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Isolation of recombinant human growth hormone and mutants expressed as inclusion bodies in *E. coli*

Masterarbeit

zur Erlangung des akademischen Grades einer
Diplom-Ingenieurin

der Studienrichtung Biotechnologie
erreicht an der
Technischen Universität Graz

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Technische Universität Graz
2011

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DANKSAGUNG

Ich widme diese Arbeit meinen Eltern, die mich während meines gesamten Studiums in jeder erdenklicher Hinsicht unterstützten, in schönen Zeiten meine Freude teilten sowie mir in schwierigen Zeiten immer zur Seite standen und mir den notwendigen Rückhalt gaben.

Bedanken möchte ich mich allen voran bei Prof. Bernd Nidetzky für die Möglichkeit diese Arbeit am Institut für Biotechnologie und Bioprozesstechnik durchzuführen. Ein besonderer Dank gilt Dipl.-Ing. Johanna Wiesbauer für ihre freundliche und motivierende Betreuung, die Einführung in das praktische Arbeiten im Labor sowie die Weitergabe ihres umfangreichen fachlichen Wissens. Bedanken möchte ich mich auch bei Dr. Stefan Leitgeb für die fachlichen Anregungen und Diskussionen.

Weiters möchte ich mich bei allen Mitarbeitern des Instituts bedanken, die mich auf die eine oder andere Weise bei der Durchführung dieser Arbeit unterstützten und mir immer freundlich und hilfsbereit zur Seite standen.

Bei Dr. Ruth Birner-Grünberger möchte ich mich für Durchführung der MALDI-TOF-MS Messungen bedanken.

Mein Dank gilt aber ganz besonders auch allen, die während meines Studiums mein privates Umfeld geprägt haben. Ich danke vor allem meinen Geschwistern Claudia und Christian, meinen Freundinnen und Freunden sowie meinem Freund Fabian für die vielen schönen und lustigen Stunden sowie aufbauenden und ermutigenden Worte.

Ein besonderer Dank gilt auch meinem Onkel Karl, der mich während meines Studiums immer wieder inspirierte und mir ermöglichte außergewöhnliche und wertvolle Erfahrungen zu sammeln.

Zuletzt möchte ich mich beim Research Center Pharmaceutical Engineering und bei Sandoz für die Aufgabenstellung und die finanzielle Unterstützung bedanken.

KURZFASSUNG

Aggregation stellt ein häufiges Problem während der verschiedenen Schritte der Produktion, des Transportes und der Lagerung von therapeutischen Proteinen dar. Die Kontrolle und Prävention von Aggregation ist eine zunehmende Herausforderung in der pharmazeutischen Forschung und Entwicklung, wobei die Untersuchung und das Verständnis von Aggregationsmechanismen eine wesentliche Rolle spielt. Vorangehende Arbeit an der Aufklärung des Aggregationsmechanismus des menschlichen Wachstumshormons (Human Growth Hormone) schlägt einen Domain Swapping Mechanismus vor, bei welchem die Entfaltung der N-terminalen Helix zur Aggregation führt. Basierend auf dieser Theorie wurde die A13C/G120C Mutante designed bei welcher durch die Bildung einer zusätzlichen Disulfidbrücke die Entfaltung der N-terminalen Helix verhindert werden soll. Die Einführung eines His-Tag und einer Proteaseschnittstelle am N-Terminus des Wachstumshormons wurde mittels Overlap Extension PCR durchgeführt um eine erleichterte Reinigung sowie die Abspaltung des Tags nach der Reinigung zu ermöglichen. Das Wachstumshormon wurde in *E. coli* BL21 (DE3) exprimiert und die Inclusion Bodies wurden nach einem beschriebenen Protokoll isoliert, denaturiert und rückgefaltet. Das Denaturierungsprozedere beinhaltete die Verwendung von 8 M Harnstoff als Denaturierungsmittel und 1% 2-Mercaptoethanol als Reduktionsmittel. Die Rückfaltung des Proteins wurde mittels Entfernung der Solubilisierungsmittel durch Dialyse durchgeführt. Imobilised Metal Ion Affintiy Chromatography (IMAC) wurde zur Reinigung des mit dem His-Tag versehenen Wachstumshormons verwendet. Das gereinigte Protein wurde anschließend mit intrinsischer und extrinsischer Fluoreszenzmessung analysiert.

ABSTRACT

Aggregation is a common problem with pharmaceutical proteins during production, refolding, purification, shipment and storage. Thus, the control and prevention is of great importance and an increasing challenge in pharmaceutical research and development. The understanding of the aggregation mechanism is of interest since it facilitates the control and prevention of aggregation issues. Preliminary work on the aggregation pathway of human growth hormone proposed a domain swapping mechanism with the unfolding of the N-terminal helix leading to aggregation. Based on this theory the A13C/G120C human growth hormone mutant was designed to prevent the unfolding of the N-terminal helix by introduction of an additional disulfide bond. The introduction of an affinity tag and a protease cleavage site to the N-terminus of the protein was performed by overlap extension PCR in order to facilitate purification and to cleave the tag after purification. Human growth hormone was expressed as inclusion bodies in *E. coli* BL21 (DE3) and the inclusion bodies were isolated, denatured and refolded according to a reported protocol. The denaturing procedure included 8 M urea as denaturing agent in the presence of 1% 2-mercaptoethanol as reducing agent. The refolding of the protein was accomplished by removal of the solubilization agents using dialysis. Immobilized metal ion affinity chromatography (IMAC) was used for purification of the tagged human growth hormone. The purified protein was analyzed by intrinsic tryptophan and extrinsic fluorescence measurement.

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ABBREVIATIONS

Ala	alanine
APS	ammonium persulfate
ANS	8-anilino-1-naphthalenesulfonic acid
bp	base pairs
BSA	bovine serum albumin
CV	column volume
Cys	cysteine
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphat
EDTA	ethylenediaminetetraacetic acid
fwd	forward
Gly	glycine
hGH	human growth hormone
His	histidine
HPLC	high pressure liquid chromatography
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB-medium	Luria-Bertani-medium
LC	liquid chromatography
LMW	low molecular weight
LTQ-FT	linear trap quadrupole-fourier transformation
MS	mass spectrometry
MW	molecular weight
PCR	polymerase chain reaction
OD	optical density
PES	polyethersulfone
PMSF	phenylmethylsulfonyl fluoride
rev	reverse
rpm	rotations per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	super optimal broth

T	temperature
TEMED	tetramethylethylenediamin
TEV	tobacco etch virus
Tm	melting temperature
UV	ultraviolet
WT	wild type

1 INTRODUCTION

1.1 General introduction

Human growth hormone is a single chain polypeptide containing 191 amino acid residues with a molecular weight of 22.1 kDa. Human growth hormone, also known as somatropin, is involved in the regulation of many metabolic and physiological processes including the stimulation of cell growth and reproduction. Thus, human growth hormone is mainly used as a pharmaceutical to treat growth hormone deficiency in hypopituitary dwarfism (Brems et al., 1990). The hormone is produced by somatotroph cells in the pituitary gland under control of the hypothalamus. Human growth hormone is stored in the pituitary gland in form of granules, secreted from these granules into the blood stream and carried to the target receptor (Jensen et al., 2008). The protein comprises a four-helix structure, where the helices run up-up-down-down which is unusual compared to the more common up-down-up-down connectivity. The N-terminal and the C-terminal helices are longer (26 and 30 residues) than the other helices (21 and 23 residues) (De Vos et al., 1992). The molecule contains two disulfide bonds between Cys^{53} and Cys^{165} (forming a large loop) and Cys^{182} and Cys^{189} (forming a small loop) (Brems et al., 1990). The structure of human growth hormone is shown in figure 1.

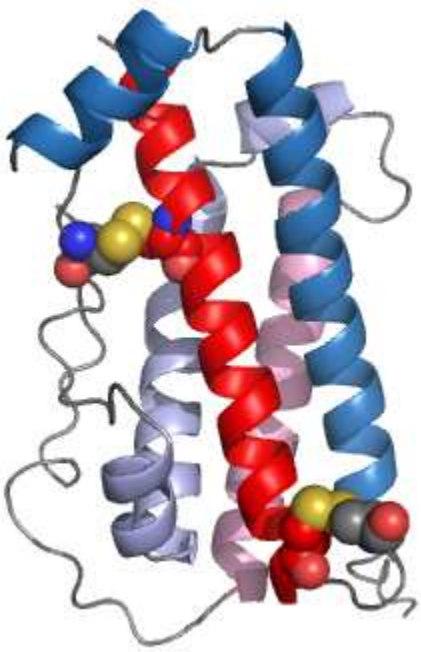

Figure 1: Structure of human growth hormone

1.2 Aggregation and stability of pharmaceutical proteins

Aggregation of pharmaceutical proteins during production, refolding, purification, shipment and storage processes is a common problem and thus the control and prevention of aggregate formation is of great importance and an increasing challenge in pharmaceutical research and development (Weiss et al., 2009). Since protein aggregates affect human health by causing immunogenic reactions and anaphylactic shocks, the minimization of aggregates present in pharmaceutical products must be ensured (St. John et al., 2001). Besides, the aggregation of proteins is responsible for a number of neurodegenerative diseases e. g. the Alzheimer's and Parkinson's disease (Jensen et al., 2008).

The term aggregation indicates the self-association of proteins which can be reversible or irreversible and is often accompanied with loss of biological activity or increased immunogenicity of the pharmaceutical. Aggregates are either soluble or insoluble, whereas insoluble aggregates are commonly referred to precipitates. Soluble aggregates include dimers, trimers and oligomers but also high molecular weight aggregates. Protein aggregates are formed by covalent or noncovalent interactions and result in different shapes and morphology. Covalent aggregates can be formed by disulfide bonds and noncovalent interactions include hydrogen bonds, hydrophobic and electrostatic interactions (Mahler et al., 2009; Weiss et al., 2009).

Protein aggregation is a complex process which is not fully understood today. Different mechanisms or pathways of protein aggregation were described, where more than one can occur for a certain protein. The complete understanding of the aggregation mechanism is not necessary for pharmaceutical development but some understanding facilitates the control and prevention of aggregation issues (Philo et al., 2009).

The initial state of a protein which aggregates differs. Various possibilities are described in the following. Reversible aggregation of the native monomer can occur, where the propensity to aggregate is intrinsic for the native form of the protein. Within the increase of protein concentration and formation of larger aggregates the aggregation often becomes irreversible. Aggregation of the monomer after transient conformational change or unfolding is common for many therapeutic proteins and can be inhibited by conditions which stabilize the native state of the protein. Aggregation of the monomer

after chemical modification can occur, where the protein undergoes chemical changes or degradation such as oxidation, deamidation or proteolysis. In this case the aggregation can be inhibited by improving the chemical stability of the protein (Philo et al., 2009).

Other mechanisms responsible for protein aggregation are the nucleation-induced and the surface-induced aggregation. In the first case, the native monomer has a low tendency to form small aggregates and the addition of native monomers to these is not thermodynamically favored. If these small aggregates reach a critical size they serve as a nucleus for aggregation, the addition of native monomers is thermodynamically favored and leads to the formation of larger aggregates. In the second case, the aggregation is induced by the binding of the native monomer to a surface, the native monomer undergoes a change of conformation and aggregates (Philo et al., 2009).

The mechanism of aggregation is depended on the intrinsic properties of a protein but also on the environmental conditions, including different types of applied stress. Aggregation can be induced by different conditions including temperature, mechanical stress such as shaking and stirring but also freezing and thawing. Surface effects are known to promote aggregation as well, both on liquid-solid but also liquid-gas surfaces. Chemical, physical and thermal stresses are applied to the pharmaceuticals during the manufacturing process, shipment and storage and therefore, understanding of the induction of aggregate formation is important in order to control and prevent aggregation (Mahler et al., 2009; Philo et al., 2009).

The required shelf life for therapeutic proteins is 18-24 months. The stability of pharmaceutical proteins in general is limited by degradation, chemical and physical. Chemical degradation includes e. g. deamidation, oxidation and disulfide shuffling. Physical degradation includes e. g. protein unfolding, aggregation and adsorption to surfaces. Thus, minimizing the aggregation in formulation is crucial and a great challenge. Factors influencing the chemical and physical stability of proteins in formulation include pH, salt type, salt concentration, preservatives and surfactants (Chi et al., 2003).

1.3 Aggregation of human growth hormone

It was reported in literature that the folding mechanism of human growth hormone is a three-state process (DeFelippis et al., 1993). The folding mechanism is depicted in figure

2, where N refers to the folded native state, U refers to the unfolded state and I refers to the monomeric folding intermediate which, under certain conditions, self-associates (DeFelippis et al., 1993).

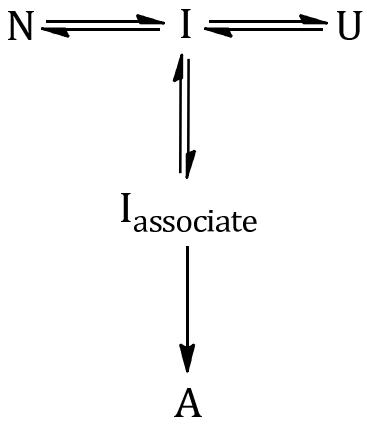

Figure 2: Aggregation mechanism of human growth hormone

The denaturation of human growth hormone was investigated at different protein concentrations and it was found that ΔG, the calculated free energy of unfolding, decreases with increasing protein concentration. This observation was explained by the presence of folding intermediates which have a tendency to self-associate. It was proposed for human growth hormone that the self-associated folding intermediates are formed due to hydrophobic interactions between the partially unfolded intermediates. In contrast, stable folding intermediates have been found for nonhuman growth hormone (Brems et al., 1990; DeFelippis et al., 1993).

To gain insight into the aggregation mechanism of a protein, the knowledge about the nature of the intermediate leading to aggregation is of great importance. Thus, the examination of the conformational change of human growth hormone preceding aggregation was an issue of interest.

The preliminary work for the investigation of the aggregation mechanism of human growth hormone was done by Dr. Rene Meier. The results of his work provided the basis for the planning and implementation of the work done within this master thesis. Dr. Rene Meier proposed a mechanism for the aggregation of human growth hormone based on literature (Jensen et al., 2008). In this literature the authors studied the self-association of human growth hormone by nitrogen-15 NMR relaxation. The authors investigated the protein-protein interaction and reported an increased rotational diffusion property with increasing concentration. The authors studied the regions

involved in the self-association and proposed that the regions on the surface close to the N- and the C-terminus are involved in intermolecular interactions. However, the chemical shift changes were too small in order to find the specific regions involved in the self-association. Based on this literature, Dr. Rene Meier proposed a domain swapping mechanism for human growth hormone. The theory of domain swapping involves unfolding of the N-terminal helix and is illustrated in figure 3.

