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Eglė STRAINIENĖ

STUDIES OF THE REFOLDING PROCESSES OF RECOMBINANT GROWTH HORMONES

DOCTORAL DISSERTATION

TECHNOLOGICAL SCIENCES,
CHEMICAL ENGINEERING (05T), BIOTECHNOLOGY (T490)

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DAKTARO DISERTACIJA

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CHEMIJOS INŽINERIJA (05T), BIOTECHNOLOGIJA (T490)

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Abstract

The recombinant expression of proteins in *E.coli* cytoplasm at high concentrations often results in the formation of insoluble aggregates known as inclusion bodies. To obtain a soluble and biologically active protein, IBs must be solubilized and refolded. The proper folding of target protein *in vitro* competes with unproductive side reaction such as misfolding and aggregation. To prevent aggregation and increase refolding yield of correctly folded protein, low molecular weight additives are used. A great number of articles on the refolding strategies of various proteins in the presence of different solution additives have encouraged us to test these additives on two model proteins: recombinant mink and porcine growth hormones, and provide some evidence for this refolding strategy. The refolding of recombinant growth hormones from *E. coli* inclusion bodies at high protein concentration and pH 8.0 was impossible in the absence of the solution additives, due to a high level of protein aggregation and precipitation.

Low molecular weight additives are mainly grouped into two groups: protein stabilizers and denaturants. However, there are solution additives, which are not placed in these two groups. They have only marginal effect on protein structure and stability, but appear capable to suppress the aggregation of protein. The group of aggregation suppressors includes such solution additives as L-arginine, L-arginine ethyl ester and cyclodextrins. L-arginine and L-arginine ethyl ester effectively suppressed the precipitation of growth hormones during refolding, but they did not inhibit soluble oligomers formation. Methyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin showed a positive effect on the aggregation suppression of both proteins. The influence of different L-arginine, L-Arginine ethyl ester, methyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin concentrations on the renaturation yield of both proteins was investigated. Moreover, those additives did not only suppress the folding-related, but also temperature-related aggregates formation of both proteins.

Since the solution additives effect on each protein was different, the main goal of the thesis was to elucidate the mechanisms by which L-arginine and cyclodextrins affect the aggregate formation of two model proteins, mink and porcine growth hormones, in two model systems: refolding from IBs and thermal denaturation.

Reziumė

Esant aukšto lygio rekombinantinių baltymų raiškai *E. coli* ląstelėse susidaro netirpūs agregatai – intarpiniai kūneliai. Norint iš jų gauti tirpų ir biologiškai aktyvų baltymą, intarpiniai kūneliai turi būti tirpdomi ir renatūruojami. Baltymų renatūracija *in vitro* konkuruoja su šalutiniais procesais – netaisyklingai susisukusių baltymo formų susidarymu ir agregacija. Mažos molekulinės masės priedai, naudojami renatūracijos procese, slopina baltymų agregaciją ir padidina jų renatūracijos išeigą. Apie baltymų renatūraciją esant mažos molekulinės masės priedams parašyta ne viena dešimtis straipsnių, tačiau siekiant aiškumo apie jų poveikį baltymų renatūracijos procesui, šie priedai buvo panaudoti renatūruojant du modelinius baltymus – rekombinantinius kiaulės ir audinės augimo hormonus. Rekombinantinių kiaulės ir audinės augimo hormonų renatūracija (pH 8,0) iš *E.coli* intarpinių kūnelių buvo neįmanoma dėl didelės baltymų agregacijos, nesant mažos molekulinės masės priedams.

Visi mažos molekulinės masės priedai skirstomi į dvi grupes: baltymų stabilizatoriai ir denatūrantai. Tačiau yra tokių priedų, kurie nepriklauso nei vienai iš šių grupių. Jie nežymiai įtakoja baltymo molekulės struktūrą, bet slopina baltymų agregaciją. L-argininas, L-arginino etilo esteris ir ciklodekstrinai yra priskiriami baltymų agregacijos slopintojų grupei. L-argininas ir L-arginino etilo esteris efektyviai slopino rekombinantinių augimo hormonų precipitatų susidarymą renatūracijos proceso metu, tačiau neinhibavo tirpių oligomerų formavimosi. Iš visų išbandytų ciklodekstrinų, tik metil-β-ciklodekstrinas and 2-hidroksipropil-β-ciklodekstrinas slopino rekombinatinių augimo hormonų agregaciją renatūracijos proceso metu. Ištirta rekombinatinių augimo hormonų renatūravimo išeigos priklausomybė nuo šių priedų koncentracijos.

Kadangi mažos molekulinės masės priedų poveikis įvairiems baltymams skirtingas, todėl šio darbo tikslas buvo ištirti dviejų modelinių baltymų, rekombinantinių audinės ir kiaulės augimo hormonų, agregaciją, slopinančių priedų, L-arginino ir ciklodekstrinų, veikimo mechanizmą dviejose modelinėse sistemose: renatūracijos iš intarpinių kūnelių bei terminės denatūracijos.

