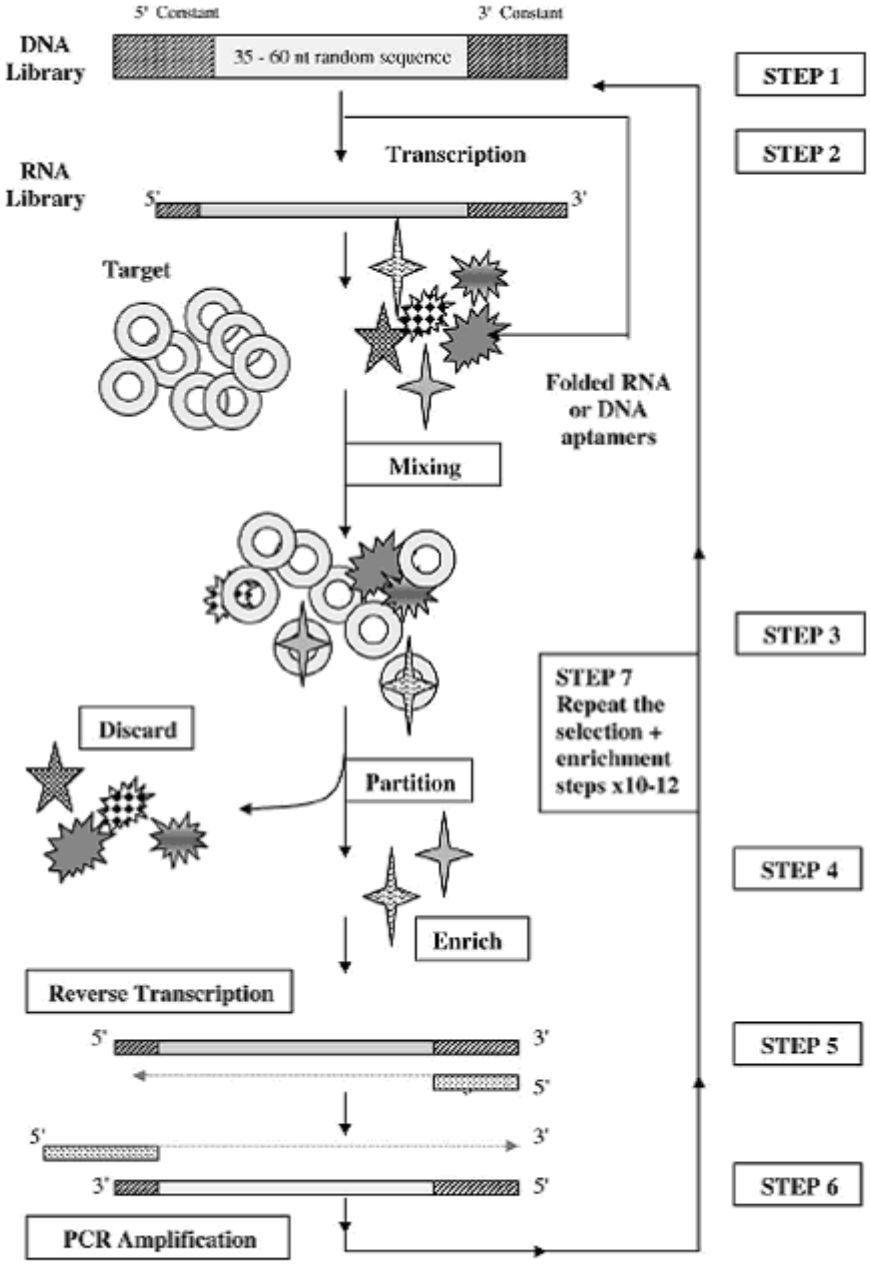

Figure 2. 1 Systematic evolution of ligands by exponential enrichment (Sampson, 2003)

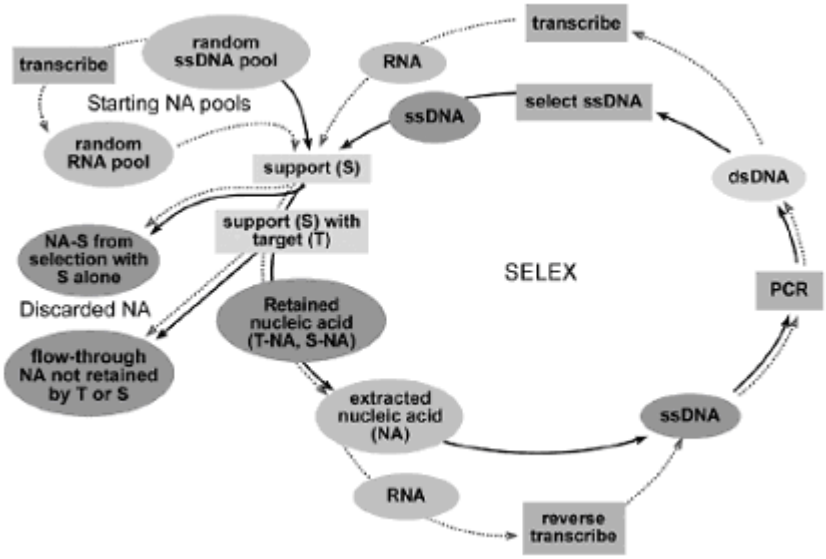

Figure 2. 2 Aptamer selection process with ssDNA and RNA libraries (Howard et al, 2006)

For the targets varying from clinically important proteins (Schneider et al., 1995; Tang et al., 2006), peptides (Rusconi et al., 2002), to small molecules, such as dyes (Ellington and Szostak, 1990, 1992), amino acids (Famulok, 1994) and drugs (Jellinek et al., 1993, 1995); the systematic evolution of aptamers (ligands) by exponential enrichment process provides a rapid isolation of binding oligonucleotide sequences to the targets from a very large nucleic acid library.

There is not any systematic evolution of aptamers (ligands) by exponential enrichment process study performed for determining aptamer sequence for human growth hormone in the literature. In this study, aptamer, which is specific to human growth hormone, is determined with a modified aptamer (ligand) selection process.

2.3.2 Aptamer-affinity chromatographic purification of proteins: Applications

The stability of aptamer protein complexes is characterized by the apparent dissociation constants (K_d); aptamers typically bind target proteins with dissociation constants in the picomolar to nanomolar range (Ravelet et al, 2006).

The high affinity of aptamers for their targets is given by their capability of folding upon binding their target molecule: they can incorporate small molecules into their nucleic acid structure or integrate into the structure of larger molecules such as proteins (Herman and Patel, 2000). In Figure 2.3, recognition in an aptamer-protein complex is illustrated; an RNA aptamer (gray) selected against MS2 bacteriophage coat protein (blue) was crystallized with its protein target (Convery et al., 1998).

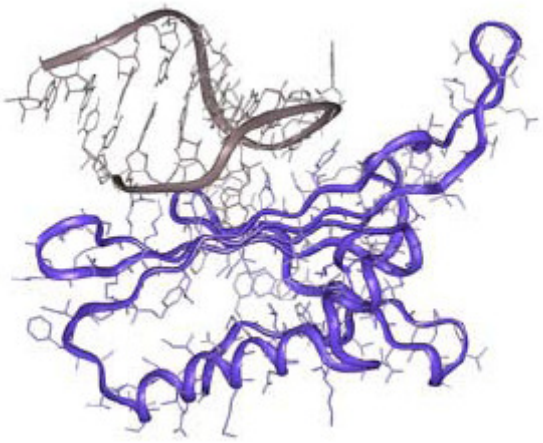

Figure 2. 3 Recognition in an APTAMER-PROTEIN complex (Convery et al., 1998)

Romig and co-workers have presented the first work concerning the use of an immobilized DNA aptamer as an affinity stationary phase (Romig et al, 1999). In Table 2.3 some of the published studies about aptamer based separation are given.

Table 2. 3 Published studies about aptamer based separation

Target	Aptamer	Support	Source
Human l-selectin	5'-biotinylated DNA aptamer	Streptavidin sepharose support	(Romig et al, 1999)
Thrombin	5'-thiol modified DNA aptamer	Bare fused-silica	(Bock et al, 1992)
HCV RNA polymerase	RNA aptamer	Magnetic beads	(Cho et al, 2004)
HCV replicase	RNA aptamer	Magnetic beads	(Chung et al, 2005)
TTF1	DNA aptamer	Magnetic beads	(Murfy et al, 2003)
Adenosine	3'-biotinylated DNA aptamer	Streptavidin support	(Deng et al, 2001)
FAD	35-base RNA aptamer	Inner walls of fused capillaries	(Clark et al, 2003)
D-enantiomer of arginine-vasopressin	55-base DNA aptamer	Streptavidin support	(Michaud et al, 2003)
Pigpen protein	Biotinylated aptamer	Magnetic Beads	(Blank et al, 2001)
Tenascin-C	Biotinylated aptamer	Streptavidin support	(Daniels et al, 2003)

There is no work in the literature reporting the purification of recombinant human growth hormone with aptamer-affinity chromatography.

2.3.3 Comparison of antibody and aptamer based separation

The use of antibodies in recognizing molecules predates the 1950's (Berson et al., 1956; Laurell, 1990; Opitz, 1990; Yalow et al., 1959). Monoclonal antibodies each recognizing a single epitope on the target molecule, theoretically allow the construction of immunoadsorbents of any desired specificity (Kohler and Milstein, 1975).

Aptamers show a very high affinity for their targets, with dissociation constants typically from the micromolar to low picomolar range, comparable to those of some monoclonal antibodies, sometimes even better (Jenison et al., 1994). Aptamers possess several clear advantages over antibodies for protein recognition. First, aptamers can be produced by chemical synthesis at a relatively high degree of purity resulting in little or no batch to batch variation and are produced through an in vitro process which does not require animals (Ravelet et al, 2006). Since oligonucleotides have more robust structures than proteins, aptamers can withstand harsher experimental conditions than antibodies and can be stored and reused without causing much degradation (Cao et al, 2005). Moreover, aptamers can also be easily labeled or modified in controllable ways for different molecular recognition applications (Cao et al, 2005). Aptamers can be easily immobilized onto solid surfaces without much expected change in their binding affinities to proteins (Cao et al, 2005); linkage of antibodies to columns that often result in couplings that are not uniform, leading to reduced capacity and/or affinity and that can allow leaching of the antibody from the column (Romig et al, 1999). Furthermore, antibodies present relative high size which limits the ligand density at the chromatographic surface. (Ravelet et al, 2006). Finally, some targets are toxic or are poorly immunogenic making the availability of the antibody problematic (Romig et al, 1999).

2.4 Human growth hormone

Human growth hormone (hGH) is a pituitary derived protein with a wide range of biological functions including protein synthesis, cell proliferation and metabolism (Tritos and Mantzoros, 1998). hGH is well known for therapeutic applications such as treatment of dwarfism, bone fractures, skin burns, bleeding ulcers and AIDS (Roehr, 2003).

2.4.1 Biochemical and physicochemical properties

Recombinant human growth hormone (hGH, somatotropin) is manufactured by recombinant DNA technology and is a 191 amino acid polypeptide (MW 22 kDa) with an amino acid sequence and two internal disulphide bridges identical to that of the major component of human pituitary growth hormone (Pearlman and Bewley, 1993). DNA and amino acid sequence of human growth hormone is given in Appendix A and three dimensional structure of hGH is given in Figure 2.4.

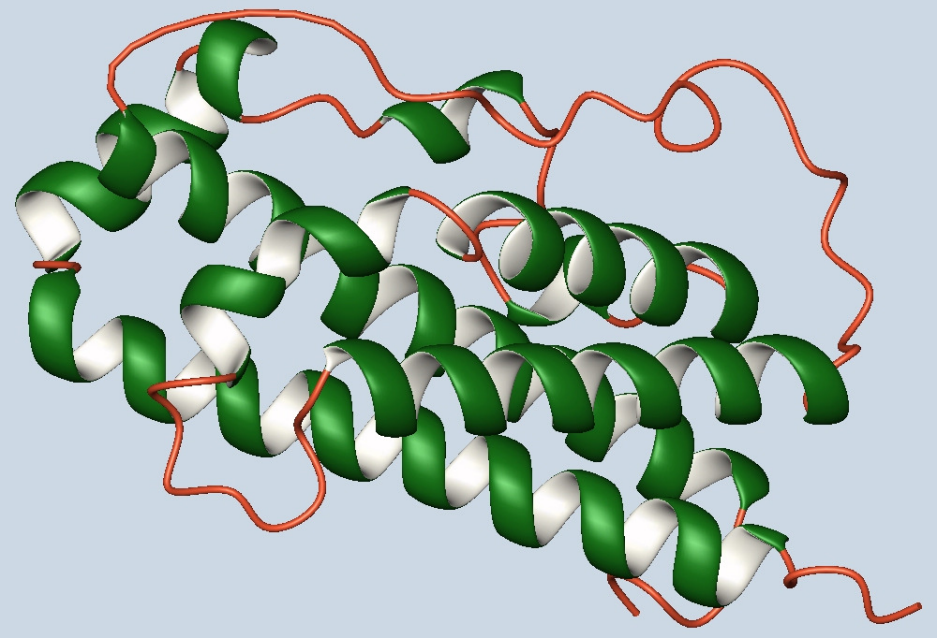

Figure 2. 4 Structure of human growth hormone

2.4.2 Separation and purification methods of human growth hormone

In the case of recombinant human growth hormone (rhGH), secreted in host cells' periplasmic space, critical contaminants include hGH related forms such as polymers, sulfoxides and desamido derivatives, bacterial endotoxins, contaminant host cell proteins (ECP), antibiotic, and bacterial DNA (Oliveira et al, 1999).

Purification of rhGH is typically performed using combinations of adsorption chromatographies such as ion exchange chromatography, hydrophobic interaction chromatography, metal chelate affinity chromatography and gel filtration chromatography. In Table 2.4, some of the published studies about purification of human growth hormone are given.

Table 2. 4 Published studies about purification of human growth hormone

Purification Method	Yield	Purity	Source
Ammonium sulfate fractionation	higher than 40%	-	(Oliveira et al, 1999)
First gel filtration chromatography			
Anion exchange chromatography			
Second gel filtration chromatography			
Hydrophobic interaction chromatography			
Acid precipitation	about 40%	99%	(Khodabandeh et al, 2003)
Immunoaffinity chromatography	-	-	(Jonsdottir et al, 1986)
Affinity chromatography	-	-	(Batternby et al, 1995)
Reversed-phase HPLC			
Gradiflow large-scale electrophoresis	90%	98%	(Derek et al, 2003)
DEAE-Sepharose ion-exchange chromatography	50%	99%	(Patra et al, 2000)
Sephacryl S-200 gel filtration chromatography			
Octyl- or phenyl-Sepharose hydrophobic interaction chromatography	28–48%	-	(Lefort et al, 1986)
Metal chelate affinity chromatography	-	-	(Mukhija et al, 1995)
Metal affinity chromatography	-	80%	(Llesmne et al, 1997)

CHAPTER 3

MATERIAL AND METHODS

3.1 Chemicals

The list of the chemicals used in the experiments is given in Appendix B with their suppliers Sigma-Aldrich GmbH, Taufkirchen, Germany and Merck KGaA, Darmstadt, Germany.