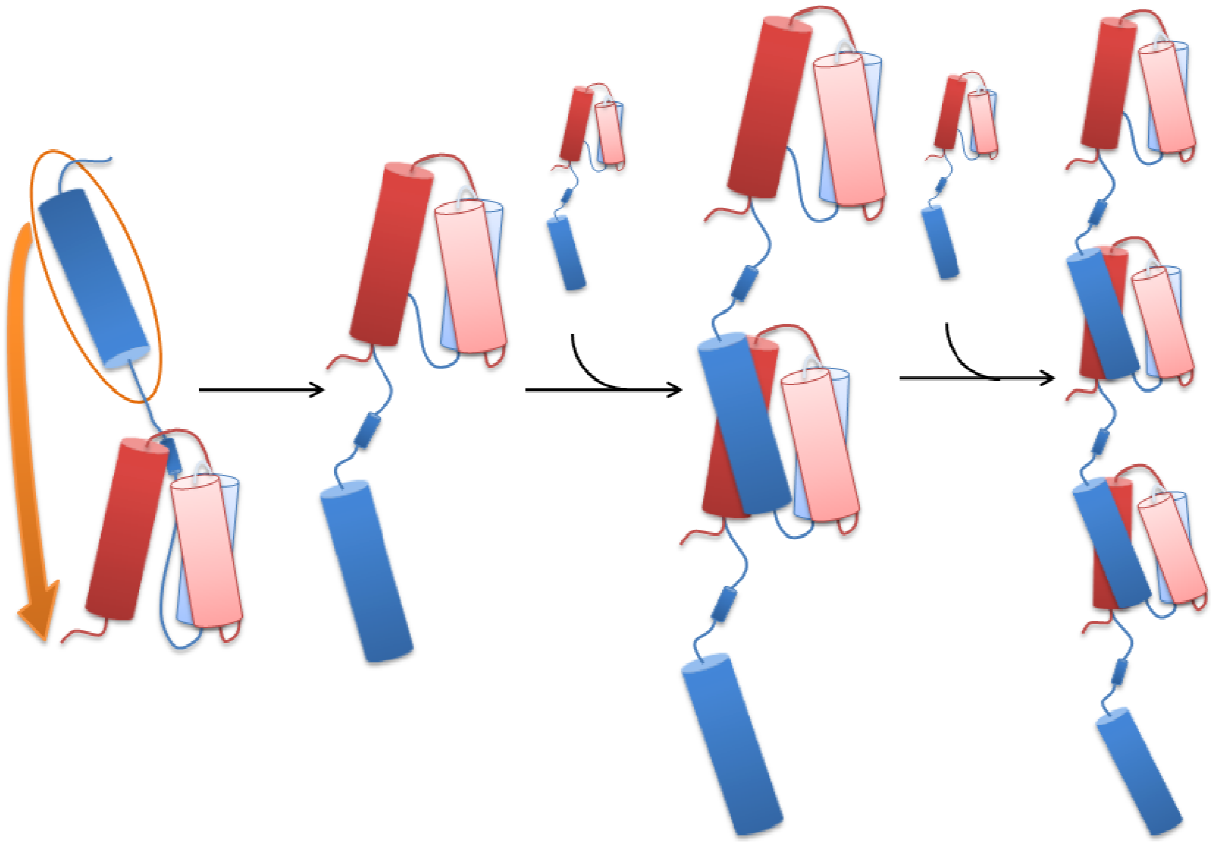

Figure 3: Domain swapping mechanism proposed by Dr. Rene Meier

Based on this domain swapping mechanism, human growth hormone mutants were designed which were supposed to have a lower tendency to unfold the N-terminal helix. The reduced tendency to aggregation of these mutants would support the theory of the domain swapping mechanism. Two mutants were proposed: the A13C/G120C and the M14C/L177C mutant, both having a disulfide bridge introduced. These two mutants were designed based on the comparison of a number of structural analogues of the human growth hormone where the location of disulfide bridges was examined. Ovine interferone tau shows a disulfide bridge connecting the N-terminal with the third helix. The human growth hormone mutant A13C/G120C was designed with a similar disulfide bridge. The human erythropoietin shows a disulfide bridge connection the N- and the C-

terminus. The human growth hormone mutant M14C/L177C was designed with a similar disulfide bridge. The analysis of the two mutants and the WT in molecular dynamic simulation showed that at room temperature all three proteins are stable with respect to backbone movement. When analyzed the mutants in molecular dynamic simulation at higher temperatures it was shown that the M14C/L177C mutant had a slightly more stable N-terminal helix compared to the WT, the overall structure was less stable. The A13C/G120C mutant showed improved overall stability, especially in the N-terminal helix. Therefore, the A13C/G120 mutant was chosen to be generated by genetic engineering.

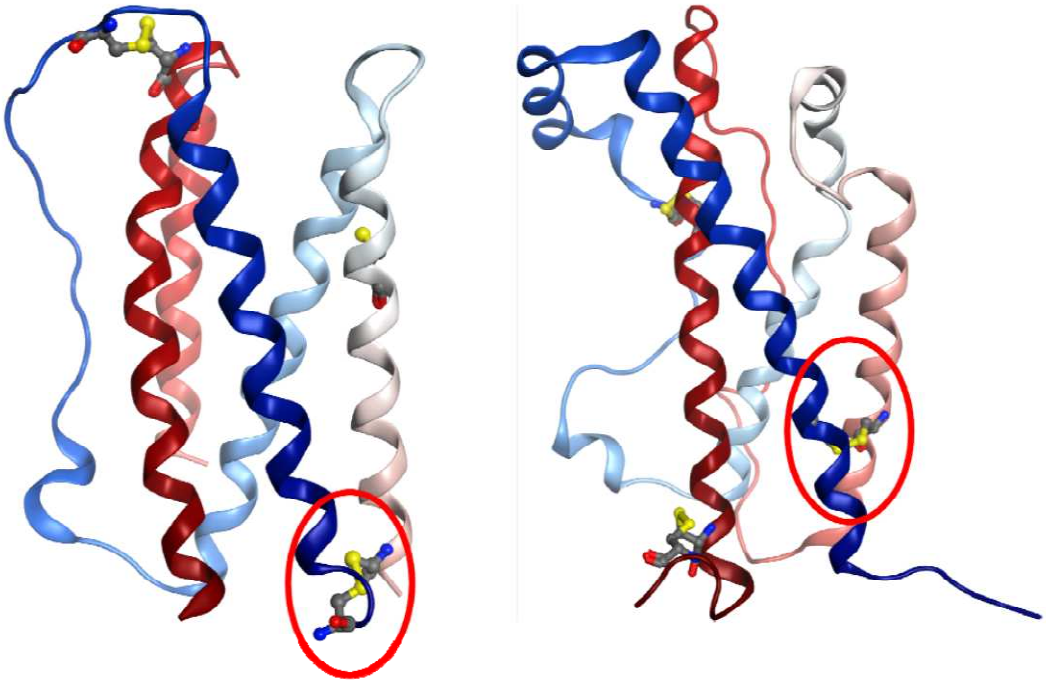

Figure 4: Structures of ovine interferone tau (left) and human growth hormone A13C/G120C mutant (right) made by Dr. Rene Meier

Additionally, the A13C single mutant was planned to be generated. On the one hand, in order to provide a starting point for the generation of the double mutant in case it is not possible to introduce the two mutations at once. On the other hand, the A13C mutant could also be a subject of investigation in terms of examining the aggregation behaviour. It was assumed that the A13C mutant might have an increased tendency to self-associate due to the presence of the additional cysteine on the N-terminal helix which might lead to intermolecular disulfide bond formation.

1.4 Refolding of inclusion bodies

The overexpression of recombinant proteins in prokaryotic organisms often results in the formation of insoluble aggregates, termed inclusion bodies. *E. coli* is a host organism frequently used for the production of recombinant proteins which does not require posttranslational modification such as glycosylation. Particularly in *E. coli* overexpression of proteins frequently lead to accumulation of these inclusion bodies within the host cell (Singh et al., 2005).

In general, the procedure of recovering bioactive proteins from inclusion bodies involves several steps: isolation and washing of the inclusion bodies, solubilization of the inclusion bodies followed by refolding of the solubilized protein (De Bernardez Clark, 1998). Inclusion bodies are isolated by centrifugation after cell disruption. The quality of the inclusion bodies can be improved by maximum cell disruption which can be achieved by combination of mechanical and other cell disruption methods (De Bernardez Clark et al., 1999). Solubilization of inclusion bodies is generally accomplished by high concentrations of denaturants such as 6 M guanidine hydrochloride or 8 M urea. In general, the use of guanidine hydrochloride is preferred over urea because of two reasons. On the one hand, guanidine hydrochloride is a stronger denaturant and on the other hand, urea forms isocyanate leading to carbamylation (Cabrita et al., 2004). Reducing agents such as 2-mercaptoethanol, dithiothreitol (DTT) or cysteine are often used along with denaturants to ensure the reduction of disulfide bonds and to prevent intra- or intermolecular disulfide bond formation (Singh et al., 2005). The dissolved inclusion bodies are usually refolded into the native conformation by removal of denaturing and reducing agents. In case of proteins containing disulfide bonds, the use of oxidizing/reducing agents promote the appropriate disulfide bond formation. The most common reagents are reduced and oxidized glutathione (GSH/GSSG) or cysteine/cystine. Protease inhibitors such as e. g. PMSF might be a component of the refolding buffer (Cabrita et al., 2004).

The purity of inclusion bodies has an influence on renaturation and refolding since contaminants promote co-aggregation. Therefore, inclusion bodies are washed with detergents such as Triton X-100 or low concentrations of denaturants such as guanidine hydrochloride or urea (Cabrita et al., 2004).

Refolding of denaturated inclusion bodies is usually performed by dilution or dialysis and chromatographic procedures are used as well (De Bernardez Clark, 1998). During dialysis of the denatured protein solution against renaturation buffer the slow removal of the denaturant takes place. The refolding procedure by dialysis often results in enhanced formation of protein aggregates (Cabrita et al., 2004).

The production of proteins as inclusion bodies has some advantages over the production of soluble proteins within the host cell or secretion of the proteins into the culture medium. Protein expression as inclusion bodies is often associated with the expression of high levels of protein. In addition, inclusion body proteins are less accessible to proteolytic degradation by cellular proteases. Furthermore, due to the high density compared to many other host cell components, inclusion bodies can be isolated by centrifugation after cell disruption and thus, inclusion bodies facilitate the separation of the desired protein. Since inclusion bodies usually contain mostly the desired protein and only few host cell proteins, a certain homogenicity is achieved and further purification can be facilitated.

However, the great disadvantage of the expression of recombinant proteins as inclusion bodies is the required refolding procedure which frequently results in poor recovery of refolded protein.

Protein aggregation during refolding is the main reason for the poor recovery of native protein. Aggregation results from hydrophobic interactions between the folding intermediates as well as the native and the denatured protein. The inhibition of hydrophobic interactions suppresses the formation of aggregates during refolding. Low molecular weight compounds were found to prevent these interactions where the mechanism of action of these additives on the folding process is not completely clear. It is assumed that these small molecules influence the solubility and the stability of the native, intermediate or denatured state of the protein. The most important additive reported is L-arginine, which is proposed to increase the solubility of folding intermediates, leading to decrease in aggregation. Polyethylene glycol (PEG) was reported to serve as a folding enhancer, where the hydrophobic parts of the additive interact with the hydrophobic side chains exposed by the denatured protein. Denaturants such as guanidine hydrochloride or urea were also found to improve the yield of folded protein when added at nondenaturing concentrations in case the native

state of the protein is stable under these conditions. Other low molecular weight compounds were found to suppress the formation of aggregates during refolding, e. g. surfactants (De Bernardez Clark et al., 1999). Other strategies to prevent aggregation during refolding include e. g. the application of molecular chaperones which facilitate protein folding. Refolding procedures are carried out at 4°C in order to prevent aggregation (Cabrita et al., 2004).

During refolding kinetic competition occurs between folding and aggregation. It was found that aggregation follows a second- or higher-order reaction whereas folding follows a first-order reaction. Because of this kinetic competition, the rate of aggregation increases with increasing concentration of the unfolded protein. Thus, refolding at low initial protein concentrations prevents the formation of aggregates and the consequent low yield of folded protein. Refolding is recommended at low protein concentrations of 0.01-0.1 mg/mL (Cabrita et al., 2004; De Bernardez Clark et al., 1999).

Three different chromatographic procedures for refolding proteins from inclusion bodies are in use: solvent exchange by size-exclusion chromatography, on-column refolding and chaperone-assisted refolding (Swietnicki, 2006). During refolding of inclusion bodies by size-exclusion chromatography, the denatured inclusion bodies are loaded on the column. The size-exclusion effect restricts the aggregation of partially unfolded proteins which have a tendency to aggregate due to the exposure of hydrophobic residues by physically isolating the protein molecules. The protein is refolded by solvent exchange whereas the protein is eluted with renaturation buffer (Swietnicki, 2006). During on-column refolding the denatured protein is reversibly immobilized to the column and the denaturant is slowly removed by solvent exchange. Reversible immobilization prevents aggregation during denaturant removal and is most commonly accomplished by using a six to ten residue histidine affinity tag (Swietnicki, 2006). During chaperone-assisted refolding, either the chaperone or the denatured protein is immobilized to a solid support and either the denatured protein or the chaperone passes over the support in order to facilitate folding (Swietnicki, 2006). Refolding procedures by chromatography usually result in higher quality of the protein and higher yields, but are more expensive compared to conventional dilution or dialysis procedures. However, these procedures might be the method of choice for proteins where refolding attempts using dilution or dialysis were not successful (Swietnicki, 2006).

2 MATERIALS AND METHODS

2.1 Chemicals

Chemicals used in this work were obtained from Carl Roth GmbH or Sigma-Aldrich. Buffers and solutions used for chromatographic methods were sterile filtered with 0.2 µm cellulose acetate filters or 0.2 µm polyamide filters (Sartorius GmbH). Antibiotic stock solutions were sterile filtered with 0.2 µm PES membrane syringe filters (VWR International GmbH). All media were autoclaved at 121°C for 20 minutes.

The plasmid containing the sequence of the human growth hormone and an ampicillin resistance was provided by Prof. Michael Hodsdon and his group in Yale. Primers used in this work were purchased from IDT (Integrated DNA Technologies). Phusion DNA polymerase and Phusion DNA polymerase buffer were purchased from Finnzymes. Pfu DNA polymerase, Pfu DNA polymerase buffer and *Dpn*I were obtained from Promega. Taq polymerase dream, Taq polymerase buffer, dNTPs, restriction enzymes and 6x DNA loading dye were purchased from Fermentas.

Human growth hormone serving as reference was provided by Sandoz.

2.2 General methods

2.2.1 Agarose gel electrophoresis

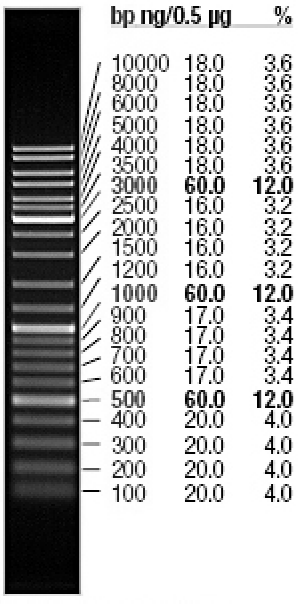

Figure 5: Gene Ruler DNA Ladder Mix (100-10000 bp)

1% agarose gel containing 0.006 µg/mL ethidium bromide and TAE-buffer, pH 8 (242 g/L Tris, 57.1 mL/L acetic acid, 18.6 mg/mL EDTA in deionized H_2O) was used. 6x DNA loading dye was added to the samples before application to the gel. TAE-buffer was used as electrophoresis buffer and DNA was separated at room temperature and 70 V. For quantification and sizing of DNA the Gene Ruler DNA Ladder Mix (100-10000 bp) was used. After electrophoresis the DNA fragments were shown with UV detection at 312 nm.

2.2.2 Purification of PCR products or DNA fragments from agarose gels

For the purification of PCR products or DNA fragments from agarose gels the NucleoSpin Ectract II isolation kit was used. The procedure was carried out according to manufactures protocol.

2.2.3 Preparation of electrocompetent *E. coli* cells

Electrocompetent *E. coli* BL21 (DE3) cells were cultivated in shaking flasks in medium containing 5 g/L yeast extract, 5 g/L NaCl and 10 g/L peptone at 37°C and 140 rpm until an OD of 0.5-0.8 was reached. The biomass was transferred into sterile, cooled centrifugation beakers. The cells were harvested by centrifugation at 4°C at 5000 rpm for 25 minutes. The pellets were resuspended in 10 mL sterile, cooled water. Approximately 200 mL sterile, cooled water was added to the resuspended pellets. The resuspended cells were centrifuged at 4°C at 5000 rpm for 25 minutes. The pellets were resuspended in 10 mL sterile, cooled 10% glycerin and pooled. Approximately 200 mL sterile, cooled 10% glycerin was added, followed by centrifugation at 4°C at 5000 rpm for 25 minutes. The pellet was resuspended in 30 mL sterile, cooled 10% glycerin and transferred into a sterile Falcon tube, followed by centrifugation at 4°C at 5000 rpm for 25 minutes. The pellet was resuspended in 4 mL 10% glycerin, aliquoted to 100 µL in Eppendorf tubes, frozen with liquid nitrogen and stored at -70°C.

