ANALYSIS AND CHARACTERIZATION OF THE PICHIA PASTORIS SECRETOME FOR PHARMACEUTICAL PROTEIN PRODUCTION

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ABSTRACT

ANALYSIS AND CHARACTERIZATION OF THE PICHIA PASTORIS SECRETOME FOR PHARMACEUTICAL PROTEIN PRODUCTION

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Human growth hormone (hGH) is used for the treatment of many diseases like short stature in children. It is generally purified by using affinity chromatography. Due to the high cost of affinity chromatography, other chromatographic techniques have been investigated for purification of recombinant human growth hormone (rhGH). The aim of this work was to explore chromatographic purification protocols for rhGH from the secretome of *Pichia pastoris*. First, the entire secretome was examined by 2-D gel electrophoresis. A major 4 unique proteins apart from rhGH were found in the secretome. The molecular weights of the other proteins were found to range between 20 and 140 kDa. The isoelectric points of most of the proteins in secretome were determined to vary between 4.4 and 5.7. To putatively identify the proteins in the secretome, the secretome of *P. pastoris* from the literature was analyzed using the software JVirGel 2.0, which forms a virtual 2D gel image. By comparing the virtual image with the experimental gel results, the possible identities for the experimental bands were suggested as the paf1 complex component, cell wall protein DAN4, protein phosphatase, lectin-like protein, putative glucanases, protein kinase, aspartic proteinase 3, repressible acid phosphatase. After the characterization of the secretome proteins, the purification of rhGH was investigated using desalting, size exclusion and

anion exchange chromatography. In the desalting column, proteins were separated from impurities like salts and peptide parts. It was found that using size exclusion chromatography, the rhGH was partially purified from proteins with molecular weight lower than 15 kDa. It was predicted that rhGH interacts with other proteins and forms agglomerates. Although partial purification of rhGH is possible by using consecutive usage of two chromatographic techniques, the separation yield is low, since sequential chromatographic techniques probably caused substantial protein loss. As the final investigation of this work, secretomes produced by two different promoters were compared for purification of rhGH. Fractions of size exclusion and anion exchange chromatography were analyzed by SDS-PAGE. It was found that the secretomes were mostly similar except for the fact that the rhGH concentration of the secretome with the modified GAP promoter was higher than that for the secretome with modified AOX. In conclusion, rhGH was partially purified by using size exclusion and anion exchange chromatography.

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Keywords: Recombinant human growth hormone, 2-D gel electrophoresis, JVirGel, Size exclusion chromatography, Ion exchange chromatography

ÖZ

PICHIA PASTORIS SEKRETOMUNUN FARMASÖTİK PROTEİN ÜRETİMİ İÇİN ANALİZİ VE KARAKTERİZASYONU

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İnsan büyüme hormonu (hGH) çocuklarda kısa boyluluk tedavisinde kullanıldığı gibi pek çok hastalığın da tedavisinde kullanılmaktadır. hGH yüksek saflıkta kullanılabildiği için afinite kromatografisi kullanılarak saflaştırılmaktadır. Afinite kromatografisinin yüksek maliyetinden dolayı diğer kromatografik teknikler kullanılarak rekombinant insan büyüme hormonunun (rhGH) saflaştırılması araştırılmıştır. Çalışmanın amacı kromatografik saflaştırma protokollerini kullanarak rhGH'in *Pichia pastoris* üretim ortamından ayrılmasıdır. İlk olarak *P. pastoris* sekretomu iki boyutlu jel elektroforez ile analiz edildi. Sekretomda rhGH dışında molekül ağırlık aralığı 20 ila 140 kDa olan 4 ana protein bulundu. Sekretomdaki proteinlerin izoelektrik noktalarının 4.4 ve 5.7 arasında değiştiği belirlendi. Sekretomdaki proteinleri tanımlamak için literatürdeki *P. pastoris* sekretomu JVirGel 2.0 programı ile analiz edildi. Program sanal olarak sekretomun iki boyutlu jel elektroforez görüntüsünü oluşturdu. Deneysel jel sonuçlarını sanal görüntü ile karşılaştırınca deneysel bantların olası kimlikleri paf1 kompleks bileşeni, hücre duvarı protein DAN4, protein fosfotaz, lektin gibi protein, glukanazlar, protein kinaz, aspartik proteinaz 3, asit fosfotaz olarak önerildi. Sekretomdaki proteinlerin karakterizasyonundan sonra rhGH saflaştırılması tuz giderici kolon, jel geçirgenlik

kolonu ve anyon değişim kolonu kullanılarak araştırıldı. Tuz giderici kolonda sekretomdaki proteinler tuz ve peptit parçaları gibi safsızlıklardan ayrıldı. rhGH'in jel geçirgenlik kolonu kullanılarak molekül ağırlığı 15 kDa olan proteinlerden kısmi olarak saflaştığı görüldü. rhGH'in sekretomdaki diğer proteinlerle etkileşip topak oluşturduğu tahmin edilmektedir. rhGH'in arka arkaya iki kromatografik teknik kullanılarak kısmi olarak saflaştırılması mümkün olsa da ayrım düşük verimle gerçekleşmektedir. Art arda iki kromatografik teknik kullanımının önemli miktarda protein kaybına neden olduğu düşünülmektedir. Bu çalışmada son olarak iki farklı promotör kullanılarak ürettirilen sekretomlar rhGH'in saflaştırması açısından birbirleriyle karşılaştırıldı. Jel geçirgenlik ve iyon değişim kolonundan alınan fraksiyonlar SDS-PAGE ile incelendi. rhGH konsantrasyonunun modifiye GAP ile üretilen sekretomda modifiye AOX ile üretilene göre daha yüksek olması dışında büyük bir fark gözlenmedi. Sonuç olarak rhGH jel geçirgenlik ve iyon değişim kolonu kullanılarak kısmi olarak saflaştırıldı

.

Anahtar Kelimeler: Rekombinant insan büyüme hormonu, 2-D jel elektroforez, JVirGel, Boyut dışlanım kromatografisi, İyon değişim kromatografisi

To my family

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ABBREVIATIONS	
AOX	Alcohol oxidase
BSA	Bovine Serum Albumin
Da	Dalton
GAP	Glyceraldehyde 3-phosphate dehydrogenase
mL	Milliliter
MW	Molecular weıght
pI	Isoelectric point
rhGH	Recombinant Human Growth Hormone

CHAPTER 1

INTRODUCTION

Biopharmaceuticals are protein products for therapeutic purposes (P. F. Stanbury, Whitaker, & Hall, 2016). Biopharmaceuticals consist of proteins, nucleic acids, cell and tissue-based drugs (Aires-Barros & Azevedo, 2016). They have some advantages like low toxicity, minimal side effects, high selectivity and low non-specific and drug-drug interactions (Lee *et al*., 2015). Seven of the top ten drugs in the US were biopharmaceuticals in 2015 (Wang *et al*., 2017). It is estimated that the global market worth of biopharmaceuticals will reach 400 billion USD in 2025 (Love, Dalvie, & Love, 2018) and further estimated that half of the drugs will be biopharmaceuticals in the near future. Biopharmaceuticals are mainly produced by recombinant DNA technology (Jozala *et al*., 2016). Vaccines, interferons, growth hormones are examples of the biopharmaceuticals (Vogl, Hartner, & Glieder, 2013).

Hormones are regulatory substances. They carry signals and cause changes in cells. Hormones are carried to their targets via the circulation system of the body (Walsh, 2003). Human growth hormone (hGH) is a four-helix bundle protein (Şahin, Öztürk, Çalık, & Özdamar, 2015), which is essential for normal body growth. The mature form of hGH contains 191 amino acids and its molecular weight is 22 kDa. It is mainly used for the treatment of short stature in kids (Song, Jiang, Wang, & Zhang, 2017; Walsh, 2003). Since 1987, hGH has been produced primarily as a biopharmaceutical using host microorganisms (Şahin, Öztürk, Çalık, & Özdamar, 2015).

Biopharmaceutical production consists of two parts. The first part is the upstream process in which the desired compounds are produced (P. Stanbury, Whitaker, & Hall, 2016). Biopharmaceuticals are generally produced by using

bacteria, yeast and mammalian cells (Aires-Barros & Azevedo, 2016). Yeasts combine the advantages of both prokaryotic and eukaryotic organisms. They can grow fast in inexpensive media and genetically modified easily like bacterial cells. Also, they can make post-translational modifications like eukaryotic cells (Tripathi & Shrivastava, 2018).

Pichia pastoris is a well-known host for the production of biopharmaceuticals among yeasts, also used for the production of recombinant hGH (Azadi *et al*., 2018). It can grow in cost-effective media rapidly to high concentrations. Also, it can secrete the recombinant proteins to the production medium with a low amount of host cell proteins. This simplifies the purification of drugs (Maleki *et al*., 2012; Schlenzig, Wermann, Ramsbeck, Moenke-Wedler, & Schilling, 2015). Viral contamination risk is low with *P. pastoris* and it has a Generally Recognized as Safe (GRAS) status. As a eukaryotic organism, it can carry out human-like post-translational modifications like proper folding of proteins, glycosylation and disulfide bonds. Therapeutics from *P. pastoris* have been approved by the U.S Food and Drug Administration (FDA) and Europa Medicine Agency (EMA) (Crowell et al., 2018). By using different type promoters like well-known alcohol oxidase (AOX) promoters and glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter, recombinant proteins are produced by *P. pastoris* with using different substrates (Schwarzhans, Luttermann, Geier, Kalinowski, & Friehs, 2017). Çalık *et al*. investigated the production of rhGH under the AOX promoter. The concentration of rhGH was found as 640 mg/L. The *P. pastoris* under the AOX promoter uses methanol as a substrate for the production of the rhGH (Çalik et al., 2013). Since methanol is toxic to the cells (Çalik *et al*., 2015), glucose and glycerol can be used as a substrate under alternative GAP promoter. The concentration of rhGH with GAP promoter was found as 611 mg/L (Çalık, Hoxha, Çalık, & Özdamar, 2018).

The second part of the biopharmaceutical production is called the downstream process. In this part, the target product is purified from all impurities, concentrated and prepared for the market (Jozala *et al*., 2016). Purification of proteins is a difficult

task. Although for industrial enzymes, 70-80 % of purity is enough, 95-99.9 % of purity is needed for proteins used for medical and diagnostic applications (Aires-Barros & Azevedo, 2016) starting from concentrations typically between 0.1 and 5 g/L immediately after fermentation (P. Stanbury *et al*., 2016). Not only the initial product concentrations are low but also the production medium consists of cell fragments, degradative enzymes and other metabolic products that may interfere with the purification (P. Stanbury *et al*., 2016). Moreover, proteins can be denatured or degraded easily if not handled correctly. Furthermore, slow oxidation and microbial contamination should be prevented (Voet, Voet, & Pratt, 2012). Also, there is some protein loss after each purification step due to the decay of the product (P. Stanbury *et al.*, 2016).

In order to choose the most suitable separation process for protein products, the properties of both the targeted product as well as the side-products should be known (P. Stanbury *et al.*, 2016). One tool is two-dimensional gel electrophoresis that separates proteins according to their isoelectric points (pI) and molecular weights. By using 2-D gel electrophoresis, up to hundreds and thousands of proteins in the production medium could be analyzed at the same time. The number of proteins, their isoforms, and post-translational modifications could be seen in a 2-D gel image (Lilley, Razzaq, & Dupree, 2001; Øye *et al.,* 2013). Also, there are programs like JVirGel that allow the generation of the virtual 2-D image of the secretome of the host. By comparing real and virtual results, the identity host cell proteins may be predicted (Hiller, Grote, Maneck, Mu, & Jahn, 2006).

Chromatographic techniques can be used for all purification steps and analysis of protein drugs during the downstream process (P. Stanbury *et al*., 2016). It can separate components with similar properties. Chromatography is preferred over liquid-liquid extraction or membrane separation because its resolution is higher than both techniques. That means it separates products with small concentrations and high yields. Resins in the column can be easily regenerated and cleaned in one place (Aires-Barros & Azevedo, 2016). Also, aggregation and misfolded proteins are separated by

using chromatography and chromatography helps misfolded proteins to turn the correct form (Carta & Jungbauer, 2010). One disadvantage of chromatography is its cost, which is high due to the cost of resins. Also, there are limitations to the scale-up of chromatography. One limitation is the backpressure of the column owing to the packing material of the column (Jozala *et al*., 2016; P. Stanbury, Whitaker, & Hall, 2016).

In this study, the purification protocol of rhGH from the secretome of *P. pastoris* was tried to be determined using chromatographic techniques. The purification protocol should be cost-effective with maximum yield. For this reason, separation with ion-exchange chromatography and size exclusion chromatography was investigated for two different secretomes of *P. pastoris* with modified GAP and AOX promoter, respectively. Also, proteins in secretome were investigated by using 2-D gel electrophoresis and their properties were found. The Identity of proteins in secretome was predicted by using JVirGel and known secretome of *P. pastoris* from literature.

In the following literature survey chapter, more detailed information is given about chromatographic separation, characterization of proteins by using JVirGel and 2-D gel electrophoresis and hGH. In the third chapter, materials, chemicals, procedures to purify rhGH from secretome and characterization of host cell proteins in secretome are described. In chapter 4, the main results are given and discussed. In chapter 5, conclusions, possible future directions of study and proposed purification protocol for rhGH is given.

CHAPTER 2

LITERATURE SURVEY

2.1. Human Growth Hormone (hGH)

The human growth hormone is a single polypeptide chain. It is also known as somatotropin. It is released from the pituitary gland (Wojtowicz-Krawiec *et al*., 2014). The mature form of hGH consists of 191 amino acids. Its molecular weight is 22 kDa. It also has an isoform having a molecular weight of 20 kDa. It has two disulfide bonds (Walsh, 2007). The amino acid sequence of the hGH is given in Appendix A. It does not have post-translational modifications like glycosylation. The pI of rhGH is 5.1 (Çalik et al., 2010; Levarski, Šoltýsová, Krahulec, Stuchlík, & Turňa, 2014). It increases anabolic activity such as tissue developments including bone formation (Wafelman, 1999). The growth hormone is released at high levels in childhood. After puberty, it is released in small amounts to maintain the muscles. Administered as a drug, hGH is used for the treatment of short stature in children, Turner syndrome, chronic renal failure, obesity and burn injuries (Walsh, 2007; Wojtowicz-Krawiec et al., 2014). It is used also as an enhancer for milk production in the dairy industry (Lee *et al.*, 2015).

hGH was produced by using deceased people's pituitary glands until 1985. It was found that the production of hGH from humans causes the Creutzfeld–Jacob disease. It is a deadly disease. After that, the production of rhGH has been done by using different microorganisms with recombinant technology and purification techniques (Azadi *et al*., 2018; Walsh, 2003). *Escherichia coli* is used for the production of rhGH. *E. coli* cannot carry out post-translational modifications. Production with *E. coli* causes the formation of inclusion bodies. The refolding process of inclusion bodies causes protein loss and adds an extra step for purification.

Due to these reasons, steps for purification of rhGH increase and production yield decreases (Levarski, Šoltýsová, Krahulec, Stuchlík, & Turňa, 2014). Yeast is used for the production of the rhGH because it can do post-translational modifications and secrete recombinant proteins in the medium (Tripathi & Shrivastava, 2018). *P. pastoris* is one of the industrial hosts for the production of rhGH (Lee *et al.*, 2015). *P. pastoris* is a methylotrophic yeast. It can utilize the methanol as a carbon source due to its strong, inducible alcohol oxidase (AOX) promoters (Choengpanya *et al.*, 2015). Although AOX promoters are used for the production of therapeutic proteins with high concentration, methanol as a carbon source may cause some health and environmental problems (Güneş & Çalık, 2016). For this reason, the glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter is an alternative promoter to AOX promoters. This is a constitutive promoter (Schwarzhans, Luttermann, Geier, Kalinowski, & Friehs, 2017) which may be used with glucose and glycerol as the carbon source. Since the GAP promoter is active during the whole fermentation process, protein production and cell mass increase simultaneously. The GAP promoter can be used for continuous fermentation (Potvin, Ahmad, & Zhang, 2012) but cannot be used for the production of proteins that toxic to its host microorganism *P. pastoris* (Çalik *et al.,* 2015). Limitations and drawbacks with promoters could be overcome like modified AOX promoters which do not need methanol as carbon source (Schwarzhans *et al.*, 2017).

Production of rhGH from *P. pastoris* is done in three or four steps with the AOX promoter. In the first step, the number of cells increases by using glycerol as a substrate at the batch phase. In the second step, to increase the cell number further, glycerol is fed the system at the fed-batch phase. In the third step, if AOX is used as a promoter, methanol is added to the system as a substrate at fed-batch mode to start the production of the rhGH production (Bayraktar, 2009). If the GAP promoter is used, the production of rhGH by using *P.pastoris* is done in two steps. Glycerol batch phase is used as the first step, glucose or glycerol is added at the fed-batch mode in the second step (Hoxha, 2016). Human growth hormone concentration is highest at the

exponential growth phase (Çalik *et al.,* 2010; Çalik, Şahin, Taşpinar, Soyaslan, & Inankur, 2011). In table 2.1, examples of rhGH production studies by *P. pastoris* are given.

Table 2.1. *Summary of the rhGH production studies with P. pastoris*

The approach of the study	**Promoter**	**Concentration of rhGH (mg/L)**	**Reference**
Effect of the carbon sources on the rhGH production with different *P. pastoris* strains investigated	AOX	110	(Orman, Çalık, & Özdamar, 2009)
Effect of pH on the rhGH production with *P. pastoris* investigated	AOX	270	(Çalik, Bayraktar, *et al.*, 2010)
Effect of different carbon sources on the rhGH production with *P. pastoris* investigated	AOX	270	(Çalik, Inankur, et al., 2010)
Effect of co-substrate sorbitol feeding strategies on the rhGH production with *P. pastoris* investigated	AOX	640	(Çalik *et al.*, 2013)
The rhGH production with *P. pastoris* investigated with the hybrid fed-batch operation.	GAP	611	(Çalık *et al.*, 2018)

For the purification of rhGH from production medium, mainly chromatographic techniques are used. Immobilized metal ion affinity chromatography (IMAC) is a type of affinity chromatography. By combining IMAC and protein parts, which is called a tag like the polyhistidine tag, rhGH is purified. Because the cost of

affinity chromatography is high, weak ion-exchange chromatography could be used for purification (Chen, He, Shi, & Yang, 2013; Mooney, Fredericks, Christensen, Bruun Schiødt, & Hearn, 2015; Tripathi & Shrivastava, 2018).

Other than chromatography, rhGH could be purified by using linear-gradient electrophoresis by using membranes (Catzel *et al.*, 2003). Çulfaz-Emecen *et al.* investigated the separation of rhGH from the secretome of *P. pastoris* by using membranes and diafiltration. rhGH could not be separated from secretome. It is suggested that rhGH forms agglomerate with other proteins due to high salt concentration in secretome (Akcan, 2017). In table 2.2, some of the examples for the purification of the rhGH are summarized.

Table 2.2. *Summary of production and purification studies for rhGH*

Host	Characterization Techniques	Purification Techniques	Reference
E. coli	SDS-PAGE	Purification: hydrophobic interaction chromatography (HIC) Concentration: anion exchange chromatography	(Wojtowicz-Krawiec *et al.*, 2014)
E. coli	SDS-PAGE Western blot MALDI-TOF mass spectroscopy	Immobilized metal ion affinity chromatography (IMAC)	(Mooney *et al.*, 2014)
E. coli	SDS–PAGE Nb2 cell line proliferation assay RP-HPLC analysis SEC Circular dichroism Endotoxin concentration assay and removal	Capture: Affinity chromatography Polishing: Anion-exchange chromatography	(Levarski, Šoltýsová, Krahulec, Stuchlík, & Turňa, 2014)
Pichia pastoris	RP-UPLC	Capture: Cation exchange chromatography Anion exchange chromatography Hydrophobic charge induction chromatography (HCIC)	(Timmick *et al.*, 2018)
Pichia pastoris	SEC RP-HPLC capillary zone electrophoresis Nb2 proliferation test	Capture: Weak anion exchange chromatography Purification: Hydrophobic interaction chromatography Strong anion exchange chromatography	(Azadi *et al.*, 2018)
Pichia pastoris	SDS-PAGE MALDI-TOF	IMAC	(Çalik *et al.*, 2008)

2.2. Chromatographic techniques

The downstream process has three steps. These steps are recovery, purification, polishing, respectively. The aim of the recovery step is to remove the solid particles like cells, recover the product and concentrate the medium and decrease the purification volume. Cell harvesting and recovery of products is the first step of the purification process for both extracellular and intracellular protein products. It includes mainly solid-liquid separation techniques based on product properties like size, shape, density. Product is recovered from production media which contains cells, other impurities and concentrated. Filtration, centrifugation, sedimentation techniques are used for the recovery part (Jozala *et al.*, 2016; Aires-Barros & Azevedo, 2016). After the removal of cells from culture media, the supernatant contains 85-95% water and a low amount of desired product. Purification step is the main step separation of product from other impurities by using different properties like size, charge, hydrophobicity. To remove most of the water and concentrate the product, membrane process, liquid-liquid extraction, precipitation, chromatography could be used. Product is purified from remaining impurities and prepared for the market in the polishing step by using chromatography, crystallization, drying (Aires-Barros & Azevedo, 2016; P. Stanbury, Whitaker, & Hall, 2016).

Chromatographic separation is based on the distribution of the product between stationary and mobile phases. The stationary phase is formed from generally uniform-sized particles and they are packed in the column. The mobile phase is the fluid that flows through the column and equilibrates with the stationary phase. The product is generally loaded to the column with the mobile phase. Separation is done either in an isocratic mode or gradient elution of the mobile phase. In isocratic mode, the mobile phase remains the same throughout the separation. In gradient elution, salt and/or pH gradients are applied along the stationary phase to release proteins from the column (Aires-Barros & Azevedo, 2016). Components that have weaker interaction with stationary phase elute faster than components which have strong interaction. Eventually, the constituents of the protein mixture are separated from each other

(Jozala *et al*., 2016). For protein purification, generally liquid chromatography is used (Tripathi & Shrivastava, 2018). Chromatographic separation is done based on size, charge, polarity, the affinity of protein to the stationary phase. The most commonly used types of chromatography are size-exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography and affinity chromatography (Stanbury, Whitaker, & Hall, 2016).

2.2.1. The solubility of proteins respect to salt concentration

Proteins are soluble in physiological conditions. The physiological condition means 0.15 -0.20 M salt concentration with neutral pH. The solubility of proteins is a combination of polar interaction with solvent and ionic interaction with the salt molecules (Scopes, 1994).