3.2 Aptamer library and PCR primers

Aptamer library and two primers for amplification of the aptamer library are purchased from Thermo Fisher Scientific (Ulm, Germany). Purchased oligonucleotides are HPLC purified and 0.2 µmol scale.

The primers are designed using primer design software (NAR, UK). The sequences of the aptamer library and the two primers are given in Table 3.1.

Table 3. 1 The sequences of aptamer library and the primers

Name	Sequence (5'→3')
AptLib03	TGAGGGAATCGGGTTT-**N21-**TAACAATAAGCTCGCA
AptLib03-F	TGAGGGAATCGGGTTT
AptLib03-R	TGCGAGCTTATTGTTA

3.3 Micro-particles

Poly-glycidylmethacrylate micro-particles with 5 µm size and NH_2 surface functionality are purchased (Micromod Partikeltechnologie GmbH, Warnemuende, Germany). Micro-particles are poly-glycidylmethacrylate particles synthesized by dispersion polymerization. In the dispersion polymerization reaction, glycidylmethacrylate is used as monomer, benzoyl peroxide is used as initiator, ethanol is used as continuous phase, and polyacrylic acid is used as stabilizer. Ammonia activation of poly-glycidylmethacrylate particles is performed via ammonia treatment for NH_2 surface functionality. 5 µm size is chosen in order to decrease centrifugation time to 1 minute at 13000 rpm.

3.4 PCR amplification and agarose gel electrophoresis

PCR amplification of target genes is performed with thermal cycler (Techne, UK). PCR contents and their final concentrations are given in Table 3.2 and PCR conditions are given in Table 3.3. The final concentrations of the PCR contents and PCR conditions are optimized for each amplification reaction. The optimization parameters are $MgCl_2$, total reaction volume, annealing temperature. *Taq.* DNA polymerase, PCR Grade Water, $MgCl_2$, dNTP mixture, 10X buffer were purchased from Fermentas Inc. (Vilnius, Lithuania). PCR primers are purchased from Thermo Fisher Scientific (Ulm, Germany). The primers are designed using primer design software (NAR, UK).

Table 3. 2 PCR contents and their final concentrations

PCR contents	Volume used (µl)	Final Concentration
PCR Grade Water	Up to 50	NA
$MgCl_2$ (25 mM stock solution)	3-5	1.5-2.5 mM
10X PCR Buffer	5	1X
dNTP's (10 mM of each dNTP)	0.5	0.1 mM
Forward primer (100 µM)	0.5	1 µM
Reverse primer (100 µM)	0.5	1 µM
Taq. DNA polymerase(5 u/µl)	0.2	0.02 u/µl
Template (50pg/µl)	5-10	5-10 pg/µl
Total Volume	**50**	

Table 3. 3 PCR conditions

Temperature (°C)	Time (sec.)	Cycle number	Steps
95	300	1	Initial denaturation
95	30-60	30-45	Denaturation
55-65	30-90		Annealing
72	20-60		Extension
72	300	1	Final extension

After PCR, the products were analyzed by agarose gel electrophoresis and molecular imaging is performed with UVP Biolmaging System, and Hamamatsu Digital CCD Camera, UK. 100bp DNA molecular weight marker, agarose, 5X TBE buffer, gel loading solution, gel staining solution were purchased from Fermentas Inc. (Vilnius, Lithuania).

3.5 Purification of PCR products

The PCR products were purified for further applications. The purification of the products was done according to the manufacturer's directions (PCR Purification Kit, Fermentas Inc., Vilnius, Lithuania):

1. On 20 µl PCR product, add 2 µl of Solution A and 22 µl of Solution B.
2. Mix the tubes gently by vortexing and leave at +4°C for an hour.
3. Centrifuge the tubes at 10,000 rpm for 10 minutes.
4. Discard supernatant and add 100 µl of 70% Ethanol wash solution.
5. Centrifuge the tubes at 13,000 for 5 minutes.
6. Discard supernatant and leave the tubes to dry for 20 minutes.
7. Resuspend the pellet with 15 µl PCR grade water and vortex.

3.6 Determination of protein and DNA concentration

Determination of protein and DNA concentration is performed by spectrophotometric readings at 280 nm and 260 nm (Thermo Spectronic, Heλiosα). The formulas used in oligonucleotide and protein quantification are given in Appendix C.

Determination of protein is also performed by using a molecular imaging system (UVP Biolmaging System, and Hamamatsu Digital CCD Camera, UK).

3.7 Ligation reaction

Amplified and purified DNA is ligated into pGEM-T vector (Figure 3.2) according to manufacturer's directions (Thermo Fisher Scientific, Ulm, Germany).

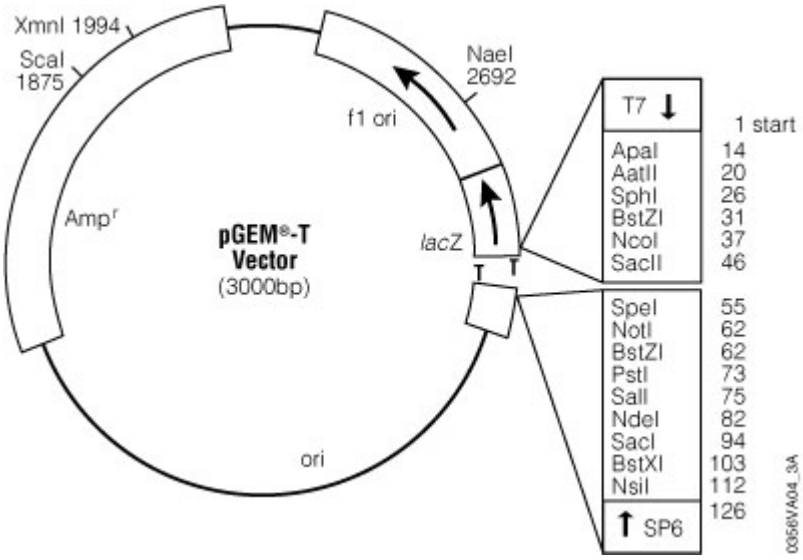

Figure 3. 1 pGEM-T Vector circle map and sequence reference points

Ligation contents and conditions are given in Table 3.4 and Table 3.5. Amount of insert DNA to be added to the reaction mixture was calculated such that insert:vector ratio of 1:3 was achieved, as given in the equation below:

20 ng vector x size of insert (bp) / size of vector (bp) x 3 = amount of insert (ng)

Table 3. 4 Ligation contents and their concentrations

Ligation contents	**Volume used (µl)**	**Final Concentration**
Nuclease free water	5	NA
10X ligation buffer	1	1X
pGEM-T vector (20 ng/µl)	1	2 ng/µl
T4 ligase (3 u/µl)	1	0.3 u/µl
PCR product	2	
Total Volume	10	

Table 3. 5 Ligation conditions

Temperature (ºC)	Time	Cycle number	Step
14	4 hours	1	Ligation

3.8 Transformation of plasmid DNA by $CaCl_2$ method to *E. coli*

LB Broth liquid, LB agar solid medium, and the *E. coli (JM109)* strain in amphicilin containing LB Broth solid medium and LB/ampicillin/IPTG/X-Gal plates are supplied from Sigma-Aldrich GmbH, Taufkirchen, Germany.

Transformation of plasmid DNA by $CaCL_2$ method to E.coli is performed with the protocol given below (Sambrook, 2001):

1. Inoculate a single colony of *E. coli* cells into 50 ml LB medium and incubate overnight at 37ºC with shaking (250 rpm).
2. In a 2 liter flask, inoculate 4 ml of the culture into 400 ml LB medium, incubate at 37ºC with shaking (250 rpm) to an OD_{600} of 0.375.
3. Aliquot culture into eight 50 ml prechilled sterile polypropylene tubes and leave the tubes on ice for 10 minutes.
4. Centrifuge at 1600 x *g* for 7 minutes at 4ºC.
5. Resuspend each pellet in 10 ml ice-cold $CaCl_2$ solution and centrifuge 5 minutes at 1100 x *g* at 4ºC.
6. Resuspend each pellet in 10 ml cold $CaCl_2$ solution and keep resuspended on ice for 30 minutes.
7. Centrifuge for 5 minutes at 1100 x g at 4ºC. Resuspend each pellet in 2 ml ice-cold $CaCl_2$ solution.
8. Aliquot 250 µl into prechilled sterile polypropylene tubes and freeze immediately at -70ºC.
9. For each ligation reaction, use two plates. Prior to plating, equilibrate the plates to room temperature.

10. Centrifuge the tubes containing ligation reactions and at the bottom of the tube, collect the contents. Add 2 µl of ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice. For determination of the competent cells, set up another tube on ice with 0.1 ng crude plasmid.
11. Remove the tubes of frozen competent cells from -70ºC storage and place in an ice bath until thawing for 5 minutes.
12. Transfer 50 µl of cells into each prepared tube and place the tubes on ice for 20 minutes. Heat shock the cells for 50 seconds in a water bath at 42ºC without shaking. Return the tubes to ice for 2 minutes.
13. Add 950 µl LB broth to the tubes containing cells transformed with ligation reactions and add 950 µl LB broth to the tubes containing cells transformed with crude plasmid.
14. Incubate for 90 minutes at 37ºC with shaking (150 rpm).
15. Plate 100 µl of transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For transformation control, use 1:10 dilution with LB broth for plating.
16. Incubate the plates overnight at 37ºC. Pick white colonies and put into 100 µl of LB broth liquid medium in a 1.5 ml centrifuge tube.

3.9 Sequencing

The sequencing primers are purchased from Thermo Fisher Scientific (Ulm, Germany). The sequences of the sequencing primers are given in Table 3.6.

Table 3. 6 Sequences of the sequencing primers

Sequencing primer name	Sequence of the sequencing primer (5'→3')
pUC/M13 Forward	GTTTTCCCAGTCACGAC
pUC/M13 Reverse	GTCCTTTGTCGATACTG

Dye-terminator sequencing kit (Amersham Biosciences, Buckinghamshire, UK) was used for sequencing reactions. 4 vials of sequencing reactions were prepared containing ddATP, ddCTP, ddGTP and ddTTP respectively at a total volume of 10 µl (Table 3.7). The sequencing thermal cycler program is given in Table 3.8. Sequencing reactions were purified by ammonium acetate purification as described by the manufacturer:

1. In each tube, add 2 µl of 7.5 M ammonium acetate and 30 µl of cold 99% ethanol.
2. Vortex the tubes gently and leave on ice for 20 minutes.
3. Centrifuge the tubes at 12 000 rpm for 25 minutes for DNA to precipitate.
4. Discard supernatant and add 200 µl of 70% ethanol wash buffer.
5. Vortex tubes gently and centrifuge at 13 000 rpm for 5 minutes.
6. Discard supernatant and leave the tubes to dry for 20 minutes.
7. Add 5 µl of formamide loading dye and vortex the tubes.
8. Store the tubes are stored at -20 °C refrigerators.

The products were run at automated sequencing system (Visible Genetics Inc., Open Gene System, Toronto, Canada).

Table 3. 7 Sequencing reaction contents and their concentrations

Sequencing reaction contents	Volume used (µl)	Final concentration
PCR grade water	6.5	NA
Reaction Buffer	0.7	NA
Primer(5µM)	1	0.5 µM
ddNTP mix	0.6	
Sequencing enzyme	0.2	
Purified PCR product	1	
Total	**10**	

Table 3. 8 Sequencing reaction conditions

Temperature (ºC)	Time (sec.)	Cycle number	Steps
94	300	1	Initial denaturation
94	30	18	Denaturation
55	30		Annealing
72	45		Extension
94	30	15	Denaturation
72	60		Extension

3.10 SDS-PAGE

Proteins were analyzed with SDS-PAGE according to the method described by Laemmli (1970), with some modifications. Gels were stained with silver staining method or coomassie brilliant blue after electrophoretic run was completed using the procedure of Blum et al., (1987). All chemicals and solutions are explained in Appendix E.

Pouring SDS-polyacrlamide Gels:

1. Clean the glasses with ethanol. And assemble the glass plates according to the manufacturer's instructions. To check whether the glasses are properly sealed, pour distilled water between glasses. If water level does not decrease, glasses are properly sealed. Then, pour out the water and let the glasses to dry.
2. In an Erlenmeyer flask, prepare appropriate volume of solutions containing the desired concentration of monomer solution for 12% separating gel, using the values given in AppendixB. Mix the solutions in order shown. Polymerization will begin as soon as the NNN'N'-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) have been added.

3. Swirl the mixture rapidly and immediately pour the solution into the gap between the glass plates. Leave sufficient space for the stacking gel. Add some water to overlay the monomer solution and leave the gel in a vertical position until polymerization is completed.
4. After 30 min, pour off the water and dry the area above the separating gel with filter paper before pouring the stacking gel Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerize 30-45 minutes.

Preparation of Samples and Running the Gel:

1. While stacking gel is polymerizing, prepare samples by diluting at least 1:1 with sample buffer and heated at 95°C for 5 minutes.
2. After polymerization is complete (30 min), mount the gel in electrophoresis apparatus and fill the reservoir with running buffer.
3. Load up 20 µl of each sample into the wells and start running with 30 mA. After the dye front has moved into the separating gel increase the applied current. The usual run time is approximately 45 minutes. This electrophoresis cell is for rapid separation and is not recommended for runs over 60 minutes long.

Staining SDS-Polyacrylamide Gels with Coomassie Brilliant Blue:

1. After running is completed, immerse the gel in 5 volumes of staining solution and place on a slowly rotating platform for 4h at room temperature.
2. Remove the stain and save it for future use. Destain the gel by soaking it in the methanol:acetic acid solution without the dye on a slowly rotating platform for 4-8h, changing the destaining solution 3-4 times. After destining store the gel in dH2O.

Staining SDS-Polyacrylamide Gels with Silver Salts:

The gels were silver stained using the procedure of Blum et al. (1987).