2.2.4 Transformation of electrocompetent *E. coli* cells

100 µl electrocompetent *E. coli* cells were mixed with 2 µl plasmid and transferred into precooled electroporation cuvettes (PEQLAB Biotechnologie GmbH). Electroporation was performed with a MicroPulser Electroporator (Bio-Rad) using the programme suitable for bacteria. 800 µl SOC medium (20 g/L peptone, 0.58 g/L NaCl, 5 g/L yeast extract, 0.18 g/L KCl, 0.625 g $MgSO_4$ supplemented with 3.46 g/L glucose) were added

immediately and mixed carefully. The transformed cells were regenerated at 37°C for 1 hour and afterwards plated on selective LB-medium (5 g/L yeast extract, 5 g/L NaCl, 10 g/L peptone, 15 g/L agar-agar, ampicillin) and cultivated over night. Transformants were picked, plated again on selective medium and plasmid DNA was isolated.

2.2.5 Isolation of plasmid DNA from *E. coli*

The GeneJET Plasmid Miniprep Kit (Fermentas) and the Wizard Plus SV Minipreps DNA Purification System (Promega) were used for isolation of plasmid DNA from *E. coli*. The procedure was performed according to manufactures protocol. The plasmid DNA concentration was determined by measuring the UV absorption at 260 nm.

2.2.6 DNA sequencing

DNA sequencing was accomplished by LGC Genomics GmbH. Sequence analysis was done with Vector NTI.

2.2.7 Bradford protein quantification

Roti-Quant protein quantification assay (Carl Roth GmbH) according to Bradford was used. The measurement was performed on the DU 800 UV/Vis Spectrophotometer (Beckman Coulter) and BSA was used for calibration.

2.2.8 SDS-PAGE

SDS gels were prepared according to the following protocol. 10 mL of separating gel containing 4.4 mL deionized water, 3 mL 40% acrylamide, 2.5 mL 1.5 M Tris-HCl buffer, pH 8.8, 50 µL 20% SDS, 50 µl 10% APS and 5 µl TEMED was prepared. 10 mL of stacking gel was prepared, containing 3.1 mL deionized water, 625 µL 40% acrylamide, 1.25 mL 0.5 M Tris-HCl buffer, pH 6.8, 25 µL 20% SDS, 25 µL 10% APS and 2.5 µL TEMED. Mini-PROTEAN TGX Precast gels, 4-20% (Bio-Rad) were used for SDS-PAGE. Protein SDS sample buffer (20 mM KH_2PO_4, 6 mM EDTA, 6% SDS, 10% glycerol, 0.05% Bromphenol Blue) or protein SDS sample buffer with 2-mercaptoethanol (9.840 mL protein SDS sample buffer supplemented with 160 µL 2-mercaptoethanol) was added to the protein solution applied to the gel. The mixture was heated at 99°C for 5 minutes before applying to the gel. The protein electrophoresis was performed using SDS-PAGE running buffer (30.3 g/L Tris, 144 g/L glycine, 10 g/L SDS, pH 8.3). LMW protein standard

(GE Healthcare) shown in figure 6 was used. The procedure was performed using the Mini-PROTEAN Tetra Cell (Bio-Rad).

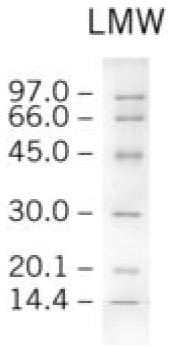

Figure 6: LMW protein standard

2.2.9 Coomassie staining protocol for SDS-PAGE gels

The gel was stained with PhastGel Blue R Coomassie R350 (GE Healthcare) for approximately one hour. For destaining a solution containing 30% ethanol, 10% acetic acid was used. For preserving the gel, incubation in 10 % acetic acid, 13% glycerol was performed.

2.2.10 Silver staining protocol for SDS-PAGE gels

The gel was incubated in the fixing solution (50% methanol, 10% acetic acid) for 30 minutes and then incubated in a 5% methanol solution. The gel was washed with deionized water three times for 5 minutes. The sensitizing solution (0.02% sodium thiosulfate) was prepared freshly and the gel was incubated for 2 minutes and afterwards again washed with deionized water three times for 30 seconds. The silver staining solution (prepared freshly) contained 0.2% silver nitrate and the gel was incubated for 25 minutes. The gel was washed in deionized water three times for one minute. The developing was done for approximately 5-10 minutes in a solution containing 3 g sodium carbonate, 50 µL 37% formaldehyde, 2 mL sensitizing solution filled to 100 mL dionized water (prepared freshly). After developing the gel was applied to a stopping solution (1.4% EDTA, pH 8) for 10 minutes. Finally, the gel was washed with deionized water three times for 5 minutes.

2.3 Genetic work

The sequence of the plasmid containing the human growth hormone sequence and an ampicillin resistance is not completely known. The sequence of the human growth hormone is shown in the appendix.

The idea was to introduce an affinity tag and a protease cleavage site at the 5'-end of the human growth hormone sequence in order to facilitate the purification and to cleave the tag after purification. The frequently used polyhistidine-tag (his-tag) was chosen to be introduced as well as the TEV protease cleavage site. It was reported that the his-tag is not immunogenic and thus not required to be cleaved from the fusion protein after purification (Mukhija et al., 1995). Nevertheless, it was decided to introduce the TEV protease cleavage site into the fusion protein. The cleavage site of the TEV protease is ENLYFQG with the cleavage occurring between Q and G (Terpe, 2003).

2.3.1 Overlap extension PCR cloning

The plasmid containing the human growth hormone sequence and an ampicillin resistence (in the following termed as phGH) was used as template DNA in the following overlap extension PCR. The addition of the *Nde*I restriction site, the his-tag and the TEV protease cleavage site to the 5'-end of the human growth hormone sequence was performed according to a reported overlap extension PCR cloning protocol (Bryksin et al., 2010). The insert containing the *Nde*I restriction site, the his-tag and the TEV protease cleavage site was cloned into the plasmid without restriction endonucleases or T4 DNA ligase. For the ligation independent cloning method the insert was employed using synthetic megaprimer containing overlapping regions with the template DNA. The sequences as well as the properties (taken from the specification sheet of IDT) of the megaprimer are shown in table 1 whereas the sequences on the 5'-end and on the 3'-end, indicated in black, are compatible with the phGH. The *Nde*I restriction site is indicated in blue, the his-tag is indicated in green, the loop providing a sufficient number of basepairs necessary for a megaprimer is indicated in orange and the TEV protease cleavage site is indicated in red. The compositions of the PCR mixtures are shown in table 2 and 3. The PCRs were performed using thermocycler (Bio-Rad).

Two different PCR mixtures (PCR fwd and PCR rev) were started for each, the forward as well as the reverse direction. The reactions were carried out under the following

conditions: initial denaturation at 100°C for 2 minutes, denaturation at 98°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 10 minutes. After 4 cycles, PCR fwd and PCR rev were mixed and additional 18 cycles of denaturation, annealing and extension were performed. Final extension at 72°C for 10 minutes was added. Template DNA was then digested with *Dpn*I at 37°C for 1 hour and the overlap extension PCR product was transformed into electrocompetent *E. coli* BL21 (DE3) cells. After regeneration in SOC medium the transformed cells were grown on LB-plates containing 100 mg/L ampicillin at 37°C over night. In the following, the generated plasmid is termed as phGHHisTEV.

Table 1: Megaprimer used for overlap extension PCR

Primer	Sequence	Properties
Megaprimer fwd	TTAAGAAGGAGATATACATATGCACCACCACCACC ACCACTCTTCTGGTGTAGATCTGGGTACCGAGAAC CTGTACTTCCAATCCTTCCCAACCATTCCCTTATCC	T_m: 71.3°C GC Content: 47.1% DNA Bases: 106
Megaprimer rev	GGATAAGGGAATGGTTGGGAAGGATTGGAAGTACA GGTTCTCGGTACCCAGATCTACACCAGAAGAGTGGT GGTGGTGGTGGTGCATATGTATATCTCCTTCTTAA	T_m: 71.3°C GC Content: 47.1% DNA Bases: 106

Table 2: Overlap extension PCR mixtures (PCR fwd and PCR rev)

PCR fwd	PCR rev
2 µL dNTPs (2 mM)	2 µL dNTPs (2 mM)
4 µL Phusion buffer (5x)	4 µL Phusion buffer (5x)
1 µL phGH template (3 ng/µL)	1 µL phGH template (3 ng/µL)
2 µL megaprimer fwd (1 pmol/µL)	2 µL megaprimer rev (1 pmol/µL)
10 µL H_20	10 µL H_20
1 µL Phusion polymerase (1:5)	1 µL Phusion polymerase (1:5)

Table 3: Final overlap extension PCR mixture

PCR final (after 4 cycles)
4 µL dNTPs (2 mM)
8 µL Phusion buffer (5x)
2 µL phGH template (3 ng/µL)
2 µL megaprimer fwd (1 pmol/µL)
2 µL megaprimer rev (1 pmol/µL)
20 µL H_20
2 µL Phusion polymerase (1:5)

2.3.2 Colony PCR

Transformants gained from overlap extension PCR cloning were cultivated on LB-medium. Colony PCR was performed in order to identify clones containing the generated plasmid. The primers used for the reaction are shown in table 4 (properties taken from the specification sheet of IDT).

Cells were prepared by suspending in 60 µl sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and heating for 5 min at 99°C. The colony PCR mixture is shown in table 5. Colony PCR was carried out under the following conditions: initial denaturation at 95°C for 4 minutes, denaturation at 95°C for 40 seconds, annealing at 55°C for 40 seconds and extension at 72°C for 1 minute for 25 cycles, with a final extension at 72°C for 5 minutes. Template DNA was then digested with *Dpn*I at 37°C for 1 hour. Gel electrophoresis was performed in order to identify the PCR products.

Table 4: Primer used for colony PCR

Primer	Sequence	Properties
NdeIhGH	TATACATATGCACCACCACCACCACCACTCTTCTG	T_m: 64.4°C GC Content: 48.5% DNA Bases: 35
HindIIIhGH	CTAATTAAGCTTCTAGAAGCCACAGCTGCCCTCC	T_m: 63.8°C GC Content: 50% DNA Bases: 34

Table 5: Colony PCR mixture

PCR
2 µL dNTPs (2 mM)
2 µL Dream Taq buffer (10x)
6 µL cell suspension
2 µL NdeIhGH primer (10 pmol/µL)
2 µL HindIIIhGH primer (10 pmol/µL)
5.5 µL H_20
0.5 µL Dream Taq polymerase

2.3.3 Site-directed mutagenesis

The A13C and the A13C/G120C mutant were generated according to a reported two-stage PCR protocol (Wang et al., 1999). In order to introduce the desired mutations, primer were designed which are shown in table 6 (properties taken from the specification sheet of IDT). The PCR mixtures are shown in table 7 and 8. The DNA was amplified under the following conditions: initial denaturation at 95°C for 1 minute, denaturation at 95°C for 50 seconds, annealing at 55°C for 50 seconds and extension at 72°C for 15 minutes for 18 cycles, with a final extension at 72°C for 10 minutes. In case of the double mutant the fwd primer were added to the reaction mixture at the beginning, 4 cycles of denaturation, annealing and extension were performed and then the rev primer were added to the reaction mixture and the DNA amplification was continued.

Table 6: Primer used for site-directed mutagenesis

Primer	Sequence	Properties
hGHA13Cfwd	CAGGCTTTTTGACAACTGTATGCTCCGCGCCCATCG	T_m: 68.2°C GC Content: 55.5% DNA Bases: 36
hGHA13Crev	GGAATAGGTCCGAAAAACTGTTGACATACGAGGCGCG	T_m: 66.5°C GC Content: 51.3% DNA Bases: 37

hGHG120Cfwd	ACCTCCTAAAGGACCTAGAGGAATGCATCCAAACGC	T_m: 65.6°C GC Content: 50.0% DNA Bases: 36
hGHG120Crev	GGATCTCCTTACGTAGGTTTGCGACTACCCCTCCGACC	T_m: 69.2°C GC Content: 57.8% DNA Bases: 38

Table 7: PCR mixture used for site-directed mutagenesis of the A13C single mutant

PCR single mutant
5 µL dNTPs (2 mM)
5 µL Pfu polymerase buffer (10x)
4 µL phGHHisTEV (50 ng/µL)
1.5 µL hGHA13Cfwd primer (10 pmol/µL)
1.5 µL hGHA13Crev primer (10 pmol/µL)
32 µL H_20
1 µL Pfu polymerase

Table 8: PCR mixture used for site-directed mutagenesis of the A13C/G120C double mutant

PCR double mutant
5 µL dNTPs (2 mM)
5 µL Pfu polymerase buffer (10x)
4 µL phGHHisTEV (50 ng/µL)
1.5 µL hGHA13Cfwd primer (10 pmol/µL)
1.5 µL hGHA13Crev primer (10 pmol/µL)
1.5 µL hGHG120Cfwd primer (10 pmol/µL)
1.5 µL hGHG120Crev primer (10 pmol/µL)
29 µL H_20
1 µL Pfu polymerase

2.4 Protein expression

Recombinant *E. coli* BL21 (DE3) cells transformed with the respective plasmid were grown in shaking flaks in a medium containing 5 g/L yeast extract**,** 5 g/L NaCl**,** 10 g/L tryptone and 100 mg/L ampicillin. The starter culture was grown overnight at 37°C at 140 rpm and the main culture was inoculated to an OD of 0.05. After induction with 1 mM IPTG at an OD of 0.6-0.8 the cells were further grown at 37°C at 140 rpm for 4 hours. Cells were harvested by centrifugation at 4°C at 5000 rpm for 30 minutes and stored at -20°C. The OD was measured using the Spectronic GENESYS 5 UV-Vis Spectrophotometer (Thermo Electron Corporation).

2.5 Fermentation

In order to gain higher yields of the A13C/G120C mutant, recombinant BL21 (DE3) *E. coli* cells were grown in a 5 L B. Braun Biotech International Biostat C fermenter in a medium containing 22 g/L glucose monohydrate, 10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, 1 g/L NH_4Cl, 0.25 g/L $MgSO_4$*7 H_20, 0.1 ml/L polypropylene glycol, 3 g/L K_2HPO_4, 6 g/L KH_2PO_4, 2 mg/L thiamine, 100 mg/L ampicillin and 1 ml/L trace element solution (4 g/L $FeSO_4$*7 H_20, 1 g/L $MnSO_4$*H_20, 0.4 g/L $CoCl_2$, 0.15 g/L $CuSO_4$* 5 H_2O, 0.1 g/L H_3BO_3, 0.2 g/L $ZnSO_4$*7 H_2O, 0.2 g/L Na_2MoO_4*2 H_2O and 0.4 g/L $FeCl_3$ dissolved in 5 M HCl). The fermentation culture was inoculated with a starter culture to an OD of 0.5 and the fermentation was carried out at 37°C. The culture was induced at an OD of 2.2 with 1 mM IPTG and further grown for 4 h at 37°C. 100 mg/L ampicillin was added again upon induction. The pH of the medium was maintained at 7.0 with 2 M KOH and 1 M H_2PO_4 and a minimum of 40% pO_2 was fixed. Polypropylene glycol was added automatically when needed. Cells were harvested at an OD of 26 by centrifugation at 5000 rpm for 30 minutes and stored at -20°C.

Samples were taken at different time points after induction in order to determine cell density, glucose consumption and the expression of the A13C/G120C mutant. The OD was measured using the Spectronic GENESYS 5 UV-Vis Spectrophotometer (Thermo Electron Corporation). The glucose consumption was measured using Diabur Test 5000 glucose test stripes (Roche Diagnostics). The expression of the A13C/G120C mutant was followed by SDS-PAGE. For SDS-PAGE the samples were centrifuged at 5000 rpm for 30 minutes and the cell pellets were suspended in 20 mM sodium phosphate buffer, pH 7.

2.6 Mass spectrometry protein sequence analysis

Sample preparation and MS analysis were performed at the Center for Medical Research, Medical University Graz.