The solubility of proteins increases with increasing salt concentration at low salt concentration. Because salt molecules prevent the attractive forces between the proteins and precipitation of proteins. This is called salting in. If more salt added to the medium, salt molecules interact with the solvent molecules. So, solvent molecules, which solve the protein molecules, decrease with high salt concentrations. This is called salting out. Proteins are precipitated under high salt concentration (Voet, Voet, & Pratt, 2012).

2.2.2. Size-based separation (Size exclusion chromatography)

This technique is also referred to as gel permeation or gel filtration chromatography (Aires-Barros & Azevedo, 2016). Proteins are separated from each other based on their hydrodynamic volumes in size exclusion chromatography (Tripathi & Shrivastava, 2018). The stationary phase consists of packed porous beads. Small molecules like salts or peptides enter the pores of beads, whereas larger molecules like protein cannot, and flow through the column. Thus small molecules stay in columns longer than large molecules, which exit from column earlier (Nelson & Cox, 2008; Aires-Barros & Azevedo, 2016). Eventually, large and small molecules are separated from each other by their size and corresponding retention time in the

column. The pore size determines the molecular weight range of proteins that can be separated. Size exclusion chromatography can be used for both protein purification and group separation. In group separation, the mixture is separated into two main groups. Desalting and buffer exchange are examples of group separation in size exclusion. Since size exclusion chromatography is a non-binding separation, the concentration of the sample does not have a significant effect on separation. Also, separation can be done with a broad pH, temperature range, different ionic strength and different additives to the mobile phase. The mobile phase remains the same through separation in the isocratic mode phase (GE Healthcare Bio-Sciences AB, 2010; Aires-Barros & Azevedo, 2016). Size exclusion column works with small sample loads because it dilutes the sample while separating (Lederman, 2018). The sample needs to be concentrated before the chromatography. Resolution of the size exclusion column increase with increasing column length. On the other hand, the flow rate of the mobile phase should be low due to the pressures needed to overcome the hydraulic resistance of the column. Due to the necessity of small sample load and low flow rates, size exclusion chromatography is not typically suitable for purification. It is mainly used for analysis (Tripathi & Shrivastava, 2018).

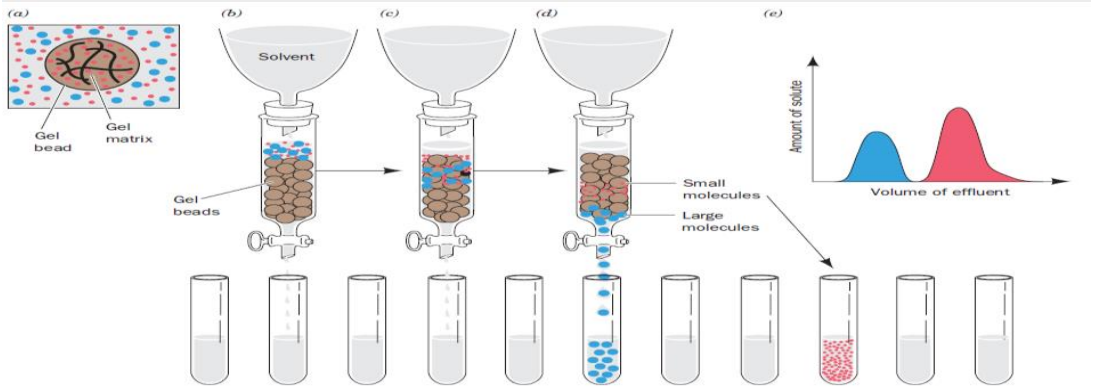


Figure 2.1. Separation with size exclusion column (Voet et al., 2012)

2.2.3. Charge-based separation (Ion-exchange chromatography)

Ion-exchange chromatography separates proteins based on their net surface charges. Proteins with similar charges in the mobile phase reversibly exchange with the mobile ions on the stationary phase in ion-exchange chromatography (Aires-Barros & Azevedo, 2016). Many biological products like amino acids, nucleic acids and proteins have a net surface charge due to their structure. The sign and charge of proteins are determined by the isoelectric point of proteins and pH of the buffer solution. The isoelectric point (pI) of protein is the pH in which the net charge of the protein is zero. If the pH of the buffer is higher than the pI of protein, protein becomes negatively charged, whereas the protein is positively charged when the pH of the buffer is lower than the pI. In an anion exchanger, fixed positively charged groups are found in the stationary phase. Negative-charged molecules exchange with the mobile anions on the exchanger. Positively charged molecules bind the cation exchanger, which has negative fixed charges on the surface of the stationary phase. By changing the ionic strength with a salt solution or the pH of the mobile phase, molecules that are bound the resins are released easily (Nelson & Cox, 2008; Voet, Voet, & Pratt, 2012). Ion-exchange chromatography is used for separation because it is suitable for working with high sample loads and flow rates (GE Healthcare Bio-Sciences AB, 2010; Lederman, 2018). Moreover, it has a lower cost compared to other chromatography techniques. For example, the cost of ion-exchange chromatography is estimated as one-fifth of affinity chromatography. (Aires-Barros & Azevedo, 2016; Tripathi & Shrivastava, 2018).

For the purification of rhGH from the medium by using ion-exchange chromatography, generally, the pH of the medium was chosen between 5 and 8.2. It is found that rhGH more stable at pH 5 but changing pH between 3 to 7 does not affect the concentration of rhGH (Bayraktar, 2009). For the cation exchange chromatography, optimum pH is 5.5 (Moore, Wong, & Rawitch, 1980). For anion exchange chromatography, it is between 6 and 8.2 (Azadi *et al.*, 2018; Sonoda & Sugimura, 2008; Timmick *et al.*, 2018).

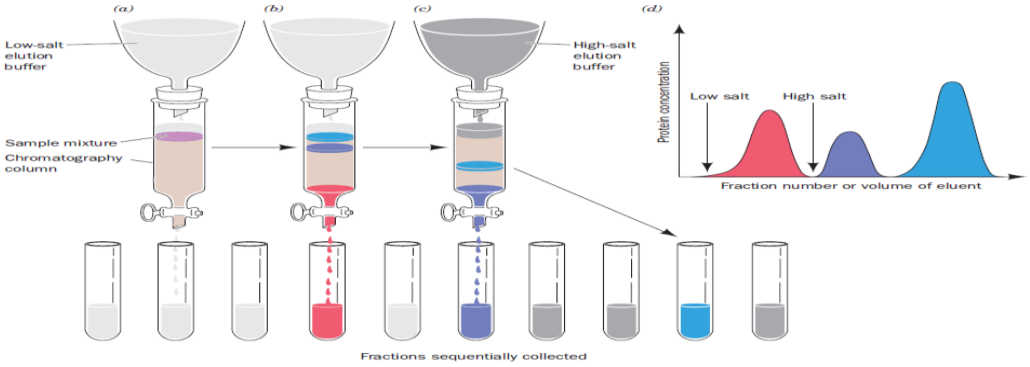


Figure 2.2. Separation with ion-exchange chromatography (Voet et al., 2012)

2.2.4. Definition of separation performance parameters by using chromatograms

Chromatograms are generally formed by injection of the three-component mixture into the column. The retention time of each component is proportional to the strength of their interaction with the stationary phase of the column. In the case of retention time, retention volume term can be used. Retention volume is calculated by multiplying retention time with the volumetric flow rate of the mobile phase in the column.

The retention factor is the comparison of the amount of solute in the stationary phase with the amount of solute in the mobile phase. It is shown by k'. The relationship between the retention factor and retention volume is given equation 2.1.

$$V_R = V_M + k' V_M \quad \text{(Eq. 2.1)}$$

V_R shows the retention volume of solute and V_M shows the volume of the mobile phase. The retention factor is also related to the distribution coefficient, K_D.

$$k' = K_D \frac{V_S}{V_M} \quad \text{(Eq. 2.2)}$$

V_S shows the volume of the stationary phase. The retention factor is proportional to the distribution coefficient and volume ratio of stationary and mobile phases. The distribution coefficient is related to the mobile phase and pore dimensions. In size exclusion chromatography, the distribution coefficient is only proportional to

the pore dimension. In ion-exchange chromatography, the distribution coefficient is not constant. It changes during ion-exchange chromatography with step and linear-gradient due to change in the mobile phase (Hagel, Jagschies, & Sofer, 2008).

The efficiency of the separation with the column is determined by the height equivalent of a theoretical plate (HETP) or plate number (N)(Carta & Jungbauer, 2010b). Plate number shows how close chromatographic separation to the ideal separation conditions. The plate number can be calculated by using equation 2.3;

$$Ni = \frac{\mu t,i^2}{\sigma t,i^2} \quad \text{(Eq. 2.3)}$$

$\mu_{t,i}$ represents the first absolute moment of the peak. It shows the concentration change within time. $\sigma_{t,i}$ represent the variance. It is calculated by using the second absolute moments of the peak (Schulte & Epping, 2005).

Parameters like plate number of the column for each component, retention times or volumes of components, symmetry and standard deviation of peaks with their moments can be calculated by using free software. Software is called as ChromatographyGUI. Software is based on MATLAB codes. It was written by Harun Koku and Bill Kolodzey in 2009.

Porosity is one of the performance parameters for the column chromatography (Carta & Jungbauer, 2010b). It shows the ratio of the volume of porous packing material covers the total column volume (Quinn, 2014).

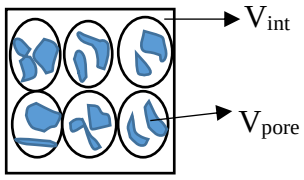


Figure 2.3. Schematic of the chromatography column with interstitial and pore volumes

Total porosity is calculated by using equation 2.4;

$$\text{Total porosity} = \frac{Vint+Vpore}{Vc} \quad \text{(Eq 2.4)}$$

V_{int} shows the interstitial volume of the column. V_{pore} shows the total pore volume of the column. Vc is the total column volume. It is hard to measure the interstitial and pore volume one by one. A combination of interstitial and pore volume can measure by injecting a small molecule to the column. Small molecule both pass through the interstitial volume and pores of packing material. The retention volume of a small molecule like acetone is the combination of interstitial and pore volumes (Schulte & Epping, 2005).

2.3. Characterization of secretome of *P. pastoris*

Secretome is the part of the proteome. Secretome includes secreted proteins from the cell to the surrounding environment. Cells may secrete proteins into a medium for different purposes, such as protection from other cells or for maintenance (Huang *et al.*, 2011). Extracellular protein products are secreted to the medium during production. To choose an efficient separation procedure for protein products, the properties of both product and other proteins in the medium should be known (Stanbury *et al.*, 2016). For characterization, electrophoretic techniques are used like two dimensional (2-D) gel electrophoresis. In 2-D gel electrophoresis, hundreds of proteins are separated from each other and visualized simultaneously. The amount, number and physicochemical properties like size and isoelectric points of proteins are determined by 2-D gel electrophoresis analysis. Also, isoforms and post-translational modifications of proteins can be seen. Proteins are protected in gels and 2-D gel electrophoresis is compatible with other characterization techniques such as mass spectroscopy. On the other hand, the reproducibility of 2-D gel electrophoresis analysis is low. Analyzing low concentration proteins, proteins with molecular weights higher than 250 kDa, hydrophobic proteins is problematic. Hydrophobic proteins do not enter gel and lost. If proteins are molecular weight above 250 kDa is

separated by the second dimension, they could exit from the gel (Brandi et al., 2018; Westermeier, 2005; Westermeier & Naven, 2002).

2.3.1. Two-dimensional gel electrophoresis (2-D)

Proteins are amphoteric. They have polar groups like amino and carboxyl groups in their structure and have net charge under an electrical field, with a net charge of zero at the isoelectric point (pI) (Voet, Voet, & Pratt, 2012). Electrophoresis separated molecules according to their electrophoretic mobilities under an electrical field (Westermeier, 2005). 2-D gel electrophoresis combines isoelectric focusing and sodium dodecyl sulfate (SDS-PAGE) gel electrophoresis. While SDS-PAGE separates proteins according to their molecular weight, isoelectric focusing separates them according to their respective isoelectric point in the medium. In isoelectric focusing, pH gradients are used. Stable pH gradients are applied to the protein mixture. The proteins migrate till the pH equal to their pI, after which the electrical field no longer affects them because their net charge is zero. Therefore proteins are separated according to their pI in the isoelectric focusing (Westermeier, 2005; Voet, Voet, & Pratt, 2012). After isoelectric focusing, SDS-PAGE is used as the second separation due to its low cost and easy to stain nature (Rabilloud, 2010; Rabilloud & Lelong, 2011). First, the gel with the finalized IEF step is equilibrated with SDS (DeWald, Adams, & Pearson, 2011) which is an anionic detergent. It binds proteins and makes them negative charged, thus masking the net charge of proteins. Also, SDS causes partially unfolding in proteins. The average shape of proteins become similar. Proteins are separated from each other according to their molecular weight in SDS-PAGE. The number of proteins in the mixture, molecular weight and isoelectric points of proteins are determined by using 2-D gel electrophoresis (Nelson & Cox, 2008; Westermeier, 2005).

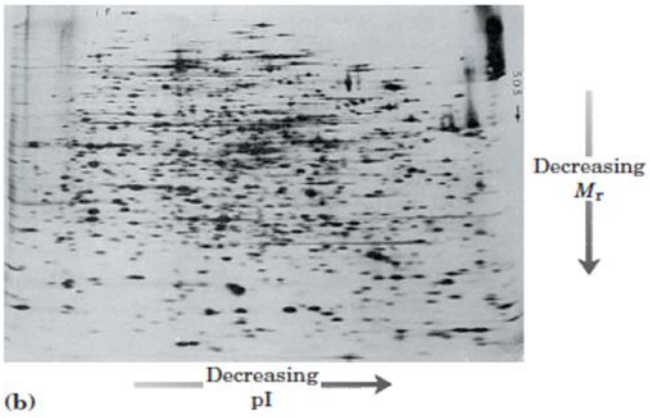


Figure 2.4. Example of 2-D gel electrophoresis (Nelson & Cox, 2008)

To characterize the secretome and observe the rhGH existence in the secretome different methods and staining techniques can be used. One of the methods is the comparison of the secretome that contains rhGH with wild type *P. pastoris* secretome by using 2-D gel electrophoresis. Comparing points in 2-D gel images of secretomes, the existence of rhGH can be found. Other than 2-D gel electrophoresis, the presence of rhGH in secretome can be ensured by using western blot analysis. Western blot analysis is used for the determination of specific protein which is found in the protein mixture. It is also called as immunoblotting because of the usage of antibodies in the analysis of specific protein or proteins in the complex protein mixtures. Western blot analysis consists of three parts. In the first part, proteins are separated according to their size by SDS-PAGE. In the second part, proteins are transferred the solid support like nitrocellulose or PVDF membrane by electro transferring. This process is called as blotting. In the last part, specific protein or proteins are detected by using suitable primary and secondary antibodies and visualized. The second antibody is labeled with an enzyme. By adding the substrate, specific protein or proteins can be seen easily (Mahmood & Yang, 2012). By using ELISA (enzyme-linked immunosorbent assay), rhGH existence can be proven. In ELISA, the sample is adsorbed on the inert media. To prevent the adsorption of the other proteins on free space of membrane, membrane treated with inexpensive protein like casein. Then, the primary antibody solution is given to the column. It binds the protein of interest. The second antibody solution is

bind to the primary antibody. The second antibody contains an enzyme. Substrate and enzyme interact and interaction causes the color change. By color change, the presence of the desired protein and its concentration could be determined (Nelson & Cox, 2008). Finally, analyzing the protein bands from the gel by using mass spectroscopy. By comparing amino acid sequence and molecular weight of protein with mass spectroscopy results. The presence of the desired protein could be determined (Encarnación *et al.*, 2005).

Mattanovich *et al.* investigated the secretome of *P. pastoris* DSMZ 70382 by using glucose as a substrate. Proteins in secretome were predicted by using genome screening and results are compared with real 2-D gel electrophoresis results. It was found that the secretome of *P. pastoris* has low-level contaminants and nearly zero protease activity. A total of 20 proteins were identified (Mattanovich *et al.*, 2009a). Huang *et al.* analyzed the secretome of *P. pastoris* with the AOX promoter. The pre-induction and post-induction phase was investigated by 2-D gel electrophoresis and mass spectroscopy. A total of 75 protein is identified. Their pI is between 3 and 7. The molecular weight of proteins was found between 10-260 kDa (Huang *et al.*, 2011).

2.3.2. Theoretical prediction of points by JVirGel 2.0

JVirGel 2.0 is a software tool that can generate virtual images of 2-D gel electrophoresis analysis for proteomic studies. It predicts the migration behavior of proteins according to their theoretical molecular weights and calculated isoelectric points. Images are formed using the FASTA formatted amino acid sequence of the proteins of the desired microorganism. The program can be used on a local computer or online. Also, the 2-D gel images of some eukaryotic and prokaryotic microorganisms could be found directly online like *E. coli* and *S. cerevisiae*. It can be downloaded free from http://www.jvirgel.de/.

The program can be used to predict the secretome from the proteome of the microorganism (Hiller, Grote, Maneck, Mu, & Jahn, 2006). Furthermore, the predictions can be helpful to determine the optimum pH range for the 2-D gel

electrophoresis in practice (Hiller *et al.*, 2003; Sankarasubramanian *et al.*, 2015). It could also be used to find signal peptides and membrane proteins.

2.4. Aim of the study

The main aim of the present study was to establish a purification protocol for rhGH from the secretome of *P. pastoris* by using chromatographic techniques. Two different secretomes of *P. pastoris* were investigated.

CHAPTER 3

EXPERIMENTAL METHODS

3.1. Materials

For chromatography applications, three columns were purchased from GE Healthcare. These columns were desalting, size exclusion and ion exchange column, respectively. The desalting column was HiPrep 26/10 Desalting. The size exclusion column was HiPrep 16/60 Sephacryl S-100 High Resolution. Its molecular weight range for separation was between 1 and 100 kDa. The ion exchange column used HiPrep Q XL 16/10. Its resin was polypropylene with quaternary ammonium. It is a strong anion exchanger. Three main chemicals for the buffer solutions were KH_2PO_4 (MW =136.08g/mole), K_2HPO_4 (MW=174.18 g/mole) and NaCl (MW=58.44 g/mole), respectively. Phosphate salts were purchased from Merck. Sodium chloride was obtained from Sigma Aldrich. To clean and maintain the column, ethanol (J.T. Baker) and NaOH (Merck) solutions were used. To observe the retention time of molecules with different molecular weight and charge, bovine serum albumin (BSA, MW=66 kDa, pI=4.7) and acetone (Merck) were injected to the chromatography columns. A low molecular weight calibration kit from GE Healthcare was used to calibrate the size exclusion column. Before chromatography, samples should be free from particles to prevent them from getting into a chromatography column. For that reason, hydrophilic polyethersulfone filters, which pore size was 0.45 and 0.20 μm, were purchased from Millipore. The mobile phase buffers for the chromatography were filtered using 0.45 μm Sartorius hydrophilic cellulose acetate membrane filters. Furthermore, 2 and 5 mL sample loops were purchased from GE healthcare to apply samples to the chromatography columns.

To characterize the secretome of *P. pastoris*, the two-dimensional gel electrophoresis method was used. A 2-D cleanup kit from Bio-Rad was used for removing excess salts from the production medium. The hGH standard, which was called Humatrope a trademark name, was purchased from Lily. The gene id of Humatrope is DB0052. Its amino acid sequence is given Appendix A. To observe the chromatography efficiency, SDS-PAGE analysis was done. SDS-PAGE preparation kit was purchased from the Bio-Rad. For staining procedure, acetic acid, potassium carbonate and sodium dodecyl sulfate were obtained from Sigma Aldrich, methanol and silver nitrate from Merck, sodium thiosulfate pentahydrate from Fluka.

3.2. Solution Preparation

Buffer for the anion exchange, size exclusion, and desalting column were prepared according to buffer recipe from Liverpool buffer recipe calculator. To make the buffer for size exclusion and desalting column, 2.68 g KH_2PO_4, 5.26 g K_2HPO_4, and 2.32 g NaCl were dissolved in the 1000 mL pure water. So, 50 mM KH_2PO_4-K_2HPO_4 and 150 mM NaCl buffer were prepared at pH 7. In the application of the ion exchange column, two different buffers were used. These buffers were start and elution buffer, respectively. Start buffer was prepared by dissolving 0.22 g of KH_2PO_4, 4.05 g of K_2HPO_4 with 0.303 g NaCl in the 500 mL up water. So, start buffer with a concentration of 50 mM KH_2PO_4-K_2HPO_4 and 150 mM NaCl was prepared at pH 8.0. Elution buffer had the same concentration of start buffer except for the additional 1 mole of NaCl. So, 0.18 g of KH_2PO_4, 4.11 g of K_2HPO_4 and 25.04 g of NaCl were dissolved in 500 mL up water to prepare elution buffer. Eventually, elution buffer was prepared with a concentration of 50 mM KH_2PO_4-K_2HPO_4 and 1000 mM NaCl at pH 8. All solutions in the chromatography should be filtered to prevent impurities from contaminating the chromatography system. Buffers were filtered with filters with pore size 0.45 or 0.20 µm. After filtration, buffers were sonicated by Sonorex sonicator by Bandelin for 15 minutes to prevent any gas bubble in liquid chromatography.

3.3. Model Proteins

BSA solutions were prepared by dissolving in ultra-pure water at 1, 5 and 10 g/L concentrations. 20 mL 5 % (v/v) acetone solutions were prepared. 125, 250, 500 and 1000 and 2000 mg/L hGH standard solutions were prepared by using ultra-pure water.

3.4. Production Medium Pretreatment

The production of rhGH was done by the researchers in Prof.Dr.Pınar Çalık's Industrial Biotechnology and Metabolic Engineering Laboratory at METU and the production medium was kindly provided. The production was done in the bioreactor (BRAUN CT2-2) that operating volume 0.8 – 2.2 mL. Production of the sample with a modified GAP was done in a two-step. In the first step, cells grown on the basal salt medium (BSM) at the glycerol batch phase. Glucose fed-batch was done in the second step for rhGH production (Hoxha, 2016). Production with modified AOX includes the three-step. The glycerol batch phase was the start of the process for cell growth. To increase cell growth further and derepress the modified AOX promoter, the glycerol-fed batch was done. In the last step, ethanol was given with fed-batch operation (Bayraktar, 2009). During the process to maintain cell growth Pichia trace minerals (PTM) were added. The composition of BSM and PTM are given in Appendix B. The operation temperature was 30 °C. The pH of the medium was kept constant at 5 during production (Hoxha, 2016). The production medium of bioreactor consisted of *P. pastoris* secretome with its cells.