Table 3. 9 Procedure for silver staining

	STEP	SOLUTION	TIME OF TREATMENT	COMMENTS
1	Fixing	Fixer	≥ 1 hr	Overnight incubation is all right
2	Washing	50% Ethanol	3 x 20 min	Should be fresh
3	Pre-treatment	Pretreatment Solution	1 min	Should be fresh
4	Rinse	Distilled water	3 x 20 sec	Time should be exact
5	Impregnate	Silver Nitrate Solution	20 min	-
6	Rinse	Distilled water	2 x 20 sec	Time should be exact
7	Developing	Developing Solution	~ 5 min	After a few minutes add some distilled water to carry on the reaction slowly. Time should be determined by observation of color development
8	Wash	Distilled water	2 x 2 min	-
9	Stop	Stop Solution	≥ 10 min	The gels can be kept in this solution overnight

Protein molecular weight marker (Fermentas Inc., Vilnius, Lithuania) is used for acquiring accurate and reliable analysis results. Molecular imaging is performed with UVP Biolmaging System, and Hamamatsu Digital CCD Camera, UK.

Aptamer affinity equilibrium experiments

Human growth hormone which has the original pH=7.0, was diluted to the desired concentration in the range C_{hGH}=0.05-0.25 mg/ml with sterile distilled water. Aptamers that are immobilized onto micro-particles have a concentration of "0.26 μmol aptamer/mg micro-particle" and aptamer containing micro-particles are used as the solid phase in equilibrium experiments which were conducted at room temperature. In order to avoid an error coming from the volume decrease, experiments were conducted in 10 parallel 500 μl microreactors containing 200 μl reaction volume and for each data point sample was collected from one of the microreactors. 5 mM acetic acid solution (pH=5.8) is used as elution buffer and distilled water is used in washing steps. Adsorbed hGH concentration was calculated using a material balance.

Batch aptamer affinity column experiments

Aptamer affinity column is purchased from Bio-Rad Laboratories, USA and filled with affinity packing. Aptamers that are immobilized onto micro-particles have a concentration of 19 μmol aptamer/mg micro-particle and aptamer containing micro-particles are used as affinity packing. Prepared affinity packing is placed into the differential aptamer affinity column (DAAC; L/D=0.3, D=0.01 m, m=0.027g micro-particle). Human growth hormone which has the original pH=7.0, was diluted to the desired concentration in the range C_{hGH}=0.25-0.55 mg/ml with distilled water and batch separation experiments were conducted at room temperature. Human growth hormone solution is pumped to the column with a volumetric flow rate of 0.142 cm^3/sec. And effluent is directed back to the

human growth hormone solution. Adsorbed hGH concentration was calculated by mass balance.

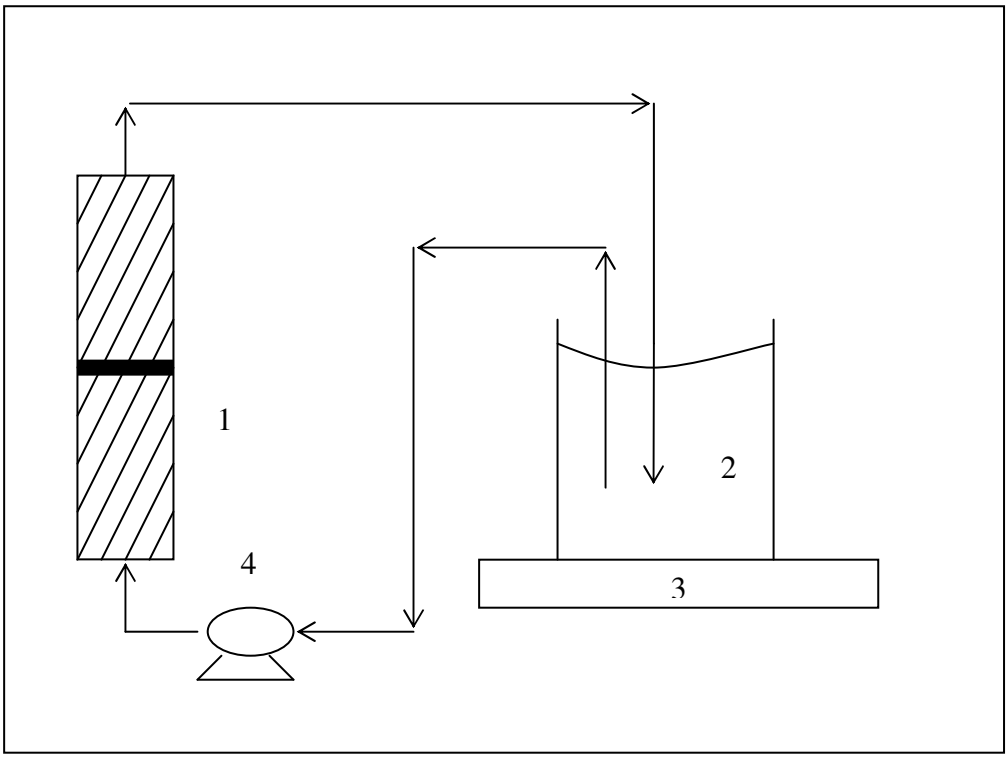

Figure 3. 2 Batch differential aptamer affinity columns (DAAC) experimental step up (1-DAAC, 2-hGH stock solution, 3- Magnetic stirrer, 4-Pump)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Aptamer library synthesis

The ligand for aptamer affinity chromatography is selected with performing several selection steps. Table 4.1 summarizes each step from aptamer selection to column experiments for human growth hormone separation. First step of the selection process is the aptamer library synthesis. Aptamer library contains a random region of 21 nucleotides in order to acquire diversity for hGH-specific aptamer selection (Table 4.2). The diversity of the random sequence is maximally 4^{21} minus the frequency of palindromes and symmetric sequences. Aptamer library (Thermo Fisher Scientific, Ulm, Germany) is diluted to 500μM concentration, and the primers for amplification of aptamer library are diluted to 100μM concentration.

4.2 Immobilization of hGH-free fermentation broth proteins

Aptamer, which is selected for hGH, must have high affinity and specificity for hGH. In order to gain specificity, aptamers having affinity to the proteins other than hGH must be eliminated. For this purpose, proteins in hGH-free fermentation broth of *B.subtilis* (*npr-, abr-*) carrying pMK4 are immobilized onto micro-particles and then incubated with aptamer library.

Table 4. 1 Aptamer selection processes

Process	Purpose
1. Aptamer library synthesis	Acquiring diversity for aptamer selection
2. Immobilization of hGH-free proteins	In order to gain specificity, aptamers having affinity to proteins other than hGH must be eliminated
3. Incubation of aptamer library with proteins other than hGH	Aptamers that bind to proteins are eliminated and free aptamers are stored for hGH specific aptamer selection.
4. hGH immobilization	To select hGH specific aptamer.
5. Incubation of hGH with the stored free aptamers	Unbound aptamers are discarded.
6. Elution of bound aptamers at different temperatures	Aptamers having low affinity to hGH are eliminated with elution at low temperatures.
7. Aptamer amplification	Aptamers having high affinity to hGH are amplified in order to be inserted into a vector.
8. Cloning and sequencing	Acquiring hGH specific aptamer sequences
9. Aptamer synthesis	Aptamer is synthesized to be used in purification trials
10. Aptamer immobilization	Aptamer is immobilized onto a solid support.
11. Incubation of aptamer with hGH fermentation medium	hGH is separated from fermentation medium.
12. hGH elution	Elution is performed by decreasing pH
13. Equilibrium experiments	Determination of the capacity of the immobilized aptamer specific to hGH
14. Aptamer affinity chromatography	A column is prepared with using the selected aptamer as ligand

Table 4. 2 The sequences of aptamer library and the primers

Name	Sequence (5'→3')
AptLib03	TGAGGGAATCGGGTTT-**N21-**TAACAATAAGCTCGCA
AptLib03-F	TGAGGGAATCGGGTTT
AptLib03-R	TGCGAGCTTATTGTTA

For covalent binding of proteins in hGH-free fermentation broth of *B.subtilis* (*npr-, abr-*) carrying pMK4, 5 μm micro-particles having NH_2 surface functionality are activated with glutaraldehyde leading to surface reactive aldehyde groups (Figure 4.1). Proteins in the hGH-free fermentation broth of *B.subtilis* (*npr-, abr-*) carrying pMK4 were immobilized onto the micro particles via NH_2 groups of proteins. After protein immobilization, glycine treatment is performed for capping uncoupled reactive aldehyde groups on 5 μm micro-particles.

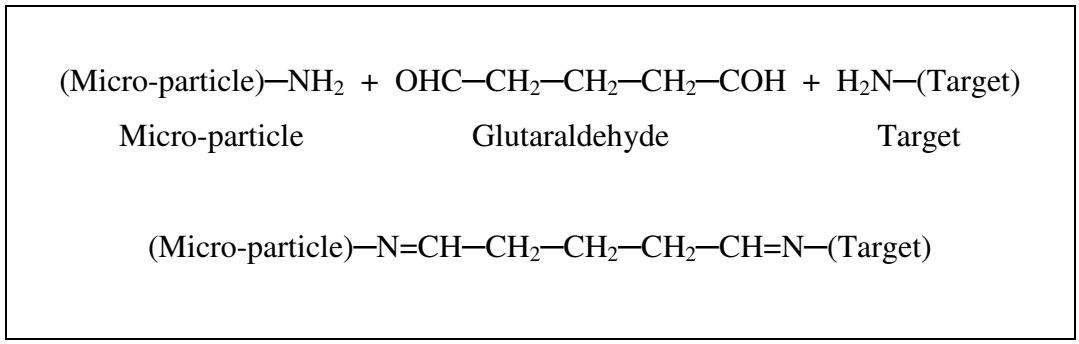

Figure 4. 1 Immobilization of target onto the micro-particles

Optimization of incubation time in protein immobilization (hGH-free fermentation broth proteins) is performed (Figure 4.2). Glutaraldehyde activation, optimized protein immobilization and glycine treatment protocols are given below.

4.2.1 Glutaraldehyde activation of micro particles

2 µl from 100 µg/µl micro-particle solution, 1 µl from %25 (v/v) gluteraldehyde solution and 50 µl distilled water is mixed in a 0.2 µl microcentrifuge tube and left for an incubation of 30 minutes at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

4.2.2 Protein immobilization reaction

The pellet (from glutaraldehyde wash step) is resuspended in 200 µl, pH=7.4, containing 0.1 µg/µl hGH-free fermentation broth of *B.subtilis.* 30 minutes incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric mass balance calculations to evaluate binding efficiencies.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

4.2.3 Glycine treatment of micro particles

After protein immobilization, glycine treatment is performed for capping uncoupled reactive aldehyde groups on 5 µm micro-particles.

The pellet (proteins immobilized onto micro-particles) is resuspended in 50 µl 1M glycine and 30 minute incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

The pellet is stored for aptamer elimination protocol in order to remove hGH unspecific aptamers at 4ºC not more than 5 days.

In protein immobilization reaction, optimization of incubation time is performed (Figure 4.1). For binding efficiency calculations, C^0_{Pro} (initial hGH-free fermentation broth protein concentration) and C_{Pro} (liquid phase hGH-free fermentation broth protein concentration at time "t") are used. "$(C^0_{Pro} - C_{Pro}) / C^0_{Pro}$ " value refers to solid phase (adsorbed) protein concentration per total protein concentration which gives the binding efficiency. No significant increase in binding efficiencies is observed for incubation times more than 30 minutes. So, protein immobilization is performed with an incubation time of 30 minutes.

The amount of protein used in immobilization is 20 µg (200 µl, 0.1 µg/µl). Using the binding efficiency value from Figure 4.2 "$(C^0_{Pro} - C_{Pro}) / C^0_{Pro} = 0.3984$" for 30 minutes incubation (data for Figure 4.2 is given in Appendix F); immobilized protein is calculated as 7.97 µg.

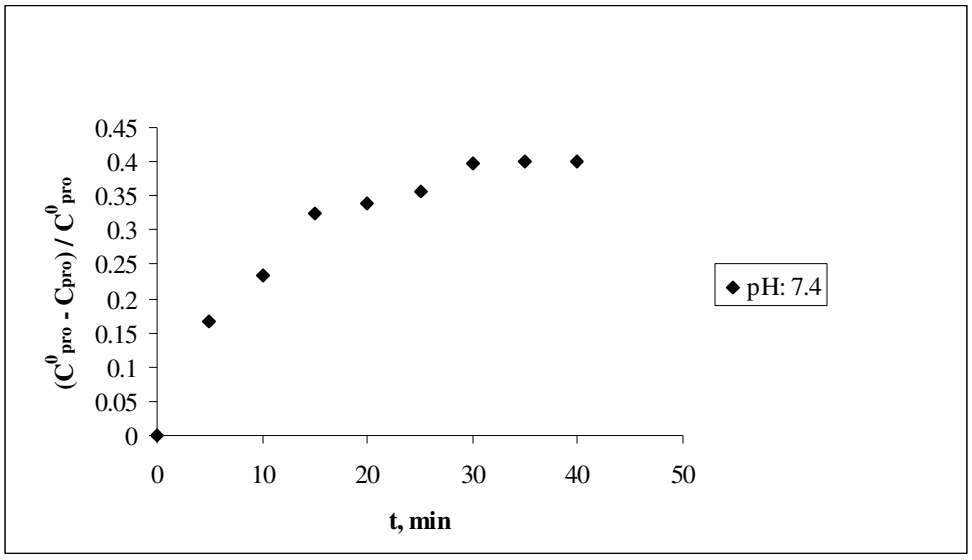

Figure 4. 2 Variation in hGH-free fermentation broth protein immobilization onto the micro-particles with time
V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg

4.3 Immobilization of pure hGH

In order to select hGH specific aptamers, pure hGH is immobilized onto micro particles.

For covalent binding of hGH, micro particles are activated with glutaraldehyde leading to surface reactive aldehyde groups. Proteins immobilized onto the micro particles via their NH_2 groups. After hGH immobilization, glycine treatment is performed for capping uncoupled reactive aldehyde groups.

Optimization of incubation time in hGH immobilization is performed (Figure 4.3). Glutaraldehyde activation, optimized hGH immobilization and glycine treatment protocols are given below.

4.3.1 Glutaraldehyde activation of micro particles

2 µl from 100 µg/µl bead solution, 1 µl from %25 (v/v) gluteraldehyde solution and 50 µl distilled water is mixed in a 0.2 µl microcentrifuge tube and

left for an incubation of 30 minutes at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant removed.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

4.3.2 hGH immobilization reaction

The pellet is resuspended in 200 µl, pH=7.4, 0.1 µg/µl pure hGH. 30 minutes incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric mass balance calculations to evaluate binding efficiencies.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

4.3.3 Glycine treatment of micro particles

The pellet is resuspended in 50 µl 1M glycine and 30 minute incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

The pellet is stored for aptamer selection protocol at 4ºC not more than 5 days.