List of abbreviations

Ac-β-CD	–	acetyl-β-cyclodextrin
L-Arg	–	L-arginine ((S)-2-amino-5-guanidinopentanoic acid)
L-ArgEE	–	L-arginine ethyl ester
BSA	–	bovine serum albumin
CD	–	cyclodextrin
DTT	–	1,4-dithiothreitol
EDTA	–	ethylenediaminetetraacetic acid
E.coli	–	*Escherichia coli*
Gly	–	glycine
GdnCl	–	guanidine hydrochloride
Hsp	–	heat shock proteins
HP-β-CD	–	2-hydroxypropyl-β-cyclodextrin
IB	–	inclusion body
IPTG	–	isopropyl β-D-1-thiogalactopyranoside

LB	–	Luria-Bertani medium
Me-β-CD	–	methyl-β-cyclodextrin
PCR	–	polymerase chain reaction
PDB	–	Protein Data Bank
PMSF	–	phenylmethylsulfonyl fluoride
rhGH	–	recombinant human growth hormone
rmGH	–	recombinant mink growth hormone
rpGH	–	recombinant porcine growth hormone
RP-HPLC	–	reversed phase high performance liquid chromatography
SDS	–	sodium dodecyl sulfate
SDS-PAGE	–	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	–	N, N, N′, N′-tetramethylethylenediamine
Tris	–	2-amino-2-hydroxymethyl-propane-1,3-diol
Trp	–	tryptophan
Wt	–	wild type

Contents

Introduction

Formulation of the problem

The formation of protein aggregates is an unproductive and undesirable phenomenon in biotechnology. Overexpression of recombinant proteins in bacteria often leads to the misfolding and formation of insoluble aggregates, i.e., inclusion bodies (IBs). The general strategy used for recovery of the biologically active protein from inclusion bodies involves: IBs isolation and washing, solubilization of the aggregated protein which usually causes denaturation, and finally, refolding of the solubilized protein (De Bernardez Clark 1998). However, the correct refolding of the target protein competes with uproductive side reactions such as misfolding and aggregation. Moreover, protein aggregation is a common issue encountered during its purification and storage.

Various techniques have been developed to prevent the aggregation and increase solubility of proteins, such as the use of a molecular chaperones and protein mutagenesis, as well as control pH, temperature, ionic strength or protein concentration (Rudolph and Lilie 1996). A practical and relatively simple approach to solve the aggregation problem is the utilization of low molecular

weight additives. Chemical additives are mainly grouped into the two groups: protein stabilizers and denaturants. However, there are some solution additives such as L-arginine, cyclodextrins, which are not placed in these two groups. It has been reported that they have marginal effect on protein structure and stability, but appear capable to suppress or disrupt the protein-protein interaction, and they are defined as aggregation suppressors (Hamada, et al. 2009; Tsumoto, et al. 2003b).

The object of research

The main target in protein refolding research is to minimize aggregation and increase refolding yield. The amino acid L-Arginine is one of the chemical additives which increased refolding yields of various proteins by suppressing their aggregation. Moreover, it has been reported that arginine is the most effective suppressor for heat-induced aggregation among natural amino acids (Shiraki, et al. 2002). L-Arginine is considered to be effective only at high concentrations (0.1–1.0 M) suggesting that weak interactions between protein and arginine are involved. However, the mechanism of arginine action has not been fully understood.

Cyclodextrins are other additives which prevent the formation of protein aggregates during the refolding and thermal denaturation processes. They are water soluble, non-toxic, stable and biocompatible. The best known types of cyclodextrin are α-, β- and γ-cyclodextrin, which consist of six, seven and eight glucosyl units, respectively. The more glucose units there are in the circle of cyclodextrin, the larger hydrophobic cavity is. To improve their solubility in water various derivatives have been synthesized, such as 2-hydroxypropyl-α-, maltosyl-β-cyclodextrin, etc. However, the ability of various cyclodextrin to prevent the protein aggregation during the refolding process varied significantly (Kim, et al. 2006b).

It is essential to mention that the solution additives which prevent protein aggregation during refolding process can often be ineffective during thermal denaturation of the same protein and vice versa. Also, proteins are different in their physical and chemical properties, thus, the additives which work well for a certain protein during the refolding process might be disadvantageous for other proteins (De Bernardez Clark, et al. 1999).

The overall goal of the thesis

The overall goal of the thesis has been to elucidate the mechanisms by which L-Arginine and cyclodextrins affect the aggregate formation of two model proteins, mink and porcine growth hormones, in two model systems: refolding from IBs and thermal denaturation.

The tasks of the thesis

1. To elucidate the effect of various solution additives on mink and porcine growth hormones refolding and thermal denaturation processes.
2. To investigate the mechanism of growth hormones aggregation and its suppression by L-Arginine and cyclodextrins.
3. To determine the effect of the Trp86Gly mutation within rmGH on the action of L-Arginine during refolding.