To remove the secretome from the medium, the production medium was centrifuged at 4000 g for 10 minutes at +4°C. Eventually, the cells were removed from the secretome which includes rhGH. After centrifugation, the secretome was filtered before chromatography and stored at -20°C. Filtration was done with hydrophilic filters to prevent the entry of large fragments into the chromatography system. Two filters were used with 0.45 and 0.20 μm pore sizes, respectively. It is found that the freeze-thaw process of rhGH causes some agglomeration (Fradkin, Carpenter, &

Randolph, 2009). The freeze-thaw cycle does not affect the rhGH until 5 freeze-thaw cycles (Livesey, Hodgkinson, Roud, & Donald, 1980; Reyna, Traynor, Hines, Boots, & Azziz, 2001)

3.5. Chromatography Experiments

Chromatography experiments were carried out with the AKTA prime plus chromatography system from G.E Healthcare. Three different pre-packed chromatography columns were used according to their separation properties with the system.

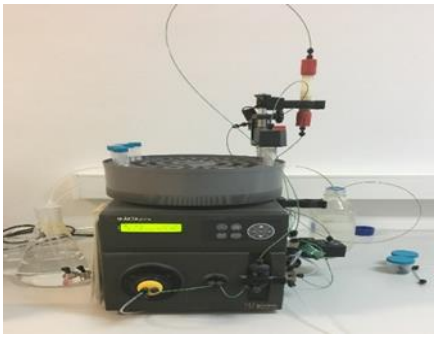


Figure 3.1. Akta prime plus chromatography system with ion-exchange column

Columns were equilibrated with their special buffer solution before and after each usage of columns. The manual run mode was chosen during all chromatography experiments. The pressure limit was set as 0.35 MPa according to the column manual. For each column, the optimum flow rate was determined. After each run, columns should be clean with up water. For the storage, the ethanol solution was used.

Absorbance values of proteins were detected by using UV lamb at 280 nm. Chromatograms were formed by unicorn software with the AKTA prime plus chromatography system. When pre-packed columns were used, the pressure drop should not exceed the 0.15 MPa. The final pressure should not exceed 0.35 MPa. Sample loops with 100 µL and 2 mL were used during experiments. Flow rates of columns in the experiments and volumes of columns are given in Table 3.1

Table 3.1. *Summary of chromatography columns and their properties with operation conditions*

Column type	**Column name**	**Buffer type**	**Optimum flow rate (mL/min)**	**Volume of column (mL)**
Desalting	HiPrep 26/10 Desalting	50 mM KH_2PO_4-K_2HPO_4 and 150 mM NaCl	5	53
Size Exclusion	HiPrep 16/60 Sephacryl S-100 High Resolution	50 mM KH_2PO_4-K_2HPO_4 and 150 mM NaCl	0.5	120
Ion (anion) exchange	HiPrep Q XL 16/10	**Start Buffer;** 50 mM KH_2PO_4-K_2HPO_4 and 150 mM NaCl pH=8.0 **Elution Buffer;** 50 mM KH_2PO_4-K_2HPO_4 and 1M NaCl pH=8.0	2.5-5	20

3.5.1. Desalting Column Experiments

Even though the secretome of *P. pastoris* was centrifuged and filtered before the chromatography, there were some excess salt ions and some impurities in it. By using a desalting column, removing excess salts and other impurities was aimed. Also, partial purification of rhGH was done by separating proteins and other impurities from each other.

In the experiments, HiPrep 26/10 Desalting column from G.E Healthcare was used. As mentioned in 2.2.2, it separates molecules according to their size, like the size exclusion column. Small particles like salts and peptides, which molecular weights are 5000 Da and below, enter the pores in the desalting column, resulting in earlier elution of higher molecular weight substances. The separation range of desalting was small, unlike the size exclusion column. Moreover, secretome was concentrated by using a desalting column

3.5.2. Anion Exchange Column Experiments

Ion-exchange chromatography was used for the partial and total purification of rhGH from secretome. HiPrep Q XL 16/10 from G.E Healthcare was used for the experiments.

Negative and positive charged proteins were separated from each other with ion-exchange chromatography by using the isoelectric point of proteins. The net charge of the protein is zero in the isoelectric point. The net charge of protein can be set by altering the pH of the buffer solution of chromatography. The isoelectric point of rhGH, which its net charge is zero, is around 5.1. For that reason, in order to get negatively charged rhGH, the pH of the buffer was selected as 8. There were two buffers in anion exchange chromatography. These were start and elution buffer. By using the start buffer, negatively charged proteins like rhGH in the secretome were captured by positively charged resin. Ionic strength was increased by using elution buffer with 1 molar NaCl. After the elution buffer, negatively charged proteins including rhGH released from the column.

3.5.3. Size Exclusion Column Experiments

It was aimed to purify rhGH from other proteins in secretome partially or totally by using its molecular weight. HiPrep 16/60 Sephacryl S-100 High-Resolution column from G.E Healthcare was used for experiments. It separates the molecules according to their molecular weight.

3.6. Characterization of *P. pastoris* secretome

3.6.1. Two-Dimensional Gel Electrophoresis (2-D Gel Electrophoresis)

Proteins are separated from each other according to their size and isoelectric point by using 2-D gel electrophoresis. One of the aims of the study was to identify proteins in the secretomes of *P. pastoris* with 2-D gel electrophoresis. The production medium with modified GAP was investigated with 2-D gel electrophoresis.

2-D gel electrophoresis was done by using the protean system from Bio-Rad. 2-D gel electrophoresis procedure consists of the main two-step. Experiments were carried out at METU central laboratory. These two steps were explained in detail below.

3.6.1.1. Rehydration and Isoelectric Focusing Procedure

Rehydration buffer was prepared by using 8 M Urea, 2% (v/v) CHAPS, 50 mM DTT, 0.2% (w/v) bio-lytes ampholytes. 5 µL sample was dissolved in 125 µL rehydration buffer for each strip. Sample and rehydration buffer mixture were applied rehydration tray from one end to another. Ready IPG strip, which length was 7 cm, was prepared by removing its cover. Then side which was protected by cover applied on the sample and rehydration buffer mixture. When the strip was placed on the mixture, the "+" side of the strip should be placed on the "+" side of the tray. Also, there should be no bubble between strip and tray. A half-hour waited for sample loading. Then the tray was covered with 1 mL of silicone oil. Active rehydration was done. Active rehydration started with 50 volt and 15 hours. Then, 250 volts was applied through the strip for 15 minutes. After that, 4000 volts was applied for 2 minutes. Finally, the current was kept constant at 20000 volt/hour for 5 hours.

3.6.1.2. Equilibration and SDS-PAGE procedure

Equilibration buffer 1 was prepared by using 6 M Urea, 0.375 M Tris-HCl pH=8.8 , 2 % SDS, 20 % Glycerol, 2 % DTT and equilibration buffer 2 was prepared by using 6 M Urea, 0.375 M Tris-HCl pH=8.8, 2 % SDS, 20 % Glycerol, 2.5 % iodoacetamide . After IEF, the strip was taken from tray and side which covered with mineral oil was cleaned by using filter paper. The side in which proteins were found is gently cleaned by using filter paper with ethyl alcohol. Strip was placed in the new tray and 2.5 mL equilibration buffer 1 was applied on the side where proteins were found for each strip during 15 minutes. After that, the strip was dried by using filter paper. The same procedure was applied for the equilibration buffer 2. After the second equilibration, the strip was washed with a Tris-HCl solution and dried. At the same

time, the SDS-PAGE gel was prepared. Before the polymerization of gel, the strip was carefully placed into the stacker gel. After that, the SDS-PAGE procedure and silver staining were applied.

3.6.2. Sodium Dodecyl Sulfate Gel Electrophoresis (SDS-PAGE)

To determine the purification level of rhGH after each chromatographic separation and molecular weight distribution in each fraction of chromatography, SDS-PAGE analyses were done for each secretome of *P. pastoris* which produced by using different promoter and production technique. Mini protean set from Bio-Rad was used for experiments. SDS-PAGE procedure was given in detail below.

3.6.2.1. Gel Preparation and Electrophoresis

Gel drying apparatus and 1 mm glasses were prepared. Stacker and resolver solutions were prepared according to the TGX Stain-Free™ FastCast™ Acrylamide Solutions. 10% (w/v) ammonium persulfate (APS) and N, N, N', N' tetramethylethylenediamine (TEMED) were added for each gel according to the kit. The first resolver was poured between the glasses. Then stacker solution was added. After that, the comb, which produces wells for sample injection, was inserted into stacker gel very carefully. Gels were left for polymerization for 50 minutes. Then, the comb was removed from stacker gel and wells were formed. After that, wells were washed with pure water and dried by filter paper. Gels were placed onto the gel electrophoresis device and the 1X running buffer was loaded onto them. Samples were prepared for SDS-PAGE by mixing 13 µL samples with 5 µL 4X Loading dye (bromophenol blue) and 2 µL 0.1 M Dithiothreitol (DTT) solution. To activate DTT and break cysteine bonds and linearize the proteins, samples were incubated at 95 °C for 5 minutes. 15 µL samples and 15 µL hGH standard were loaded onto wells.2 µL protein ladder was loaded in first well. The gels were run at 200 volts for 50 minutes.

3.6.2.2. Staining Procedures

After running, gels were stained according to staining procedures above for different dyes.

Silver Staining Procedure

Fixer Solution (1hour): 100 ml methanol, 24 ml acetic acid, 100 µL 37% formaldehyde were mixed and ultra-pure water was added up to 200 mL.

50% ethanol solution (3 x 20 minutes): 125 mL ethanol and water were mixed with each other.

Pure water (3x 20 seconds): Gel was washed by ultra-pure water to remove any remaining molecules from the fixer and ethanol solution.

Pre-treatment solution (1 minute): 0.05 g $Na_2S_2O_3.5H_2O$ was dissolved in 200 ml distilled water.

Pure water (3x 20 seconds): Gel was washed by ultra-pure water to remove any remaining molecules from the pre-treatment solution.

Silver nitrate solution (20 minutes): 0.2 g silver nitrate was put into a bottle and 100 ml ultra-pure water and 75 µl 37% formaldehyde was added.

Pure water (3x 20 seconds): Gel was washed by ultra-pure water to remove any remaining molecules from silver nitrate solution.

Developing Solution (5 minutes): 2.25 g potassium carbonate was dissolved in 100 mL ultra-pure water. 2 ml from pretreatment solution and 75 µl 37% formaldehyde were added.

Stop Solution (storage): 50 ml methanol, 12 ml acetic acid were mixed and ultra-pure water was added up to 100 mL.

Coomassie Brillant Blue Staining Procedure

Fixing Solution (1 hour): 0.8 mL 25 % glutaraldehyde and 30 mL ethanol. The volume was completed to 100 mL. Finally, 2.73 g sodium acetate trihydrate was added.

Coomassie Blue Dye (1 hour): 1 g of dye was dissolved in 300 mL methanol.650 ml distilled water and 50 mL acetic acid was added. The solution was stirred 2 hours and filtered.

Destain solution 1 (Change periodically until the background is clear): 300 mL methanol, 50 mL acetic acid and 650 mL distilled water were mixed.

Destain solution 2 (storage): 880 mL distilled water, 70 mL acetic acid and 50 mL methanol were mixed.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Model Proteins and hGH standard

The secretome of *P. pastoris* is a complex medium that consists of salts, rhGH, and other host cell proteins. Model proteins and molecules were injected in each chromatography column to determine optimum conditions and observe the performance of the chromatography columns. Optimum working conditions of columns, average retention volumes of model proteins were determined according to the response of the column to model proteins and molecules. In Table 4.1, a summary of experiments with model proteins and hGH standard are given for different chromatography columns.

Chromatography experiments were begun with the desalting column. BSA (MW=66 kDa) was loaded to the column at 2, 5 and 10 mg/mL concentrations. The flow rate of analysis was chosen as 5 mL/min. The retention volume of BSA was found as 13 mL, corresponding to 3 minutes. The chromatograms are given in Appendix C.

Table 4.1. *Summary of runs for model proteins and hGH standard*

Run no	**Sample**	**Characterization /Purification Technique**	**Column**	**Notes**
1	BSA solutions	Size exclusion chromatography	Desalting	The optimum flow rate was determined.
2	hGH standard	Size exclusion chromatography	Desalting	The retention volume of rhGH was determined.
3	Acetone solution	Size exclusion chromatography	Desalting	The working volume of the column for a small molecule was determined. The plate number of the column was determined.
4	BSA solutions	Ion exchange chromatography	Anion exchange	The correct working of the column was ensured and retention volume of BSA in the column was determined.
5	hGH standard	Ion exchange chromatography	Anion exchange	The retention volume of rhGH was determined.
6	Acetone solution	Size exclusion chromatography	Size exclusion	The working volume of the column for a small molecule was determined. The plate number of the column was determined.
7	BSA solutions	Size exclusion chromatography	Size exclusion	Column performance was validated.
8	Proteins from low molecular weight calibration kit	Size exclusion chromatography	Size exclusion	The retention volume range of rhGH was determined and column performance was observed.
9	hGH standard	Size exclusion chromatography	Size exclusion	The retention volume of rhGH was determined.
10	NaCl	Size exclusion chromatography	Size exclusion	The retention volume of NaCl was determined and the calibration curve for size exclusion column was formed.

Subsequently, as the main aim of the study was the purification of rhGH from secretome, hGH standard (MW=22 kDa) prepared at 1 g/L, was loaded to the same column. Two different flow rates of desalting column buffer, 5 and 6.5 mL/min, were used. The retention volume of the hGH standard was found 13 mL at a 5 mL flow rate. It was found that the retention volume of BSA or hGH standard did not change with altered concentrations of the sample. Proteins with different molecular weights like BSA and hGH standard exited from the column at the same retention volume because the desalting column could not differentiate large proteins from each other since their molecular weights were above both 5 kDa. This is an expected result for the desalting column because the main aim of the desalting column is removing the small particles like salts and amino acids. Molecules with a molecular weight below 5000 Da can enter the pores of desalting column whereas higher molecular weight molecules like BSA and hGH standard do not. To observe the retention volume of molecules with molecular weight lower than 5000 Da, acetone (MW= 58.08 g/mole = 58.08 Da) was injected into the column. The retention volume of acetone was found as 39 mL. By using the retention volume of acetone, the porosity of the column was found as 75 % of the total column volume by using equation 2.4. Also, the plate number, which shows the resolution of the column was found as approximately 150 by using equation 2.3. Comparing with the plate number of ion exchange and size exclusion, it was very low.

After the desalting column runs, the separation performance of the anion exchange column was investigated. The chromatograms of model proteins and molecules are given in Appendix C. A 5 mg/mL BSA solution was injected into the column. For BSA to be retained by the anion-exchange column, the pH of the ion exchange buffer was selected as 8 to make sure that the net charge of BSA becomes negative. The BSA solution was loaded to the column with the start buffer and was eluted from the column by the application of elution buffer at a higher ionic strength. The retention volume of BSA was observed at 62 mL.

After BSA experiments, the hGH standard with 1 g/L concentration was fed to the anion exchange column to find the approximate retention volume of rhGH. The plate number of anion exchange column was found as approximately 2000 by using the rhGH standard according to equation 2.3. The pI of the hGH standard is 5.1 thus for the mobile medium pH of 8, the net charge of the hGH standard is also negative. The hGH standard exit from the column after the application of elution buffer like BSA. The retention volume of the hGH standard was found at 100 mL. According to the results, it is concluded that the application of elution buffer shows its effect after 1.5 column volume (30 mL) of the buffer has flowed through the column.

Finally, size exclusion column experiments were carried out. A 5 % acetone solution was given the column to determine the porosity and plate number of the column. Since acetone is a very small molecule, its retention volume is equal to the total pore volume of the column. Acetone solution was given the column by using different flow rates. The retention volume of acetone was 100 mL for both flow rates. According to the result, porosity was estimated at 83 % of the column volume by using equation 2.4. Also, the plate number of size exclusion chromatography was found as 1500 by using equation 2.3. Then, BSA solution with 10 mg/mL concentration was given the column and the results are compared with results with the column manual. According to the manual, the retention volume of BSA should be 46 mL and the peak of BSA should be one sharp peak. The observed retention volume of BSA was found as 43 mL. It is very close to the retention volume from the manual but the peak is not sharp. Due to the BSA stored a very long time, it could have degraded.

To prepare the calibration curve of the size exclusion column, a mixture of proteins was injected into the column from the low molecular weight calibration kit. The molecular weight of chosen proteins for the experiment should be around the molecular weight of hGH. Proteins, their molecular weights, their observed retention volumes, and retention volumes from manufacturer manual are given in Table 4.2. It was observed that except conalbumin, retention volumes from manual and experimental retention volumes of the other two proteins are close to each other. The

difference between the retention volume from the manufacturer manual and the observed retention volume of conalbumin has been occurred due to the degradation of protein during size exclusion chromatography. The experiment was repeated to find the approximate retention volume of rhGH because the molecular weight range of three proteins in the first experiment was not close enough to the rhGH. In this experiment, the molecular weight range of chosen three proteins around the rhGH was narrowed. Instead of ribonuclease A, carbonic anhydrase was used for the second experiment. By comparing the results of these two experiments in Table 4.2, the retention volume range of rhGH was determined as 55 and 70 mL. After that, 1 and 2 g/L hGH standards were injected to the size exclusion column to observe the retention volume of hGH. The retention volume of hGH standard in size exclusion column was observed as 63 mL for different hGH standard concentration. This is expected also retention volume of hGH standard was between the determined range from other experiments. After the samples were loaded the size exclusion column, it was observed that the calibration curve of the size exclusion column is not enough especially small molecules with lower molecular weight. For that reason, the NaCl solution was injected into the column and its retention volume was tried to be determined. The absorbance value of NaCl could not be measured by the UV lamp. So, in order to determine the retention volume of NaCl, conductivity was observed. NaCl is ionized in water and one retention volume was observed by using conductivity for NaCl. By comparing the molecular weight of acetone (MW=58.08 Da) and NaCl (MW=58.44 Da), the exit volume of acetone should be later than the exit volume of NaCl. However, the retention volume of NaCl was observed at 105 mL and the retention volume of acetone was found as 100 mL. As NaCl was ionized, its molecular weight decreased to approximately half and ions exited from the column at the same time because their molecular weight was similar. For that reason, only one retention volume was found for NaCl and its exited volume later than acetone. The hydrodynamic radius of proteins was calculated from the equation (Carta & Jungbauer, 2010a);

$$\text{Hydrodynamic radius (nm)} = 0.081 \times (\text{molecular weight})^{\frac{1}{3}} \quad \text{(Eq 4.1)}$$

Table 4.2. *Molecular weight, hydrodynamic radius and retention volume values of proteins and molecules for size exclusion calibration graph*

Species	**Molecular Weight(Da)**	**Hydrodynamic radius (nm)**	**Retention volume(mL) (Observed)**	**Retention volume(mL) (Manufacturer manual)**
NaCl	29	0.159 (Hussain, Abashar, & Al-Mutaz, 2006)	105	-
Acetone	58.08	0.18 (Golubev, Gurina, & Kumeev, 2018)	100.5	-
Ribonulease A	13700	1.94	68.09	65
hGH standard	22000	2.27	62.51	-
Carbonic Anhydrase	29000	2.49	57.31	57
Ovalbumin	43000	2.84	47.51	47
BSA	66000	3.27	43.04	46
Conalbumin	75000	3.42	29.85	43

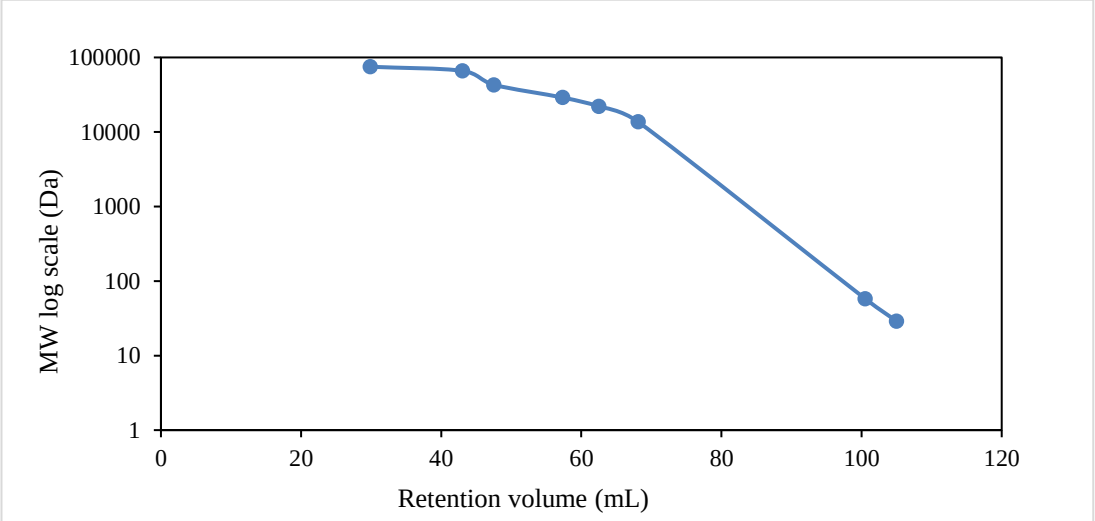


Figure 4.1. Calibration curve of size exclusion chromatography HiPrep 16/60 Sephacryl S-100 High Resolution (a)

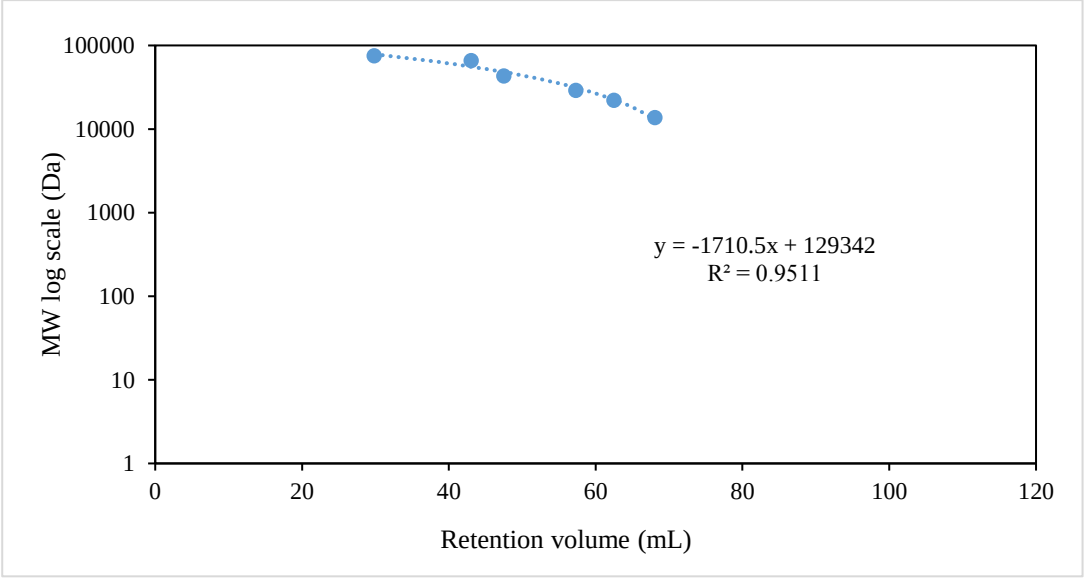


Figure 4.2. Calibration curve of size exclusion chromatography HiPrep 16/60 Sephacryl S-100 High Resolution except for acetone and NaCl (b)

In Figures 4.1 and 4.2, calibration curves for the size exclusion column are given. In calibration curves, retention volumes of proteins are shown against the molecular weights. By using the calibration curve, the determination of the molecular weight of protein that corresponds to the retention volume was aimed. In Figure 4.1,

the calibration curve includes all proteins and molecules that given the column. Due to the separation range of size exclusion column is between 1 and 100 kDa and molecular weights of acetone and salt are lower than 1 kDa, the second calibration curve without salt and acetone was drawn in Figure 4.2. It is observed that there was a linear relationship between the retention volumes of proteins with their molecular weights. By using equation;

$$MW = -1710.5 \times V_R + 129342 \quad \text{(Eq. 4.2)}$$

The molecular weight of fractions that exit from the size exclusion column can be determined.