Protein identification and internal sequence information was received from LC-MS/MS. Protein bands stained with Coomassie Brillant Blue R350 were excised from SDS gels and reduced, alkylated and digested with Promega modified trypsin according to a reported method (Shevchenko et al., 1996). Digests were separated by nano-HPLC (Agilent 1200 system, Vienna, Austria) equipped with a Zorbax 300SB-C18 enrichment column (5 µm, 5 x 0.3 mm) and a Zorbax 300SB-C18 nanocolumn (3.5 µm, 150 x 0.075 mm). 40 µl of sample were injected and concentrated on the enrichment column for 6 minutes using 0.1% formic acid as isocratic solvent at a flow rate of 20 µL/min. The column was then switched in the nanoflow circuit, and the sample was loaded on the nanocolumn at a flow rate of 300 nL/min. Separation was carried out using the following gradient, where solvent A was 0.3% formic acid in water and solvent B was a mixture of acetonitrile and water (4:1) containing 0.3% formic acid: 0-6 min: 13% B; 6-35 min: 13-28% B; 35-47 min: 28-50% B; 47-48 min: 50-100% B; 48-58 min: 100% B; 58-59 min: 100-13% B; 59-70 min: re-equilibration at 13% B. The sample was ionized in the nanospray source equipped with nanospray tips (PicoTipTM Stock FS360-75-15-D-20, Coating: 1P-4P, 15+/- 1µm Emitter, New Objective, Woburn, MA, USA). It was analyzed in a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operated in positive ion mode, applying alternating full scan MS (m/z 400 to 2000) in the ion cyclotron and MS/MS by collision induced dissociation of the 5 most intense peaks in the ion trap with dynamic exclusion enabled. The LC-MS/MS data were analyzed by searching the amino acid sequences of the human growth hormone proteins with Mascot 2.2 (MatrixScience, London, UK). A maximum false discovery rate of 0.05 using decoy database search, an ion score cut off of 20 and a minimum of 2 identified peptides was chosen as identification criteria.

2.7 Isolation, denaturation and refolding of inclusion bodies

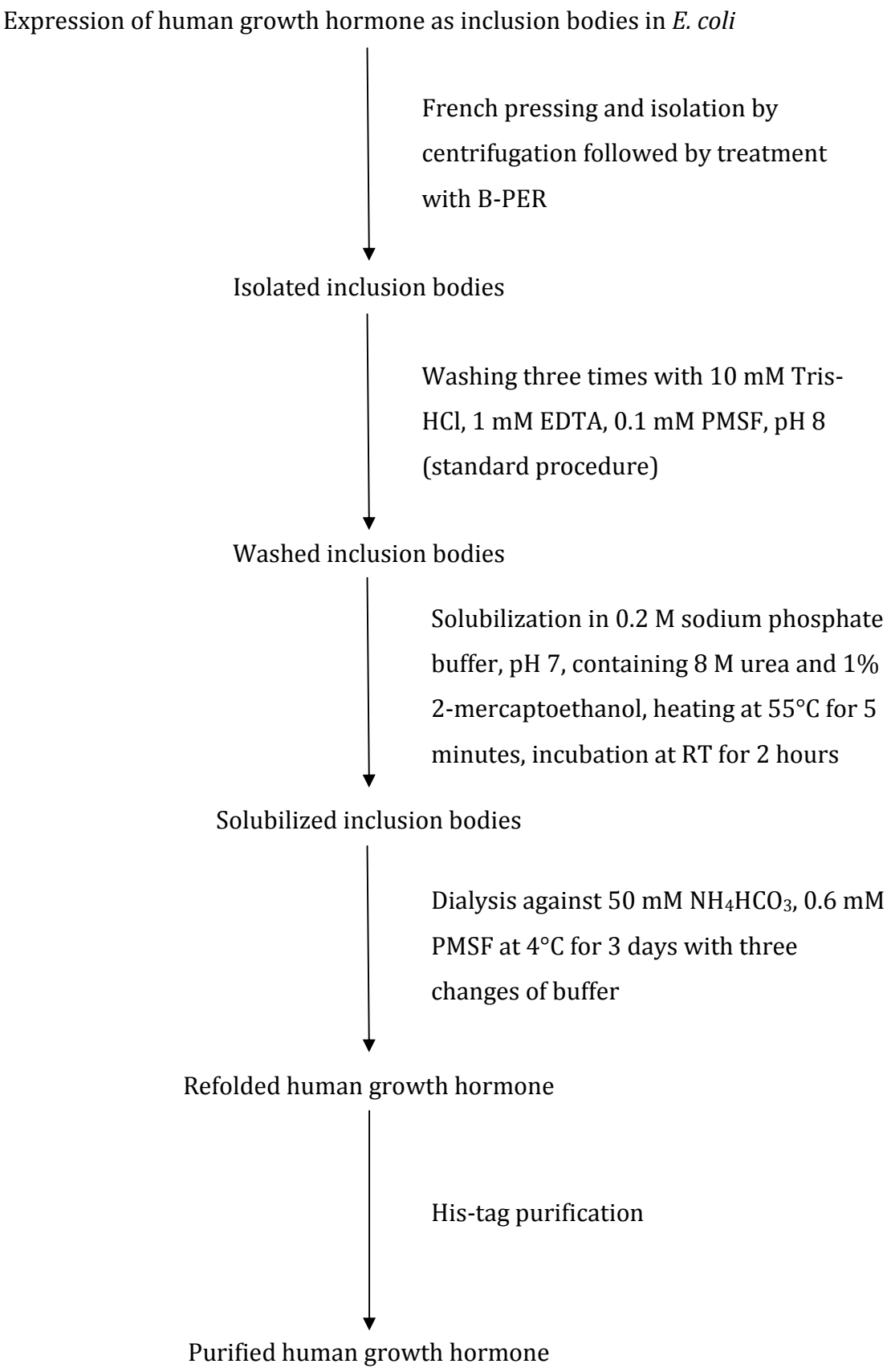

Figure 7: Recovery of native human growth hormone from inclusion bodies expressed in *E. coli*

The harvested cells from cultivation in shaking flasks and from fermentation were dissolved in 20 mM sodium phosphate buffer, pH 7. Cells were disrupted by French pressing at 18000 psi (Aminco French Press, American Instruments) and inclusion bodies were isolated by centrifugation either at 4°C at 13200 rpm for 30 minutes using a microcentrifuge or at 30000 rpm at 4°C for 30 minutes using an ultracentrifuge. The supernatant was discarded and the pellets were treated with B-PER bacterial protein extraction reagent (Thermo Scientific). The protein extraction procedure was performed according to manufactures protocol. Washing and refolding of the inclusion bodies followed a protocol described for human growth hormone and human prolactin (Jensen et al., 2008; Paris et al., 1990).

The inclusion bodies were washed three times with 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE buffer) including 0.1 mM PMSF. Afterwards, they were dissolved in 0.2 M sodium phosphate buffer, pH 7, containing 8 M urea and 1% 2-mercaptoethanol. The dissolved inclusion bodies were heated at 55°C for 5 min and then incubated at room temperature for 2 hours. The solution was diluted to a concentration of 0.1 mg/mL with 0.2 M sodium phosphate buffer, pH 7, containing 8 M urea and 1% 2-mercaptoethanol and renaturated by dialysis against 50 mM NH_4HCO_3, 0.6 mM PMSF at 4°C for 3 days with three changes of buffer. The precipitates formed during dialysis were removed by centrifugation at 14000 rpm for 15 minutes at 4°C.

2.7.1 Alternative washing procedures

In the following, the washing procedure described above is indicated as standard washing procedure.

Two alternative washing procedures were carried out using the same conditions as described above but different washing solutions. Apart from the washing step, the isolation and refolding of the inclusion bodies was done according to the protocol described above.

2.7.2 Alternative washing procedure including urea

The inclusion bodies were washed first with the standard washing solution 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE buffer) including 0.1 mM PMSF supplemented with 4 M urea. Afterwards, they were washed again three times with the standard washing solution.

2.7.3 Alternative washing procedure including triton X-100

The inclusion bodies were washed first with a solution containing 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8, supplemented with 0.5% triton X-100. Afterwards, they were washed two times with 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.

2.8 Purification

Since the concentration of the solution containing the refolded inclusion bodies was very low a concentrating step prior to purification was necessary. Therefore, devices such as Vivaspin 20 or Vivaflow 50 (Sartorius GmbH) were used. Purified peak fractions were pooled and concentrated with these devices as well. For purification attempts material gained from fermentation was isolated, refolded, concentrated and loaded on the column.

2.8.1 His-tag purification

Immobilized metal ion affinity chromatography (IMAC) was used for purification of the his-tagged human growth hormone. Two different columns were used, a 15 mL Cu-Chelate-Sepharose column and a 5 mL Ni-Chelate-Sepharose column (HisTrap HP column, GE Healthcare). The purification procedure was carried out using the ÄKTAprime plus system. The equilibration buffer (buffer A) used was 50 mM sodium phosphate buffer, pH 7, 30 mM imidazol. The elution buffer (buffer B) used was 50 mM sodium phosphate buffer, pH 7, 400 mM imidazol.

The protein solution was sterile filtered with a 0.2 µm PES membrane syringe filters (VWR International GmbH) and applied to the column at a flow rate of 2 mL/min. His-tagged human growth hormone was eluted using a linear gradient from 30 to 400 mM imidazol at a flowrate of 3 mL/min.

In case of the runs using the HisTrap HP column (GE Healthcare) the purification was carried out using the following gradient where buffer A was 50 mM sodium phosphate buffer, pH 7, 30 mM imidazol and buffer B was 50 mM sodium phosphate buffer, pH 7, 400 mM imidazol: 0-10 min: 100% A (load, flowrate 2 mL/min); 10-30 min: 100% A (inject, flowrate 1 mL/min); 30-45 min: 100% A (load, flowrate 1 mL/min); 45-70 min: 100% A (load, flowrate 3 mL/min); 70-170 min: 100-0% A (load, flowrate 3 mL/min).

The UV absorbance was measured at 280 nm. Peak fractions were pooled, concentrated and checked with SDS-PAGE.

2.8.2 DEAE ion-exchange purification

The protein solution was loaded on a 50 mL DEAE-sepharose column. Elution was carried out using increasing salt concentration from 0 to 1 M NaCl in 50 mM sodium phosphate buffer, pH 7, over 20 CV with a flow rate of 5 mL/min.

2.9 Fluorescence measurement

Fluorescence measurements were performed on a Fluorescence Spectrophotometer F-4500 (Hitachi). Quartz cuvettes (Hellma GmbH) were used. Intrinsic tryptophan fluorescence was measured by excitation at 280 and 295 nm and the emission spectrum was read between 300 and 500 nm. Solutions containing 0.1 mg/mL human growth hormone were measured. ANS fluorescence was excitated at 388 nm and the emission spectrum was read between 400 and 650 nm. 50 µL of 1 mM ANS were added to 500 µL human growth hormone (0.1 mg/ml). The used photomultiplier voltage was 950 V and the used slit width was 5 nm. The spectrum was recorded three times per sample and each smoothed afterwards.

3 RESULTS AND DISCUSSION

3.1 Genetic work

3.1.1 Overlap extension PCR

The identification of positive transformants containing the plasmid generated by overlap extension PCR was performed by colony PCR as shown in figure 8. The Gene Ruler DNA Ladder Mix (100-10000 bp) is shown in lane 1 (from left to right). The amplified fragments are indicated on the gel and were identified in lane 2, 3, 4, 6, 7, 8, 9 and 10 (from left to right). The amplified fragments were supposed to contain about 660 bp which corresponded to the size observed on the gel.

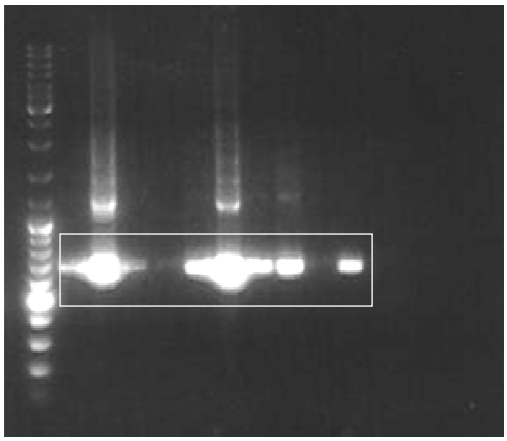

Figure 8: Gel electrophoresis of the colony PCR products

The DNA sequence of the positive transformants according to colony PCR was analyzed. The insertion of the megaprimer including the his-tag and the TEV protease cleavage site into the human growth hormone plasmid by overlap extension PCR was verified. Positive transformants were chosen and used for further experiments.

3.1.2 Site directed mutagenesis

The presence of the introduced mutations was verified by DNA sequence analysis. The DNA sequence of the WT and the A13C/G120C mutant is shown in the appendix. The relevant parts of the chromatograms of DNA sequence analysis of the A13C and the A13C/G120C mutant are appended as well.

3.2 Protein expression

Recombinant BL21 (DE3) cells harbouring the WT, the A13C mutant or the A13C/G120C mutant were grown in shaking flasks. The cultivation details are described in the methods. The cells were harvested after 4 hours of expression at an OD of about 1.9-2.2. The cell wet weight determined after harvesting was about 1.2-2.6 g/L.

3.3 Fermentation

High-level production of recombinant human growth hormone as inclusion bodies in *E. coli* was described in literature. Recombinant human growth hormone was expressed during fed-batch fermentation, where the fermentation was carried out at 37°C. Upon induction with 1 mM IPTG, the cells were cultivated for 4 hours, and then harvested. It was described that after 3 hours of induction the level of recombinant human growth hormone expression reached a certain level which was maintained after 4 hours of induction (Patra et al., 2000).

During batch fermentation the A13C/G120C mutant was expressed in BL21 (DE3) *E. coli* cells as inclusion bodies. The cell growth and the glucose consumption were followed during fermentation as shown in figure 9.

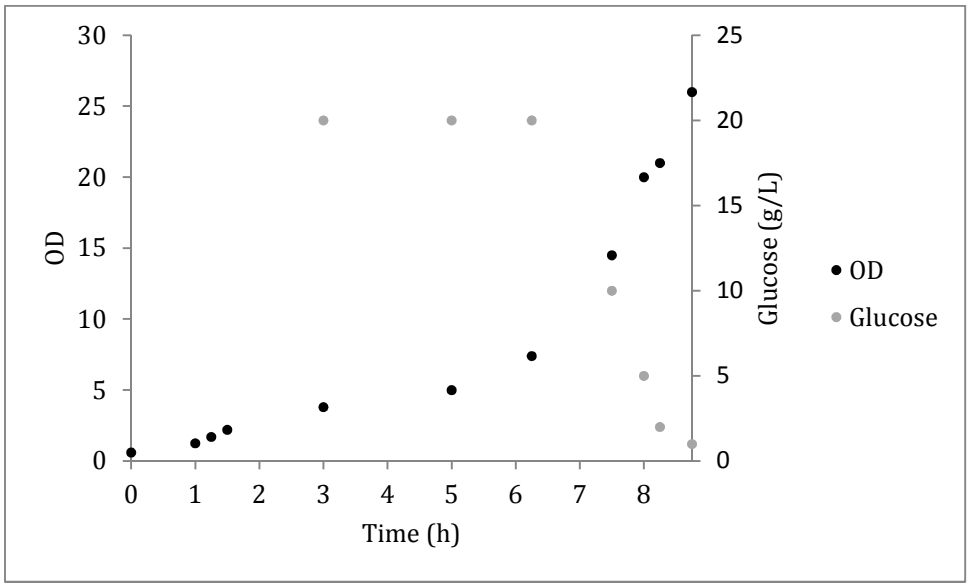

Figure 9: Cell growth and glucose consumption during fermentation

The cells were induced at an OD of 2.2 after 1.5 hours and the glucose consumption was first measured after 3 hours of fermentation. The cells were harvested upon depletion of glucose at an OD of 26.

The expression of the recombinant human growth hormone mutant was followed by SDS-PAGE as shown in figure 10. SDS-PAGE shows the overexpression whereas it seems that after 3.5 hours of induction the level of expression decreases. Since it is known from literature that the level of expression maintained after 3 hours of induction it might be assumed that this is the case for this fermentation as well. But the fact that the level of expression decreased might lead to the assumption that the expressed human growth hormone mutant was degraded upon 3.5 hours after induction. Further investigation of this assumption would be necessary in order to gain evidence.