In pure hGH immobilization reaction, optimization of incubation time is performed (Figure 4.3). For binding efficiency calculations, C^0_{hGH} (initial hGH concentration) and C_{hGH} (liquid phase hGH concentration at time "t") are used. "$(C^0_{hGH} - C_{hGH}) / C^0_{hGH}$ " value refers to solid phase (adsorbed) hGH concentration per total hGH concentration which gives the binding efficiency. No significant increase in binding efficiencies is observed for incubation times

more than 30 minutes. So, pure hGH immobilization is performed with an incubation time of 30 minutes.

The amount of hGH used in immobilization is 20 μg (200 μl, 0.1 μg/μl). Using the binding efficiency value from Figure 4.3 $(C^0_{hGH}-C_{hGH})/ C^0_{hGH}=0.3$ for 30 minutes incubation (data for Figure 4.3 is given in Appendix F); immobilized protein is calculated as 6 μg.

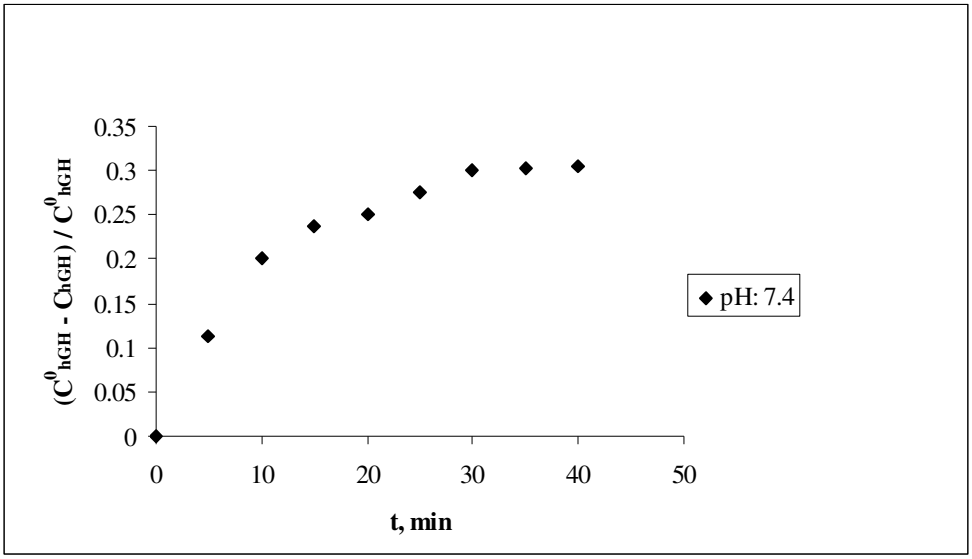

Figure 4. 3 Variation in hGH immobilization onto the micro-particles with time V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg

4.4 Selection of aptamers specific to hGH

As discussed above, for selection of aptamers with desired specificity, aptamer library is first incubated with immobilized proteins of hGH-free fermentation broth and aptamers with having no affinity to these proteins are used for incubation with micro-particles on which pure rhGH is immobilized.

Since the objective of the study is not only finding a ligand for human growth hormone but also establishing specifity for binding; possible cross reactions have to be eliminated. For this purpose, aptamers having affinity to other proteins should not be used in aptamer selection experiments. Fermentation broth, not containing human growth hormone is used for avoiding cross reactions. Thus, aptamers having affinity to these proteins were first eliminated.

4.4.1 Incubation of aptamer library with hGH-free micro particles

Optimization of pH and incubation time is performed (Figure 4.4). Optimized protocol is given below.

The pellet of micro particles on which proteins of r-hGH-free fermentation broth are immobilized, is resuspended with 200 µl, pH=7.0, 500 µM aptamer library and 5 minutes incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric mass balance calculations to evaluate binding efficiencies. After spectrophotometric readings, supernatant is taken for incubation with the pellet of micro particles on which pure r-hGH is immobilized.

In aptamer elimination reaction, optimization of pH and incubation time is performed (Figure 4.4). For binding efficiency calculations, C^0_{Apt} (initial aptamer concentration) and C_{Apt} (liquid phase aptamer concentration at time "t") are used. "$(C^0_{Apt}–C_{Apt})/C^0_{Apt}$ " value refers to solid phase (adsorbed) aptamer concentration per total aptamer concentration which gives the binding efficiency. No significant increase in binding efficiencies is observed for incubation times more than 5 minutes for all pH values. At pH=7.0, highest binding efficiencies are obtained. Binding efficiencies at pH=8.0 are close to the ones at pH: 7.0 but binding efficiencies are relatively low at pH=6.0 meaning that binding reaction favor basic pH values. So, aptamer elimination reaction is performed with an incubation time of 5 minutes at pH=7.0.

Micro particles, on which 7.97 µg protein is immobilized, are incubated with 100 nmol aptamer library. Using the binding efficiency value from Figure

4.3 "$(C^0_{Apt}–C_{Apt})/C^0_{Apt}$ = 0.0049" for 5 minutes incubation at pH=7.0 (data for Figure 4.3 is given in Appendix F); adsorbed aptamer is calculated as 490 pmol.

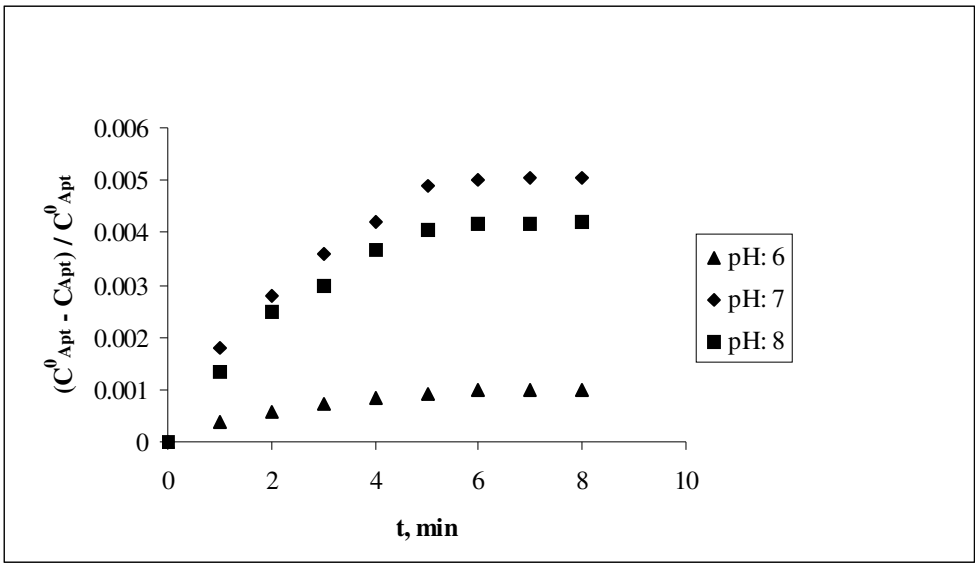

Figure 4. 4 Effect of pH and incubation time on hGH non-specific aptamer elimination V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg

4.4.2 Incubation of aptamer library with beads having r-hGH

The pellet of micro particles on which pure r-hGH is immobilized, is resuspended with supernatant of incubation of aptamer library with hGH-free micro particles. 5 minutes incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric mass balance calculations to evaluate binding efficiencies.

For the selection of hGH specific aptamer, pH and incubation time is optimized (Figure 4.5). For binding efficiency calculations, C^0_{Apt} and C_{Apt} are used. No significant increase in binding efficiencies is observed for incubation

times more than 5 minutes for all pH values. At pH=7.0, highest binding efficiencies are calculated. Binding efficiencies at pH=8.0 are close to the ones at pH: 7.0 but binding efficiencies are relatively low at pH=6.0 meaning that binding reaction favor basic pH values. So, aptamer selection reaction is performed with an incubation time of 5 minutes at pH=7.0.

Micro particles, on which 6 μg hGH is immobilized, are incubated with 99.51 nmol aptamer library having no affinity to *B. subtilis* extracellular proteins. Using the binding efficiency value from Figure 4.5 "$(C^0_{Apt}-C_{Apt})/ C^0_{Apt}$ = 0.00102" for 5 minutes incubation at pH=7.0 (data for Figure 4.5 is given in Appendix F); adsorbed aptamer amount is calculated as 95 pmol.

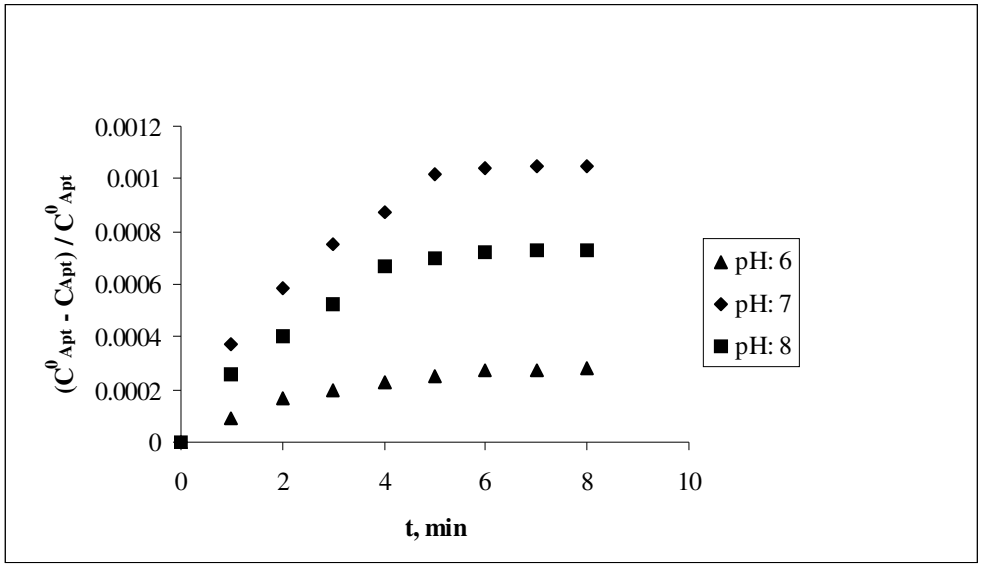

Figure 4. 5 Effect of pH and incubation time on hGH specific aptamer selection
V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg

4.5 Elution of selected aptamers

Since oligonucleotides are stable to high temperatures, elution of aptamers is performed with a temperature gradient. Aptamers having low affinity to hGH are eluted at low temperatures and aptamers having high affinity to hGH are eluted at high temperatures. Elution is performed with 10ºC increments. The amounts of eluted aptamers at each temperature are given in Figure 4.6.

4.5.1 Aptamer elution; incubation at 55ºC

The pellet is resuspended with 50 µl distilled water. The solution is incubated at 55ºC for 5 minutes. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric quantification calculation.

4.5.2 Aptamer elution; incubation at 65ºC

The pellet is resuspended with 50 µl distilled water. The solution is incubated at 65ºC for 5 minutes. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric quantification calculation.

4.5.3 Aptamer elution; incubation at 75ºC

The pellet is resuspended with 50 µl distilled water. The solution is incubated at 75ºC for 5 minutes. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric quantification calculation.

4.5.4 Aptamer elution; incubation at 85ºC

The pellet is resuspended with 50 µl distilled water. The solution is incubated at 85ºC for 5 minutes. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric quantification calculation.

4.5.5 Aptamer elution; incubation at 95ºC

The pellet is resuspended with 50 µl distilled water. The solution is incubated at 95ºC for 5 minutes. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric quantification calculation.

Aptamers, which are bound to hGH, are eluted at different temperatures as seen in Figure 4.6. At 55ºC nearly half of the bound aptamers are eluted as a conclude of their low affinity to hGH. At 95ºC, almost no aptamers have been eluted. Eluted aptamers at 85ºC are calculated as 0.16µM (data for Figure 4.6 is given in Appendix F) and these aptamers are used for further applications since these aptamers have highest affinity to hGH compared to the ones eluted at lower temperatures.

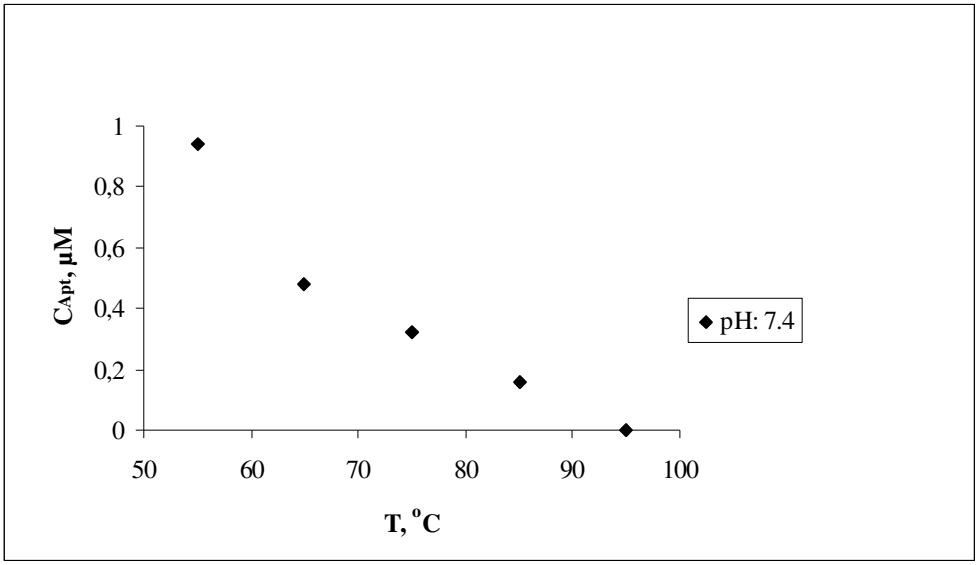

Figure 4. 6 Effect of temperature on aptamer elution
V_R=0.05ml reaction volume, V=0.5ml reactor volume, T=25ºC, micro-particle amount=0.2 mg

4.6 Amplification of selected aptamers

Eluted aptamer solution is diluted to 1 pM concentration. Polymerase chain reaction (PCR) was performed to obtain double stranded DNA from single stranded DNA in a total volume of 20 µl, containing 2.5 mM $MgCl_2$, 10 pmol of each primer, 1U of *Taq*. polymerase, and 0.2 mM of dNTP's mix (Table 4.1). The thermal cycler program for PCR was set as follows: an initial denaturation step at 94ºC for 30 seconds, 18 cycles of denaturation at 94ºC for 10 seconds, annealing at 55ºC for 10 seconds, extension at 72ºC for 10 seconds (Table 4.3). After PCR, the products were analyzed by 3% agarose gel electrophoresis (Figure 4.7).