4.2. Recombinant hGH

The main aim of the study was the purification of rhGH from the secretome of *P. pastoris* with different promoters. To decide on an effective purification protocol for rhGH, other proteins and molecules should be identified in the secretome.

4.2.1. Characterization of the Production Medium with modified GAP promoter

After centrifugation of the bioreactor medium, the secretome with a modified GAP promoter from the medium was obtained. The secretome was analyzed by the 2D gel electrophoresis procedure described in section 3.6.1 to determine the number of proteins in the secretome, and their molecular weights and isoelectric points. According to the SDS-PAGE result, the concentration of rhGH was determined as 200 mg/L. In Table 4.3, a summary of runs for characterization of secretome with modified GAP is given.

Table 4.3. *Summary of experiments of secretome with modified GAP promoter for characterization*

Experiment no	Sample	Characterization /Purification Technique	Notes
1	Secretome of *P. pastoris* with modified GAP	2-D gel electrophoresis	Protein number, molecular weight and isoelectric points of proteins in secretome were determined.
2	Secretome of *P. pastoris* with modified GAP	JVirGel and Protpi	The identity of proteins was predicted.

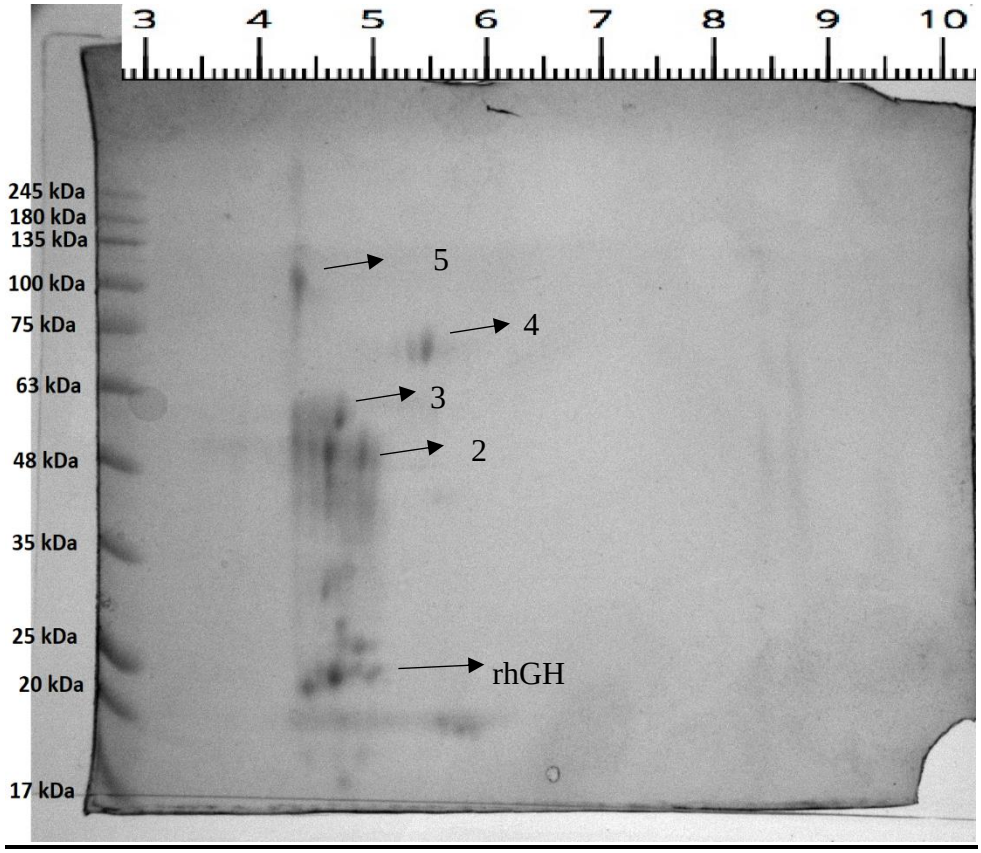


Figure 4.3. 2-D gel electrophoresis image of the secretome of modified GAP promoter with silver stain

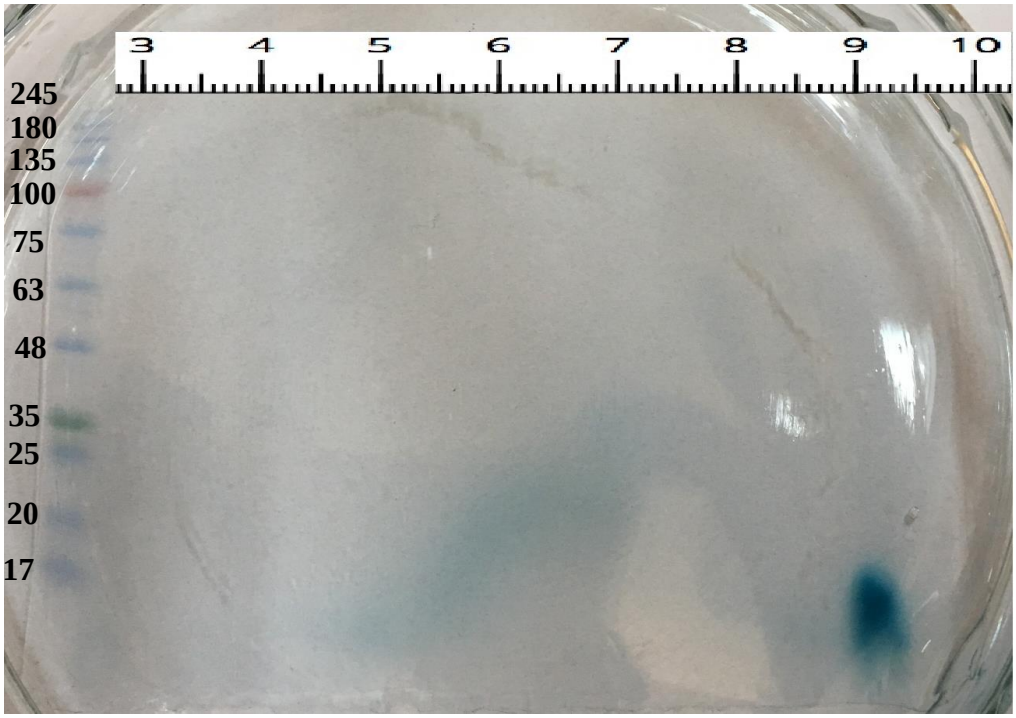


Figure 4.4. 2-D gel electrophoresis image of the secretome of modified GAP promoter with coomassie blue stain

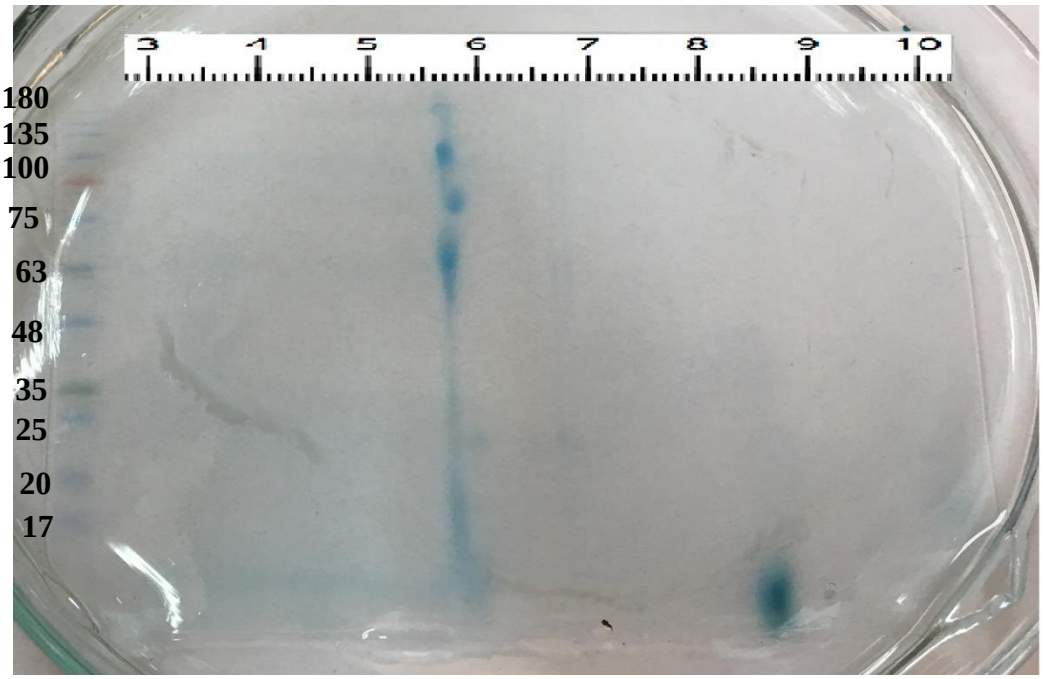


Figure 4.5. 2-D gel electrophoresis image of the secretome of modified GAP promoter with coomassie blue stain (before the analysis of excess salts and other impurities which prevent isoelectric focusing removed by using acetone, tris, and chloroform treatment)

Table 4.4. *Results of 2-D gel electrophoresis with different staining techniques*

Secretome of modified GAP promoter treated with silver staining			
Band no	Predicted Molecular weight (kDa)	Predicted Isoelectric point	Predicted protein
1	25	4.8	rhGH
2	51	4.6	
3	56	4.7	
4	70	5.5	
5	118	4.4	
Secretome of modified GAP promoter treated with coomassie blue staining			
Band no	Predicted Molecular weight (kDa)	Predicted Isoelectric point	Predicted protein
1	10	9	
2	22	6	smear
Secretome of modified GAP promoter treated with coomassie blue staining (concentrated with acetone, tris and chloroform mixture)			
Band no	Predicted Molecular weight (kDa)	Predicted Isoelectric point	Predicted protein
1	10	8.8	
2	22	5.7	rhGH
3	63	5.7	
4	75	5.7	
5	135	5.7	

In table 4.4, molecular weights of major protein bands and their isoelectric points are summarized for 2-D gel electrophoresis results. In Figures 4.3, 4.4 and 4.5, the pI ruler placed on the 2-D gel images where immobilized pH gradient gel (IPG) strips placed. The IPG strips have a linear pI range between 3 and 10. It was found that apart from rhGH there are 4 major distinct spots, which presumably correspond to secretome proteins other than rhGH considering Figures 4.3 and 4.5. The distinct separation of the proteins in the secretome was observed with a 2- D gel image with silver staining. Result of 2-D gel image of secretome with silver staining compared to the 2-D gel images of the same secretome with coomassie blue staining. In figure 4.3, the isoelectric point of rhGH was found as 4.8. In figure 4.5, the isoelectric point of

rhGH was found as 5.7. The isoelectric point range in secretome was found between 4.4 and 5.7 in figure 4.3. However, the isoelectric point range of secretome was found between 5.7 and 8.8 in figure 4.5. The difference in the isoelectric point range for the same sample in Figures 4.3 and 4.5 could be caused by the different salt concentrations in the secretome. The high salt concentration of the sample causes changes in the current. Also, ions in the sample migrate through the separation and form another electrical field. Both current and ions affect the isoelectric focusing process. For that reason, the pI of the proteins could be seen differently in the image from their actual pI values (Mao & Pawliszyn, 1999). Çulfaz-Emecen *et. al.* also analyzed two different secretomes of *P. pastoris* by using 2-D gel electrophoresis. These secretomes also called BR and BD. Isoelectric point range of BR between the 5 and 9. Six or more proteins were present in BR. The isoelectric point range of BD was between 3 and 6. There were six or more proteins in BD (Akcan, 2017). Molecular weights of the proteins in both Figures 4.3 and 4.5 were different from each other and rhGH. On the other hand, isoelectric points of proteins were close to each other. There is one protein band with its molecular weight below the 15 kDa. Its isoelectric point is 9 in Figures 4.4 and 4.5. It is predicted that this is a combination of other proteins degraded parts in the secretome. It is not considered a protein. After considering 2-D gel electrophoresis images in Figures 4.3, 4.4 and 4.5, the average molecular weight of rhGH was found as 23.5 kDa and its average isoelectric point was determined as 5.1. These results are very close to the properties of the rhGH standard. Molecular weight and isoelectric point of hGH standard are 22 kDa and 5.1, respectively.

4.2.2. Theoretical Prediction of 2D Gel Bands: JVirGel 2.0

Another aim of the study, determine the identities of unknown proteins in secretome without using other analysis like mass spectroscopy. For that reason, JVirGel software was used. JVirGel forms the virtual 2-D gel images of secretomes with different microorganisms. First of all, the list of proteins of *P. pastoris* secretome was found from literature (Huang *et al.*, 2011). Proteins from the list were collected to form one fasta file by using UniProt. Fasta file was loaded onto JVirGel and the

virtual 2-D image of *P. pastoris* was produced. By using the program, molecular weights and isoelectric points of proteins in secretome were calculated. While the program calculating the molecular weights and isoelectric points of proteins, it considers the interactions of proteins to each other in secretome and their migration behavior. To compare molecular weights and isoelectric points of proteins from JVirGel, molecular weights and isoelectric points of proteins also calculated by using Prot pi. Molecular weight and isoelectric points were calculated individually in the Prot pi. Eventually, Table 4.5 is formed.

In Figure 4.6, a 2-D gel electrophoresis image of the secretome of *P. pastoris* is given. Molecular weights and isoelectric points were calculated by JVirGel to form a virtual 2-D gel image.

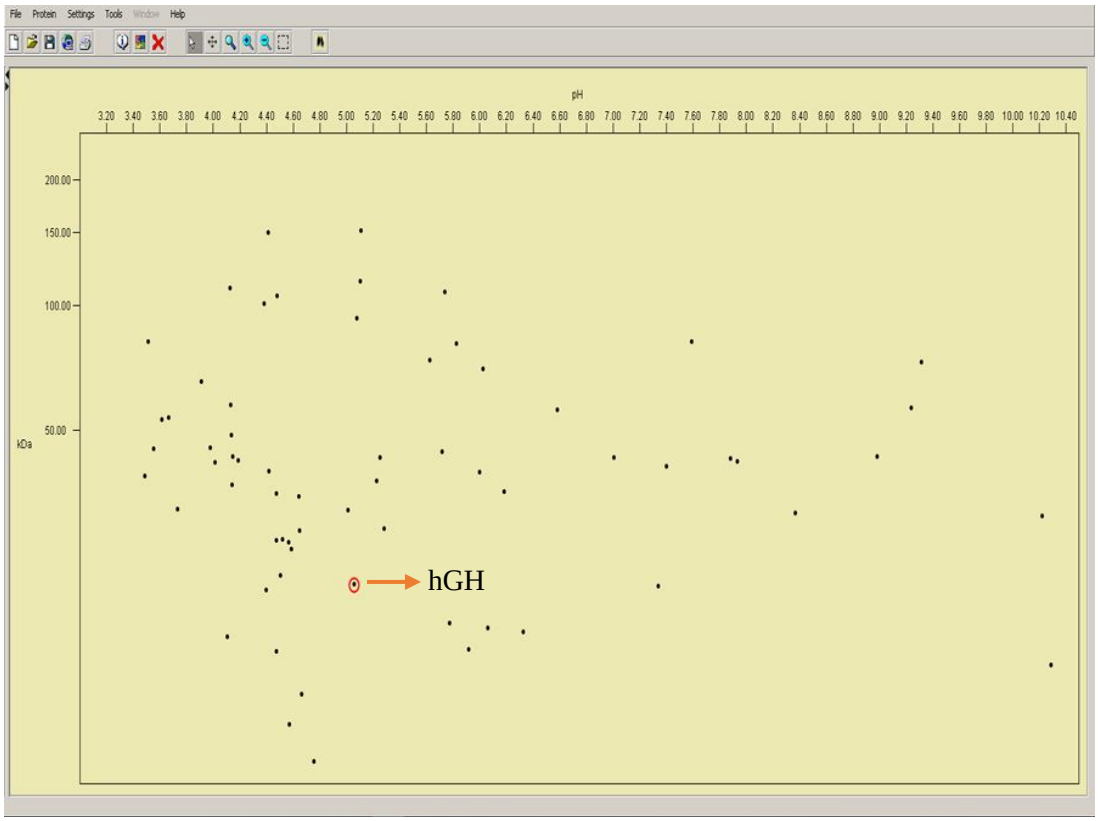


Figure 4.6. Virtual 2-D gel image of the secretome of P. pastoris and rhGH by JVirgel

Table 4.5. *Comparison of molecular weights and isoelectric points from Prot pi and JVirJel*

Protein names	MW(kDa)	pI	MW(JVirJel)	pI(JVirJel)
Sulfite reductase beta subunit	161.2	5.33	151.79	5.11
Vesicle-mediated ER to Golgi transport protein	160.29	4.66	149.75	4.41
Paf1 complex component	120.14	5.33	113.9	5.1
Cell wall protein DAN4 (Delayed anaerobic protein 4)	118.36	4.38	111.1	4.12
Protein phosphatase	115.41	5.87	107.82	5.74
Similar to Saccharomyces cerevisiae YKR102W FLO10 Lectin-like protein with similarity to Flo1p, thought to be involved in flocculation	114.06	4.72	106.81	4.48
Putative glucanases, secreted	109.63	4.66	103.62	4.38
Heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70)	100.302	5.31	92.82	5.08
Mucin family member	88.61	3.83	83.51	3.51
Cobalamin-independent methionine synthase	85.84	5.96	80.96	5.82
Transcription activator MSS11 (Multicopy suppressor of STA genes protein 11)	85.05	7.35	81.76	7.59
Ferric reductase and cupric reductase	80.47	8.92	75.59	9.32
Protein kinase	78.15	5.8	73.61	5.63
Alcohol oxidase 1 (AOX 1)	73.9	6.12	70.16	6.02
Ferro-O2-oxidoreductase	70.91	4.23	65.71	3.91
Ferrioxamine B transporter	70.31	8.87	64.2	9.24
Aspartic proteinase 3	62.95	4.47	59.78	4.13
1,3-beta-glucanosyltransferase	58.3	4.01	54.7	3.61
Catalase (EC 1.11.1.6)	57.86	6.63	55.82	6.58
1,3-beta-glucanosyltransferase	57.2	4	54.7	3.61
Repressible acid phosphatase	52.69	4.42	50.53	4.14
Glycosidase (EC 3.2.-.-)	49.49	4.32	47.22	3.98
Putative glucanase	49.02	4.45	44.94	4.15
Glycosidase (EC 3.2.-.-)	48.77	3.95	46.84	3.55
D-serine dehydratase	46.86	5.83	44.36	5.72
Enolase 1	46.53	5.47	42.9	5.25
Aspartate aminotransferase (EC 2.6.1.1)	45.51	7.52	42.67	7.88
Aspartate aminotransferase (EC 2.6.1.1)	45.43	6.94	42.82	7.01

Protein names	MW(kDa)	pI	MW(JVirJel)	pI(JVirJel)
Protein of the SUN family that may participate in DNA replication	45	4.38	43.18	4.01
Mitochondrial outer membrane protein OM45 (Outer membrane protein of 45 kDa)	44.58	8.36	43.1	8.98
Vacuolar aspartyl protease (Proteinase A)	44.32	4.65	41.6	4.42
Ketol-acid reductoisomerase, mitochondrial (Acetohydroxy-acid reductoisomerase) (Alpha-keto-beta-hydroxylacyl reductoisomerase)	44.31	7.48	42.15	7.93
Alanine/glyoxylate aminotransferase 1	44.1	7.12	40.92	7.4
Cell wall exo-1,3-beta-glucanase	44.02	4.51	42.21	4.19
Cell wall protein	43.38	3.87	40.54	3.49
3-phosphoserine aminotransferase	42.34	6.1	39.51	6
Probable family 17 glucosidase SCW10 ((Soluble cell wall protein 10)	40.47	4.511	38.77	4.14
BA75_02955T0	39.7	5.43	37.66	5.22
BA75_02990T0	39.05	4.74	37.46	4.47
Thiamine thiazole synthase (Thiazole biosynthetic enzyme)	37.07	6.26	35.48	6.18
Cell wall biogenesis involved protein	35.73	4.91	34.6	4.64
Thioredoxin reductase (EC 1.8.1.9)	34.38	5.25	32	5.01
Protein ECM11 (Extracellular mutant protein 11)	34.11	7.89	31.51	8.57
Endo-beta-1,3-glucanase	33.95	4.09	32.13	3.73
Citrate/oxoglutarate carrier protein	33.502	9.63	31.01	10.22
Cell wall mannoprotein PIR3 (Covalently-linked cell wall protein 8) (Protein with internal repeats 3)	33	5.39	30.85	4.65
BA75_00070T0	31.72	5.61	30.7	5.28
Eisosomes component	31.67	4.9	30.42	4.52
Cell wall mannoprotein HSP150 (150 kDa heat shock glycoprotein) (Covalently-linked cell wall protein 7) (Protein with internal repeats 2)	30.53	5.32	30.85	4.65

Protein names	MW(kDa)	pI	MW(JVirJel)	pI(JVirJel)
BA75_02994T0	30.22	4.93	27.84	4.59
Protein ROT1	28.42	4.75	27.17	4.47
BA75_03280T0	25.55	4.78	24.07	4.51
Somatotropin (Growth hormone) (GH) (GH-N) (Growth hormone 1) (Pituitary growth hormone)	24.85	5.3	23.94	5.06
Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	24.69	4.76	23.11	4.4
Putative uncharacterized protein YAL004W	23.74	5.27	22.27	5.78
20S proteasome subunit beta type-4	21.97	7.04	20.95	7.34
Thioredoxin peroxidase	21.45	4.85	20.09	4.48
Phosphatidylglycerol/phosphatidylinositol transfer protein	19.13	4.51	17.77	4.11
6,7-dimethyl-8-ribityllumazine synthase (DMRL synthase) (EC 2.5.1.78)	17.73	6.37	16.26	6.33
Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	17.64	6.13	14.03	4.66
Nucleoside diphosphate kinase (EC 2.7.4.6)	16.97	6.2	16.06	4.75
Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	15.68	6	14.77	5.92
Peptidylprolyl isomerase (EC 5.2.1.8)	15.16	5.42	14.03	4.66
Ubiquitin-60S ribosomal protein L40	14.57	9.67	13.55	10.28
Acyl-CoA-binding protein	9.84	4.83	9.72	4.57

Although JVirGel calculated the pI and molecular weight of proteins in one step, molecular weight and pI of proteins were computed one by one with Prot pi. Due to JVirGel calculate properties of proteins based on predicted interaction of proteins in the secretome, there could be some difference in the properties of proteins. The general change pattern of molecular weights and isoelectric points of proteins was observed by comparing results from JVirGel and Prot pi in Table 4.5. It was observed that the molecular weight values of proteins from JVirGel were slightly lower than the molecular weights from the Prot pi. Moreover, the pI values of proteins from JVirGel

are lower than the pI values of Prot pi until pI values reach 10. After the pI values of proteins reach 10, the pI values of proteins from JVirGel are higher than the pI values of Prot pi.