Methodology of research

Methodology of research includes the expression of recombinant proteins, isolation and solubilization of IBs, oxidative refolding, chromatographic purification, cloning and mutagenesis, thermal denaturation procedures.

Scientific novelty

Solution additives are widely used in biotechnology to stabilize proteins, to increase the rate of protein folding, to reduce solvent accessibility and conformational mobility, to increase solvent viscosity and decrease aggregation probability. The aggregation studies of the effects of various solution additives on recombinant growth hormones refolding of from IBs and thermal unfolding processes revealed the additives which affect protein association while having little or no effect on protein folding. It was demonstrated that the refolding efficiency of mink and porcine growth hormones depended on the solution additives (L-Arg, L-ArgEE and β-cyclodextrins) and the addition of those reagents enabled to refold biologically active proteins in high yield. It was shown that L-ArgEE prevented refolding-induced aggregation of rmGH more effectively than L-Arg. It was the first time when it was shown that the efficiency of cyclodextrins during refolding of rmGH and rpGH was not only

related to the cavity size, but also to the substituents and their molar substitution degree on the ring of cyclodextrin.

The rational choice of the solution additive requires a detailed knowledge of the molecular mechanism employed to achieve a desired result. Based on the assumption that L-Arginine acts as aggregation suppressor due its guanidinium group interaction with hydrophobic side chain of amino acid tryptophan within the protein, the mutation of single tryptophan within rmGH was introduced. Mutational study revealed for the first time that L-Arginine prevented protein (W86G_rmGH) aggregation during the refolding process which possessed no tryptophans per polypeptide chain. Thermal denaturation studies of recombinant growth hormones in the presence of L-Arginine and cyclodextrins revealed that they did not act as stabilizators, but affected temperature induced unfolding and aggregation.

Practical value

Proteins in their functional native structure are involved in promoting or controlling every process in the living cells. However, many proteins lose their native structure and biological functions under various stresses. The production of recombinant proteins in biotechnology is interfered by protein aggregation, which competes with proper protein folding and leads to low yields of a biologically active protein. It is well known that certain natural organisms can live under extreme conditions, such as high temperature, pressure, etc., and function normally under them. In this thesis, the developed refolding method of recombinant growth hormones in the presence of natural and chemically modified solution additives might be used for the refolding of other proteins. The identification of the intrinsic and extrinsic factors during refolding and thermal denaturation processes contributes to our understanding of the aggregation mechanisms and provides some valuable information for biotechnology.

The major findings presented for defence

1. The renaturation of rmGH and rpGH, produced in *E. coli* as inclusion bodies, at pH 8.0 is achieved only in the presence of solution additives, due to a high level of protein aggregation-precipitation.

2. L-arginine, L-arginine ethylester, 2-hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin increase the refolding yield of recombinant mink and porcine growth hormones by suppressing their aggregation.

3. Mutation of rmGH (W86G_rmGH) does not affect the beneficial effect of L-arginine on the refolding process of recombinant growth hormones from *E. coli* inclusion bodies.

4. L-arginine and 2-hydroxypropyl-β-cyclodextrin do not increase the thermal stability of recombinant growth hormones, but suppress the aggregation of thermally unfolded proteins.

Approval of the results

The material presented in the dissertation was published in two scientific articles in the journals indexed in the list of Institute for Scientific information ISI Web of Science. The results of the dissertation were discussed at 5 international and national scientific conferences:

1. The 8th International Conference on Protein stabilisation *"ProStab2009"*. 2009, Graz, Austria.
2. The young scientists and students international scientific conference *"Modern problems of microbiology and biotechnology"*. 2007, Odesa, Ukraine.
3. The 7th International Conference on Protein stabilisation *"ProtStab2007"*. 2007, Exeter, UK.
4. 10th scientific conference *„Biochemistry and systems biology"*. 2008, Tolieja, Lithuania.
5. 9th scientific conference *„Biochemistry: science and knowledge-based society"*. 2006, Tolieja, Lithuania.

Structure of the dissertation

The dissertation consists of the introduction, three main chapters, general conclusions, list of references, and list of the author's publications. In the first chapter of thesis I have analysed and summarized available literature data on the formation, structure and solubilization of inclusion bodies, the strategies of protein refolding in order to provide a theoretical background of the experimental data obtained in this work. The second chapter describes the research methods which have been used in this thesis. The third chapter presents an efficient refolding strategy for two model proteins, mink and porcine growth hormones, in the presence of low molecular weight additives. The scope of the work is 124 pages, 36 figures and 9 tables. 252 sources of literature have been used in the dissertation.