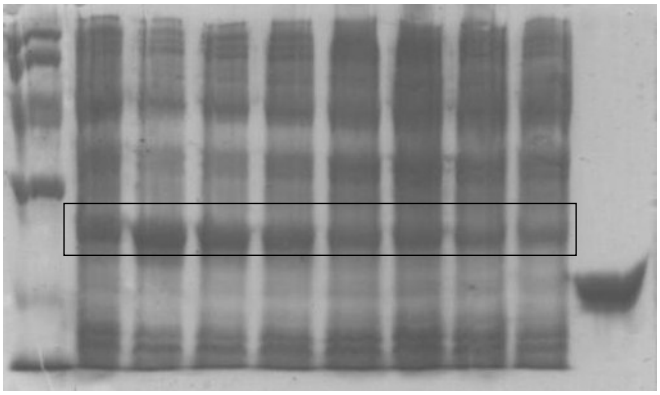

Figure 10: SDS-PAGE of the expression of recombinant human growth hormone during fermentation

Lane 1 (from left to right) shows the LMW standard; lane 2 to 9 (from left to right) show the induced cells after 0, 1.5, 3.5, 4.75, 6, 6.5, 6.75 and 7.25 hours after induction, respectively; lane 10 shows the reference human growth hormone. The difference between the reference human growth hormone and the expressed protein results from the difference in MW because of the his-tag, the TEV protease cleavage site and other amino acids added to the generated human growth hormone. The difference in MW is 2.3 kDa which corresponds to the difference observed on the gel. The MW of the reference human growth hormone is 22.1 kDa and the MW of the generated human growth hormone is 24.4 kDa.

The cells were harvested and the cell wet weight of 31 g/L was determined. The fermentation resulted in an about 15-fold higher yield compared to the cultivation in shaking flasks.

3.4 Mass spectrometry protein sequence analysis

Protein identification results received from LC-MS/MS of the human growth hormone expressed during fermentation showed that a mixture of the WT and A13C/G120C mutant was present. Related data is not shown in this master thesis. However, the material gained from fermentation was used for all following refolding and purification experiments.

By reason of these results, in order to gain the A13C and the A13C/G120C mutant in pure form, a new transformation was performed. Therefore, the presence of the mutations was verified by DNA sequence analysis as shown in the appendix. The positive transformants were cultivated in shaking flasks and the sequence of the expressed proteins was analysed by mass spectrometry. The overview of the related data received from LC-MS/MS is appended. The protein view shows the sequence coverage of the protein where the matched peptides are shown in bold red. Furthermore, the list of the found peptides with the respective delta mass and ions score is shown. Peptides with ions score below 20 are not shown as matched. The data shows that the A13C as well as the A13C/G120C mutant was obtained in pure form.

3.5 Denaturation of human growth hormone in the presence of urea

3.5.1 Denaturation of human growth hormone in the presence of 8 M urea

The influence of different denaturing conditions including 8 M urea on human growth hormone was investigated by intrinsic fluorescence measurement. The intrinsic fluorescence was excitated at 295 nm (tryptophan fluorescence). Intrinsic tryptophan fluorescence provides information about changes in protein folding and conformation. The red-shift of the emission maximum indicates the exposure of the tryptophan (in the inside of the native human growth hormone) to the hydrophilic environment (Brems et al., 1990).

Samples containing 0.5 mg/mL reference human growth hormone (in 0.2 mM sodium phosphate buffer, pH 7) were denatured using the following conditions: 8 M urea, 0.2 M sodium phosphate buffer (heated at 55°C for 10 minutes followed by incubation at room temperature for 2 hours); 8 M urea, 0.2 M sodium phosphate buffer (incubated at room

temperature for 2 hours); 8 M urea, 1% 2-mercaptoethanol, 0.2 M sodium phosphate buffer (heated at 55°C for 10 minutes followed by incubation at room temperature for 2 hours); 8 M urea, 1% 2-mercaptoethanol, 0.2 M sodium phosphate buffer (incubated at room temperature for 2 hours); native human growth hormone in 0.2 M sodium phosphate buffer. The intrinsic tryptophan fluorescence of the native and the respective denatured human growth hormone is shown in figure 11.

The emission spectrum of native human growth hormone (F) shows a maximum at 335 nm. (A) and (B) show a maximum at the same wavelength. This means, that although the intensity is quenched, 8 M urea in the absence of 1% 2-mercaptoethanol does not lead to complete unfolding of the native protein, neither with heating, nor without. Denaturation with 8 M urea and 1% 2-mercaptoethanol without heating (C) shows even more quenching and a red-shift of 4 nm (from 335 to 339 nm). Denaturation under the same conditions but heating (D) shows a red-shift of 5 nm (from 335 to 340 nm).

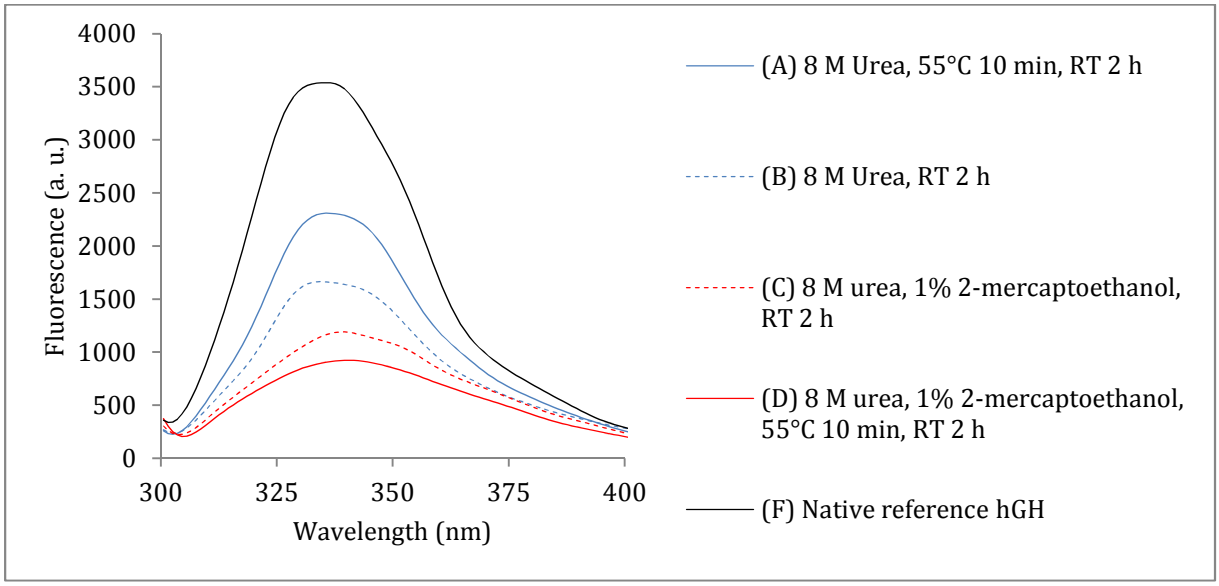

Figure 11: Intrinsic tryptophan emission spectra of native and denatured human growth hormone

Addition of 1 M urea to 0.5 mg/mL human growth hormone in 0.2 mM sodium phosphate buffer, pH 7, followed by heating at 55°C for 10 minutes shows a decrease of intrinsic fluorescence intensity compared to native human growth hormone (0.5 mg/mL in 0.2 mM sodium phosphate buffer). Further addition of urea under the same conditions neither decreased the intrinsic fluorescence intensity further nor resulted in a red-shift of the emission maximum as illustrated in figure 12. Only addition of 8 M urea along with 1% 2-mercaptoethanol to 0.5 ml/mL human growth hormone (followed by

heating at 55°C for 10 minutes) resulted in significant changes of the emission spectrum. The emission maximum shifted from 335 to 341 nm and a significant decrease of fluorescence intensity was observed compared to native human growth hormone as well as human growth hormone treated with different urea concentrations and heating in the absence of 1% 2-mercaptoethanol.

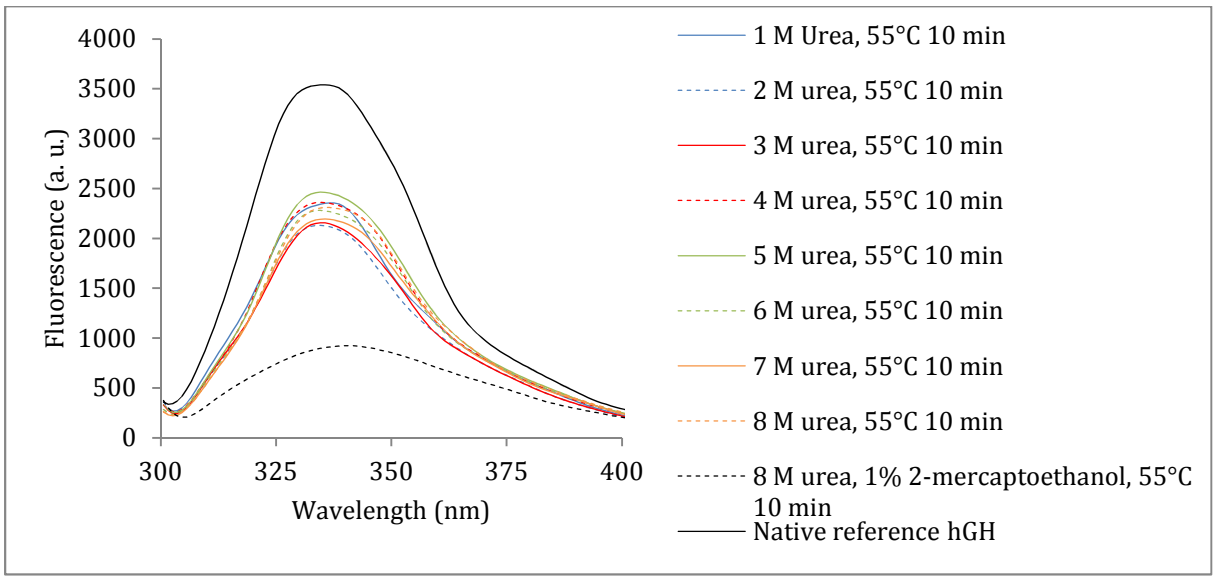

Figure 12: Intrinsic tryptophan emission spectra of native and denatured human growth hormone

According to these observations it was decided that for further denaturation procedures 8 M urea, 1% 2-mercaptoethanol, 0.2 M sodium phosphate, heated at 55°C for 10 minutes followed by incubation at room temperature for 2 hours was used.

Due to the fact that the human growth hormone is not completely denatured with urea without heating and in the absence of 1% 2-mercaptoethanol it was considered that urea can be used for washing of the inclusion bodies since it does not solubilise the human growth hormone but might solubilise other proteins and thus can serve as pre-purification agent. The purity of inclusion bodies has an influence on renaturation and refolding since contaminants promote co-aggregation. Therefore, inclusion bodies are washed with low concentrations of denaturants or detergents such as Triton X-100 (Cabrita et al., 2004).

3.6 Washing procedures for inclusion bodies

3.6.1 Standard washing procedure

The standard washing procedure is shown in figure 13. Lane 1 (from left to right) shows the LMW standard; lane 2 shows the crude extract from French pressing; lane 3 shows the crude extract from B-PER treatment; lane 4 shows the inclusion bodies after the first washing step; lane 5 shows the inclusion bodies after the second washing step; lane 6 shows the inclusion bodies after the third washing step; lane 7 shows the denatured inclusion bodies.

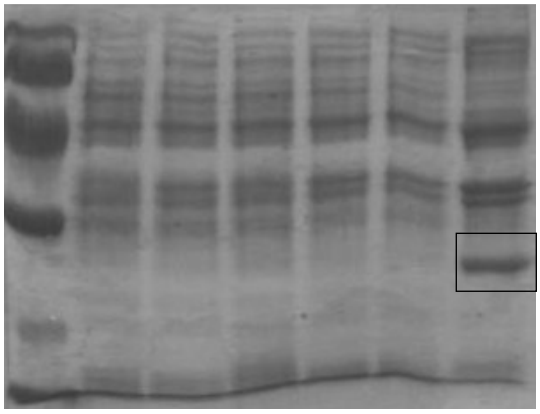

Figure 13: SDS-PAGE of the standard washing procedure

3.6.2 Alternative washing procedures

Alternative washing procedures might include low concentrations of denaturing agents, such as urea or guanidine hydrochloride, or detergents, such as Triton X-100. The purpose of washing of the inclusion bodies is the removal of contaminant proteins, that accumulate on the hydrophobic surface of inclusion bodies which might decrease the protein yield during refolding (De Bernardez Clark, 1998).

3.6.3 Alternative washing procedure including urea

The SDS-PAGE of the washing procedure including urea is shown in figure 14. Lane 1 (from left to right) shows the LMW standard; lane 2 shows the crude extract from French pressing; lane 3 shows the crude extract from B-PER treatment; lane 4 shows the inclusion bodies after the first washing step (washing solution supplemented with urea); lane 5 shows the inclusion bodies after the second washing step; lane 6 shows the

inclusion bodies after the third washing step; lane 7 shows the denatured inclusion bodies; lane 8 shows the refolded protein.

To compare the protein pattern of the denatured inclusion bodies in figure 13 with figure 14, no significant difference was observed. Thus, the anticipated pre-purification by including urea in the washing procedure was not observed.

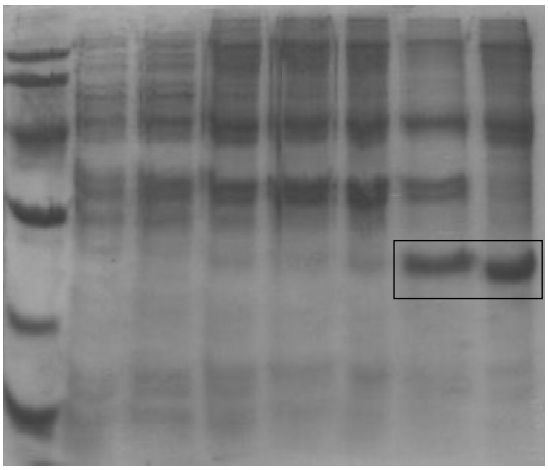

Figure 14: SDS-PAGE of the washing procedure including urea

3.6.4 Alternative washing procedure including triton X-100

The SDS-PAGE of the washing procedure including triton X-100 is shown in figure 15. Lane 1 (from left to right) shows the crude extract from French pressing; lane 2 shows the crude extract from B-PER treatment; lane 3 shows the inclusion bodies after the first washing step (washing solution supplemented with triton X-100); lane 4 shows the inclusion bodies after the second washing step; lane 5 shows the inclusion bodies after the third washing step; lane 6 shows the denatured inclusion bodies; lane 7 shows the refolded protein; lane 8 shows the LMW standard.

Again, no significant difference between the protein pattern of the denatured inclusion bodies between figure 13 and figure 15 is observed. Additionally, the indicated human growth hormone bands in figure 15 show decreased intensity.

In respect of these results the standard washing procedure was used for all further experiments.

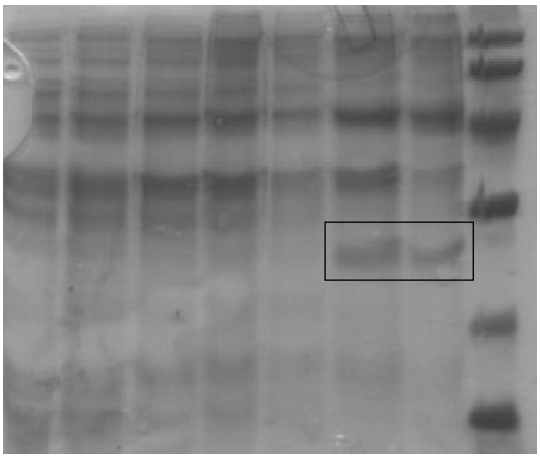

Figure 15: SDS-PAGE of the washing procedure including triton X-100

3.7 Purification

Since the refolding solution contained a low protein concentration, concentrating using Vivaspin 20 and Vivaflow 50 was necessary, where a general problem observed was the loss of protein during the procedures. Additionally, protein was lost during the filtration with 0.2 µm PES membrane syringe filters. These problems restricted the amount of protein available for loading on the column.

3.7.1 His-tag purification

IMAC was used for the purification of the his-tagged human growth hormone. IMAC is based on the interaction between a transition metal ion (Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}) immobilized on a matrix and specific amino acid side chains, in this case histidine residues (Terpe, 2003).

Initially, the purification was attempted with a 15 mL Cu-Chelate-Sepharose column. Several attempts were made but in general, the purification with this column was not successful. Problems occurred including the washout of copper from the column and loss of total protein over the whole procedure.