By using results from Table 4.4 and 4.5, comparing both molecular weight and isoelectric point values of proteins of the secretome of *P. pastoris* from Prot pi and JVirGel, the identities of four major unknown proteins were predicted. Five possible proteins were determined, which have a molecular weight between 110 and 140 kDa and their isoelectric point between 4.4 and 5.7. These proteins were paf1 complex component, cell wall protein DAN4, protein phosphatase, lectin-like protein with similarity to Flo1p and putative glucanases. Proteins, which have a molecular weight higher than 100 kDa, is not secreted but it could find in secretome due to cell lysis. One possible protein was found for the protein whose molecular weight was 70 kDa and isoelectric point is around the 5.6. This protein could be a protein kinase. Three proteins were determined which have molecular weights were between 50 and 60 kDa and their isoelectric points were between 4.6 and 4.7. These proteins were aspartic proteinase 3, putative glucanase and repressible acid phosphatase. All possible proteins are shown in the red above. To ensure the predicted identities of proteins in the secretome, further, analyze like mass spectroscopy is needed.

4.2.3. Separation of rhGH from *P. pastoris* secretome with modified GAP promoter

Separation with chromatography depends on the difference between the properties of proteins from each other like size, charge, and affinity. For purification of rhGH, mainly size exclusion and ion exchange chromatography were used. In Table 4.6, a summary of experiments of secretomes with modified GAP or AOX promoter is given for different chromatography columns.

Table 4.6. *Summary of runs for secretome of P. pastoris with different promoters by using chromatographic techniques (a)*

Run no	Sample	Characterization /Purification Technique	Column	Notes
1	Secretome of *P. pastoris* with modified GAP	Size exclusion chromatography	Desalting	Proteins were separated from impurities like salt and protein parts like peptide
2	Secretome of *P. pastoris* with modified GAP	Size exclusion chromatography	Size exclusion	Proteins were tried to separate according to their size.
3	Secretome of *P. pastoris* with modified GAP	Size exclusion chromatography	Desalting + Size exclusion	Proteins in the desalting column were given to size exclusion column for further separation
4	Secretome of *P. pastoris* with modified GAP	Ion exchange chromatography	Anion exchange	Proteins were tried to separate according to their net surface charge at pH 8 by using step elution
5	Secretome of *P. pastoris* with modified GAP	Ion exchange chromatography	Anion exchange	Proteins were tried to separate according to their net surface charge at pH 8 by using linear gradient elution

Table 4.6. *Summary of runs for secretome of P. pastoris with different promoters by using chromatographic techniques (cont.) (a)*

Run no	Sample	Characterization /Purification Technique	Column	Notes
6	Secretome of *P. pastoris* with modified GAP	Size exclusion + Ion exchange chromatography	Desalting + Anion exchange	Proteins in the desalting column were given to anion exchange column for further separation
7	hGH standard	Size exclusion chromatography	Size exclusion	The calibration curve of the hGH standard was formed.
8	Secretome of *P. pastoris* with modified AOX	Size exclusion chromatography	Size exclusion	Proteins were tried to separate according to their size.
9	Secretome of *P. pastoris* with modified AOX	Size exclusion + Ion exchange chromatography	Size exclusion + Anion exchange	Proteins in size exclusion column were given to anion exchange column for further separation by using step elution
10	Secretome of *P. pastoris* with modified AOX	Size exclusion + Ion exchange chromatography	Size exclusion + Anion exchange	Proteins in size exclusion column were given to anion exchange column for further separation by using linear gradient elution

Table 4.6. *Summary of experiments for secretome of P. pastoris with different promoters by using chromatographic techniques (b)*

Experiment no	Sample	Characterization Technique	Notes
1	Secretome of *P. pastoris* with modified GAP	SDS-PAGE	The purification level of each fraction from columns was observed.
2	Secretome of *P. pastoris* with modified AOX	SDS-PAGE	The purification level of each fraction from columns was observed.

4.2.3.1. Size Based Separation

It is found that the molecular weights of proteins were different from each other and rhGH from 2D gel electrophoresis. For that reason, the size-based separation was used to purify the rhGH from the secretome of *P. pastoris.* Size based separation experiments began with the desalting column. In this study, the desalting column was mainly used for the concentrate the proteins from secretome and eliminate the excess salt.

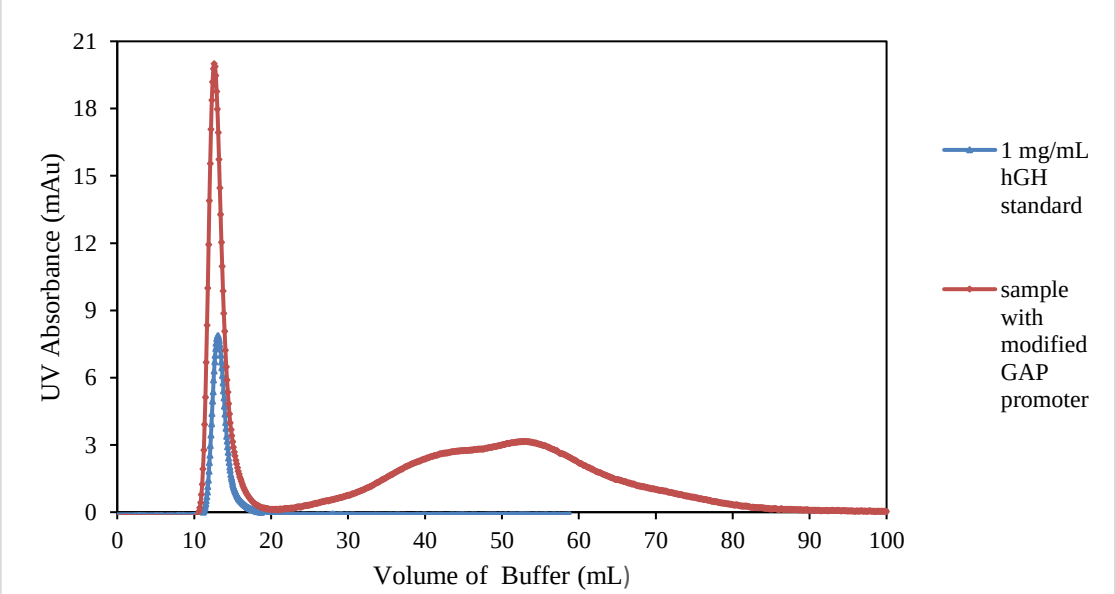


Figure 4.7. Comparison of the secretome of *P. pastoris* and hGH standard in desalting column

In Figure 4.7, the chromatogram of the proteins in secretome and 1 g/L hGH standard solution is shown. Both proteins in secretome and hGH standard exited from the column at the same retention volume. It is concluded that either all proteins in the mixture or only one protein solution exit from the desalting column at the same retention volume which is between 10 and 20 mL. Due to the molecular weight of the smallest protein higher than the 5000 Da, the desalting column could not differentiate proteins from each other. For that reason, proteins cover the same distance in the column and exit from the column at the same retention volume. Also, comparing the areas under the first peak of the secretome in the desalting column and the peak of the hGH standard, the total protein concentration of secretome was found as 3.15 g/L.

Since the resolution of the desalting column was not enough to separate rhGH from the secretome of *P. pastoris* with a modified GAP promoter, a high-resolution size exclusion column with separation range 1- 100 kDa was used for purification of rhGH from secretome.

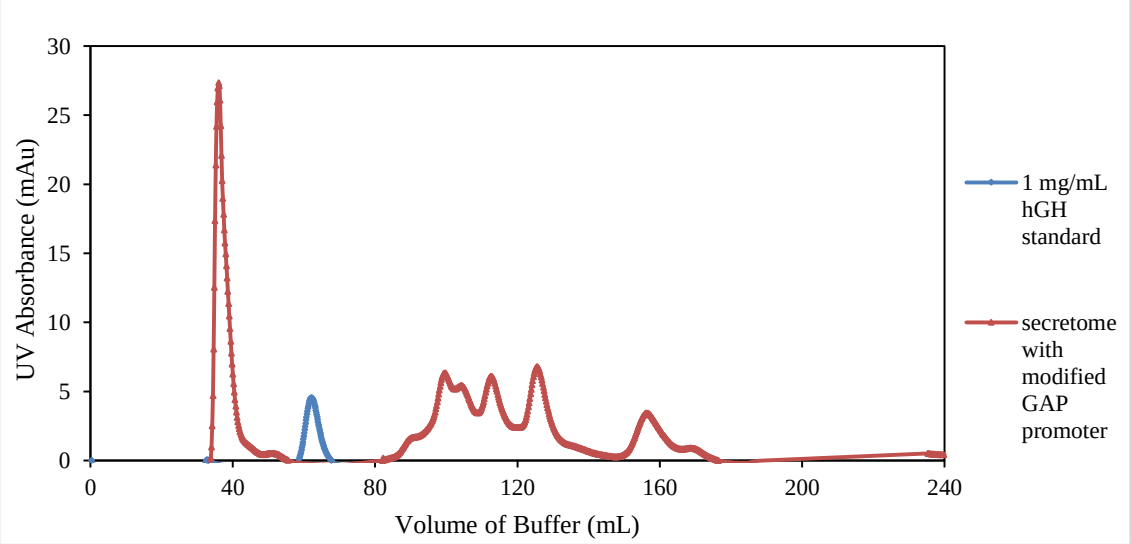


Figure 4.8. Comparison of the secretome of *P. pastoris* and hGH standard in size exclusion column

The result of the secretome of *P. pastoris* with a modified GAP promoter and the result of 1 g/L hGH standard for size exclusion column were plotted together in Figure 4.8. It was found that the retention volume of the hGH standard was 62.5 mL

in the size exclusion column. Proteins in secretome exited from the size exclusion column as two main fractions. The first fraction exited the column at 38 mL and the second fraction exited from the column between 80 and 180 mL. Owing to rhGH is found at protein mixture at secretome, at least one peak is expected at 62.5 mL for a result of the secretome in size exclusion column. However, no peak at 62.5 mL was observed for secretome. Also, the average molecular weight of the first fraction calculated by using equation 4.2 from the calibration curve of the size exclusion column. The molecular weight of the first fraction of the size exclusion column was 64.3 kDa. It is concluded that the molecular weight of the second fraction should be below 50 Da. So, the second fraction should consist of molecules smaller than proteins, like amino acids, peptides. The second fraction of the size exclusion column could have belonged to degraded parts of proteins. It could be considered as noise. This result is not expected because the molecular weight range of proteins in secretome is found as 10 – 140 kDa by using 2-D gel electrophoresis. So, the column should separate proteins in secretome and purify the rhGH. Retention volumes of proteins in secretome are expected between 0 - 80 mL of the column. rhGH consists of nonpolar amino acids at high concentrations. It is relatively hydrophobic. It could interact with the hydrophobic part of the other proteins and they could form agglomerates in the secretome. Also, the salt concentration in secretome could cause the interaction of proteins in secretome and the formation of the agglomerates. It is predicted that rhGH exited from the size exclusion column together with the first fraction of the size exclusion column. On the other hand, the concentration of rhGH was small in the secretome. It could give a small peak due to its low concentration. It could exit from the column with the second fraction but peak could be mixed with or suppressed by other peaks. For that reason, rhGH could not exit from the column at the expected retention volume. Also, some proteins could have decomposed during the chromatography, resulting in small fragments.

By using the desalting column, secretome was concentrated and proteins were partially purified from other impurities in the secretome. The first fraction of the desalting column consists of most of the proteins in the secretome with 0.15 M NaCl.

To separate the proteins from each other in the first fraction of the desalting column, it was injected into the size exclusion column for further purification of rhGH. Also, the effect of salt concentration of secretome on the separation with the size exclusion column was investigated.

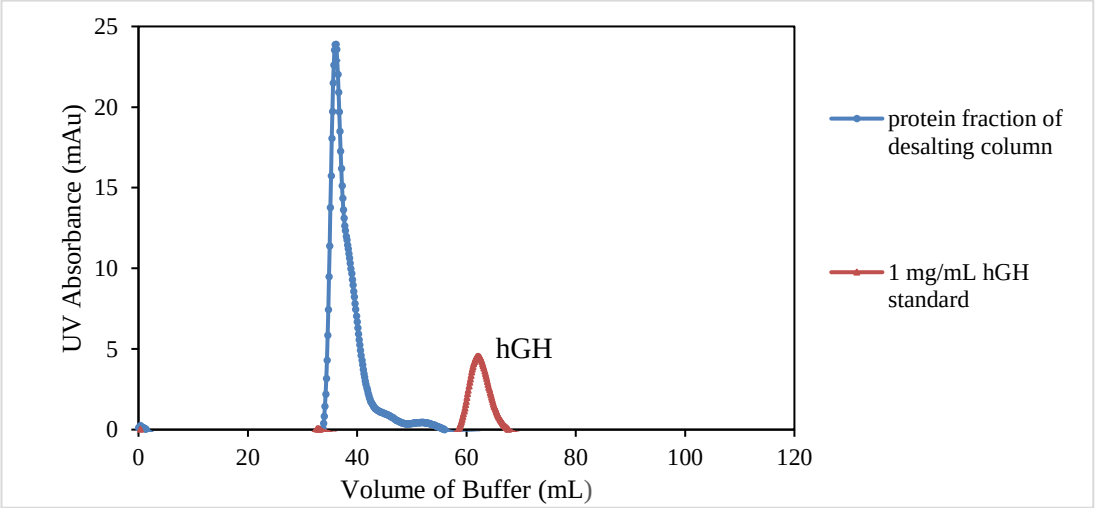


Figure 4.9. Comparison of the fraction of combination of desalting column and size exclusion column with hGH standard in size exclusion column

The first fraction of the secretome of *P. pastoris* with a modified GAP promoter from the desalting column was given the size exclusion column. It is observed that proteins in the first fraction of the desalting column are not separated from each other in Figure 4.8. This is not expected. Although all proteins exit from the desalting column at the same retention volume due to the low resolution of the desalting column, proteins in the first fraction of the desalting column should be separated from each other by using the size exclusion column. The size exclusion column has high resolution. It is predicted that proteins in the first fraction somehow interact with each other and form an agglomerate. Also, the second fraction of the size exclusion column, which was found at 80-180 mL from Figure 4.8, was eliminated after the desalting column application. Comparing the initial concentration of the first fraction of the desalting column and final concentration after the application of the size exclusion column, the concentration decreased 1/5 of the initial concentration of proteins. There is high protein loss during size exclusion chromatography.

4.2.3.2. Charge Based Separation

Since rhGH could not be separated from other proteins by using size-based separation, charge based separation experiments were done by using anion exchange chromatography. The isoelectric point of rhGH was found between 4.8 and 5.7 from 2-D gel electrophoresis analysis. Although pI values of other proteins in secretome was close to rhGH and their pI range between 4.4 and 5.7, purification of rhGH was investigated by using anion exchange chromatography.

Due to the anion exchange column was used for experiments, the pH of the buffer solution is chosen as 8. All proteins in secretome become negatively charged because of their pI smaller than the pH of the buffer. Step elution and linear gradient elution were applied.

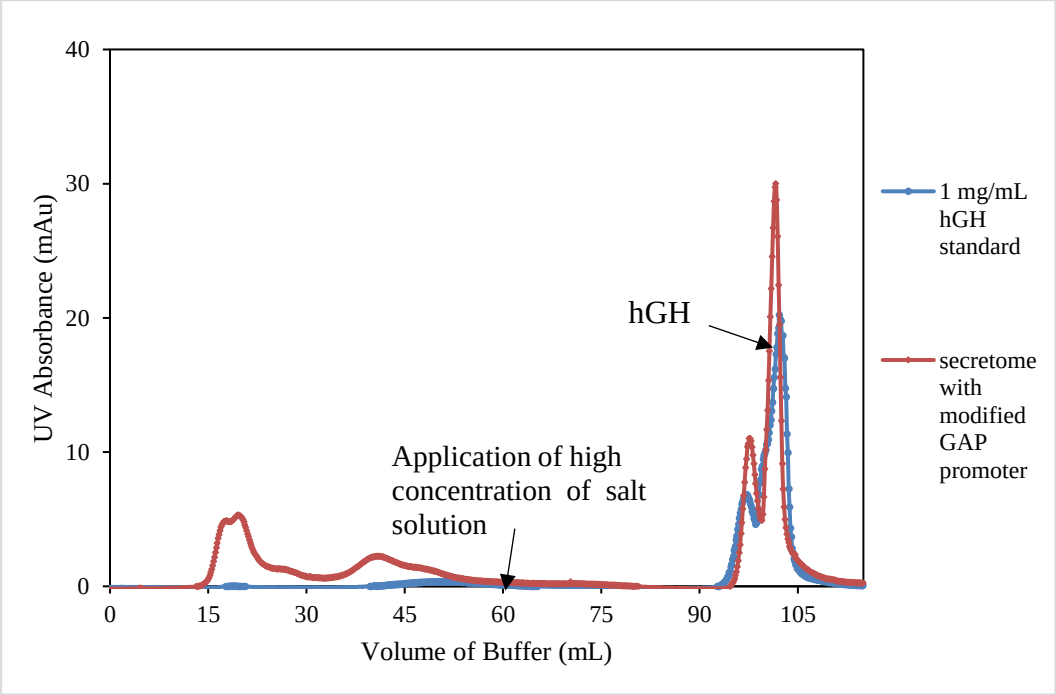


Figure 4.10. Comparison of the secretome of *P. pastoris* and hGH standard in anion exchange column with step elution condition

Secretome and hGH standard were given to the column with start buffer. There was a small salt concentration in the start buffer. So, negatively charged proteins exchanged with a negatively charged group on the positively charged matrix. If there

are positive-charged proteins in the secretome, positively charged proteins are repulsed by positively charged matrix and exit from the column at first 60 mL. After the application of start buffer during the first 60 mL, elution buffer with high salt concentration was flown through the column. Negatively charged proteins like rhGH are excluded from the column by increasing salt concentration and ionic strength. The pI of the hGH standard is 5.1. It becomes negatively charged at pH 8. It exits from the column after the application of the elution buffer. This is expected because the hGH standard captured by a positively charged matrix and released from the column after increasing ionic strength. The retention volume of the hGH standard was found as 101 mL. There was one broad peak of secretome at first 60 mL of the column. It is expected because pI values of one of the protein bands in secretome are bigger than 8.8 and their net charge should be positive according to 2-D gel electrophoresis result. There is one peak of secretome with two heads after the application of elution buffer. Its retention volume was 100 mL. This peak humps to negatively charged proteins of secretome like rhGH. Due to the column used in the experiment before was very old, the peak of the hGH standard could exit from the column with two humps instead of one peak. The retention volume of hGH standard and negatively charged proteins were close to each other. Although positive-charged proteins were separated from negatively charged proteins in the anion exchange column, resolution of the anion exchange column was not enough for the purification of rhGH from other negative charged proteins in the secretome. It could use partial purification of rhGH. By considering the molecular weight difference of proteins from secretome, size exclusion chromatography is more suitable for the purification of rhGH.

To increase the resolution of anion exchange chromatography, buffer application strategy to column changed. Results from Figure 4.10 are taken by applying one buffer without mixing to another buffer. So, starter buffer applied column first then it is changed to elution buffer. This is called a step elution mode. Instead of this strategy, the linear-gradient strategy was used with buffers for anion exchange column. The elution buffer was added to the start buffer with a linear

gradient during anion exchange chromatography. Linear gradient strategy decreases the analysis time and increases the sharpness of peaks and make peaks more distinct.

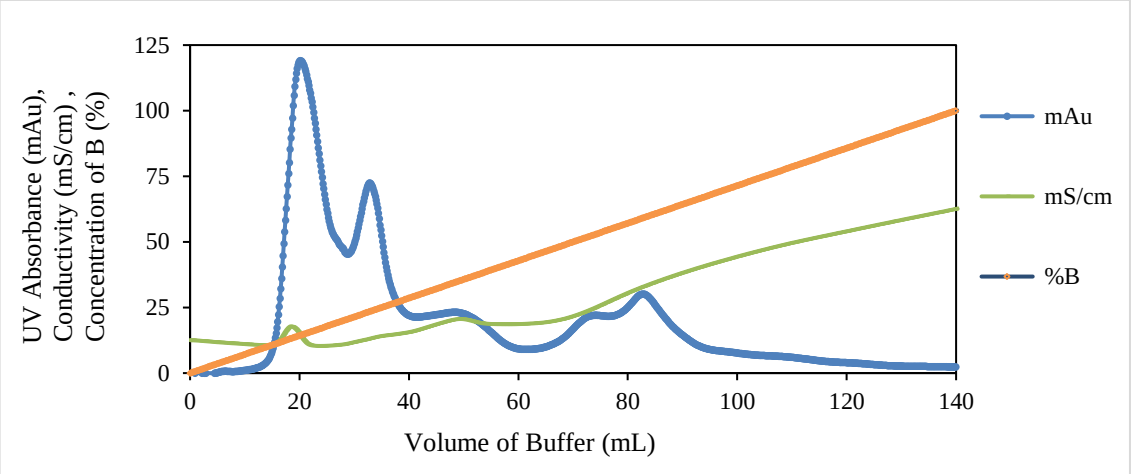


Figure 4.11. Secretome of *P. pastoris* with modified GAP in anion exchange column with the linear gradient

A hundred percent of the linear gradient was applied to the column during 140 mL. The analysis begins with zero percent of the elution buffer in the start buffer. The percentage of additional elution buffer in start buffer increased with increasing column volume linearly. The effect of additional elution buffer in start buffer could be observed by the change of conductivity during analysis. Due to the big difference between the salt concentration of the start and elution buffers, conductivity increased significantly during analysis. After the application of linear-gradient, peaks in Figure 4.11 become more prominent and separate than peaks in Figure 4.10. This is expected. On the other hand, differentiation of the peak of negatively charged proteins from the peak of the positively charged proteins becomes more difficult since the addition of elution buffer starts with the beginning of the analysis. To distinguish peaks, conductivity values are observed during analysis. High conductivity value with the constant increase in conductivity shows the effect of elution buffer. It was found that elution buffer effects the column after the 60 mL in linear gradient condition. So, the first peak with two heads should have belonged to the positive charge proteins. Its retention volume was found as 29 mL. The second peak should have belonged to the negatively charged proteins like rhGH. Its retention volume was found as 80 mL.