1

Literature overview

The production of large quantities of proteins became possible with the advent of recombinant DNA technology, and the subsequent expression of recombinant proteins in different host systems. (Arakawa, et al. 2002). Although a number of different host systems are available today, *Escherichia coli* (*E. coli*) and other prokaryotes are still the first choice for host especially if posttranslational modifications are not required for protein function (Fahnert, et al. 2004b). Recombinant proteins are produced at high concentrations in a very different environment than that in which they are normally expressed. Their structure, stability and solubility are affected by this environment and can be quite different than that of the native protein (Arakawa, et al. 2002). Thus, the recombinant expression of proteins in bacterial hosts often results in the formation of insoluble aggregates known as inclusion bodies (IBs) (Mayer and Buchner 2004; Ventura and Villaverde 2006).

In the first part of the literature review I have analysed available literature data on the formation, structure and solubilization of inclusion bodies. In the second part of the review I have summarized the available literature data on the refolding strategies in order to provide a background of the experimental data obtained in this work.

1.1. Inclusion bodies (IBs)

1.1.1. Inclusion bodies (IBs) formation

IBs have been found in all microbial expression systems as well as in lower eukaryotic organisms, such as yeasts. These aggregates usually contain the product in a non-native conformation, but the product is found in high concentrations and can reach more than 50 % of the total cellular protein. The formation of IBs mainly depends on the kinetic competition between protein-specific folding and aggregation rates connected to the synthesis rate (Fahnert, et al. 2004b; Ventura and Villaverde 2006).

Aggregation is a predominant feature in very strong expression systems, but it also increases with high inductor concentration and a short induction time (2–5 h), with the use of complex growth media and at higher cultivation temperature, as well as high cell density (Rudolph and Lilie 1996).

Rudolph came to conclusion that the IB formation depends on the specific folding behaviour rather than on the general characteristics of a protein such as size, fusion partners, subunit structure and relative hydrophobicity. However, folding-rate-limiting structural characteristics such as disulphide bonds and certain point mutations can significantly promote the formation of aggregates. That is why even small changes in the primary structure of a protein may affect its solubility. Proteins consisting of different subunits, such as antibody Fab fragments – containing both, intermolecular and intramolecular disulphide bonds – have been traditionally produced as IBs (Rudolph and Lilie 1996; Fahnert, et al. 2004b).

The formation of the correct disulphide bonds is usually the rate-limiting reaction for the formation of the native structure of a disulphide bond containing proteins. In the cytosol of *E. coli* proteins containing disulphide bond are often accumulated as IBs if they are produced in this reducing compartment (Ventura and Villaverde 2006).

There are many methods which can be tested to reduce aggregation and to optimise the *in vivo* production of the soluble, active form of the protein, e.g., cultivation at low temperature and limited induction, fusion of the protein to solubilising partners, coexpression of chaperones and foldases, expression of the protein in different cellular compartments and expression in mutant strains. However, the optimal process for a specific product is still unpredictable due to the many factors influencing the synthesis and folding of proteins in the cellular environment (De Bernardez Clark, et al. 1999; Thomas and Baneyx 1996; Misawa and Kumagai 1999).

Over-expression of membrane proteins has been a complicated challenge. Membrane proteins do not only aggregate, but they are also considered to be

toxic to the *E. coli* cell and difficult to express. Membrane proteins are mostly accumulated in small amounts, but in spite of that they are aggregating due to their surface-exposed hydrophobic regions (Vallejo and Rinas 2004).

Many therapeutically interesting proteins from eukaryotes are glycosylated. However, glycosylation is often not an assumption for function, but influences activity and degradation characteristics, such as thermostability. Therefore many therapeutic proteins can be produced in bacterial hosts lacking the eukaryotic glycosylation system, but extensive tests are necessary to verify the function. Sometimes, the recombinant proteins produced in *E. coli* may even have beneficial properties compared to the glycosylated forms. For example, a non-glycosylated recombinant variant of human tissue plasminogen activator obtained by refolding from *E. coli* IBs showed a longer half-life and lower clearance rate in rats. On the other hand, glycosylation may affect folding behaviour and solubility. Therefore, glycosylated proteins may be prone to aggregation and formation of IBs if produced in *E. coli* (Shental-Bechor and Levy 2008; Fahnert, et al. 2004b).

Prokaryotic organisms are characterised by a fast translation process of up to 20 amino acids per second. Therefore protein folding occurs in most cases posttranslationally, although the translation rate can also be slowed down if the mRNA contains a high number of rare codons. In contrast to prokaryotic organisms, protein folding in eukaryotic cells often begins cotranslationally and continues posttranslationally after the release of the protein from the ribosome. The rate of translation in eukaryotes is slower than in *E. coli*, but varies depending on the cell type and protein. Furthermore, it has been suggested that the slower rate of translation in eukaryotic cells plays an important part in the proper folding by permitting the sequential folding of individual domains during the translation process. Similarly it is known that *in vivo* a high synthesis rate of the target protein in microbial expression systems positively affects aggregation and that one way to decrease the aggregation probability is to slow down the synthesis rate, by lowering the cultivation temperature or the inducer concentration (Sorensen and Pedersen 1991; Carrio and Villaverde 2000, 2002).