Table 4. 3 PCR contents and their final concentrations

PCR contents	Volume used (µl)	Final Concentration
PCR Grade Water	14.5	NA
$MgCl_2$ (25 mM stock solution)	2	2.5 mM
10X PCR Buffer	2	1X
dNTP's (10 mM of each dNTP)	0.4	0.2 mM
Forward primer (100 µM)	0.1	0.5 µM
Reverse primer (100 µM)	0.1	0.5 µM
Taq. DNA polymerase(5 u/µl)	0.2	0.05 u/µl
Aptamer	1	
Total Volume	**20**	

Table 4. 4 PCR conditions

Temperature (ºC)	Time (sec.)	Cycle number	Steps
94	30	1	Initial denaturation
94	10	18	Denaturation
55	10		Annealing
72	10		Extension

Eluted aptamers at 85ºC are amplified with PCR (Figure 4.6). Lanes 1-5 are the aptamer amplification amplicons, lane 6 is the negative control and lane 7 is the molecular weight marker (50 bp ladder, Fermentas Inc. Vilnius, Lithuania).

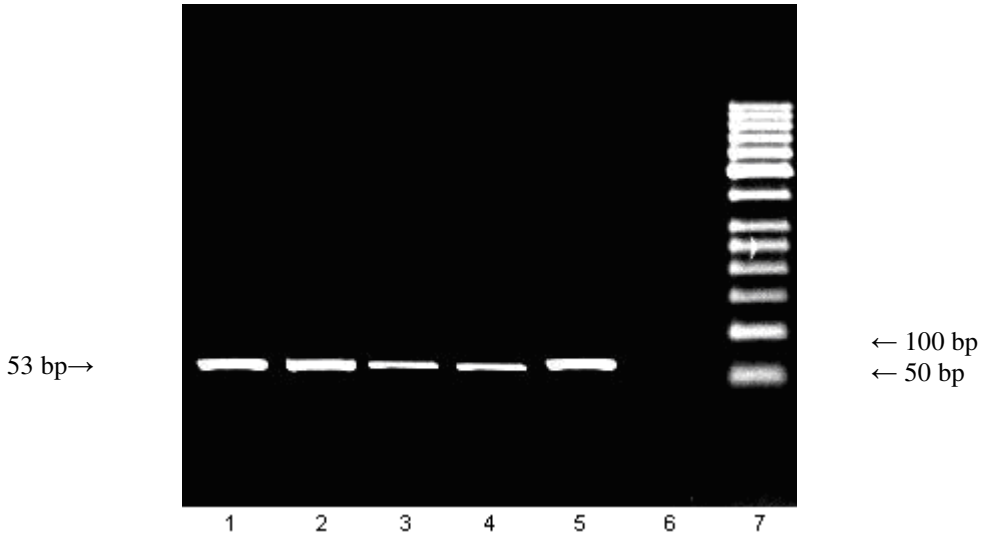

Figure 4. 7 Aptamer library amplification
Lanes 1-5: aptamer amplification amplicons, lane 6: negative control, lane 7: 50 bp ladder

4.7 PCR product purification

The PCR products were purified for cloning. The purification of the PCR products was done by PCR purification kit according to the manufacturer's directions (Qiagen PCR Purification Kit).

4.8 Ligation reaction and transformation

Selected aptamers can not be directly sequenced after amplification and purification because selected aptamers do not have a single nucleotide sequence. As explained before, aptamer library contains 10^{14} to 10^{15} random sequences. Selection of target-specific aptamers does not lead to a single nucleotide sequence. For that reason, amplified aptamers are cloned before sequencing.

Amplified and purified aptamers are ligated into pGEM-T vector; ligation contents and conditions are given in Table 4.5 and Table 4.6.

Table 4. 5 Ligation contents and their concentrations

Ligation contents	Volume used (µl)	Final Concentration
Nuclease free water	5	NA
10X ligation buffer	1	1X
pGEM-T vector (20 ng/µl)	1	2 ng/µl
Bacteriophage T4 ligase(3 u/µl)	1	0.3 u/µl
PCR product (0.5 ng/µl)	2	0.1 ng/µl
Total Volume	10	

Table 4. 6 Ligation conditions

Temperature (ºC)	Time	Cycle number	
14	4 hours	1	Ligation

Ligation reaction medium was transformed to *E.coli* by $CaCl_2$ method as described in section 3.8.

4.9 Plasmid isolation and detection of the plasmid by PCR

For detection of the plasmid by PCR, plasmid isolation from the picked single colonies was performed. PCR reaction included 2.5 mM $MgCl_2$, 50 pmol of each primer, 0.1 mM of each dNTP's and 5 µl of DNA at a total volume of 50 µl (Table 4.7). Thermal cycler program is set to an initial denaturation step at 95°C for 5 minutes, 30 cycles including 30 seconds at 95°C, 30 seconds of annealing at 55°C, 1 minutes of extension at 72°C and a final extension of 5 minutes at 72°C (Table 4.8). The products were analyzed by agarose gel electrophoresis. The 2% agarose gel was run at 120 V for 30 minutes.

Table 4. 7 PCR contents and their final concentrations

PCR contents	Volume used (µl)	Final Concentration
PCR Grade Water	33.5	NA
$MgCl_2$ (25 mM stock solution)	5	2.5 mM
10X PCR Buffer	5	1X
dNTP's (10 mM of each dNTP)	0.5	0.1 mM
Forward primer (100 µM)	0.5	1 µM
Reverse primer (100 µM)	0.5	1 µM
Taq. DNA polymerase(5 u/µl)	0.2	0.02 u/µl
Plasmid	5	
Total Volume	**50**	

Table 4. 8 PCR conditions for aptamer amplification from putative plasmids

Temperature (°C)	Time (sec.)	Cycle number	Steps
95	300	1	Initial denaturation
95	30	30	Denaturation
55	30		Annealing
72	60		Extension
72	300	1	Final extension

Plasmid isolation is performed for picked 8 colonies. Isolated plasmids are amplified with PCR (Figure 4.7). The primers (Table 4.10) perform an amplification of a region (294 bp) of the plasmid containing the insert. Lane 1 is the molecular weight marker (100 bp ladder, Fermentas Inc. Vilnius, Lithuania), lane 2 is the negative control, and lanes 3-10 are the plasmid amplification amplicons of colonies 1-8.

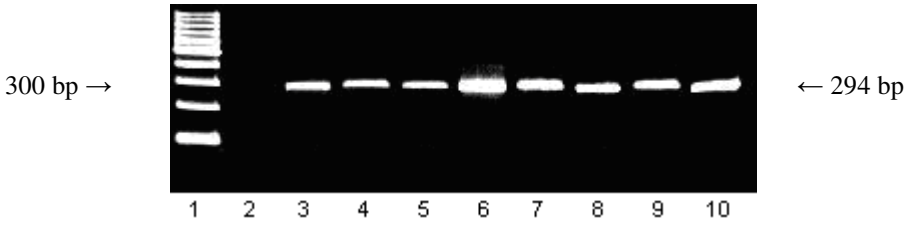

Figure 4. 8 Aptamer DNA amplification from the putative plasmids
Lane 1: 100 bp ladder, Lane 2: negative control,
Lanes 3-10: aptamer amplification from the colony plasmids 1-8

4.10 Purification of PCR products and sequencing

The PCR products were purified and sequenced. The primers used for sequencing are pUC/M13 Forward sequencing primer and pUC/M13 Reverse sequencing primer (Table 4.9).

Table 4. 9 Sequences of the sequencing primers

Sequencing primer name	Sequence of the sequencing primer (5'→3')
pUC/M13 Forward	GTTTTCCCAGTCACGAC
pUC/M13 Reverse	GTCCTTTGTCGATACTG

Dye-termination method was used for sequencing. Amersham dye-terminator sequencing kit was used for sequencing reactions. 4 vials of sequencing reactions were prepared containing ddATP, ddCTP, ddGTP and ddTTP respectively at a total volume of 10 µl (Table 4.10). The sequencing thermal cycler program is as follows: 5 minutes at 94ºC, for 18 cycles; 30 seconds at 94ºC, 30 seconds at 55ºC, 45 seconds at 72ºC and for 15 cycles; 30 seconds at 94ºC and 1

minute at 72ºC (Table 4.11). Sequencing reactions were purified by ammonium acetate purification as described by the manufacturer.

In each tube 2 µl of 7.5 M ammonium acetate and 30 µl of cold 99% ethanol are added. The tubes are vortexed gently and left on ice for 20 minutes. The tubes are centrifuged at 12 000 rpm for 25 minutes for DNA to precipitate. Supernatant is discarded and 200 µl of 70% ethanol wash buffer is added, tubes are vortexed gently and centrifuged at 13 000 rpm for 5 minutes. Supernatant is discarded and the tubes are left to dry for 20 minutes. 5 µl of formamide loading dye is added and the tubes are vortexed. The tubes are stored at -20 °C refrigerators.

The products were run at the automated sequencing system; Visible Genetics and the data were collected accordingly.

Table 4. 10 Sequencing reaction contents and their concentrations

Sequencing reaction contents	Volume used (µl)	Final concentration
PCR grade water	6.5	NA
Reaction Buffer	0.7	NA
Primer(5µM)	1	0.5 µM
ddNTP mix	0.6	
Sequencing enzyme	0.2	
Purified PCR product	1	
Total	**10**	

Table 4. 11 Sequencing reaction conditions

Temperature (ºC)	Time (sec.)	Cycle number	Steps
94	300	1	Initial denaturation
94	30	18	Denaturation
55	30		Annealing
72	45		Extension
94	30	15	Denaturation
72	60		Extension

Sequencing reaction is performed for 8 colonies; sequence of random region of an aptamer from these 8 colonies is given in Table 4.12.

Table 4. 12 Selected aptamer sequence

Source of plasmid	Selected sequences flanked by known primer sequences
Colony 7	NNNNNNNTAGCAGANNNNNNN

4.11 Synthesis of 5'NH_2 modified aptamer

After sequencing, the aptamer sequence from colony 7 is randomly chosen for purification trials. In order to be used in purification trials, aptamer has to be immobilized onto the beads. For this purpose, aptamer with 5'NH_2 modification is purchased from Thermo Fisher Scientific (Ulm, Germany). The sequence of the purchased aptamer is given below:

NH_2TGAGGGAATCGGGTTTT***NNNNNNNTAGCAGANNNNNNN***TAACAATAAGCTCGCA

4.12 Immobilization of aptamer onto the micro particles

For covalent binding of aptamer, micro particles are activated with glutaraldehyde leading to surface reactive aldehyde groups. Aptamers immobilized onto the micro particles via their NH_2 groups. After aptamer immobilization, glycine treatment is performed for capping uncoupled reactive aldehyde groups.

Optimization of incubation time in aptamer immobilization is performed (Figure 4.9). Glutaraldehyde activation, optimized aptamer immobilization and glycine treatment protocols are given below.

4.12.1 Glutaraldehyde activation of micro particles

2 µl from 100 µg/µl bead solution, 1 µl from %25 (v/v) gluteraldehyde solution and 50 µl distilled water is mixed in a 0.2 µl microcentrifuge tube and left for an incubation of 30 minutes at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant removed.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

4.12.2 Aptamer immobilization reaction

The pellet is resuspended in 200 µl, pH=7.4, 500 µM aptamer solution. 30 minutes incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is taken for spectrophotometric mass balance calculations to evaluate binding efficiencies.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

4.12.3 Glycine treatment of micro particles

After aptamer immobilization, glycine treatment is performed for capping uncoupled reactive aldehyde groups.

The pellet is resuspended in 50 µl 1M glycine and 30 minute incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellet is resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed. This washing step is performed for three times.

Aptamer sequence, which is gained from colony 7, is synthesized with 5' NH_2 modification and immobilized onto micro particles. In aptamer immobilization reaction, optimization of incubation time is performed (Figure 4.9). For binding efficiency calculations, C^0_{Apt} and C_{Apt} are used to determine $(C^0_{Apt}-C_{Apt})/ C^0_{Apt}$ value that refers to solid phase (adsorbed) aptamer concentration per total aptamer concentration which gives the binding efficiency. No significant increase in binding efficiencies is observed for incubation times more than 30 minutes. So, aptamer immobilization is performed with an incubation time of 30 minutes.

The amount of aptamer used in immobilization is 100 nmol. Using the binding efficiency value from the Figure 4.9 $(C^0_{Apt}-C_{Apt})/ C^0_{Apt} = 0.525$ for 30 minutes incubation (data for Figure 4.9 is given in Appendix F); immobilized aptamer is calculated as 52.5 nmol.

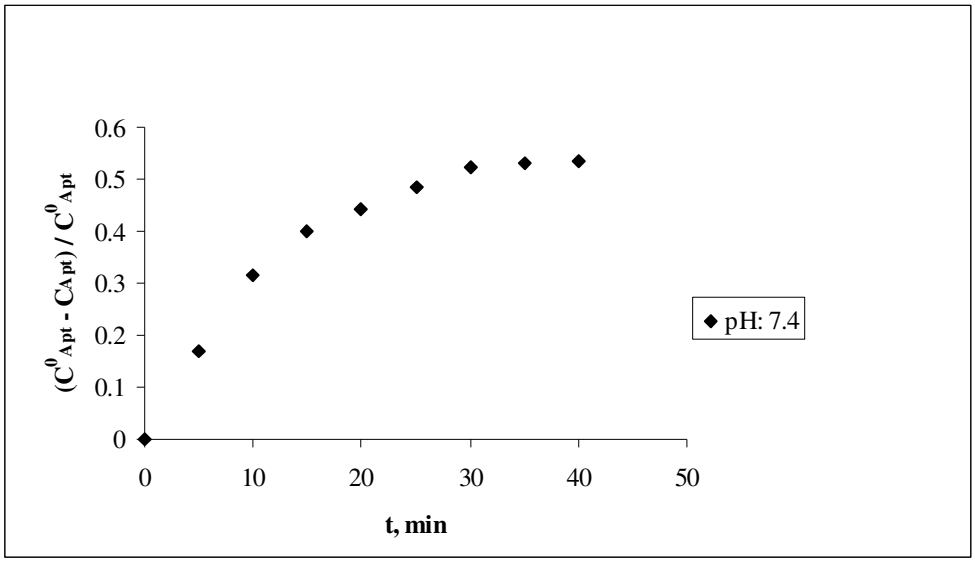

Figure 4. 9 Variation in aptamer immobilization onto the micro-particles with time V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg

4.13 Adsorption isotherms and solute elution

Small scale batch experiments were conducted for elution experiments and calculating adsorption isotherm coefficients. Human growth hormone which has the original pH=7.0, was diluted to the desired concentration in the range 0.05-0.25 mg/ml with distilled water. Micro-particles that have immobilized aptamer concentration of 0.26 μmol/mg are used as solid phase and equilibrium experiments were conducted at room temperature. 5 mM acetic acid solution (pH: 5.8) is used as elution buffer and distilled water is used in washing steps. Adsorbed hGH concentration was calculated by mass balance.