Although peaks become sharper and distinct from each other by applying linear-gradient, the increase in the resolution of anion exchange chromatography by using the linear-gradient was not enough for purification of rhGH from secretome.

Although the resolution of anion exchange chromatography was not enough for the purification of rhGH from secretome, it could be used as the second purification step. To investigate the possibility, the first fraction of the desalting column that contains partially purified and concentrated proteins of secretome was given the anion exchange column. Also, the effect of salt concentration of secretome on the separation with anion exchange chromatography was tried to observe.

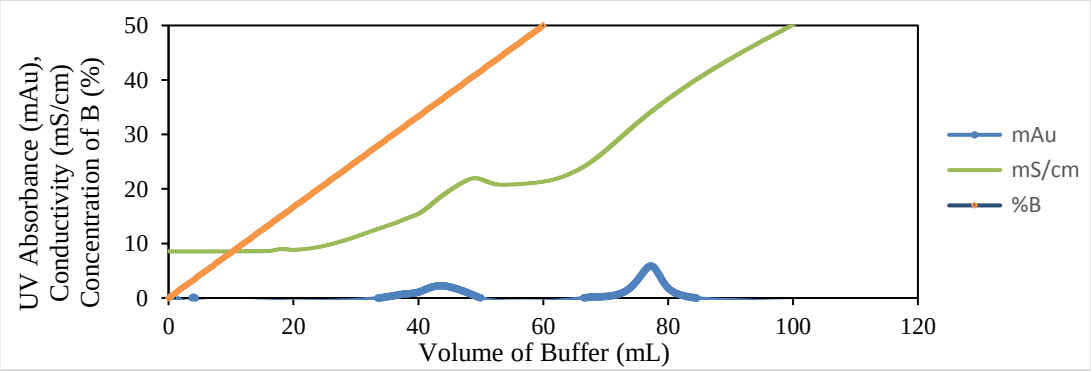


Figure 4.12. The first fraction of desalting column that contains proteins from secretome in anion exchange column with the linear gradient

The first fraction of the desalting column, which consists of proteins from secretome, was injected into the anion exchange column to further purification of rhGH. It is observed that the first fraction in Figure 4.12 is nearly zero. This peak belongs to positively charged proteins because it exits before 60 mL. Its retention volume was found as 43 mL. Positively charged proteins could be considered as impurities because the first peak in Figure 4.11 is nearly lost after desalting column application. The retention volume of the second peak was found as 77 mL. The second peak belongs to negatively charged proteins like rhGH. Although the second peak in Figure 4.12 is prominent its absorbance value is lower than the second peak in Figure 4.11. This is expected because, after each purification step, there are some losses. When the initial concentration of the first fraction in the desalting column is compared

with the final concentration of the ion exchange column, protein concentration is decreased to zero.

The pH of buffer kept constant at pH 8 during the charge-based separation experiments because the isoelectric points of proteins were very close to each other. There is not enough difference for the separation by using the pI of proteins.

4.2.4. Determination of purification of rhGH from secretome of *P. pastoris* with modified GAP promoter

To determine the purification level of rhGH in the *P. pastoris* secretome after each chromatographic step, SDS-PAGE analysis was done. In the first two wells of the SDS-PAGE image belong to molecular weight marker and hGH standard, respectively. Each remained wells belonged to secretome with modified GAP and its fractions from different chromatography columns after each chromatographic separation step. By comparing protein bands of each fraction with marker, the molecular weight distribution of each fraction is determined. Also, comparing fractions with hGH standard, the existence of rhGH is ensured.

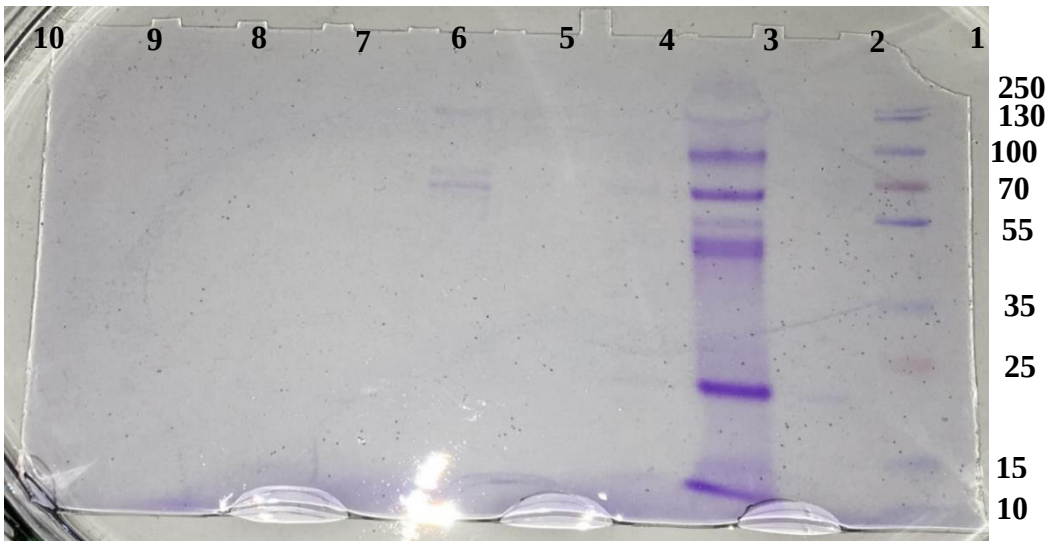


Figure 4.13. SDS-PAGE image of fractions from each chromatographic separation step with coomassie blue stain 1- Ladder 2-hGH standard 3-Sample with modified GAP 4-First fraction of desalting column 5- Second fraction of desalting column 6- First fraction of size exclusion column 7- Second fraction of size exclusion column 8- Fraction of combination of desalting column with size exclusion column 9- First fraction of anion exchange chromatography column 10- Second fraction of anion exchange chromatography

Table 4.7. *Sample definition of each well with coomassie blue staining*

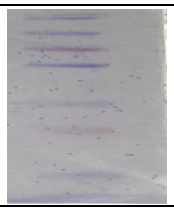
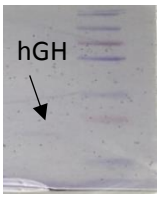
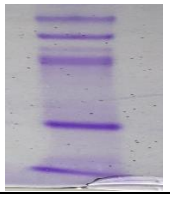
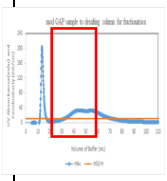
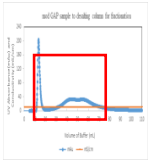
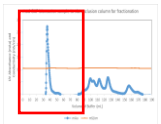
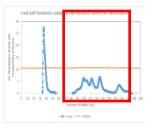
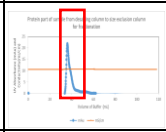
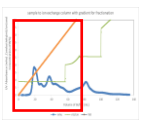
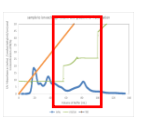
Wells					
	1	2	3	4	5
Definition	Marker	50 mg/L hGH standard	Sample with modified GAP	The first fraction of desalting column which consists of proteins	The second fraction of the desalting column which consists of impurities
Picture of well				No contrast was observed	No contrast was observed
Chromatogram	-	-	-		
Molecular weight distribution (kDa)		22	100-10	-	-
Number of bands	-	1	5	-	-

Table 4.7. *Sample definition of each well with coomassie blue staining (cont.)*

Wells					
	6	7	8	9	10
Definition	The first fraction of size exclusion column (30-60 mL)	The second fraction of size exclusion column (80-130 mL)	The fraction of combination of desalting with size exclusion column	The first fraction of the anion exchange column	A second fraction of the anion exchange column
Picture of well		No contrast was observed	No contrast was observed	No contrast was observed	No contrast was observed
Chromatogram					
Molecular weight distribution (kDa)	100-55	-	-	-	-
Number of bands	2	-	-	-	-

SDS-PAGE image in Figure 4.13 was stained by using coomassie brilliant blue. It is found that except lane 3, no bands were observed. Coomassie brilliant blue is used for the staining of concentrated protein mixture which consists of 36-47 ng of proteins (Bio-Rad, 2019). It can be noted that the standard itself resulted only in a faint band. Although the bioreactor sample with a modified GAP promotor has a relatively high concentration, protein concentration decreases after each purification step. Moreover, the sample that has 2 mL volume was diluted in the columns. Proteins in the sample ten times diluted by the chromatographic process. Due to protein loss after

each purification step and dilution of proteins, concentrations of the other fractions are probably not enough for the coomassie brilliant blue staining. So, except for the bioreactor sample, other samples were not stained sufficiently.

Table 4.8. *Molecular weight distributions for each fraction with coomassie blue staining*

3.secretome with modified GAP promoter			6. The first fraction of size exclusion column (30-70 mL)		
Band no	Predicted molecular weight (kDa)	predicted protein	Band no	Predicted molecular weight (kDa)	Predicted protein
1	10		1	55	
2	22	rhGH	2	70	
3	50		3	130	
4	55				
5	70				
6	100				

In Table 4.8, the molecular weight distribution of proteins in lane 3 is given. Six protein bands are observed in Figure 4.13. By comparing the molecular weight of protein bands from the SDS-PAGE image with 2-D gel image, both numbers of protein bands and their molecular weights are very close to each other. So, a total of four main proteins are present in the secretome of *P. pastoris* with a modified GAP promoter except for rhGH. Also, their molecular weight ranges are between 10-140 kDa. Other than third well, three very pale protein bands were observed for the first fraction of the size exclusion column. These proteins have the molecular weights at 55, 70 and 130 kDa, respectively. Due to concentrations of proteins in the fractions were low because of the dilution of samples and protein loss in the columns, proteins in the fractions could not be visualize correctly.

Due to coomassie blue is used for staining of the high concentration protein mixture, a satisfactory result was not obtained. For this reason, silver staining was used because silver staining is utilized for the low concentration of protein mixture which consists of 0.6-1.2 ng of proteins (Bio-Rad, 2019).

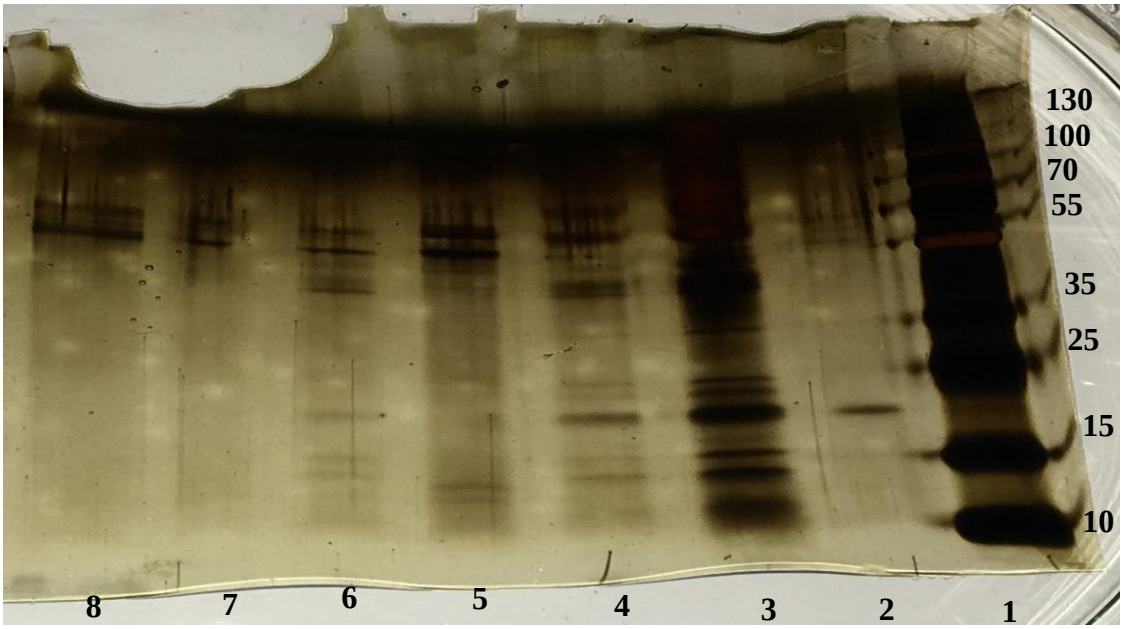


Figure 4.14. SDS-PAGE image of fractions from each chromatographic separation step with silver staining 1-Ladder 2- hGH standard 3- First fraction of desalting column 4- First fraction of size exclusion column 5- Second fraction of size exclusion column 6- Fraction of combination desalting column with size exclusion column 7- First fraction of combination of desalting column with anion exchange chromatography 8- Second fraction of combination of desalting column with anion exchange chromatography

Table 4.9. *Sample definition of each well with silver staining*

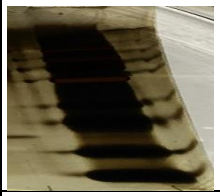
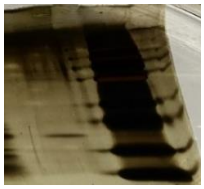
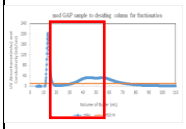
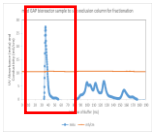
Wells				
	1	2	3	4
Definition	Marker	10 mg/L hGH standard	The first fraction of desalting column that consists of proteins	The first fraction of the size exclusion column (30-60 mL)
Picture of well				
Chromatogram	-	-		
Molecular weight distribution (kDa)	-	22	100-10	100-10
Number of bands	-	1	10	4

Table 4.9. *Sample definition of each well with silver staining (cont.)*

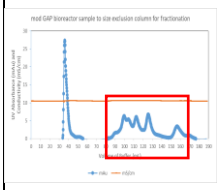
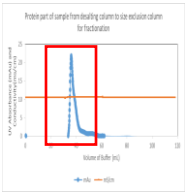
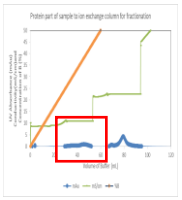
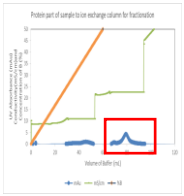
Wells				
	5	6	7	8
Definition	The second fraction of size exclusion column (80-130 mL)	The fraction of combination desalting and size exclusion column	The first fraction of combination desalting and ion-exchange column	The second fraction of combination desalting and ion exchange column
Picture of well				
Chromatogram				
Molecular weight distribution (kDa)	70-10	100-10	70-50	70-50
Number of bands	4	6	2	2

Table 4.10. *Molecular weight distributions for each fraction with silver staining*

3. The first fraction of desalting column			4. The first fraction of size exclusion column (30-70 mL)		
Band no	Predicted molecular weight (kDa)	predicted protein	Band no	Predicted molecular weight (kDa)	predicted protein
1	10		1	22	rhGH
2	12		2	55	
3	15		3	70	
4	22	rhGH	4	100	
5	25		5		
6	35		6		
7	50				
8	55				
9	70				
10	100				
5. The second fraction of size exclusion column (80-130 mL)			6. The fraction of combination desalting column and size exclusion column		
Band no	Predicted molecular weight (kDa)	predicted protein	Band no	Predicted molecular weight (kDa)	predicted protein
1	55		1	22	rhGH (very faint)
2	70		2	50	
3	15		3	55	
4	10		4	70	
7. The first fraction of combination desalting column with ion-exchange column			8. The second fraction of combination desalting with ion-exchange column		
Band no	Predicted molecular weight (kDa)	predicted protein	Band no	Predicted molecular weight (kDa)	predicted protein
1	55		1	55	
2	70		2	70	

It is observed that the number of total protein bands in the third well with silver staining is twice the total number of bands with coomassie brilliant blue staining. The silver stain could show proteins with low concentration. Isoforms of proteins and other impurities could increase the number of protein bands in the gel. Ten protein bands

are seen in the third well. The molecular weight of each band is given in Table 4.10. In the fourth well, four protein bands were observed. One of the band belongs to rhGH. The molecular weights of the other three bands were 55, 70 and 100 kDa, respectively. It was observed that rhGH is partially purified from proteins whose molecular weight is lower than 15 kDa by using the size exclusion column. In fifth well, four protein bands were seen. The molecular weights of bands were 70, 55, 15 and 10 kDa, respectively. This is not expected because the fifth lane belongs to impurities that molecular weights were lower than 50 Da. Protein bands in the fifth lane should belong to the gel preparation based impurities. In sixth well, four protein bands were seen. One of the very pale protein band belongs to the rhGH. The molecular weights of other protein bands were 50, 55 and 70, respectively. In chromatograms of the sixth sample, one peak whose molecular weight corresponds to 64.3 kDa was observed. According to these results, it is concluded that proteins interacted with each other and form an agglomerate. Due to this reason, only one peak is seen in chromatograms. Although rhGH is partially purified, it is observed that a high amount of rhGH is lost after each step of the chromatographic separation. In the last two wells, two protein bands were observed. Their molecular weights were 55 and 70 kDa, respectively. rhGH should be shown at last well but it did not. Due to the sample was diluted after each chromatographic separation and concentration of rhGH decrease with dilution, hGH could not be seen in the last lane. Protein bands, which molecular weights are 70 and 55 kDa, should be impurities.

4.2.5. Characterization of the Production Medium with modified AOX promoter

It was observed that only rhGH was present in the secretome with modified AOX that produced by shake flask bioreactor analysis from Dr. Çalık's group. Since rhGH has not separated from proteins in secretome with modified GAP promoter, purification of rhGH from secretome with modified AOX promoter was investigated. Secretome was acquired by using centrifugation of bioreactor medium. To determine the protein number in the secretome and molecular weights of proteins, SDS-PAGE is done.

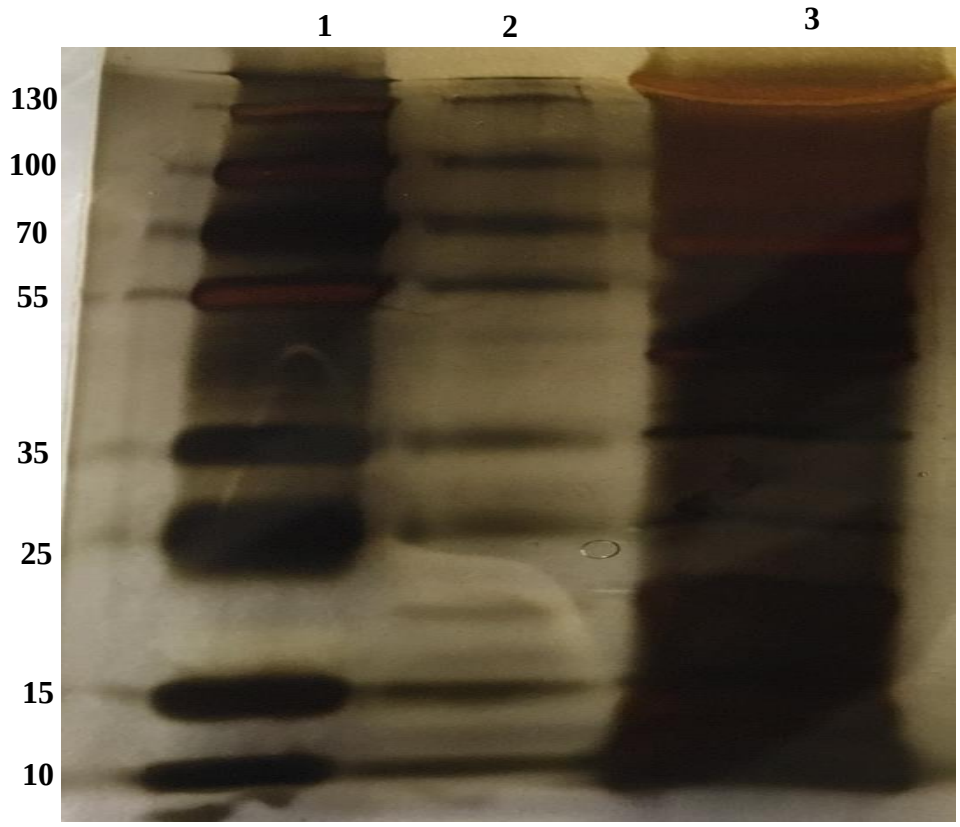


Figure 4.15. SDS-PAGE image of the secretome of *P. pastoris* with modified AOX promoter 1- Molecular weight marker 2- hGH standard 3-Sample with modified AOX

In Figure 4.15, the first two well belonged to the molecular weight marker and 10 mg/L hGH standard, respectively. In third well, the secretome of modified AOX was found. The concentration of rhGH was determined as 74.7 mg/L from SDS-PAGE. An ELISA test was carried out by Omar Masri from Dr. Çalık's Laboratory. In the ELISA, the concentration of rhGH is found between 32.9 and 43.6 mg/L. The concentration of rhGH in secretome with a modified AOX promoter is lower than the concentration of rhGH in secretome with a modified GAP promoter.

Also, molecular weights of other proteins were found as 100, 70, 55, 50,35,25,15 and 10 kDa for secretome with modified AOX promotor along with rhGH. Due to silver stain could stain proteins with low concentration, some bands could belong to the isoform of proteins and gel preparation based impurities. The

molecular weight distribution of proteins in secretome with modified AOX was nearly the same as secretome with modified GAP.

4.2.6. Separation of rhGH from *P. pastoris* secretome with modified AOX promoter

In this part, mainly separation with size exclusion and ion exchange chromatography were investigated for secretome which produced by a using modified AOX promoter.