The decision to supplement the equilibration buffer with 30 mM imidazol was made because during previous purification runs it was observed that at 10 mM imidazol a peak containing contaminated proteins occurred. The purification was attempted with a 5 mL His Trap HP column (GE Healthcare). 1.8 mg protein was applied to the column. The chromatogram is illustrated in figure 16, showing the UV absorption at 280 nm and the % of buffer B. The human growth hormone was eluted at about 120 mM imidazol.

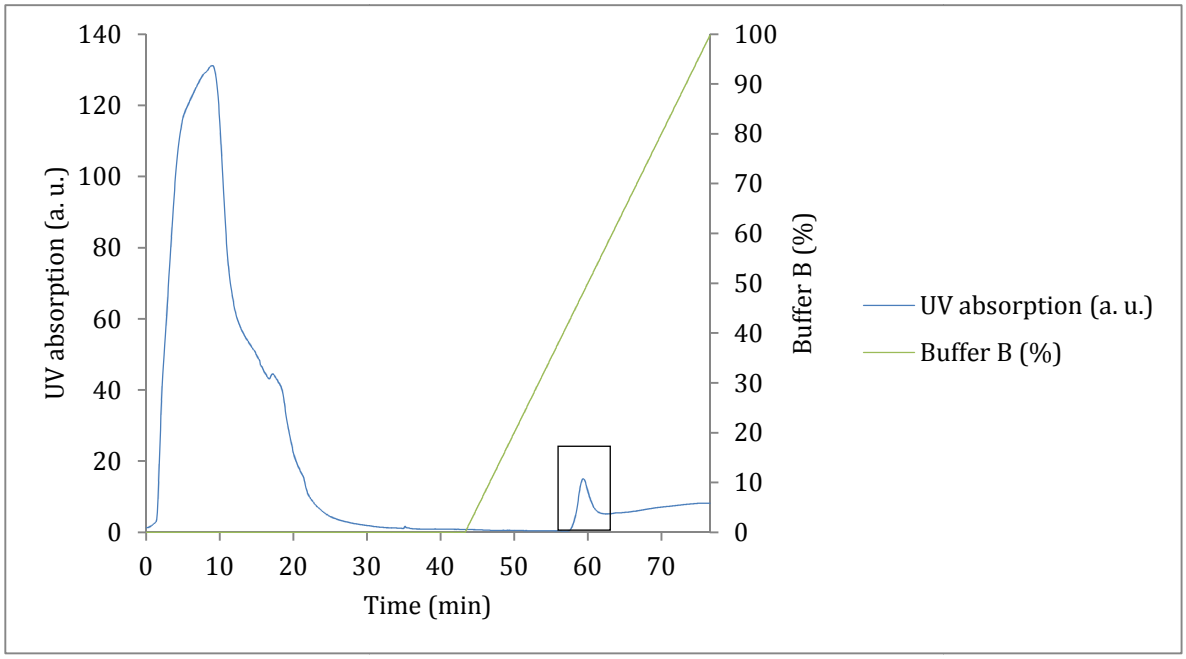

Figure 16: His-tag purification with a 5 ml His Trap HP column and a linear imidazol gradient

The SDS-PAGE of the relevant fractions of the purification is shown in figure 17. Lane 1 (from left to right) shows the LMW standard; lane 2 shows the protein solution applied to the column (before purification); lane 3 and lane 4 show the fractions of the loading peak; lane 5 shows the fraction of interest (indicated in the chromatogram); lane 6 shows again the LMW standard. In the fraction of interest almost, but not completely pure human growth hormone was found. The protein concentration of this fraction was determined and the total amount of protein of about 0.38 mg was calculated. Further purification steps could be considered but were not possible in our case because of the very small amount of protein.

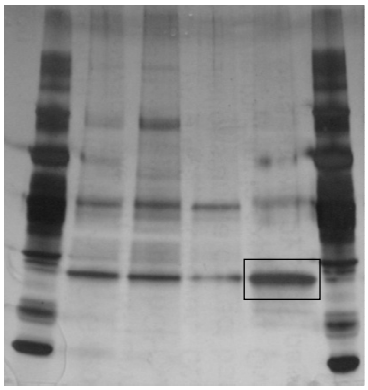

Figure 17: SDS-PAGE of the his-tag purification with a 5 ml His Trap HP column and a linear imidazol gradient

Proteins bind to the column depending on the presence of surface histidine, cysteine and tryptophan residues which have an affinity for chelated metal ions. Thus, other proteins

but the his-tagged human growth hormone might also bind to the column. Factors which affect the purification of his-tagged proteins include the position and the length of the tag. Since the SDS-PAGE shows that his-tagged human growth hormone was found in the loading peak it was assumed that the his-tag was not completely accessible for binding to the column.

3.7.2 DEAE ion-exchange purification

The purification of recombinant human growth hormone (without a tag and a protease cleavage site) by ion-exchange chromatography was reported in literature. In literature, recombinant human growth hormone was purified with a DEAE-sepharose ion-exchange column where human growth hormone was eluted between 14 and 16 mS/cm conductivity (Patra et al., 2000; Singh et al., 2005).

The attempt to purify with DEAE ion-exchange column was not successful.

3.8 Protein yield during the overall isolation, denaturation, refolding and purification process

During the overall process of protein isolation, washing of inclusion bodies, denaturation, refolding and purification there were several steps identified where loss of protein occurred.

First of all, the amount of inclusion bodies isolated depends on the quality of the cell disruption. During the washing procedure, inclusion bodies are washed out. The loss of protein during these steps was assumed not to address the problem but it was found that the following steps are responsible for the main loss of protein. The formation of aggregates during refolding leads to low protein yields. It was found that approximately 25-35% of protein was recovered during the refolding procedure due to aggregate formation. The loss of protein during concentrating steps as well as the formation of aggregates during storage of the concentrated solution which led to a decreased concentration of soluble protein was a great problem.

An example of a whole process, from protein isolation to protein purification (purification run described above) is illustrated in table 9. The cell wet weight of 7.9 g was disrupted for this example. In general, about 5.5 mg protein per g of cell wet weight was obtained.

Table 9: Protein yield during the overall isolation, denaturation, refolding and purification process

	Protein concentration (mg/mL)	Volume (mL)	Protein (mg)	Recovery (%)
hGH denatured	0.083	520	43.2	100
hGH refolded	0.027	520	14.0	33
hGH refolded and concentrated	0.326	35	11.4	26
hGH refolded, centrifuged and concentrated after a few days	0.180	10	1.8	4
hGH purified	0.126	3	0.4	1

Since 144 g of cell wet weight was obtained from 5 L fermentation a high amount of 790 mg protein could be obtained. However, the problem was that the amount of protein used for the refolding procedure was restricted due to the need of low concentrations of protein in the refolding solution which leads to high volumes. For practical reasons, not more than 600 ml of protein solution were refolded at once. The overall yield of purified human growth hormone out of the denatured inclusion bodies was only 1%. Thus, the overall process was not considered to be successful in terms of human growth hormone yield which led to the conclusion that an alternative strategy of recovering human growth hormone out of inclusion bodies must be developed.

3.9 Fluorescence measurements of the purified human growth hormone

3.9.1 Intrinsic tryptophan fluorescence spectra of purified human growth hormone

Human growth hormone has one tryptophan residue which is pointing towards the inside of the native protein. Studying the tryptophan fluorescence of a protein gives information about conformational changes.

The following fluorescence spectra show the purified human growth hormone in comparison with the native reference human growth hormone. 0.1 mg/mL human growth hormone was measured for each, the reference as well as the purified human growth hormone. The tryptophan fluorescence of the purified human growth hormone was found to be significantly quenched as shown in figure 18. The intensity was decreased by about 55% compared to the reference human growth hormone. Figure 19 shows the normalized fluorescence spectra, whereas a red-shift from 331.6 nm to 334.6 nm occurred.

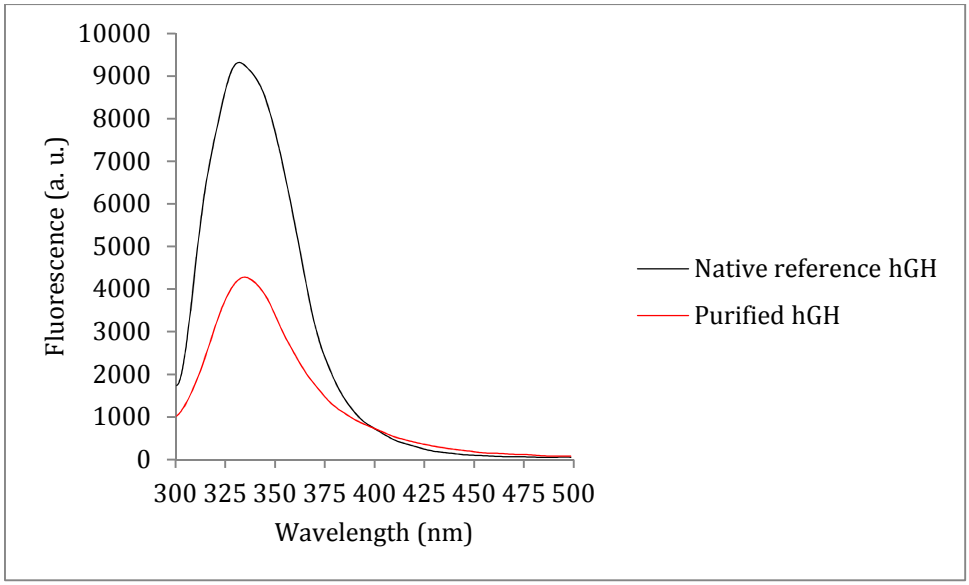

Figure 18: Fluorescence emission spectra of native reference and purified human growth hormone excitated at 280 nm

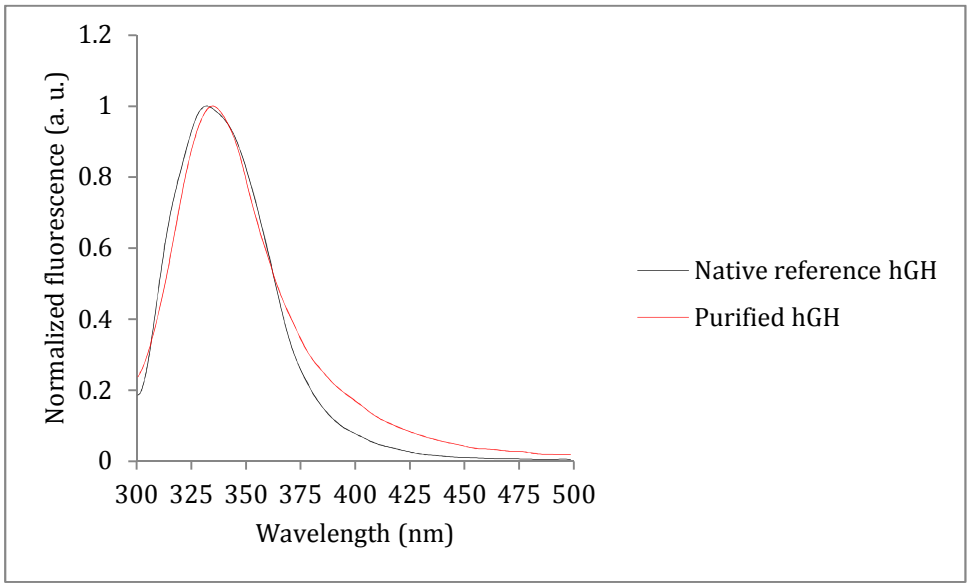

Figure 19: Normalized fluorescence emission spectra of native reference and purified human growth hormone excitated at 280 nm

The intrinsic tryptophan fluorescence spectra induced at 295 nm of the native reference human growth hormone compared with the human growth hormone gained from purification are shown in figure 20. The normalized spectra are shown in figure 21. The intrinsic tryptophan fluorescence of the purified human growth hormone shows a significant decrease of about 65% compared to the native reference protein. Furthermore, the fluorescence emission maximum of the purified human growth hormone was observed at 341 nm compared to the native reference human growth hormone where the fluorescence emission maximum was observed at 333 nm. The red-shift of 8 nm indicates the exposure of the tryptophan residues to the solvent. According to these results it was indicated, that the refolded and purified human growth hormone was not present in the native conformation where the tryptophan residue is in the inside of the folded protein. However, this conclusion could not be made, because the analyzed human growth hormone was not completely pure and the influence of the impurities on the fluorescence spectrum is not known.

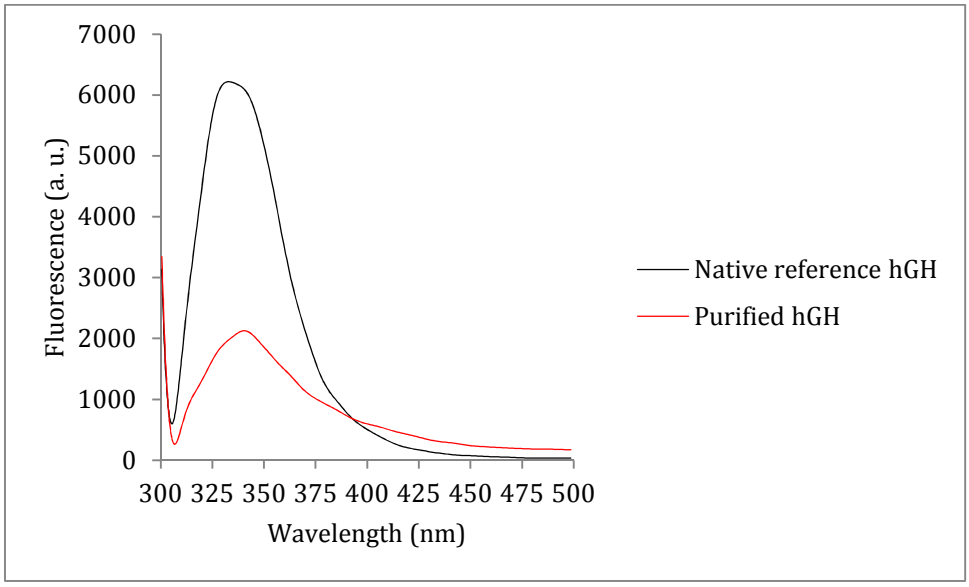

Figure 20: Fluorescence emission spectra of native reference and purified human growth hormone excitated at 295 nm

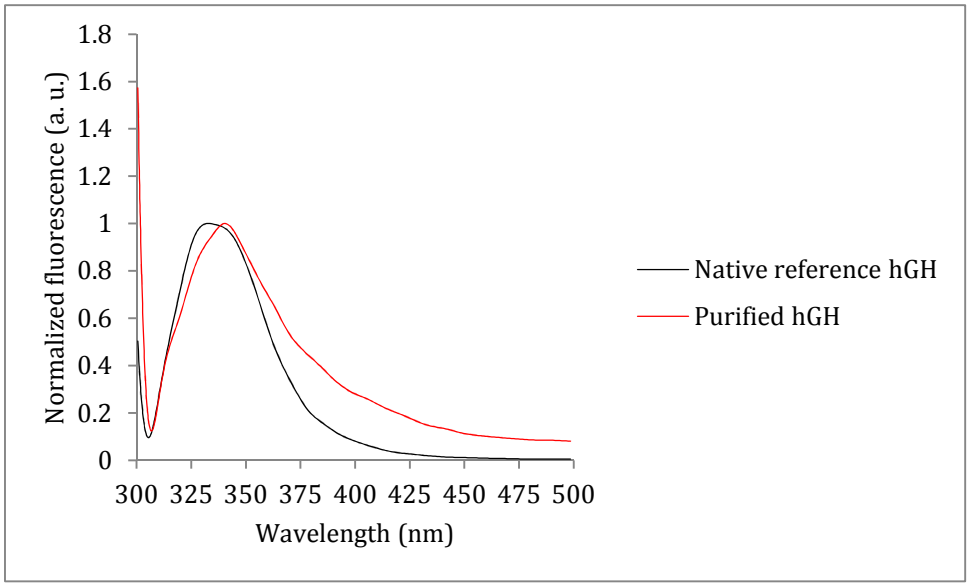

Figure 21: Normalized fluorescence emission spectra of native reference and purified human growth hormone excitated at 295 nm

3.9.2 ANS fluorescence

ANS is a charged hydrophobic fluorescent dye which is widely used for studying partially folded proteins. ANS binds to hydrophobic amino acids, normally present in the inside of the native protein. Partially folded states of proteins expose hydrophobic side chains to the solvent and thus provide binding sites for ANS (Ali et al., 1999; Swietnicki, 2006).