The eukaryotic protein disulphide isomerase (PDI) is the first catalyst for disulphide bond formation *in vivo*. PDI is a part of the complex machinery responsible for the formation and isomerization of disulphide bonds in the eukaryotic endoplasmic reticulum. While in bacteria the natural folding environment is different in many aspects. The disulphide bridges of the polypeptides expressed in *E. coli* are formed only after their translocation from a reducing cytoplasm to an oxidizing periplasm. The catalysis of the formation of disulphide bonds is more complicated in the periplasm than in the endoplasmic reticulum of eukaryotes, because its oxidising milieu (redox potential) is affected by the environment of the cell. Small molecules are constantly diffusing through

the cell envelope (Missiakas and Raina 1997). In the prokaryotic periplasm, the disulphide bond forming function is carried out by the Dsb family of proteins. DsbA oxidises the thiolgroups of proteins within the periplasm and is reoxidised by DsbB. DsbC, E and G repair the non-native bonds and are reduced again by DsbD. In conclusion it is not known when the disulphide bonds are formed (co- or post-translationally) and whether the involved cysteines are chosen specifically (De Marco 2009).

Due to the differences between disulphide bond formation in the natural eukaryotic environment and the recombinant prokaryotic host, the most target proteins in prokaryotic hosts are misfolded and accumulated in IBs. Moreover, the cytoplasm as the most important compartment for the production of IBs is reducing. Thus, the IBs formed there might be mainly due to the absence of the correct disulphide bonds (Carrio and Villaverde 2002).

1.1.2. Structure and composition of IBs

In actively producing recombinant *E. coli* cells, IBs are seen as refractile particles, usually occurring in the cytoplasm, although secretory proteins can also form IBs in the periplasm. Due to their refractile character, they can be observed directly in the living host cell by phase-contrast microscopy and their size varies up to 2 μm (Fig. 1). Scanning images of these purified particles reveal cylindrical or ovoid shapes, with differently smoothed surfaces (Mayer and Buchner 2004).

The cytosolic microbial overexpression of a recombinant protein leads to accumulation of large particles (IBs). IBs are characterized by a relatively high specific density of about 1.3 mg/ml and mainly accumulated at the proximal ends of a bacterium (Mukhoaphyay A. 1997).

The electron microscopic visualisation suggested that many primary micro-aggregates fuse to a larger amorphous body if the concentration of the unfolded protein is increasing. However the co-expression of two proteins from genes carried on the same plasmid results in the formation of two types of cytoplasmic aggregates and each of them is enriched in one type of a recombinant protein (Carrio and Villaverde 2002).

In most cases cytoplasmic IBs consist predominantly of the incorrectly folded recombinant product. The stabilisation of IBs may possibly occur mainly via hydrophobic interactions of folding intermediates. Thus, the target protein in IBs can be partially structured. Interestingly, the decreases in α-helix- and increases in β-sheet-contents have been detected in IBs in comparison to the native product. IBs can also be formed from native protein, but this seems to be an exception. For example, the Fourier Transform Infrared Spectroscopy (FTIR) measurements showed that the secondary structure of interleukin-1β in IBs is

very similar to the secondary structure of the native protein indicating that those IBs contain a folding intermediate with a native-like secondary structure (Mayer and Buchner 2004; Oberg, et al. 1994).

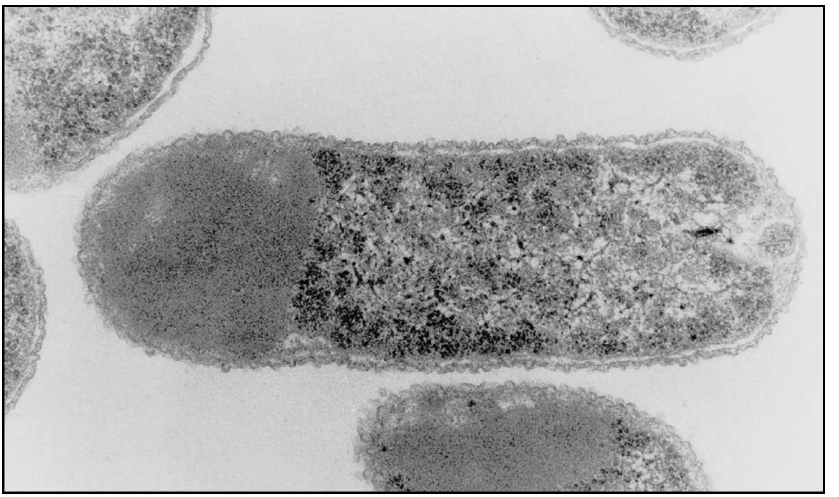

Fig. 1. *E. coli* cells containing inclusion bodies. The recombinant protein deposits are the dark spherical particles in the bacterial cytoplasm (Mayer and Buchner 2004)

Thus, IBs consist predominantly of the recombinant product. However, IB preparations often contain a significant part of other cellular proteins (membrane proteins, RNA polymerase, ribosomal subunit proteins), ribosomal RNA and DNA. These compounds are mainly integrated due to the co-precipitation of cell debris components during the process of IB preparation. The protein charge can also have a major influence on the composition of IBs (Chaturvedi, et al. 2000; Vallejo and Rinas 2004).