4.2.6.1. Size Based Separation

After first size-based separation experiments were done for secretome with modified GAP promoter, it was concluded that the desalting column could not be used for purification of rhGH from secretome. Although the desalting column separates protein mixture from other impurities like salts, it has low resolution and causes the loss. So, experiments started with the high-resolution size exclusion column to purify rhGH from secretome with a modified AOX promoter.

If only rhGH present in secretome and exit from the size exclusion column, a calibration curve of the hGH standard for size exclusion column could be used to find the approximate concentration of rhGH from the chromatogram. The hGH standards with different concentrations were given the size exclusion column. The concentration of the hGH standard was calculated by using the area under the peak. The area under the peak represents the zeroth moment (m0). By using data, a calibration curve of the hGH standard is produced.

Table 4.11. *Concentration and absorbance values of hGH standard from size exclusion column*

The concentration of hGH standard (mg/L)	The peak concentration of hGH standard (m0)
0	0
125	2.7
250	8.9
500	14.1
1000	22.1

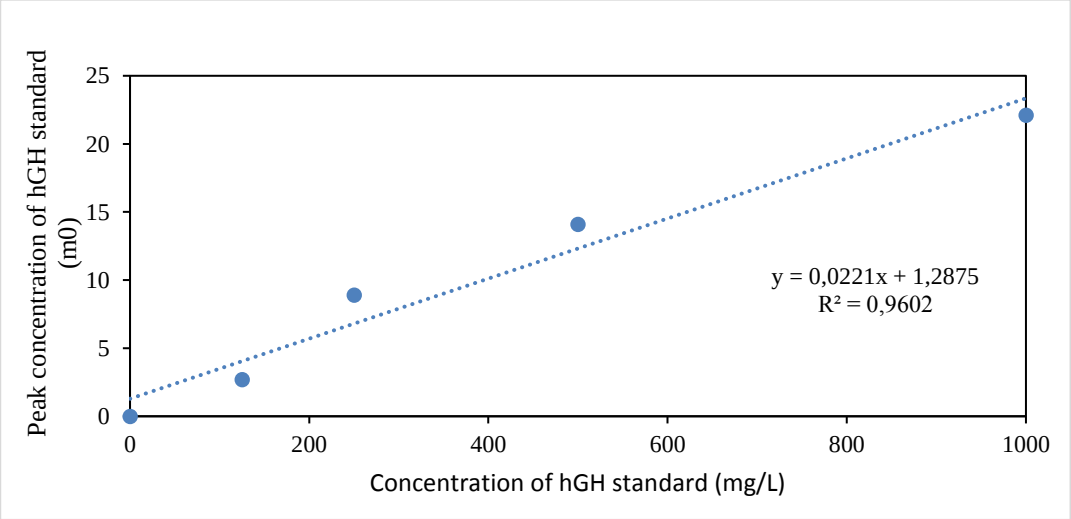


Figure 4.16. Calibration curve of human growth hormone

After the formation of the calibration curve, secretome with modified AOX was given the size exclusion column to the purification of rhGH.

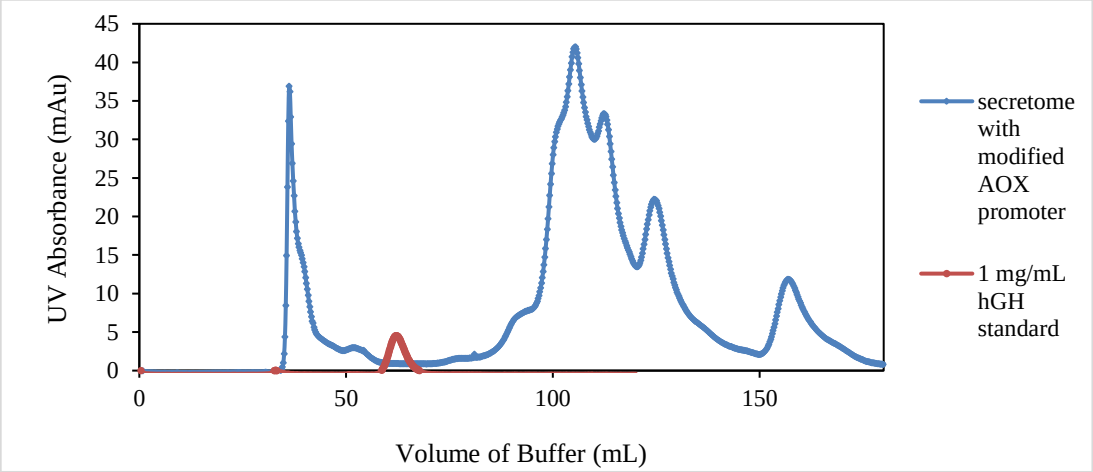


Figure 4.17. Comparison of the secretome of *P. pastoris* and hGH standard in size exclusion column

In Figure 4.17, combined results of the secretome of *P. pastoris* with a modified AOX promoter and 1 g/L hGH standard were given for the size exclusion column. Proteins in secretome exited from the size exclusion column as two main fractions like proteins in secretome with modified GAP promoter. The first fraction exited from the column at 40 mL and the second fraction exited from the column between the 80 and 180 mL. By comparing Figure 4.8 and 4.17, two figures are similar to each other. It is concluded that rhGH should be found in the first fraction. To purify rhGH from secretome, separation of the first fraction of the size exclusion column was aimed by using anion exchange chromatography.

4.2.6.2. Charge Based Separation

In anion exchange chromatography, rhGH was tried to separate from other proteins in the first fraction of the size exclusion column. To separate rhGH, step elution and linear-gradient mode of buffer were investigated.

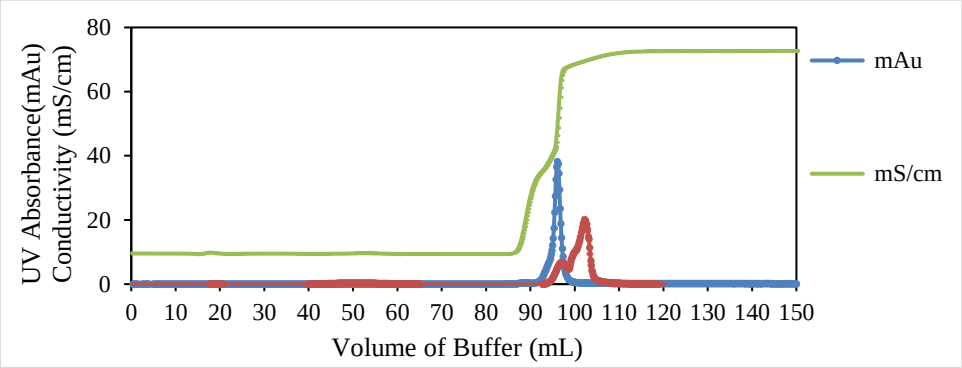


Figure 4.18. The first fraction of size exclusion column from secretome injected into the anion exchange column with step elution mode

In Figure 4.18, it is observed that the first fraction exited from the column as a single peak at 95.9 mL. It is expected because the pI of most of the proteins in secretome is lower than 8. Proteins are negatively charged. They captured during the application of start buffer and release from the column with elution buffer. The exit of proteins in the first fraction at the same retention volume was not expected. The pI values of the proteins were very close to each other. Because of this reason proteins exited from the column at the same time. Absorbance values of the first fraction in Figure 4.17 and 4.18 were very close to each other. Comparing their initial concentration from under the area of peaks, the concentration of the first fraction is reduced to half.

During 140 mL, 100 % linear gradient was applied. By using linear-gradient, increasing the sharpness of peaks and making peaks separate from each other are aimed.

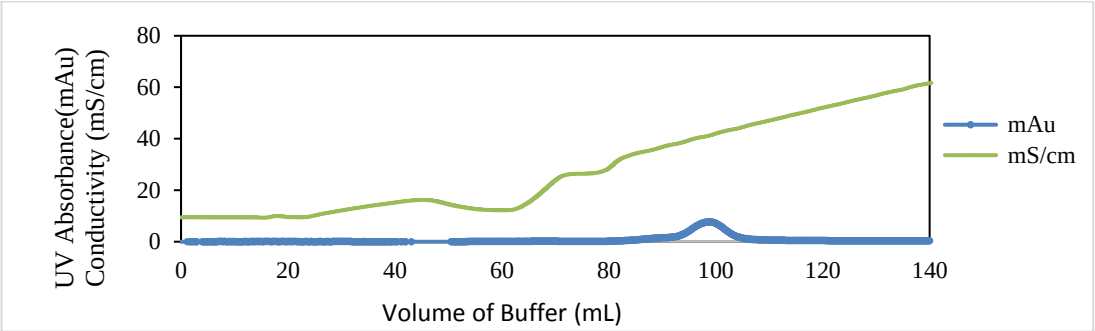


Figure 4.19. The first fraction of size exclusion column from secretome in anion exchange column with the linear gradient

In Figure 4.19, the peak of the first fraction exited from the column at 98 mL. Although linear-gradient increases the sharpness of peaks, the peak of the first fraction becomes broad than before. The initial concentration of peak was nearly the same with the initial concentration of peak with the isocratic condition.

4.2.7. Determination of purification of rhGH from secretome of *P. pastoris* with modified AOX promoter

To determine the purification level of rhGH in the *P. pastoris* secretome and molecular weight distribution of fractions after each chromatographic step, SDS-PAGE analysis was done.

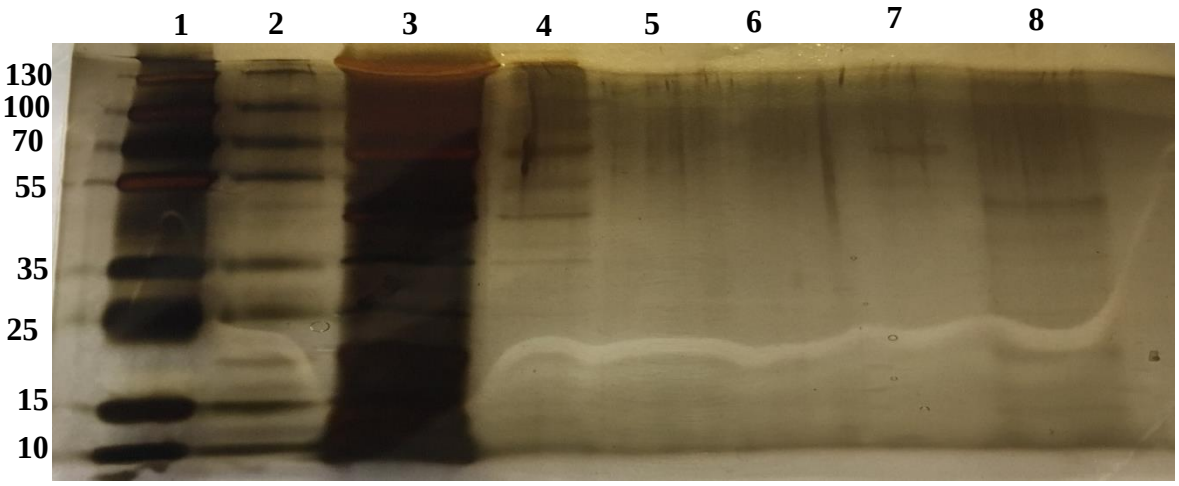


Figure 4.20. SDS-PAGE image of fractions from each chromatographic separation step with silver staining for secretome with modified AOX 1-Ladder 2- hGH standard 3- sample with modified AOX 4- First fraction of size exclusion column 5- First fraction of size exclusion column with anion exchange column by step elution 6- First fraction of size exclusion column with anion exchange column by linear gradient 7- First fraction of anion exchange chromatography with modified GAP sample 8- Second fraction of anion exchange chromatography with modified GAP sample

Table 4.12. *Sample definition of each well with silver staining*

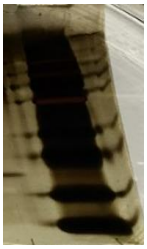
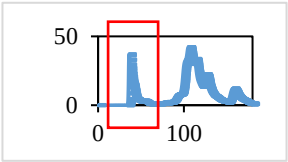
Wells				
	1	2	3	4
Definition	Marker	10 mg/L hGH standard	Modified AOX sample	First fraction of size exclusion column (30-60 mL)
Picture of well				
Chromatogram	-	-	-	
Molecular weight distribution (kDa)	-	22	10-130	70 -50
Number of bands	-	1	9	3

Table 4.12. *Sample definition of each well with silver staining (cont.)*

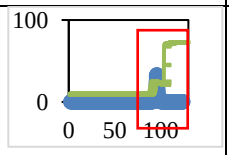
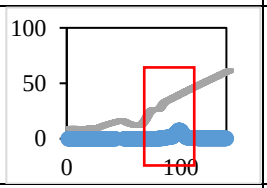
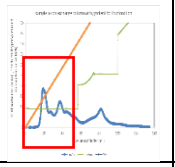
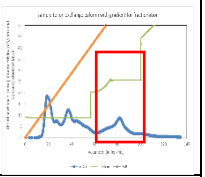
Wells				
	5	6	7	8
Definition	The first fraction of size exclusion column is given to anion exchange column (isocratic)	The first fraction of size exclusion column is given to anion exchange column (linear gradient)	The first fraction of the anion exchange column with modified GAP	The second fraction of the anion exchange column with modified GAP
Picture of well				
Chromatogram				
Molecular weight distribution (kDa)	-	-	70 – 50	50-10
Number of bands	-	-	3	6

Table 4.13. *Molecular weight distributions for each fraction with silver staining*

3. Modified AOX sample			4. First fraction from size exclusion column(30-60 mL)		
Band no	Predicted molecular weight (kDa)	predicted protein	Band no	Predicted molecular weight (kDa)	predicted protein
1	10		1	50	
2	15		2	55	
3	22	rhGH	3	70	
4	25		4		
5	35		5		
6	50		6		
7	55				
8	70				
9	100				
7. The first fraction of the anion exchange column with modified GAP			8. The second fraction of the anion exchange column with modified GAP		
Band no	Predicted molecular weight (kDa)	predicted protein	Band no	Predicted molecular weight (kDa)	predicted protein
1	50		1	10	
2	55		2	15	
3	70		3	22	rhGH
			4	25	
			5	35	
			6	50	

It was observed that nine protein bands are seen in the third well. The molecular weight of each band is given in Table 4.13. In the second lane, molecular weight marker diffused in the second lane and there are protein bands of marker other than hGH standard in the second lane. In the fourth well, three protein bands were observed. Band, which belongs to rhGH, is not observed. Due to the decrease of concentration after each chromatographic separation and dilution effect of the column on the concentration of proteins, the rhGH protein band could not be seen. The

molecular weights of three bands are the 50, 55 and 70 kDa, respectively. In fifth and sixth wells any protein bands could not be observed. According to these results, it is concluded that a high amount of rhGH and other proteins are lost after two consecutive steps of the chromatographic separation. Other than that concentration of proteins in fractions decrease due to dilution of samples in the column. Bands could not be seen owing to the combination of the dilution effect on sample concentration in the column and loss after each purification step. In the last two wells, fractions of the anion exchange column for the modified GAP sample investigated. A protein band of rhGH was found at the second fraction. This is expected because the second fraction belongs to eluted negatively charged proteins. It was found that by using anion exchange, high molecular weight proteins like 70 kDa could be eliminated.

4.2.8. Comparison of secretome of *P. pastoris* with different promoters

In this study, the separation behavior of rhGH from the secretome of *P. pastoris* was investigated. Secretomes were produced by using a modified GAP and modified AOX promoter. When secretomes are compared with each other, it is found that the number of secreted proteins and molecular weight distribution of proteins was very similar. The molecular weights of proteins were 120, 70, 56, 51 and 10 kDa. It was concluded that similar host cell proteins are released to the secretome along rhGH. Also, the concentration of rhGH from the secretome of modified GAP was higher than rhGH from modified AOX.

4.2.9. Comparison of results and suggestion of an effective purification protocol

Purification of rhGH from the secretome of *P. pastoris* was investigated by using chromatographic techniques. Especially, it was focused on separation with size exclusion and anion exchange chromatography. It was found that proteins in secretome were exited from the size exclusion column with two fractions. The first fraction could belong to proteins with molecular weight distribution between 70 and 22 kDa. The second fraction could belong to decayed protein parts. In anion exchange chromatography, most proteins exit with the eluted fraction after application of elution

buffer. The molecular weight distribution of eluted fractions was between 70 and 15 kDa. Eluted fraction includes the rhGH. By using size exclusion, proteins with molecular weight lower than 15 kDa were eliminated. By using anion exchange, proteins with a molecular weight higher than 70 kDa were eliminated. By using these two chromatographic techniques consecutively, rhGH could be partially purified from secretome. It was found that consecutive usage of these two chromatographic techniques causes protein loss. Half of the protein concentration lost in the sample after the combination of chromatographic techniques. Also, it was found that rhGH could interact with other proteins and form of the agglomerates. It is possible agglomerates form due to interaction nonpolar part of proteins with each other. The rhGH could be captured by using anion exchange chromatography. Anion exchange chromatography could be used as a capture step. It concentrates proteins for size exclusion chromatography. Fractions from anion exchange chromatography could be separated by using size exclusion. By this method purification of rhGH is possible but the yield of separation could very low.

Other than chromatography, Çulfaz-Emecen *et al.* investigated the separation of rhGH from the secretome *of P. pastoris* by ultrafiltration. First, the size-based separation was investigated by using 100, 30, 10 and 5 kDa cut-off filters. It was observed that rhGH passed the filter with 100 and 30 kDa with high retention. There was no distinct separation with 10 and 5 kDa filters. Secondly, proteins were tried to separate by changing the pH of the medium. It was observed that while rhGH could not enter the pores, proteins with high molecular weights like 70 kDa could enter the permeate side. After that, rhGH was tried to separate by changing the membrane's net surface charge. Both positive and negative modifications, rhGH rejected by the membrane. Finally, the effect of salt concentration was investigated and high rejection of hGH was found. It was suggested that the high rejection of hGH could be explained by precipitation of hGH (Akcan, 2017).

Cramer *et al.* investigated the purification of rhGH from *P. pastoris* secretome by using chromatographic techniques based on removing host cell proteins. Different

chromatography resins in the industry like multi-model anion and cation exchanger, hydrophobic charge induction and salt-tolerant anion exchanger were investigated. Cell culture was analyzed by using each resin and fractions are analyzed by RP-UPLC. By using data and mathematical models, the Matlab program was formed. This program predicted the possible three chromatographic purification steps by considering scores. It was found that rhGH was separated with 80 % recovery by using multi-model cation exchange, salt-tolerant anion exchange and hydrophobic charge induction chromatography, respectively (Timmick *et al.*, 2018).

To increase yield, another chromatographic technique could be used in case of size exclusion. Size exclusion is not suitable for purification because it works slow flow rates due to the backpressure of the column. It is not suitable for continuous purification. Due to separation time is long with the size exclusion column, it could cause the degradation of proteins. In the case of size exclusion, hydrophobic interaction could be used to separate the agglomerate. The combination of anion exchange and hydrophobic interaction chromatography could be used for purification.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

The main aim of the study was to determine an affordable and efficient purification protocol for the rhGH from the secretome of *P. pastoris* by using chromatographic separation techniques. Size exclusion and anion exchange chromatographies were used for purification of rhGH. Initially, BSA, hGH standard, ovalbumin, conalbumin, carbonic anhydrase, and ribonuclease A were used as model proteins to calibrate and optimize the chromatographic separation setup and procedure. The resolutions of columns were approximately determined by using the plate number of each column by using model proteins. The plate number of desalting, size exclusion and anion exchange columns were found as 150, 1500 and 2000, respectively. So, the highest plate number and resolution belong to the anion exchange column. The retention volumes of rhGH were determined for each column by using the hGH standard. The retention volume of proteins was found as 13 mL for the desalting column. The retention volume of rhGH was determined as 62.5 mL for size exclusion chromatography. The retention volume of rhGH in anion exchange column could change with the application of elution buffer. The calibration curve of the size exclusion column was formed by using retention volumes and molecular weights of proteins.

After first experiments with model proteins, separation of rhGH from secretome with different promoters was investigated by using chromatography. Secretome with a modified GAP promoter was separated from the bioreactor medium by using centrifugation. Approximately four major proteins were found in secretome except for rhGH from 2-D gel electrophoresis. The molecular weights of these proteins were approximately 120, 70, 56, 51 kDa. The pI range most of the proteins were between 4.4 and 5.7. After 2-D gel electrophoresis, proteins of the secretome of *P.*

pastoris from literature used to the formation of virtual 2-D gel images of the secretome by using JVirGel. By using the virtual gel image, approximate identities of proteins were predicted for unknown proteins. Possible proteins were Paf1 complex component, cell wall protein DAN4, protein phosphatase, lectin-like protein, putative glucanases, protein kinase, aspartic proteinase 3, repressible acid phosphatase. After the characterization of secretome, size exclusion and anion exchange chromatography experiments were conducted. In chromatograms, two main fractions were presented instead of six distinct peaks. While the first fraction of the desalting column belonged to the proteins, the second fraction belonged to the impurities like salts, amino acids, and peptides. The first fraction of size exclusion chromatography could belong to rhGH and the other proteins. Molecular weights of other proteins were 70, 55 and 50 kDa. By using the calibration curve of the size exclusion column and equation 4.1, the molecular weight of the first fraction was determined as 64.3 kDa. It is possible that the rhGH interact with other three proteins and form agglomerates. The retention volume of the second fraction of the size exclusion column was between 80 and 180 mL. It should belong to impurities like amino acids and protein parts because the second fraction is totally eliminated after the first fraction of the desalting column is given the size exclusion column. While the first fraction belongs positively charged proteins in the anion exchange column, the second fraction belongs to the negatively charged proteins like rhGH. The molecular weight distribution of the second fraction of the anion exchange column between 70 and 10 kDa. To find the purification level of rhGH, SDS-PAGE analysis was done. It is found that rhGH is partially purified from secretome. Proteins, which molecular weight is below then 15 kDa and higher than 70 kDa, are eliminated by using size exclusion and anion exchange chromatography, separately. By combining these chromatographic techniques is possible but nearly half of the protein concentration was lost.

Secretome with a modified AOX promoter was investigated for the purification of rhGH. The rhGH concentration was determined as 32.9 – 43.6 mg/L by the ELISA test. The concentration of rhGH with modified AOX was lower than

rhGH with modified GAP. By using SDS-PAGE analysis, molecular weight distribution and the number of proteins were found. It is similar to secretome with modified GAP. In size exclusion chromatography, two main fractions were observed. The first fraction of size exclusion chromatography could belong to rhGH and other proteins in secretome like a modified GAP sample. Molecular weights of proteins were 70, 55 and 50 kDa. It is possible that hydrophobic parts of proteins are interacted and form an agglomerate. The rhGH is relatively hydrophobic due to its consist of a high concentration of nonpolar amino acids. Due to the first fraction consist of rhGH, the first fraction was analyzed with anion exchange chromatography by using step elution and linear-gradient mode. It was observed that the first fraction from size exclusion could not separate by using anion exchange chromatography. To understand the purification level of rhGH, SDS-PAGE analysis was done. It is concluded that after combination size exclusion and anion exchange chromatography, protein concentration decreased the lower than 0.6 ng which is the lower detection limit of silver staining. The results of SDS-PAGE were not reliable because samples are nearly ten times diluted during chromatography. For that reason, some proteins that present in secretome could not be visualized correctly by SDS-PAGE analysis.