Consequently, the increase of ANS fluorescence intensity of the purified human growth hormone compared to the native reference human growth hormone as shown in figure 22 indicates structural changes. The purified human growth hormone was present in a partially folded or unfolded state. The emission maximum of ANS at 510 nm was observed for native reference human growth hormone. Decrease of intensity and change in wavelength of the emission maximum to 487 nm was observed for the purified human growth hormone. The results were obtained at a protein concentration of 0.1 mg/mL.

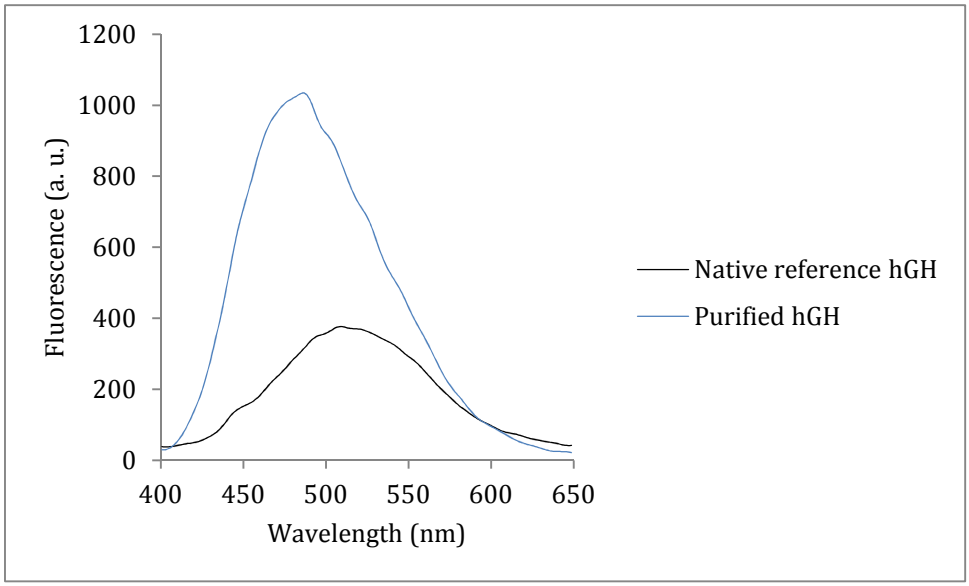

Figure 22: ANS fluorescence spectra excitated at 388 nm

3.9.3 Conclusion regarding the fluorescence measurements

The intrinsic as well as the ANS fluorescence measurements demonstrate that the refolded and purified human growth hormone was not found to be identical with the native reference human growth hormone. Thus, it was indicated that the refolded and purified human growth hormone was not recovered in the native conformation. However, this conclusion could not be made, because the analyzed human growth hormone was not completely pure and the influence of the impurities on the fluorescence spectrum is not known. Additionally, a modified form of the human growth hormone containing an additional amino acid sequence on the N-terminus of the protein was analyzed, where the influence of the modification on the fluorescence measurements is not known. Further analysis (e. g. CD spectroscopy, MS) would be necessary in order to gain evidence. In hindsight, the conclusion gained from figure 11, where the unfolding of the reference human growth hormone under the denaturing conditions was investigated, was reconsidered as described in the following.

3.10 Denaturation of human growth hormone in the presence of 6 M guanidine hydrochloride

The intrinsic tryptophan fluorescence spectra of the reference human growth hormone treated with urea under different conditions were compared with the spectrum of

reference human growth hormone treated with 6 M guanidine hydrochloride. Figure 23 shows that a significant difference occured.

It was reported elsewhere that the intrinsic tryptophan fluorescence emission maximum of human growth hormone undergoes a red-shift of 15 nm upon denaturation with 6 M guanidine hydrochloride (Brems et al., 1990).

Figure 23 shows a red-shift of the emission maximum of 18 nm (from 335 to 353 nm) when using 6 M guanidine hydrochloride. Thus it was assumed, that the human growth hormone is present in the completely unfolded state by use of 6 M guanidine hydrochloride, which might not be the case when the denaturing conditions of 8 M urea, 1% 2-mercaptoethanol, heating at 55°C for 5 minutes followed by incubation at RT for 2 hours were used. These observations might be the reason, why the refolded and purified human growth hormone could not be recovered in the native conformation.

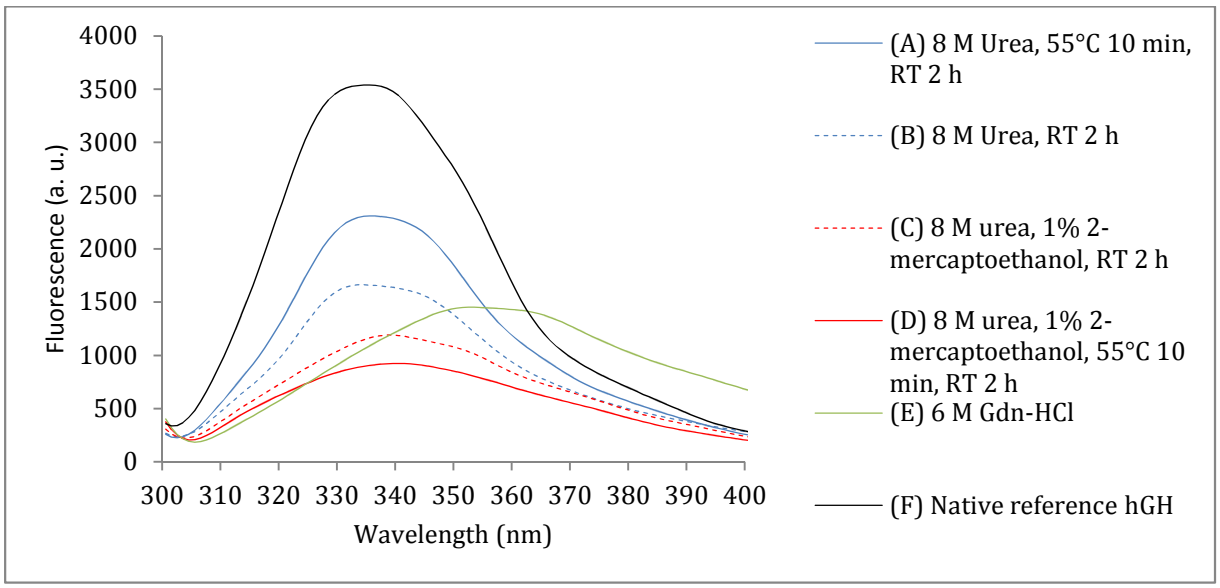

Figure 23: Intrinsic tryptophan emission spectra of native and treated human growth hormone under different conditions

4 CONCLUSION AND OUTLOOK

Since human growth hormone is a protein that is not glycosylated, several examples of the expression in prokaryotic systems such as *E. coli* were reported (Chang et al., 1987; Goeddel et al., 1979; Mukhija et al., 1995; Patra et al., 2000).

However, the expression of human growth hormone without a signal peptide in *E. coli* BL21 (DE3) leads to the formation of inclusion bodies. Problems come along with the required procedures of isolation, solubilization and refolding of the inclusion bodies. Since it was indicated that the refolded and purified human growth hormone was not present in the native conformation as shown by intrinsic tryptophan as well as extrinsic fluorescence, several steps in the procedure of recovering the native protein from inclusion bodies must be reconsidered. To gain evidence that the purified protein was not present in the native conformation further characterizations would be necessary. These characterizations might include circular dichroism (CD) in order to gain information about the secondary structure of the protein and SAXS measurements in order to gain structural information. Since the amount of protein available for these characterizations was too small, these characterizations were not done during this master thesis. Furthermore, MS measurement could be done in order to identify the presence or absence of the correct disulfide bonds in the protein.

The reasons for choosing *E. coli* as an expression system for the production of recombinant human growth hormone and mutants were on the one hand the presence of literature about the expression as inclusion bodies in *E. coli* (Jensen et al., 2008; Patra et al., 2000) and the refolding of the inclusion bodies (Jensen et al., 2008; Paris et al., 1990). On the other hand, it was planned for the future to investigate the self-association of human growth hormone by ^{15}N NMR relaxation. Therefore, it was planned to grow *E. coli* BL21 (DE3) on ^{15}N enriched medium which was reported in literature (Jensen et al., 2008).

Since it was found during this master thesis that with the applied methods of refolding and purification it was not possible to isolate a sufficient amount of human growth hormone other strategies of these critical steps of refolding and purification could be considered in the future. For the refolding procedure, the denaturation with guanidine hydrochloride could be considered due to the results found with intrinsic fluorescence

measurements where a significant difference between the fluorescence spectra of human growth hormone treated with 6 M guanidine hydrochloride was found in comparison to the protein treated with 8 M urea, 1% 2-mercaptoethanol, heating at 55°C for 5 minutes followed by incubation at RT for 2 hours. An alternative denaturation and refolding procedure using 2 M urea and an increased pH of 12.5 was reported in literature (Patra et al., 2000; Singh et al., 2005).

Working with inclusion bodies is time consuming and in most cases it is difficult to establish the proper conditions for denaturing and refolding (Cabrita et al., 2004). Thus, another strategy of expressing human growth hormone might be the use of eukaryotic expression systems, where *Pichia pastoris* becomes an alternative host organism. The expression of human growth hormone in *Pichia pastoris* was described in literature (Ecamilla-Treviño et al., 2000; Çalık et al., 2008).

In case of obtaining sufficient amounts of human growth hormone and mutants the implementation of experiments giving insight into the aggregation mechanism would in the best case allow the confirmation or disproval of the proposed domain swapping theory. These experiments might include differential scanning fluorimetry (DSF) or differential scanning colorimetry (DSC) in order to examine ΔG of unfolding for the WT compared to the mutants. The mutants might also be applied to mechanical stresses and the subsequent aggregation behavior might be studied.

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6 FIGURES

7 TABLES

8 APPENDIX

8.1 DNA sequence analysis

The DNA sequence of the WT human growth hormone including the his-tag (indicated in green) and the TEV protease cleavage site (indicated in blue) is shown in the following.

CACCACCACCACCACCACTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTACTTCCAATCC
TTCCCAACCATTCCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACC
AGCTGGCCTTTGACACCTACCAGGAGTTTGAAGAAGCCTATATCCCAAAGGAACAGAAGTAT
TCATTCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCTCCAACA
GGGAGGAAACACAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGT
CGTGGCTGGAGCCCGTGCAGTTCCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTACGGCGCCT
CTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAGGCATCCAAACGCTGATGGGGA
GGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACA
CAAACTCACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGG
ACATGGACAAGGTCGAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGTG
GCTTC

The DNA sequence of the A13C/G120C human growth hormone mutant including the his-tag (indicated in green) and the TEV protease cleavage site (indicated in blue) is shown in the following. The exchanged base pairs are indicated in red.

CACCACCACCACCACCACTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTACTTCCAATCC
TTCCCAACCATTCCCTTATCCAGGCTTTTTGACAACTGTATGCTCCGCGCCCATCGTCTGCACC
AGCTGGCCTTTGACACCTACCAGGAGTTTGAAGAAGCCTATATCCCAAAGGAACAGAAGTAT
TCATTCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCTCCAACA
GGGAGGAAACACAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGT
CGTGGCTGGAGCCCGTGCAGTTCCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTACGGCGCCT
CTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAATGCATCCAAACGCTGATGGGGA
GGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACA
CAAACTCACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGG
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GCTTC

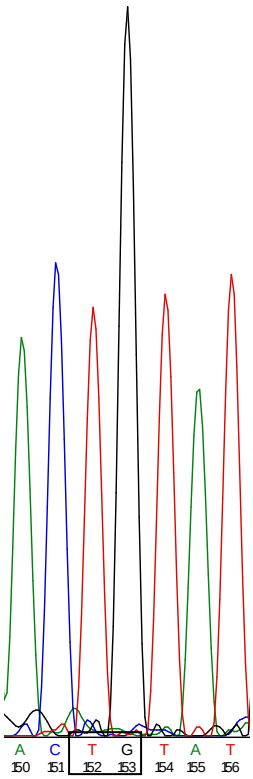


Figure 24: DNA sequence analysis of the A13C mutant

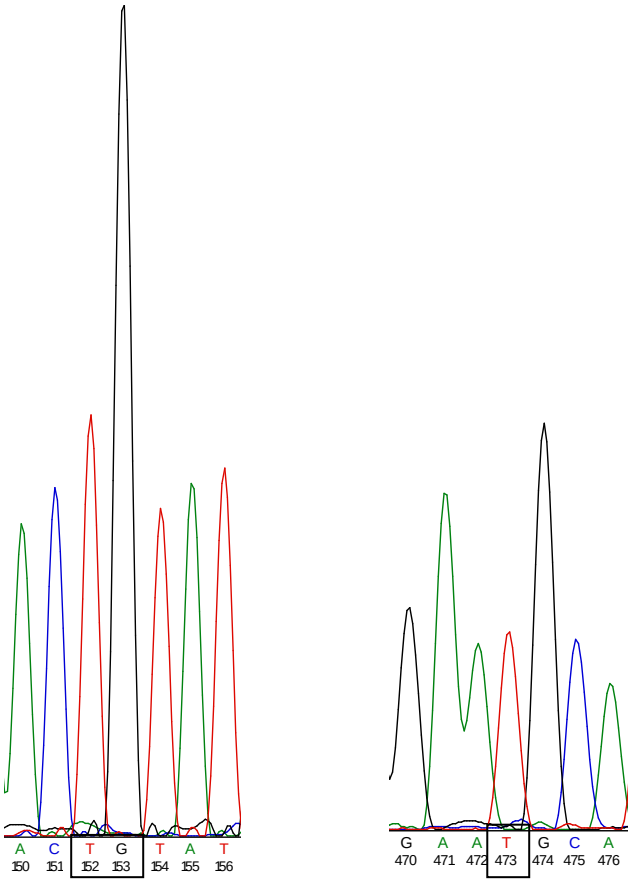


Figure 25: DNA sequence analysis of the A13C/G120C mutant

8.2 Mass spectrometry protein sequence analysis

The protein sequence of the WT human growth hormone is shown in the following, where the his-tag is indicated in green, the TEV protease cleavage site is indicated in blue and the human growth hormone sequence is indicated in black. The Ala^{13} and the Gly^{120} are indicated in red.

HHHHHHSSGVDLGTENLYFQSPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYS
FLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSN
VYDLLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDK
VETFLRIVQCRSVEGSCGF

The protein sequence of the A13C human growth hormone mutant is shown in the following, where the his-tag is indicated in green, the TEV protease cleavage site is indicated in blue and the human growth hormone sequence is indicated in black. The Cys^{13} is indicated in red.

HHHHHHSSGVDLGTENLYFQSPTIPLSRLFDNCMLRAHRLHQLAFDTYQEFEEAYIPKEQKYS
FLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSN
VYDLLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDK
VETFLRIVQCRSVEGSCGF

The protein sequence of the A13C/G120C human growth hormone mutant is shown in the following, where the his-tag is indicated in green, the TEV protease cleavage site is indicated in blue and the human growth hormone sequence is indicated in black. The Cys^{13} and the Cys^{120} is indicated in red.