4.13.1 Solute elution

Different concentrations of hGH solutions are purified with the protocol explained in section 4.15. Sampling is performed with taking supernatant and determining solid phase solute concentration with spectrophotometric readings

and solute mass balances. Approximately 5 minutes is needed for all hGH solutions at all concentrations to reach an equilibrium (Figure 4.10; data is given in Appendix F)

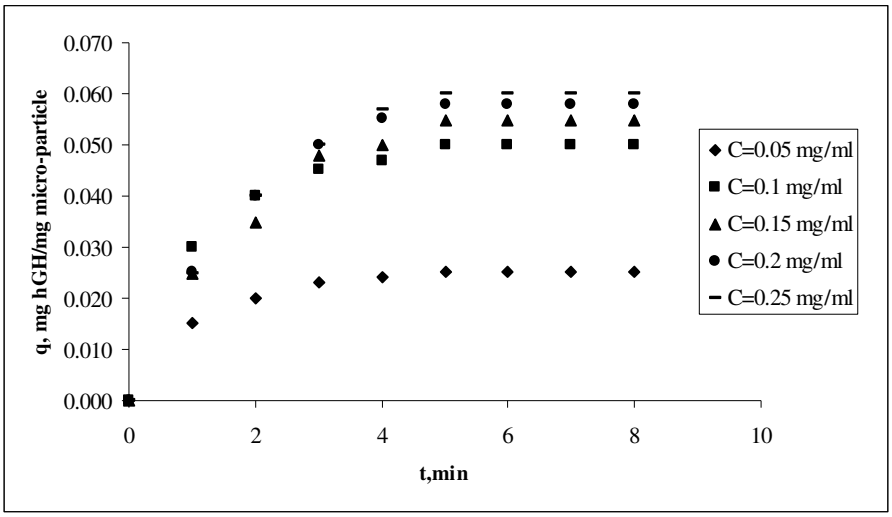

Figure 4. 10 Effect of hGH concentration on equilibrium
V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg, aptamer concentration=0.26 µmol aptamer/mg micro-particle, pH=7.0

At the end of 8 minutes, the supernatants are removed and the pellets containing adsorbed solute are used for elution experiments. 5 mM acetic acid solution (pH: 5.8) is used as elution buffer. For all concentrations, it is decided that 1 minute incubation is suitable for elution (Figure 4.11; data is given in Appendix F). Elution efficiency decreases with high solid phase solute concentration, as expected.

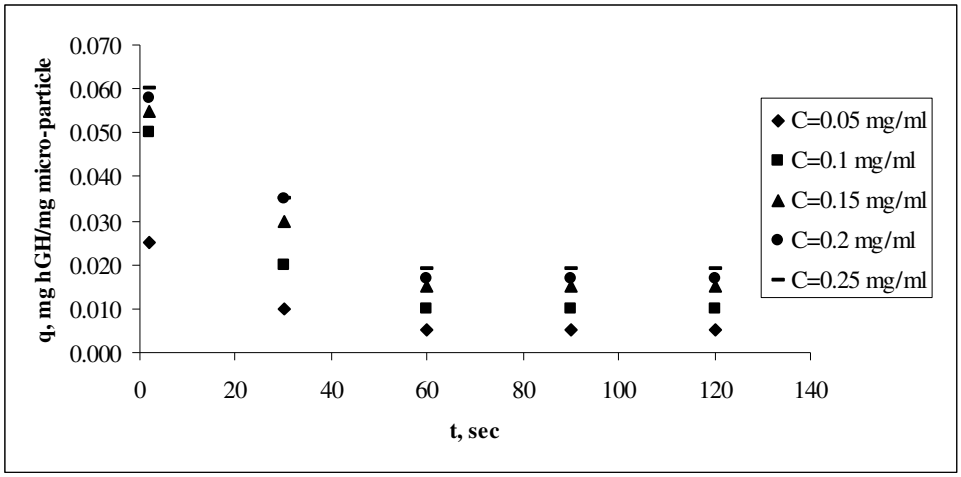

Figure 4. 11 Effect of hGH concentration on elution
V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg, aptamer concentration=0.26 µmol aptamer/mg micro-particle, pH=5.8

4.13.2 Adsorption isotherms and constants

Equilibrium based adsorption analysis is performed by using the measured liquid and solid phase solute (hGH) equilibrium concentrations (Figure 4.10; data is given in Appendix F). In Figure 4.12 (data is given in Appendix F) adsorption isotherm is plotted. The isotherm followed Langmuir type isotherm.

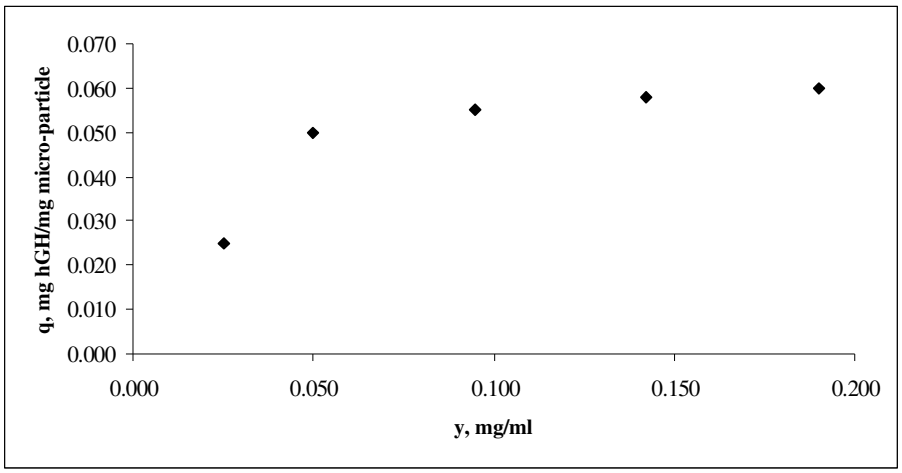

Figure 4. 12 Adsorption isotherm for batch equilibrium experiments

In order to verify the isotherm type to be Langmuir, a plot of q^{-1} versus y^{-1} is analyzed (Figure 4.13; data is given in Appendix F). Since R^2 value (0.92) is close to 1, the isotherm can be said to be Langmuir type. Equation seen in Figure 4.13 (data is given in Appendix F) can be used to evaluate saturation constant (q_0) and affinity constant for Langmuir isotherm. The intercept is q_0^{-1} and slope is K/q_0. Thus, saturation constant is found to be;

q_0=0.088 mg hGH/mg micro-particle

Saturation constant can be expressed in terms of mg hGH/µmol aptamer by dividing saturation constant with aptamer concentration on micro-particles:

q_0=0.088 mg hGH/mg micro-particle / 0.26 µmol aptamer/mg micro-particle

Finally, saturation constant and affinity constant are evaluated to be:

q_0=0.338 mg hGH/ µmol aptamer

K=0.059 mg hGH/ml

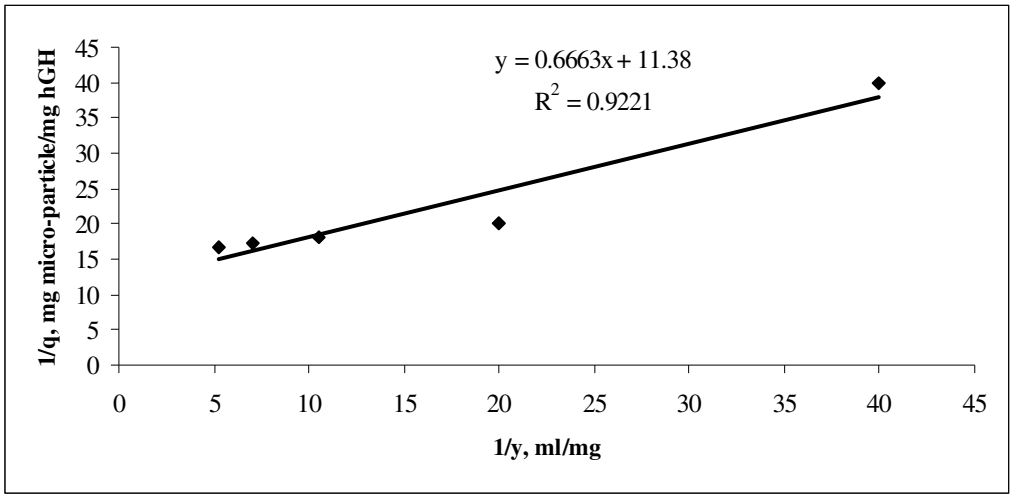

Figure 4. 13 Determination of isotherm type and constants

4.14 Aptamer affinity purification

Aptamer affinity column is purchased from Bio-Rad Laboratories, USA and filled with affinity packing. For this purpose, selected aptamer is immobilized onto particles as explained in section 4.12. Aptamer concentration was spectrophotometrically calculated to be 19 μmol aptamer/mg micro-particle. Prepared affinity packing is placed into the differential aptamer affinity column (DAAC; L/D=0.3, D=0.01 m, m=0.027g micro-particle, C_{Apt}=19 μmol/mg micro-particle). 8.1 ml, 0.40 mg/ml human growth hormone solution is prepared and pumped to the column with a volumetric flow rate of 0.142 cm^3/sec. And effluent is directed back to the human growth hormone solution. Concentration of human growth hormone solution is determined by mass balance using the data obtained at 280 nm by UV-spectrophotometry. Solid phase solute concentration (q) is determined (Figure 4.14; data is given in Appendix F). Equilibrium is observed only after 2 minutes. For that reason, another experiment is conducted with C_{hGH}=0.55 mg/ml with same volumetric flow rate but solid phase solute concentration (q) is calculated for every 20 second (Figure 4.14; data is given in

Appendix F). Another experiment with C_{hGH}=0.25 mg/ml is performed with decreasing sampling time intervals (Figure 4.14; data is given in Appendix F).

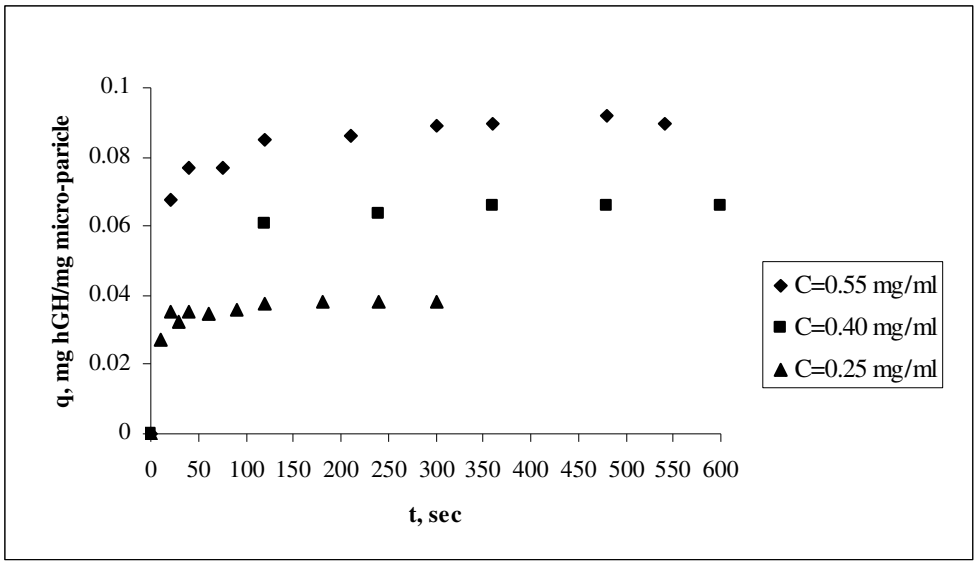

Figure 4. 14 Aptamer affinity chromatographic purification of hGH
V_R=8.1ml reaction volume, V=10.0ml reactor volume, T=25°C, micro-particle amount=0.027 g, aptamer concentration=19 µmol aptamer/mg micro-particle, pH=7.0

4.14.1 Adsorption isotherms and constants

Equilibrium based adsorption analysis is performed by using the liquid and solid phase solute equilibrium concentrations of Figure 4.14 (data is given in Appendix F). In Figure 4.15 (data is given in Appendix F) adsorption isotherm is plotted. The isotherm is Langmuir type isotherm.

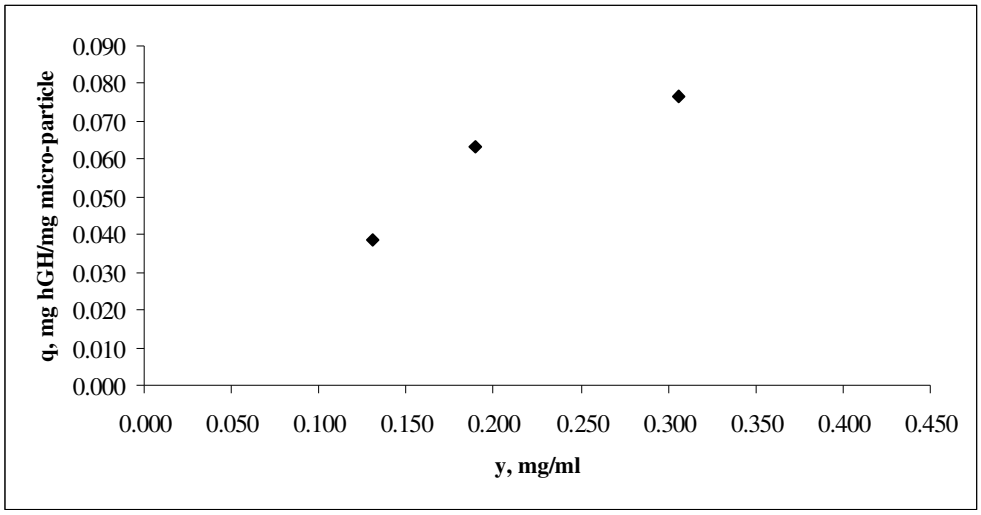

Figure 4. 15 Adsorption isotherm for affinity purification

In order to verify the isotherm type to be Langmuir, a plot of q-1 versus y-1 is analyzed (Figure 4.16; data is given in Appendix F). Since R^2 value (0,9251) is close to 1, the isotherm can be said to be Langmuir type. Equation seen in Figure 4.16 (data is given in Appendix F) can be used to evaluate saturation constant (q_0) and affinity constant (K) for Langmuir isotherm. The intercept is q_0^{-1} and slope is K/q_0. Thus, saturation constant is found to be;

q_0=0.51 mg hGH/mg micro-particle

Saturation constant can be expressed in terms of mg hGH/µmol aptamer by dividing saturation constant with aptamer concentration on micro-particles:

q_0=0.51 mg hGH/mg micro-particle / 19 µmol aptamer/mg micro-particle

Finally, saturation constant and affinity constant are evaluated to be:

q_0=0.027 mg hGH/ µmol aptamer

K=1.543 mg hGH/ml

Calculated affinity constant is close to the affinity constants in the published hGH affinity studies: 0.22 mg hGH/ml (Liesiene et al., 1997), 0.445 mg hGH/ml (Maria et al., 2000), 0.45 mg hGH/ml (Jonsdottir et al., 1986), 1.14

mg hGH/ml (Sabuory et al., 2005), 0.117 mg hGH/ml (Stefano et al., 2007), 0.011 mg hGH/ml (Yoshihide et al., 1998).