In many cases, the presence of the contaminating proteins in IBs is related to incomplete purification after cell lysis. However, even after stringent washing of IBs with detergents some *E. coli* proteins have still been found in a significant concentration. The major part of these proteins belong to the groups of membrane proteins such as OmpF, OmpC, OmpA and OmpT. These membrane components could appear as a result of the cell lysis and washing procedure leading to further protein aggregation. Other proteins can also be detected in IBs such as the molecular chaperones GroEL, DnaK, the small heat shock proteins (HSP) as HSP can be found among the first cellular detectors of misfolded proteins and they act as aggregation controllers (Carrio and Villaverde 2003; Hart, et al. 1990).

IBs formation is mainly supported by tight hydrophobic interactions and they are highly stable and resistant to proteases *in vivo*. Although IBs mostly contain incorrectly folded proteins, in some cases a native like secondary structure can be observed. Thus, IBs containing a folding intermediate are prone to the proteolysis and may disappear either due to the proteolysis or further protein functionalisation (Carrio, et al. 1999; Mukhoaphyay A. 1997).

The large set of polypeptides forming bacterial IBs are not related to each other, either structurally or sequentially and include small, large, monomeric, multimeric, prokaryotic or eukaryotic proteins. Thus, aggregation inside 'bacterial factories' has long been considered to be non-specific process, resulting in formation of disordered intracellular precipitates. However, Ventura and Villaverde suggested that IBs formation is promoted by high local concentration of the polypeptide, the accumulation of unstructured protein fragments, wrong interactions with the bacterial folding machinery, the lack of the post-translational modifications and the prevention of proper disulphide pairing in the reducing cytoplasmic environment (Ventura and Villaverde 2006).

1.1.3. Isolation and solubilisation of IBs

In general IBs are recovered by low speed centrifugation (e.g., 10000 × g for 10 minutes, at 4 ºC) of bacterial cells mechanically disrupted either by using ultrasonication for small, French press for medium, or high pressure homogenization for large scale production. The main protein contaminants in the crude inclusion body fraction are proteins from the cell envelope and outer membrane. These proteins are not integral inclusion body contaminants but they coprecipitate together with other insoluble cell material during inclusion body recovery. The addition of detergents such as Triton X-100 (e.g., 0.5 %) and/or low concentrations of chaotropic compounds (e.g., 3 M urea) either prior to the mechanical cell breakage or during washing crude inclusion body preparations allow the membrane proteins or other nonspecifically adsorbed cell material to be removed (Mukhopadhyay U. K., et al. 2002, Vallejo and Rinas 2004).

And, if the expression level of target protein is sufficiently high, collected IBs yields a rather homogeneous preparation. It may contain the recombinant protein with a purity of up to 90 %. In this case, the proteins can be renatured directly after solubilisation without a further purification of the recombinant protein (Lilie, et al. 1998).

Although IB proteins may contain a native like secondary structure content, they do not readily desintegrate under physiological solvent conditions (Rudolph and Lilie 1996). A variety of methods can be used for solubilization of IBs. In most cases, strong denaturants such as GdnCl or urea are used. The denaturants are usually employed at high concentrations (6–8 mol/l) to ensure complete

solubilization and unfolding of the IB proteins. GdnCl is preferable to urea, because urea solutions may contain or spontaneously produce cyanate, which can carbamylate the amino groups of the protein. In addition, inclusion body solubilization by urea is pH dependent and optimum pH conditions must be determined for each protein (Vallejo and Rinas 2004; Bennion and Daggett 2003).

As mentioned above, some proteins retain most of their secondary structure during aggregation into IBs. And it is ideal if refolding of the protein is carried out from this pre-existing structure rather than from completely unfolded protein molecules. IBs solubilization at low denaturants concentrations (1–2 mol/l) is an efficient method since other impurities of IBs are not solubilized under these mildly denaturing conditions (Patra, et al. 2000; Middelberg 2002).

Besides denaturants, amino acid L-arginine (L-Arg) can also be used for mild solubilization. Solubilisation of fluorescently active protein (GFP) from insoluble particles was achieved by L-arginine. It is noted that L-Arg was stronger in solubilising insoluble GFP than GdnCl below 2 M concentration (Tsumoto, et al. 2003a). In all these cases, solubilization of protein aggregates using mild denaturation results in improved recovery of bioactive protein.

Another class of denaturants solubilising IBs is detergents. Cetyl trimethylammonium salts (CTAC or CTAB) have been used for solubilisation of IBs of human and porcine growth hormones (Puri, et al. 1991, 1992; Patra, et al. 2000; Cardamone, et al. 1995). In some cases detergents can be advantageous compared to GdnCl and urea, but they may interfere with the following protein renaturation and subsequent purification.