HHHHHHSSGVDLGTENLYFQSPTIPLSRLFDNCMLRAHRLHQLAFDTYQEFEEAYIPKEQKYS
FLQNPQTSLCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSN
VYDLLKDLEECIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDK
VETFLRIVQCRSVEGSCGF

Mascot Search Results

Protein View

Match to: **P01241m1|A13C_Mutante-hGH** Score: **2437**
mit His-Tag und Tev-Proteaseschnittstelle
Found in search of E:\Data\Projects\RCPE3\RCPE 3 III\RCPE3_III_1.RAW

Nominal mass (M_r): **24705**; Calculated pI value: **5.69**
NCBI BLAST search of P01241m1|A13C_Mutante-hGH against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **59%**

Matched peptides shown in **Bold Red**

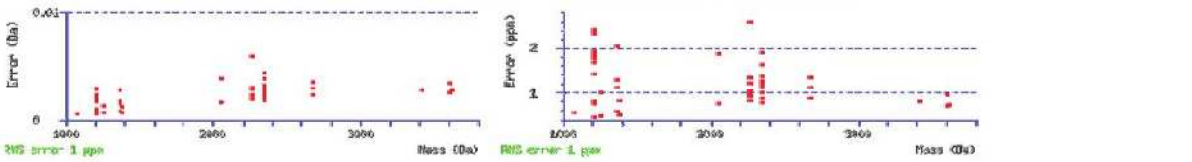
```
     1 HHHHHHSSGV DLGTENLYFQ SPTIPLSRLF DNCMLRAHRL HQLAFDTYQE
    51 FEEAYIPKEQ KYSFLQNPQT SLCFSESIPT PSNREETQQK SNLELLRISL
   101 LLIQSWLEPV QFLRSVFANS LVYGASDSNV YDLLKDLEEG IQTLMGRLED
   151 GSPRTGQIFK QTYSKFDTNS HNDDALLKNY GLLYCFRKDM DKVETFLRIV
   201 QCRSVEGSCG F
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
29 - 36	534.7522	1067.4898	1067.4892	0.0006	0	R.LFDNCMLR.A	(Ions score 68)
40 - 58	781.3834	2341.1285	2341.1266	0.0018	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 82)
40 - 58	781.3835	2341.1287	2341.1266	0.0020	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 82)
40 - 58	781.3836	2341.1290	2341.1266	0.0023	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 27)
40 - 58	781.3837	2341.1293	2341.1266	0.0027	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 67)
40 - 58	1171.5720	2341.1294	2341.1266	0.0028	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 128)
40 - 58	1171.5721	2341.1296	2341.1266	0.0030	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 139)
40 - 58	1171.5721	2341.1296	2341.1266	0.0030	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 148)
40 - 58	781.3839	2341.1298	2341.1266	0.0032	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 75)
40 - 58	1171.5725	2341.1304	2341.1266	0.0038	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 99)
40 - 58	1171.5728	2341.1310	2341.1266	0.0044	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 107)
62 - 84	891.7594	2672.2564	2672.2541	0.0023	0	K.YSFLQNPQTSLCFSESIPTPSNR.E	(Ions score 35)
62 - 84	1337.1358	2672.2570	2672.2541	0.0030	0	K.YSFLQNPQTSLCFSESIPTPSNR.E	(Ions score 81)

62 - 84	1137.1361	2672.2576	2672.2541	0.0036	0	K.YSFLQNFQFSLCFSESIPFPSNR.E	(Ions score 67)
62 - 90	1139.5412	3415.6018	3415.5990	0.0027	1	K.YSFLQNFQFSLCFSESIPFPSNRHHTQQX.S	(Ions score 100)
98 - 114	685.7387	2054.1944	2054.1928	0.0016	0	R.ISLLLIQSWLEPVQFLR.S	(Ions score 71)
98 - 114	1028.1056	2054.1966	2054.1928	0.0038	0	R.ISLLLIQSWLEPVQFLR.S	(Ions score 86)
115 - 135	1131.5690	2261.1234	2261.1216	0.0019	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 35)
115 - 135	1131.5691	2261.1236	2261.1216	0.0021	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 81)
115 - 135	1131.5692	2261.1238	2261.1216	0.0023	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 88)
115 - 135	754.7152	2261.1239	2261.1216	0.0024	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 23)
115 - 135	754.7154	2261.1244	2261.1216	0.0028	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 97)
115 - 135	1131.5696	2261.1246	2261.1216	0.0031	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 79)
115 - 135	1131.5710	2261.1274	2261.1216	0.0059	0	R.SVFANSLVYGASDSNVYDLLK.D	(Ions score 95)
115 - 147	1202.2670	3603.7732	3603.7767	0.0035	1	R.SVFANSLVYGASDSNVYDLLKDLEEGIQTLMGE.L	(Ions score 56)
115 - 147	1202.2673	3603.7801	3603.7767	0.0034	1	R.SVFANSLVYGASDSNVYDLLKDLEEGIQTLMGE.L	(Ions score 49)
115 - 147	1207.5987	3618.7743	3618.7716	0.0027	1	R.SVFANSLVYGASDSNVYDLLKDLEEGIQTLMGE.L	Oxidation (M) (Ions score 55)
136 - 147	681.3405	1360.6664	1360.5656	0.1008	0	K.DLIEGIQTLMGR.L	(Ions score 72)
136 - 147	681.3409	1360.6672	1360.5656	0.1015	0	K.DLIEGIQTLMGR.L	(Ions score 35)
136 - 147	1361.6747	1360.6674	1360.5656	0.1018	0	K.DLIEGIQTLMGR.L	(Ions score 33)
136 - 147	1361.6757	1360.6694	1360.5656	0.1038	0	K.DLIEGIQTLMGR.L	(Ions score 56)
136 - 147	689.3379	1376.6613	1376.5606	0.1007	0	K.DLIEGIQTLMGR.L	Oxidation (M) (Ions score 77)
136 - 147	1377.6690	1376.6617	1376.5606	0.1012	0	K.DLIEGIQTLMGR.L	Oxidation (M) (Ions score 33)
179 - 187	603.2925	1204.5704	1204.5699	0.0005	0	K.NYGLLYCFR.K	(Ions score 63)
179 - 187	1205.5781	1204.5708	1204.5699	0.0009	0	K.NYGLLYCFR.K	(Ions score 46)
179 - 187	603.2927	1204.5709	1204.5699	0.0010	0	K.NYGLLYCFR.K	(Ions score 73)
179 - 187	1205.5789	1204.5726	1204.5699	0.0027	0	K.NYGLLYCFR.K	(Ions score 30)
179 - 187	1205.5792	1204.5719	1204.5699	0.0020	0	K.NYGLLYCFR.K	(Ions score 37)
179 - 187	1205.5791	1204.5720	1204.5699	0.0021	0	K.NYGLLYCFR.K	(Ions score 40)
179 - 187	1205.5794	1204.5721	1204.5699	0.0022	0	K.NYGLLYCFR.K	(Ions score 43)
179 - 187	1205.5795	1204.5722	1204.5699	0.0023	0	K.NYGLLYCFR.K	(Ions score 40)
179 - 187	1205.5800	1204.5727	1204.5699	0.0028	0	K.NYGLLYCFR.K	(Ions score 23)
179 - 187	1205.5801	1204.5728	1204.5699	0.0029	0	K.NYGLLYCFR.K	(Ions score 33)
189 - 198	627.3137	1252.6128	1252.5122	0.1006	1	K.LMDKVETFLR.I	(Ions score 61)
189 - 198	1253.6207	1252.6134	1252.5122	0.1013	1	K.LMDKVETFLR.I	(Ions score 43)



Mascot: http://www.matrixscience.com/

Mascot Search Results

Protein View

Match to: **P01241m|A13C/G120C_Mutante-hGH** Score: **3231**
mit His-Tag und Tev-Proteaseschnittstelle
Found in search of E:\Data\Projects\RCPE3\RCPE 3 III\RCPE3_III_3.RAW

Nominal mass (M_r): **24808**; Calculated pI value: **5.69**
NCBI BLAST search of P01241m|A13C/G120C Mutante-hGH against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **59%**

Matched peptides shown in **Bold Red**

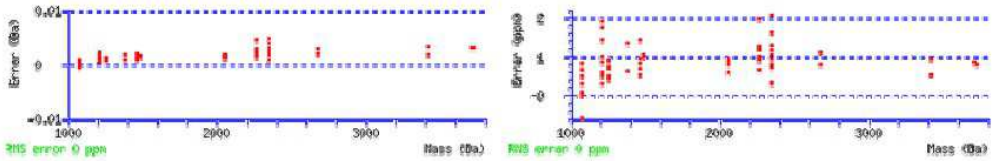
```
     1 HHHHHHSSGV DLGTENLYFQ SPTIPLSPLF DNCMLRAHRL HQLAFDTYQE
    51 FEEAYIPKEQ KYSFLQNPQT SLCFSESIPT PSNREETQQK SNLELLRISL
   101 LLIQSWLEPV QFLRSVFANS LVYGASDSNV YDLLKDLEEC IQTLMGRLED
   151 GSPRTGQIFK QTYSKFDTNS HNDDALLKNY GLLYCFRKDM DKVETFLRIV
   201 QCRSVEGSCG F
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
29 - 36	534.7516	1067.4886	1067.4892	-0.0006	0	R.LFDNCMLR.A	(Ions score 68)
29 - 36	534.7519	1067.4892	1067.4892	0.0000	0	R.LFDNCMLR.A	(Ions score 68)
29 - 36	534.7519	1067.4893	1067.4892	0.0001	0	R.LFDNCMLR.A	(Ions score 75)
29 - 36	534.7520	1067.4895	1067.4892	0.0003	0	R.LFDNCMLR.A	(Ions score 75)
29 - 36	534.7522	1067.4898	1067.4892	0.0006	0	R.LFDNCMLR.A	(Ions score 75)
29 - 36	1068.4972	1067.4899	1067.4892	0.0007	0	R.LFDNCMLR.A	(Ions score 34)
29 - 36	534.7523	1067.4901	1067.4892	0.0009	0	R.LFDNCMLR.A	(Ions score 71)
40 - 58	781.3831	2341.1274	2341.1266	0.0007	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 43)
40 - 58	781.3831	2341.1274	2341.1266	0.0008	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 72)
40 - 58	781.3834	2341.1283	2341.1266	0.0016	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 85)
40 - 58	781.3834	2341.1285	2341.1266	0.0019	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 82)
40 - 58	1171.5717	2341.1288	2341.1266	0.0022	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 116)
40 - 58	781.3836	2341.1291	2341.1266	0.0025	0	R.LHQLAFDTYQEFEEAYIPK.E	(Ions score 78)

40 - 58	781.3837	2341.1294	2341.1266	0.0028	0	R.LHQLAFDTYQEFEEAYIPK.E (Ions score 64)
40 - 58	1171.5721	2341.1296	2341.1266	0.0030	0	R.LHQLAFDTYQEFEEAYIPK.E (Ions score 139)
40 - 58	1171.5725	2341.1304	2341.1266	0.0038	0	R.LHQLAFDTYQEFEEAYIPK.E (Ions score 125)
40 - 58	1171.5725	2341.1304	2341.1266	0.0038	0	R.LHQLAFDTYQEFEEAYIPK.E (Ions score 94)
40 - 58	1171.5730	2341.1314	2341.1266	0.0048	0	R.LHQLAFDTYQEFEEAYIPK.E (Ions score 107)
62 - 84	891.7593	2672.2562	2672.2541	0.0021	0	K.YSFLQNPQTSLCFSESIPTPSNR.E (Ions score 43)
62 - 84	1337.1358	2672.2570	2672.2541	0.0030	0	K.YSFLQNPQTSLCFSESIPTPSNR.E (Ions score 82)
62 - 90	1139.5409	3415.6009	3415.5990	0.0018	1	K.YSFLQNPQTSLCFSESIPTPSNREETQQK.S (Ions score 44)
62 - 90	1708.8084	3415.6022	3415.5990	0.0032	1	K.YSFLQNPQTSLCFSESIPTPSNREETQQK.S (Ions score 50)
98 - 114	1028.1043	2054.1940	2054.1928	0.0012	0	R.ISLLLIQSWLEPVQFLR.S (Ions score 56)
98 - 114	1028.1045	2054.1944	2054.1928	0.0016	0	R.ISLLLIQSWLEPVQFLR.S (Ions score 59)
98 - 114	1028.1046	2054.1946	2054.1928	0.0018	0	R.ISLLLIQSWLEPVQFLR.S (Ions score 86)
98 - 114	685.7388	2054.1947	2054.1928	0.0019	0	R.ISLLLIQSWLEPVQFLR.S (Ions score 68)
115 - 135	1131.5688	2261.1230	2261.1216	0.0015	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 86)
115 - 135	1131.5691	2261.1236	2261.1216	0.0021	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 62)
115 - 135	1131.5692	2261.1238	2261.1216	0.0023	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 93)
115 - 135	754.7153	2261.1242	2261.1216	0.0027	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 50)
115 - 135	754.7154	2261.1243	2261.1216	0.0027	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 88)
115 - 135	1131.5695	2261.1244	2261.1216	0.0029	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 78)
115 - 135	1131.5703	2261.1260	2261.1216	0.0045	0	R.SVFANSLVYGASDSNVYDLLK.D (Ions score 57)
115 - 147	1236.6036	3706.7890	3706.7858	0.0031	1	R.SVFANSLVYGASDSNVYDLLKDLEECIQTLMGR.L (Ions score 48)
115 - 147	1241.9352	3722.7838	3722.7808	0.0030	1	R.SVFANSLVYGASDSNVYDLLKDLEECIQTLMGR.L Oxidation (M) (Ions score 42)
136 - 147	732.8451	1463.6756	1463.6748	0.0008	0	K.DLEECIQTLMGR.L (Ions score 72)
136 - 147	732.8452	1463.6758	1463.6748	0.0010	0	K.DLEECIQTLMGR.L (Ions score 80)
136 - 147	732.8453	1463.6760	1463.6748	0.0012	0	K.DLEECIQTLMGR.L (Ions score 87)
136 - 147	732.8454	1463.6762	1463.6748	0.0014	0	K.DLEECIQTLMGR.L (Ions score 80)
136 - 147	1464.6842	1463.6769	1463.6748	0.0021	0	K.DLEECIQTLMGR.L (Ions score 29)
136 - 147	740.8429	1479.6712	1479.6698	0.0015	0	K.DLEECIQTLMGR.L Oxidation (M) (Ions score 74)
136 - 147	1480.6786	1479.6713	1479.6698	0.0016	0	K.DLEECIQTLMGR.L Oxidation (M) (Ions score 32)
179 - 187	603.2924	1204.5703	1204.5699	0.0004	0	K.NYGLLYCFR.K (Ions score 69)
179 - 187	603.2924	1204.5703	1204.5699	0.0004	0	K.NYGLLYCFR.K (Ions score 64)
179 - 187	603.2925	1204.5705	1204.5699	0.0006	0	K.NYGLLYCFR.K (Ions score 56)
179 - 187	603.2926	1204.5706	1204.5699	0.0007	0	K.NYGLLYCFR.K (Ions score 63)
179 - 187	603.2926	1204.5706	1204.5699	0.0007	0	K.NYGLLYCFR.K (Ions score 63)
179 - 187	1205.5782	1204.5709	1204.5699	0.0010	0	K.NYGLLYCFR.K (Ions score 42)
179 - 187	1205.5784	1204.5711	1204.5699	0.0012	0	K.NYGLLYCFR.K (Ions score 36)
179 - 187	1205.5789	1204.5716	1204.5699	0.0017	0	K.NYGLLYCFR.K (Ions score 51)
179 - 187	1205.5793	1204.5720	1204.5699	0.0021	0	K.NYGLLYCFR.K (Ions score 38)
179 - 187	1205.5795	1204.5722	1204.5699	0.0023	0	K.NYGLLYCFR.K (Ions score 43)
188 - 198	691.3613	1380.7080	1380.7071	0.0009	2	R.KDMDKVETFLR.I (Ions score 81)
188 - 198	461.2436	1380.7090	1380.7071	0.0019	2	R.KDMDKVETFLR.I (Ions score 49)
189 - 198	627.3136	1252.6127	1252.6122	0.0005	1	K.DMDKVETFLR.I (Ions score 52)
189 - 198	1253.6201	1252.6128	1252.6122	0.0007	1	K.DMDKVETFLR.I (Ions score 43)
189 - 198	627.3138	1252.6130	1252.6122	0.0008	1	K.DMDKVETFLR.I (Ions score 57)
189 - 198	418.5450	1252.6131	1252.6122	0.0009	1	K.DMDKVETFLR.I (Ions score 51)
189 - 198	627.3139	1252.6133	1252.6122	0.0011	1	K.DMDKVETFLR.I (Ions score 61)
189 - 198	627.3139	1252.6133	1252.6122	0.0012	1	K.DMDKVETFLR.I (Ions score 61)



Mascot: http://www.matrixscience.com/