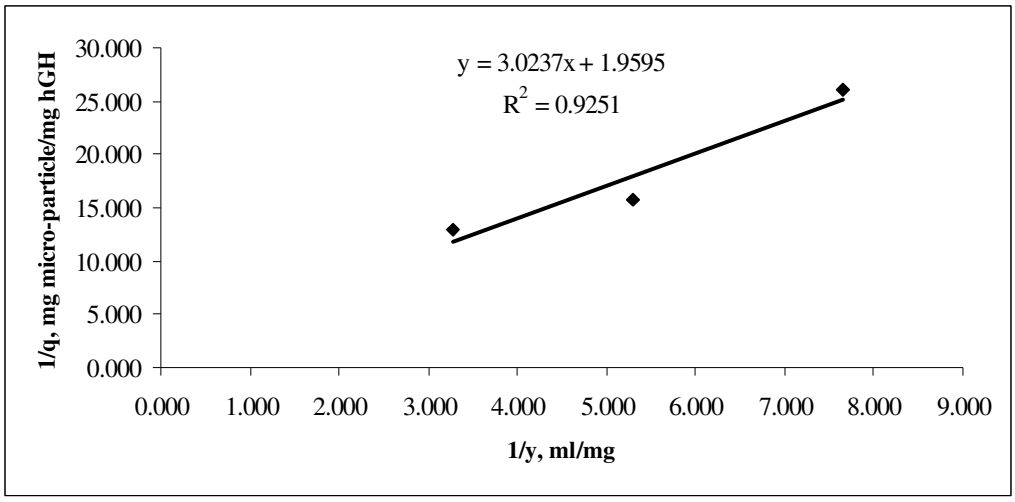

Figure 4. 16 Determination of isotherm type and constants

4.15 Separation of rhGH from fermentation broth

Optimization of incubation time and pH is performed (Figure 4.17). Optimized protocol is used for separation of hGH from fermentation broth. In the controlled separation experiment, distilled water is used as negative control and pure hGH is used as positive control. The protocol of the controlled experiment is given below. Elution of the bound solute is readily achieved by changing such parameters as salt concentration or pH, or by addition of a competitive inhibitor in solution (Cuatrecasas et al, 1968). Elution at high temperatures is not suitable for protein elution since proteins are not as stable as aptamers. Thus, 5 mM acetic acid solution (pH=5.8) is used as elution buffer. Acetic acid solution, which is used as elution buffer in the developed protocol, can easily be removed by

lyophilization. By using this elution buffer, additional purification steps, for removing elution agents, are avoided.

4.15.1 Incubation of fermentation broth with micro particles

20 µl of the fermentation broth of *B.subtilis* (*npr-, abr-*) carrying pMK4*::pre(sub*C*)::hGH* containing recombinant human growth hormone was used for SDS-PAGE. The pellets having aptamer were resuspended with 200 µl, pH:7.0, 0.1 µg/µl recombinant human growth hormone containing fermentation broth and 5 minute incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellets are resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed, supernatant is taken and 20 µl of the supernatant is used for SDS-PAGE. This wash step is performed two more time without taking supernatants for SDS-PAGE.

The pellets are resuspended in 200 µl elution buffer. 1 minute centrifugation at 13000 rpm is performed, supernatant is taken and 20 µl of the supernatant is used for SDS-PAGE.

4.15.2 Negative control: incubation of distilled water with micro particles

20 µl distilled water is taken for SDS-PAGE. The pellet having aptamer is resuspended with 200 µl distilled water and 5 minute incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellets are resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed, supernatant is taken and 20 µl of the supernatant is used for SDS-PAGE. This wash step is performed two more time without taking supernatants for SDS-PAGE.

The pellets are resuspended in 200 µl elution buffer. 1 minute centrifugation at 13000 rpm is performed, supernatant is taken and 20 µl of the supernatant is used for SDS-PAGE.

4.15.3 Positive control: incubation of pure r-hGH with micro particles

20 µl, pH=7.0, 0.1 µg/µl pure r-hGH is taken for SDS-PAGE. The pellet having aptamer is resuspended with 200 µl pure r-hGH and 5 minute incubation is performed at room temperature. 1 minute centrifugation at 13000 rpm is performed and supernatant is removed.

The pellets are resuspended in 200 µl distilled water. 1 minute centrifugation at 13000 rpm is performed, supernatant is taken and 20 µl of the supernatant is used for SDS-PAGE. This wash step is performed two more time without taking supernatants for SDS-PAGE.

The pellets are resuspended in 200 µl elution buffer. 1 minute centrifugation at 13000 rpm is performed, supernatant is taken and 20 µl of the supernatant is used for SDS-PAGE.

Elution at high temperatures is not suitable for protein elution since proteins are not as stable as aptamers. Acetic acid solution (5mM, pH=5.8) is used for elution.

Optimization of pH and incubation time were performed for hGH purification trials (Figure 4.17). For binding efficiency calculations, C^0_{hGH} (initial hGH concentration) and C_{hGH} (liquid phase rhGH concentration at time "t") were used. "$(C^0_{hGH} - C_{hGH}) / C^0_{hGH}$" value refers to solid phase (adsorbed) hGH concentration per total hGH concentration which gives the binding efficiency. No significant increase in binding efficiencies was observed for incubation times more than 5 minutes for all pH values. At pH=7.0, highest binding efficiencies are calculated. Binding efficiencies at pH=8.0 is close to the ones at pH=7.0 but binding efficiencies are relatively low at pH=6.0 meaning that binding reaction favors basic pH values. So, purification reactions were performed with an incubation time of 5 minutes at pH=7.0.

Micro particles, on which 52.5 nmol aptamer was immobilized, were incubated with 20 µg (200µl, 0.1 µg/µl) pure hGH. Using the binding efficiency value from the Figure 4.17 $(C^0_{hGH}-C_{hGH})/ C^0_{hGH}$ = 0.4067 for 5 minutes

incubation at pH=7.0 (data for Figure 4.17 is given in Appendix F); adsorbed rhGH was calculated as 8.134 μg.

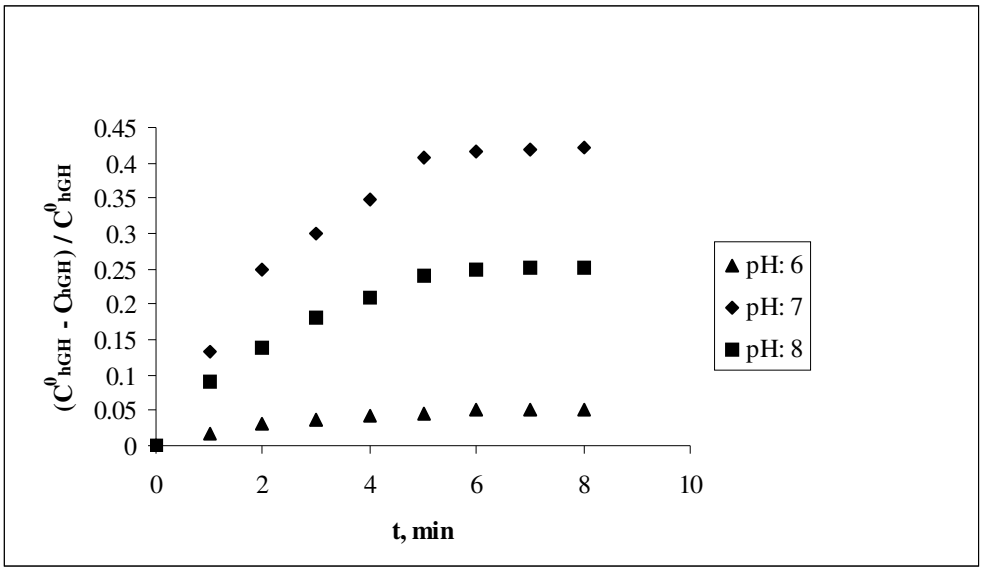

Figure 4. 17 Variation in hGH purification with pH and time
V_R=0.2ml reaction volume, V=0.5ml reactor volume, T=25°C, micro-particle amount=0.2 mg

4.15.4 SDS-PAGE

The glasses are cleaned with ethanol and assembled. The sealing is checked by pouring distilled water between the glasses. Ensuring water level does not decrease, glasses are sealed and water is poured out. Using the values given in Appendix E, solutions containing the desired concentration of monomer solution for 12% separating gel are prepared. And the solutions are mixed in order shown. With adding NNNN-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) polymerization immediately starts. The mixture is

poured into the gap between glasses and space is left for stacking gel. The gel is left in a vertical position until the end of the polymerization.

A comb is placed in the gel sandwich and managed that the teeth are at a slight angle. The gel is left for polymerization 30-45 minutes.

Samples are prepared with making a dilution with sample buffer (1:1) and heated for 5 minutes at 95C. After polymerization completion (30 min), the gel is mounted in electrophoresis apparatus and the reservoir is filled with running buffer.

30 µl of each sample is loaded into the wells and running is started with 30 mA. After the tracking dye reach into the separating gel, the applied current is increased.

After running is completed (30-40 minute), the gel is placed in excess volume of staining solution and left on a slowly rotating platform for 4h at room temperature.

Liquid phase is removed. Destaining is performed by soaking the gel in the methanol: acetic acid solution on a slowly rotating platform for 4-8h.

The gel is treated with fixer solution for 1 hour. Washing is performed with %50 ethanol solution for 30 minute twice. Pretreatment is performed with pretreatment solution for a minute. Three times of rinsing with distilled water is performed for 20 seconds each. Treatment with silver nitrate solution is performed for 20 minutes. Rinsing in distilled water is performed for 20 seconds twice. Treatment of developing solution is performed for 5 minutes. Washing with distilled water is performed twice for 2 minutes. Stop solution is added and the gel is kept in the solution at much a day.

Results of purification experiments are given in Figure 4.18 and 4.19. In Figure 4.18, lane 1 is the protein weight marker (Fermentas Inc. Vilnius, Lithuania), lanes 2-3-4 are negative control group (negative control itself, wash supernatant, elution supernatant), and lanes 5-6-7 are positive control group (positive control itself, wash supernatant, elution supernatant).

In Figure 4.19, lane 1 is the protein weight marker (Fermentas Inc. Vilnius, Lithuania), lanes 2-3-4 are hGH purification group (fermentation broth itself, wash supernatant, elution supernatant), and lanes 5-6 are positive control group (elution supernatant, positive control itself).

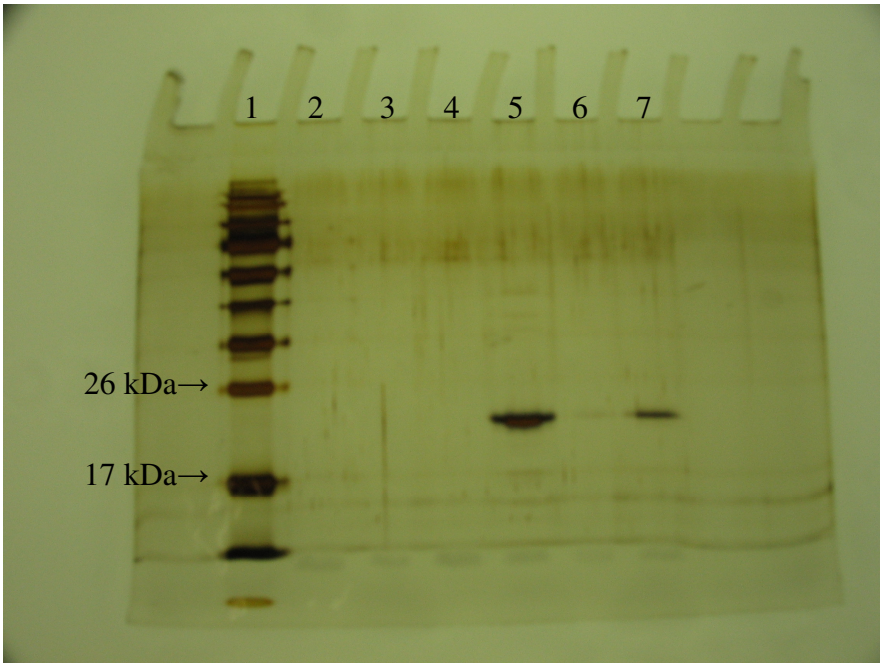

Figure 4. 18 Purification of hGH; positive and negative controls

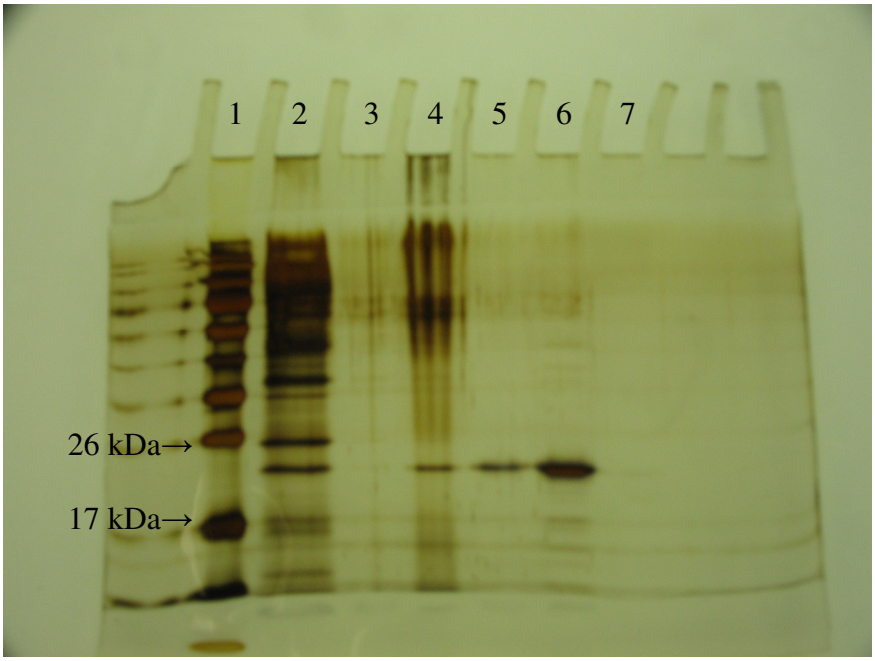

Figure 4. 19 Separation of hGH from fermentation broth

Figure 4.18 and 4.19 verify the specific purification of human growth hormone. Molecular imaging of the SDS-PAGE was performed with UVP Biolmaging System, and Hamamatsu Digital CCD Camera, UK. Intensity analysis was performed using known concentration pure human growth hormone intensity as reference (Table 4.13).