Besides denaturing agents, extremes of pH have been used to solubilise IBs. Alkaline solutions (pH $\geq$ 12) have been utilised in the preparation of growth hormones, proinsulin, aldolase, enolase, polyketide synthase, pyruvate kinase, superoxide dismutase. (Patra, et al. 2000; Singh, et al. 2008; Qiao, et al. 2003). Although pH-induced solubilization of IBs is a very simple method, it is problematic because at extreme pH values certain residues of the polypeptide chain of a protein may become chemically modified.

In the case of proteins containing cysteine, the isolated IBs usually contain a certain amount of interchain disulphide bonds, which reduce the solubility of the IBs in the absence of reducing agents. The addition of low-molecular weight thiol reagents such as dithiothreitol, glutathione, cysteine or mercaptoethanol in combination with chaotropic agents allows the reduction of the non-native inter- and intra- molecular disulphide bonds possibly formed by air oxidation during cell disruption and also keeps the cysteines in their reduced state. Optimum conditions for disruption of existing disulphide bonds are found at mild alkaline pH since the nucleophilic attack on the disulphide bond is carried out by the thiolate anion (Mukhopadhyay A. 1997; Vallejo and Rinas 2004).

Although IBs can be a source of a relatively pure protein, they should be solubilised and applied for *in vitro* refolding procedures. It is important to emphasize that *in vitro* refolding procedures are not universal and need to be adapted to each specific protein (Ventura and Villaverde 2006).

1.2. Refolding of proteins from IBs

1.2.1. Protein folding

Protein folding occurs quite rapidly, but one or more partially folded intermediate states often exist along the path to the final structure. The structures of these intermediates are not as well characterized as the native structures. They have a lot of the secondary structure elements of the native protein without the closely packed interior and full complement of weak interactions (Petsko and Ringe 2004; Yon 1997).

The amino acid sequences of proteins tend to be mixtures of polar and nonpolar residues. However, the environment in which a protein is synthesized and allowed to fold, is a significant determinant of its final structure. When such a sequence is allowed to fold e.g., in aqueous solutions, it cannot remain as a fully extended polymer. The polar and charged side chains, and the polar peptide groups form hydrogen bonds with water, but the nonpolar side chains do not. Their presence disrupt the hydrogen bonded structure of water without making any compensating hydrogen bonds with the solvent. In order to minimize this effect on water structure, these side chains tend to cluster together. This hydrophobic effect – the clustering of hydrophobic side chains from different parts of the polypeptide sequence causes the polypeptide to become compact. From an energetic point of view the compactness produces two favorable results: it minimizes the total hydrophobic surface area in contact with water, and it brings hydrophobic groups close to each other, allowing van der Waals interactions between them. Polar side chains do not need to be shielded from the solvent because they can form a hydrogen bond with water, so they will tend to be distributed on the outside of hydrophobic residues. However, the nonpolar side chains come together and form a hydrophobic core, they simultaneously drag their polar backbone amide groups into the interior of the protein. This hydrophobic folding has a negative consequence, too. These polar groups which made hydrogen bonds with water when the chain was extended, cann't do this now. The result is that most peptide N-H and carbonyl groups of folded proteins are hydrogen bonded to each other. This tendency of the amide groups of polypeptide chains to satisfy their hydrogen bonding potential through self-

interactions results in secondary structure (Petsko and Ringe 2004; Torshin and Harrison 2003).

Although most hydrophobic side chains in a protein are buried, some are found on the surface of the folded polypeptide chain in contact with water. Presumably, this unfavorable situation is a result of the numerous favorable interactions that provide a net stability for the folded protein. Such clusters of hydrophobic side chains on the protein surface are important for protein-protein recognition, protein oligomerization and protein-DNA interactions (Petsko and Ringe 2004).

1.2.2. Protein refolding strategies

Refolding is a change in protein conformation from unfolded to folded-native state. In general, IBs can be solubilized only by strong denaturants. The solubilized proteins from IBs are unfolded-disordered. The extent of protein unfolding depends on both the protein and the type of the denaturant used (Misawa and Kumagai 1999).

The solubilized proteins are highly flexible and well solvated. In aqueous buffers, proteins are folded, rigid, and compact. Ideally, the transfer of protein molecules from a high denaturant concentration to an aqueous buffer should lead to refolding, i.e., the transfer of protein molecules from the denaturant solution to the aqueous solvent will force them to transit into a native structure. However, such a drastic process usually does not work, since it is leading to misfolding and aggregation. Once misfolded or aggregated, in the absence of denaturants protein molecules have no flexibility to disaggregate and refold into the native structure (Tsumoto et al. 2003b).