To sum up, partial purification of rhGH from the secretome of *P. pastoris* is possible with very low yield. By using size exclusion chromatography, proteins, which molecular weight lower than 15 kDa, could be eliminated. Proteins, which molecular weight is higher than 70 kDa, could be eliminated by anion exchange. But protein loss is huge by combining these two chromatographic techniques. Instead of size exclusion chromatography, other chromatographic techniques can be used for the purification of rhGH from secretome such as hydrophobic interaction chromatography.

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APPENDICES

A. The amino acid sequences of rhGH and hGH standard

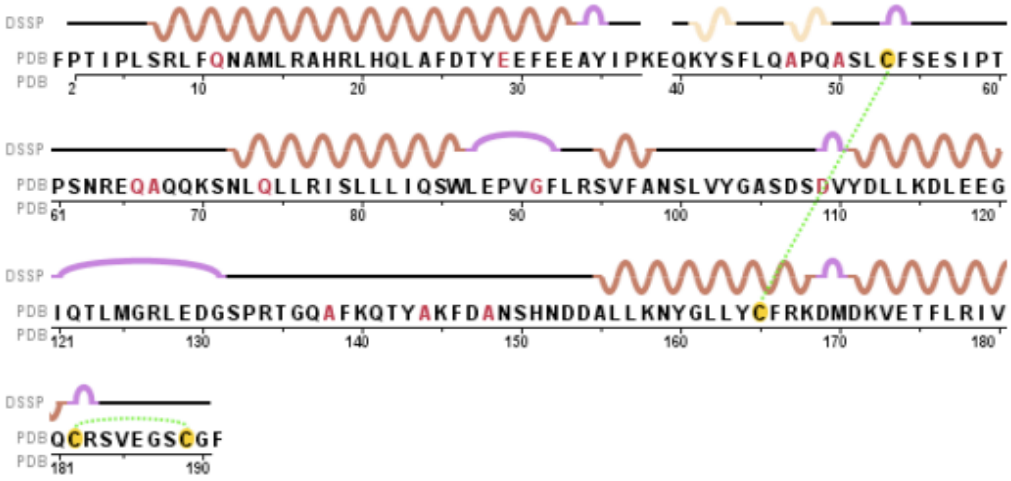

Figure A.1. The amino acid sequence of hGH (Inankur, 2010)

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FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT
PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEG
IQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIV
QCRSVEGSCGF
```

Figure A.2. The amino acid sequence of Humatrope in Fasta Format (https://www.drugbank.ca/drugs/DB00052)

B. The concentration of production medium and trace minerals for *P. pastoris*

Table B.1. *The basal salt medium for P. pastoris (Hoxha, 2016)*

Components	**Concentration (g/L)**
Glycerol (86 %)	50 ml
85% H_3PO_4	26.7ml
K_2SO_4	18.2
$MgSO_4.7\ h_2O$	14.9
KOH	4.13
$CaSO_4.2\ h_2O$	1.17
Chloromphenicol*	1mL
PTM1 *	4.35mL
10% antifoam*	1 mL
dH_2O	to 1 L

*Added after autoclaving

Table *B.2. The composition of Pichia trace minerals (Hoxha, 2016)*

Components	Concentration g/L
$CuSO_4.5\ h_2O$	6
H_3BO_3	0.02
NaI	0.08
$Na_2MoO_4.2\ h_2O$	0.2
MnSO4.H2O	3
$ZnCl_2$	20
$FeSO_4.7\ h_2O$	65
$CoCl.6\ h_2O$	0.916
H_2SO_4	5 mL
Biotin	0.2 mL
dH_2O	to 1 L

C. Chromatograms for model proteins and molecules for different chromatography columns

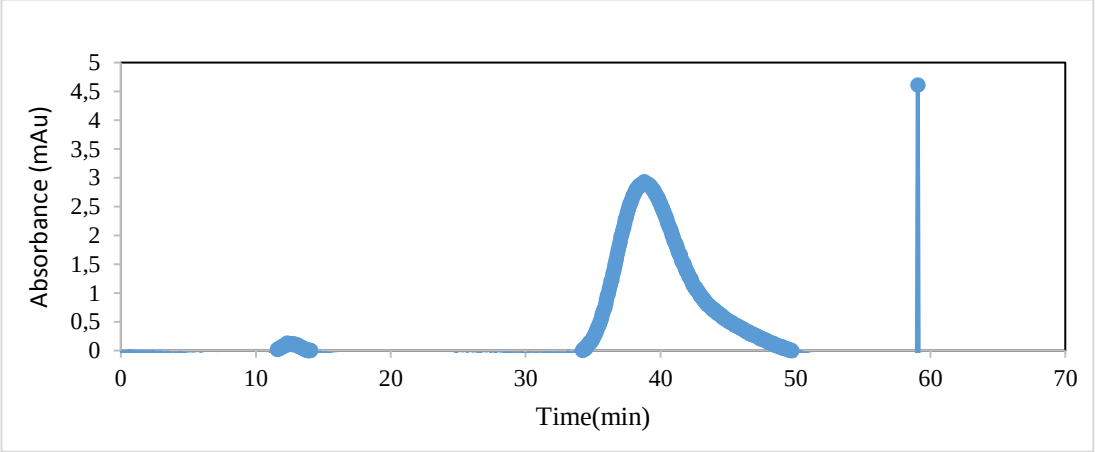

Figure C.1. Chromatogram of % 5 acetone solution in desalting column at 1 mL/min

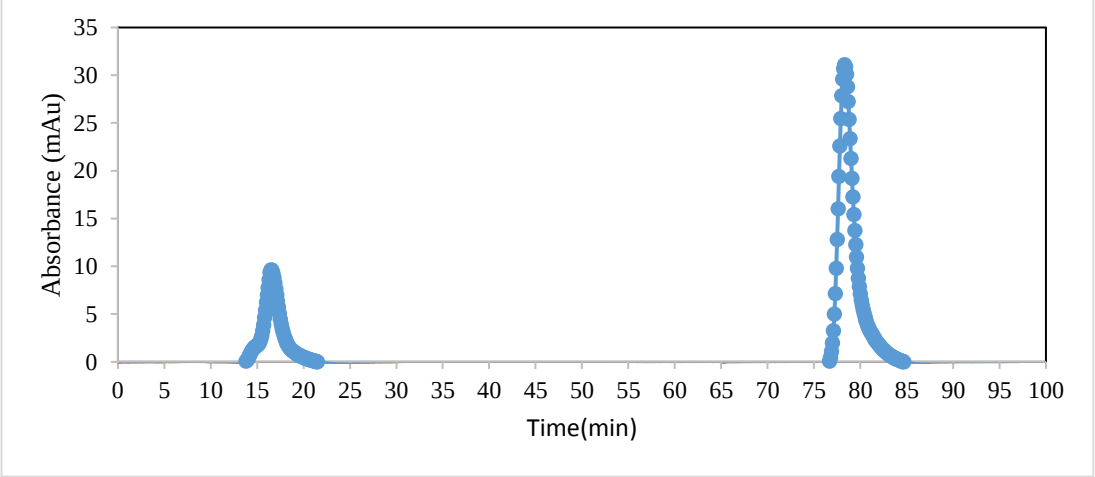

Figure C.2. Chromatogram of 2 and 5 mg/mL BSA solution in desalting column at 5 mL/min

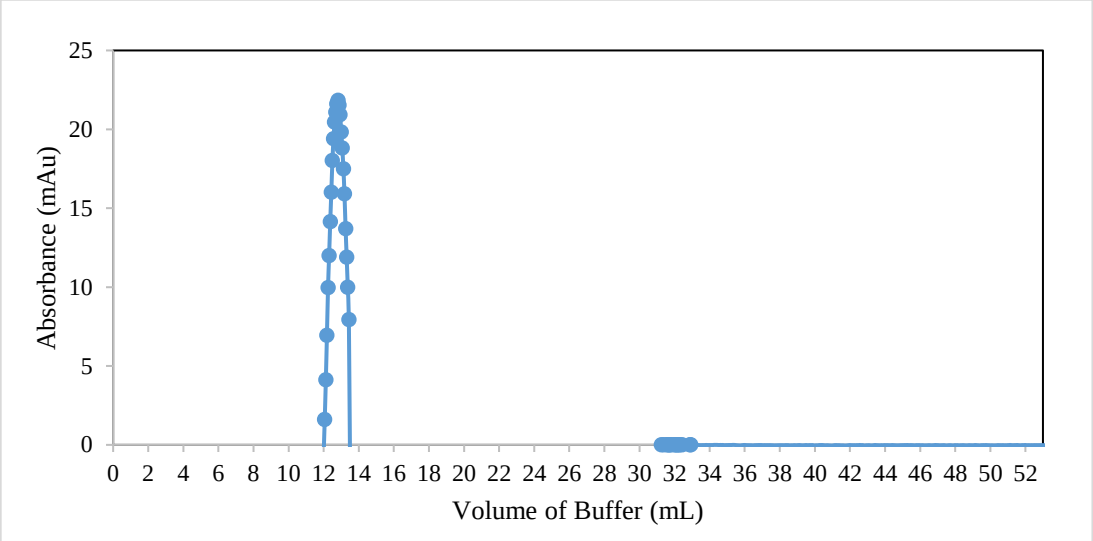

Figure C.3. Chromatogram of 10 mg/mL BSA solution in desalting column at 5 mL/min

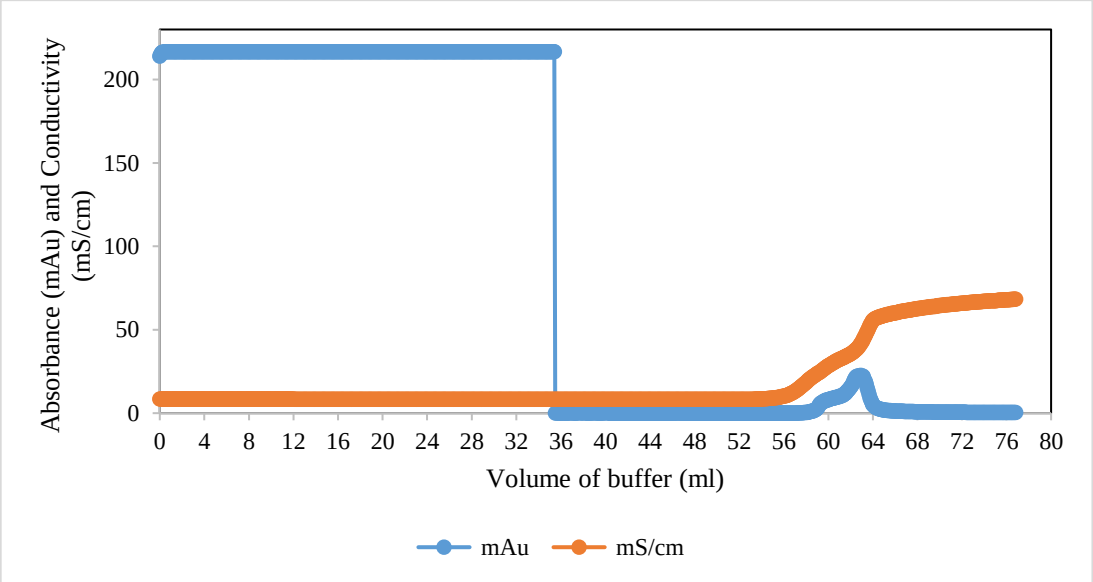

Figure C.4. Chromatogram of 5 mg/mL BSA solution in ion exclusion column

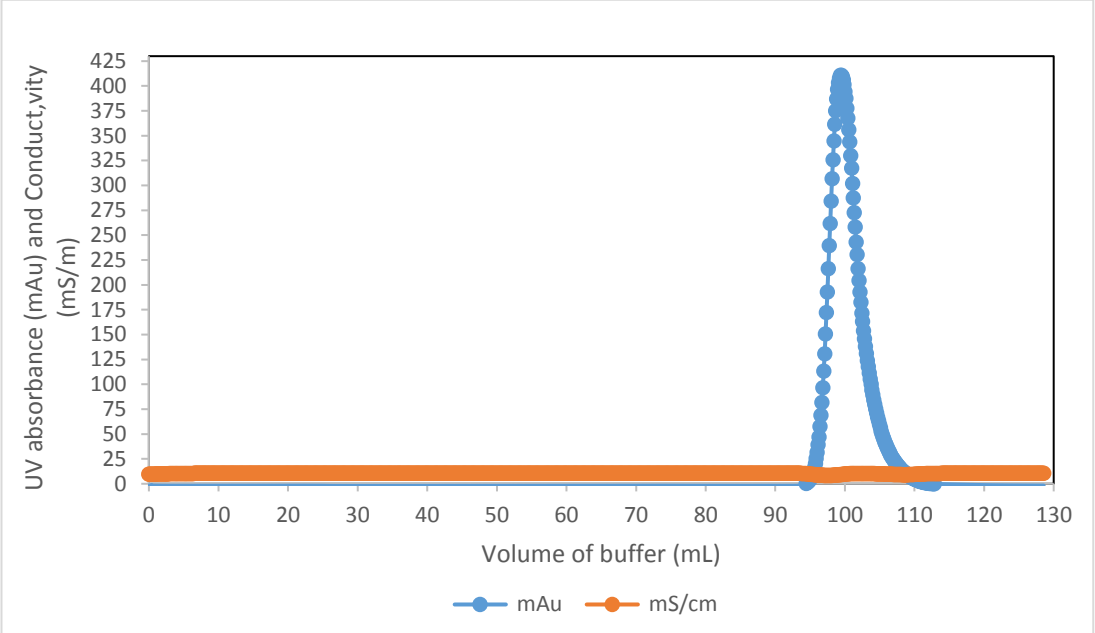

Figure C.5. Chromatogram of % 5 acetone solution in size exclusion column

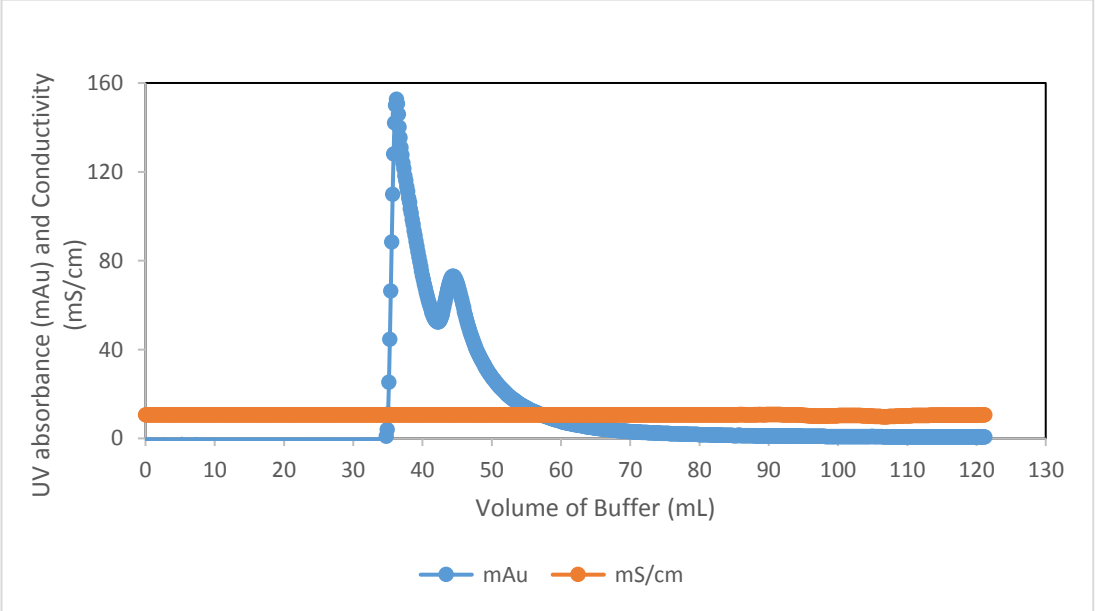

Figure C.6. Chromatogram of 10 mg/mL BSA solution in size exclusion column

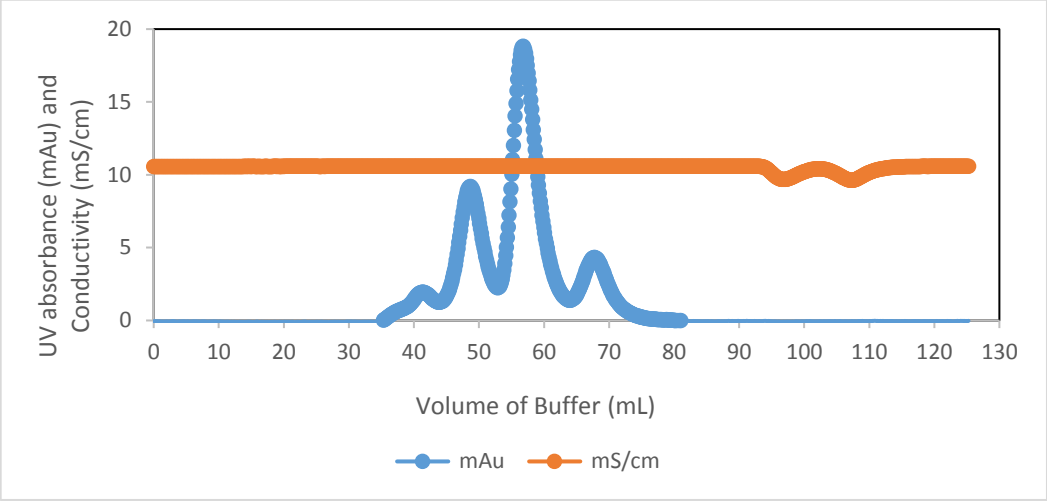

Figure C.7. Chromatogram of Ovalbumin ,Carbonic anhydrase and Ribonuclease A mixture for size exclusion chromatography

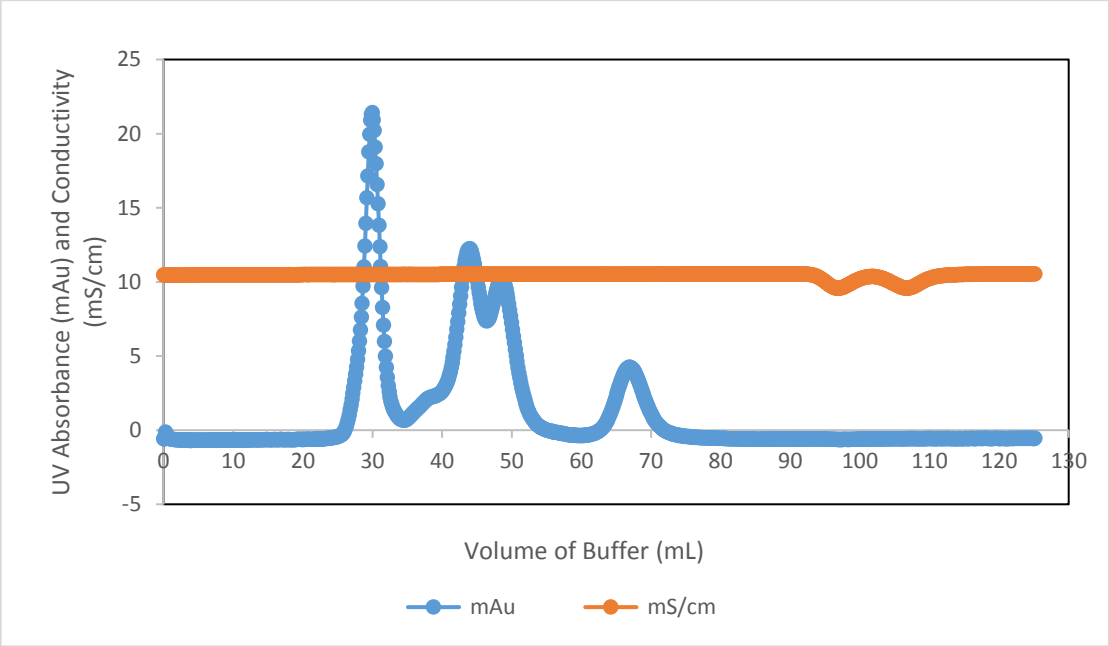

Figure C.8. Chromatogram of Ovalbumin ,Conalbumin and Ribonuclease A mixture for size exclusion chromatography

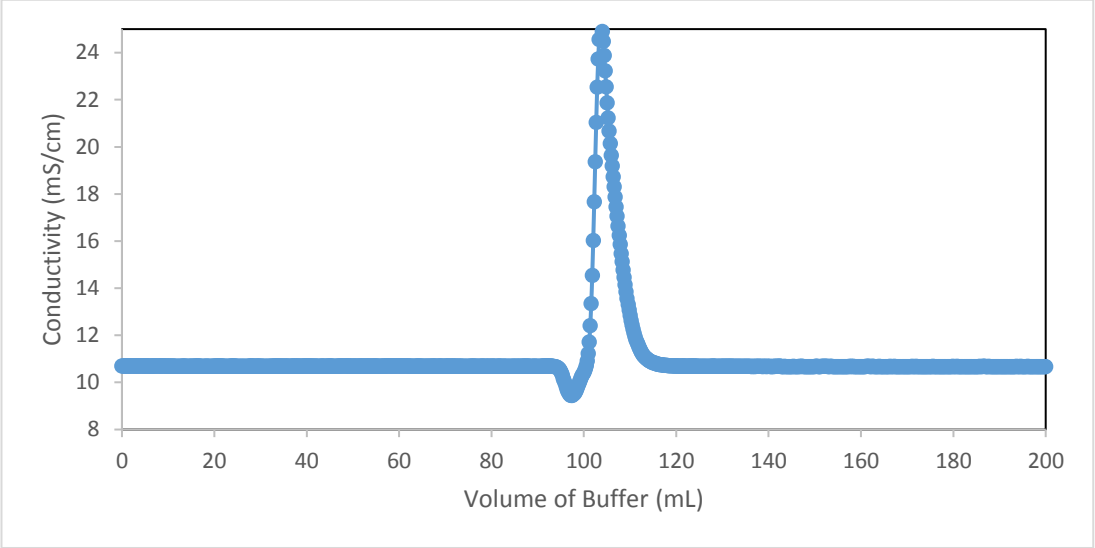

Figure C.9. Chromatogram of 0.5 M NaCl solution in size exclusion column