Table 4. 13 Purity and overall yield values for aptamer affinity purification

Purification step	Total protein (µg)	rhGH (µg)	Purity of rhGH (%)	Overall yield (%)
Culture supernatant	20.530	1.024	4.98	100.00
Aptamer affinity purification	0.421	0.420	99.76	41.02

Purity of rhGH (99.76%) gained in aptamer affinity purification is close to the purity percentages of rhGH in the published studies: 99% (Khodabandeh et al, 2003), 98% (Derek et al, 2003), 99% (Patra et al, 2000), 80% (Llesmne et al, 1997). Similiarly, overall yield (41.02%) gained in aptamer affinity purification is close to the overall yields in the published studies: 40% (Oliveira et al, 1999), 40% (Khodabandeh et al, 2003), 50% (Patra et al, 2000), 28–48% (Lefort et al, 1986)

CHAPTER 5

CONCLUSIONS

In this study, an aptamer specific for human growth hormone is selected among an aptamer library and human growth hormone is separated from the fermentation broth using aptamer affinity chromatography.

Cross reactions of aptamer with the other fermentation broth proteins are avoided by performing a modified selection process based on performing an initial incubation of the aptamer library with the extracellular proteins of *B.subtilis* other than hGH.

For selection of aptamers with high affinity to hGH, elution with a temperature gradient is applied. Selection of aptamers with this elution method is a suitable alternative for conventional aptamer selection protocols.

It is possible that, with the aid of bioinformatics, conservative hGH binding regions can be derived by performing sequencing reactions for more aptamer insert containing colonies.

A single step, aptamer affinity chromatography with 99.76% purity and 41.02% overall yield was successfully performed. The equilibrium data obtained was described by Langmuir type isotherm where saturation constant (q_0) and affinity constant (K) are calculated as 0.338 mg hGH/µmol aptamer and 0.059 mg hGH/ml, respectively. Equilibrium data obtained using aptamer affinity column was described by Langmuir type isotherm where saturation constant (q_0)

and affinity constant (K) are calculated as 0.027 mg hGH/μmol aptamer and 1.543 mg hGH/ml, respectively.

It is probable that, selected aptamer sequence can be used for purification of bulk amounts of recombinant hGH using affinity chromatography techniques.

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APPENDIX A

GENE SEQUENCE OF hGH GENE

ACCESSION K02382
VERSION K02382.1 GI:208527

```
   1 cgatatgttc ccaactattc cactgagtcg cctgttcgat aacgcgatgc tgcgtgcgca
  61 tcgtctgcac caactggctt tcgacactta ccaggagttc gaagaagcat acatcccgaa
 121 agaacagaaa tacagcttcc ttcagaaccc acagacctcg ttgtgtttct ctgaaagtat
 181 cccgacccct tctaaccgcg aagagaccca gcagaaatcg aaccttgaac tgcttcgtat
 241 ctcgctgctt ctcattcagt cgtggctgga gccagtacag ttcctgcgtt cggttttcgc
 301 aaactcactg gtttacggtg cgtctgacag taacgtttac gacctgctga aagaccttga
 361 agaagggatc cagaccctga tgggtcgcct ggaagatggt tcaccacgca ctggtcagat
 421 cttcaaacag acttactcca aattcgatac taactctcat aacgatgatg ctctgctgaa
 481 aaactacggc ctgctgtact gtttccgtaa agatatggat aaagttgaaa ctttcctgcg
 541 tatcgttcag tgtcgttctg ttgaagggtc gtgtggcttc taatag
```

/translation="MFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQK
YSFLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLR
SVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYS
KFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEGSCGF"

APPENDIX B

CHEMICALS USED IN THE EXPERIMENTS

Table B. 1 The chemicals used and the suppliers for the chemicals

Reagent	Supplier
Gluteraldehyde	Merck
Glycine	Merck
Ampicillin	Sigma
Silver nitrate	Merck
N',N',N',N'-Tetramethylethilenediamine	Sigma
Ammonium persulphate	Sigma
Glucose	Sigma
Tris hydrochloride	Sigma
Ethilene diamine tetraacetic acid	Sigma
Sodium hydroxide	Merck
Sodium dodecyl sulphate	Sigma
Potassium acetate	Merck
Coomassie brilliant blue	Sigma
Methanol	Merck
Formaldehyde	Merck
Sodium thiosulphate	Merck
Potassium carbonate	Merck

APPENDIX C

OLIGONUCLEOTIDE AND PROTEIN QUANTIFICATION

Table C. 1 Physical properties of phosphoramedites

Residue	ε (M^{-1} cm^{-1})	A_{max} (nm)	MW (g/mol)
Adenine (dAMP)	15,200	259	313.21
Guanine (dGMP)	12,010	253	329.21
Cytosine (dCMP)	7,050	271	289.18
Thymidine (dTMP)	8,400	267	304.20

ε_{hGH} = 1.18 ml $mg^{-1}cm^{-1}$

$\varepsilon_{Aptamer} = (n_{dAMP} \times 15{,}200) + (n_{dGMP} \times 12{,}010) + (n_{dCMP} \times 7{,}050) + (n_{dTMP} \times 8{,}400)$

$MW_{Aptamer} = (n_{dAMP} \times 313.21) + (n_{dGMP} \times 329.21) + (n_{dCMP} \times 289.18) + (n_{dTMP} \times 304.20)$

$T_m = 64.9 + 41 \times (n_{dGMP} + n_{dCMP} - 16.4) / (n_{dAMP} + n_{dGMP} + n_{dCMP} + n_{dTMP})$

$OD_\lambda = DF \times A_\lambda = \varepsilon \times C \times L$

APPENDIX D

PREPARATION OF SOLUTIONS FOR PLASMID ISOLATION

Glucose/Tris/EDTA (GTE) (Alkaline Lysis Solution I)

50mM Glucose

25mM Tris-HCl pH:8.0

10mM EDTA

TV:50 ml

Autoclaved for 15 minutes and stored at +4°C.

NaOH/SDS (Alkaline Lysis Solution II)

0.2N NaOH

%1 SDS

Total Volume: 5ml

Stored at room temperature.

5M Potassium Acetate (Alkaline Lysis Solution III)

5M Potassium acetate 60 ml

Glacial acetic acid 11.5 ml

dH_2O 28.5 ml

Stored at +4°C. Cooled before use on ice.

APPENDIX E

PREPARATION OF GELS AND SOLUTIONS FOR SDS-PAGE

Table E. 1 Stacking gel preparation

Reagents	Stacking Gel		Separating Gel	
	5%	**7.5%**	**10%**	**12%**
30% acrylamide mix	1.67 ml	2.5 ml	3.33 ml	4 ml
dH_2O	5.68 ml	4.85 m	4.05 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5 ml	2.5 ml	2.5 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml	-	-	-
10% (w/v) SDS	100 µl	100 µl	100 µl	100 µl
10% (w/v) ammonium persulfate	60 µl	50 µl	50 µl	50 µl
TEMED	15 µl	10 µl	10 µl	10 µl

Staining of SDS-Polyacrylamide Gels with Coomassie Brilliant Blue

A. Staining Solution:

Dissolve 0.25g Coomassie Brilliant Blue in 100 ml methanol: acetic acid solution.

B. Methanol: Acetic Acid Solution:

Combine 900 ml of methanol:H_2O (500 ml of methanol and 400 ml of H_2O) and 100 ml of glacial acetic acid.

Staining of SDS-Polyacrylamide Gels with Silver Salts

A. Fixer

Mix 150 ml methanol + 36 ml acetic acid + 150 µl 37% formaldehyde and complete to 300 ml with distilled water. This solution can be used several times.

B. 50% Ethanol

Mix 600 ml pure ethanol + 600 ml distilled water. This solution should always be prepared freshly.

C. Pretreatment Solution

Dissolve 0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 400 ml distilled water by mixing with a glass rod. Take 8 ml and set aside for further use in developing solution preparation.

D. Silver Nitrate Solution

Dissolve 0.8 g silver nitrate in 400 ml distilled water and add 300 µl 37% formaldehyde.

E. Developing Solution

Dissolve 9 g potassium carbonate in 400 ml distilled water. Add 8 ml from pretreatment solution and 300 µl 37% formaldehyde.

F. Stop Solution

Mix 200 ml methanol + 48 ml acetic acid and complete to 400 ml with distilled water.

APPENDIX F

DATA OF FIGURES IN CHAPTER 4

Table F. 1 Data of Figure 4.1

t, min	$(C_0-C) / C_0$
0	0
5	0.166
10	0.2324
15	0.3237
20	0.3403
25	0.3569
30	***0.3984***
35	0.40006
40	0.40172

Table F. 2 Data of Figure 4.2

t, min	$(C_0-C) / C_0$
0	0
5	0.1125
10	0.2
15	0.2375
20	0.25
25	0.275
30	***0.3***
35	0.3025
40	0.30375

Table F. 3 Data of Figure 4.3

t, min	$(C_0-C) / C_0$		
	pH: 6.0	**pH: 7.0**	**pH: 8.0**
0	0	0	0
1	0.00037494	0.0018	0.001328
2	0.00058324	0.0028	0.00249
3	0.00072905	0.0036	0.002988
4	0.0008332	0.0042	0.003652
5	0.00091652	***0.0049***	0.004067
6	0.00099984	0.005	0.00415
7	0.001008172	0.00504	0.0041832
8	0.001012338	0.00506	0.0042164

Table F. 4 Data of Figure 4.4

t, min	$(C_0-C) / C_0$		
	pH: 6.0	**pH: 7.0**	**pH: 8.0**
0	0	0	0
1	0.000088	0.00037494	0.000261
2	0.000165	0.00058324	0.000406
3	0.000198	0.00074988	0.000522
4	0.000231	0.00087486	0.000667
5	0.000253	***0.00102067***	0.000696
6	0.000275	0.0010415	0.000725
7	0.0002772	0.001049832	0.0007308
8	0.0002794	0.001045666	0.0007279

Table F. 5 Data of Figure 4.5

T, ºC	C, µM
55	0.94
65	0.48
75	0.32
85	***0.16***
95	0

Table F. 6 Data of Figure 4.9

t, min	$(C_0-C) / C_0$
0	0
5	0.168
10	0.315
15	0.399
20	0.441
25	0.483
30	***0.525***
35	0.5292
40	0.5334

Table F. 7 Data of Figure 4.10

t, min	q, mg/mg				
	C=0.05 mg/ml	C=0.1 mg/ml	C=0.15 mg/ml	C=0.2 mg/ml	C=0.25 mg/ml
0	0.000	0.000	0.000	0.000	0.000
1	0.015	0.030	0.025	0.025	0.025
2	0.020	0.040	0.035	0.040	0.040
3	0.023	0.045	0.048	0.050	0.050
4	0.024	0.047	0.050	0.055	0.057
5	0.025	0.050	0.055	0.058	0.060
6	0.005	0.050	0.055	0.058	0.060
7	0.005	0.050	0.055	0.058	0.060
8	0.005	0.050	0.055	0.058	0.060

Table F. 8 Data of Figure 4.11

t, sec	q, mg/mg				
	C=0.05 mg/ml	C=0.1 mg/ml	C=0.15 mg/ml	C=0.2 mg/ml	C=0.25 mg/ml
2	0.025	0.050	0.055	0.058	0.060
30	0.010	0.020	0.030	0.035	0.035
60	0.005	0.010	0.015	0.017	0.019
90	0.005	0.010	0.015	0.017	0.019
120	0.005	0.010	0.015	0.017	0.019

Table F. 9 Data of Figure 4.12

q, mg/mg	y, mg/ml
0.025	0.025
0.050	0.050
0.055	0.095
0.058	0.142
0.060	0.190

Table F. 10 Data of Figure 4.13

1/q, mg/mg	1/y, ml/mg
40	40
20	20
18	11
17	7
17	5

Table F. 11 Data of Figure 4.14

t, sec	q, mg/mg		
	C=0.55 mg/ml	C=0.40 mg/ml	C=0.25 mg/ml
0	0	0	0
10	-	-	0.0272
20	0.0679	-	0.0353
30	-	-	0.0326
40	0.0770	-	0.0353
60	-	-	0.0347
75	0.0769	-	-
90	-	-	0.0356
120	0.0850	0.0607	0.0376
180	-	-	0.0382
210	0.0859	-	-
240	-	0.0634	0.0383
300	0.0891	-	0.0380
360	0.0896	0.0657	-
420	-	-	-
480	0.0920	0.0659	-
540	0.0896	-	-
600	-	0.0661	-

Table F. 12 Data of Figure 4.15

q, mg/mg	y, mg/ml
0.063	0.189
0.077	0.306
0.038	0.131

Table F. 13 Data of Figure 4.16

1/q, mg/mg	1/y, ml/mg
15.783	5.291
13.011	3.268
26.082	7.645

Table F. 14 Data of Figure 4.17

t, min	$(C_0-C) / C_0$		
	pH: 6.0	**pH: 7.0**	**pH: 8.0**
0	0	0	0
1	0.016	0.1328	0.09
2	0.03	0.249	0.14
3	0.036	0.2988	0.18
4	0.042	0.3486	0.21
5	0.046	***0.4067***	0.24
6	0.05	0.415	0.25
7	0.0504	0.41832	0.252
8	0.0508	0.42164	0.251