The concentration of the denaturant is the key factor in the refolding, when denaturant concentration is low enough the protein molecules reorganize their structures. In other words, intermediate concentration of denaturants (usually 0.5–2 M GdnCl or 1–4 M urea) can induce folding and still maintain solubility and flexibility during refolding. Which intermediate concentration works depends on the proteins and how the denaturant concentration is reduced (Tsumoto et al. 2003b; Fahnert 2004b, Cardamone et al. 1995).

Cardamone et al. demonstrated that the high recovery of 85 % monomeric correctly refolded recombinant pGH can be obtained in a refolding environment containing critical concentration of urea (3.5 M), 10 mM β-ME and a pH of 9.1 at a protein concentration 0.5 mg/ml (Cardamone et al. 1995).

Therefore, the refolding is initiated by the controlled removal of excess denaturant. In most cases, this is allowed to occur in the presence of a suitable redox system and other folding promotion agents according to one of the different models.

1.2.3. Refolding by dilution

Direct dilution

The simplest refolding procedure is to dilute the concentrated protein-denaturant solution into a refolding buffer. Dilution forces the unfolded protein to a rapid folding by passing the intermediate denaturant concentration. Both the denaturant and protein concentrations increase, as the unfolded protein in a concentrated denaturant is delivered into a refolding buffer (Tsumoto et al. 2003b; Hevehan and De Bernardez Clark 1997). This means that the earlier part of dilution (where denaturant concentration is close to zero) is very different from the later part (where denaturant concentration is close to 1–2M). Dilution into the refolding buffer means a transition into a rigid structure which cannot fluctuate or convert to the native structure, without the presence of low concentration of denaturant. Therefore, it is recommended to include a certain level of denaturant, the concentration of which depends on the stability of the protein to be refolded. For oligomeric proteins, the earlier part of dilution means low protein concentration during refolding. Therefore, a slow dilution may result in insufficient concentration of refolded, monomeric state for a prolonged period, and hence, rapid dilution may be recommended. However, the protein concentration has to be carefully controlled to prevent aggregation. Also, dilution is a time and buffer consuming process, thus, not optimal for a large scale production. A method called “pulsed-dilution” may work better and give higher yields (Li et al. 2004; Tsumoto et al. 2003b).

Reverse dilution

Reverse dilution is performed by adding refolding buffer into the unfolded protein containing concentrated denaturant. Therefore both the denaturant and protein concentrations decrease simultaneously. Thus, the unfolded protein and/or folding intermediate are exposed to a descending denaturant concentration for a prolonged period of time. Unlike in the direct dilution, protein concentration is high at an intermediate denaturant concentration. Such conditions sometimes result in aggregation and precipitation of the protein. However, if the intermediate structure is soluble in the intermediate denaturant concentration and refolding requires slow structure rearrangement, this protocol may be desirable (Tsumoto et al. 2003b; He J. et al. 2008).

The reverse dilution has been successfully used for refolding of growth hormones (mink and porcine), staphylokinase (Sak) Variant Y1-Sak by the addition of low molecular weight additives (Bajorunaite et al. 2007, 2009; He, et al. 2008).

Mixing at a constant ratio

Refolding buffer and unfolded protein solution are mixed at a constant ratio. In contrast to the direct or reverse dilution methods, the concentrations of protein and denaturant during refolding process are kept constant using this method. The course of protein folding is similar to that in a direct dilution procedure, i.e., mixing leads to a rapid transit of the protein into an intermediate structure (Tsumoto K. et al. 2003b).

Pulsed dilution

The solubilised, denatured protein is added in pulses or continuously into the refolding buffer. The addition of a concentrated protein-denaturant mixture should occur at rates slower than the rate-determining folding step of the target protein. This will avoid the accumulation of high concentration of aggregation-prone folding intermediates that occur in the one-step dilution. For the pulse addition it has been recommended that the maximum refolding yield should reach 80 % before adding the next pulse. Also this procedure has an advantage as the folded structure does not aggregate with the unfolded or folding intermediates (Tsumoto et al. 2003b; Vallejo and Rinas 2004; De Bernardez Clark et al. 1999).

1.2.4. Refolding by dialysis, diafiltration or gel filtration chromatography

One-step dialysis

Denatured, unfolded protein samples in a concentrated denaturant buffer are dialyzed against a refolding buffer, and hence, exposed to a descending concentration of the denaturant. The denaturant concentration decreases with time to the concentration of a refolding buffer. As the concentration of the denaturant is decreased, the rate of folding into the intermediate and native structures increases. However, the rate of misfolding or aggregation will also increase. In particular, aggregation can be greatly enhanced, if the rate of folding is slow, since a low concentration of denaturant may not be enough to keep the unfolded or intermediate structures soluble. In dialysis refolding, the intermediate structure can be exposed to the intermediate denaturant concentration for a prolonged period of time. Additionally, refolding yields can be negatively affected by non-specific adsorbtion of protein to membrane. However, high refolding yields at high protein concentrations can be obtained for some proteins e.g., dialysis against 0.05 M Tris-HCl allowed to recover 42 % of carbonic anhydrase (from a starting concentration of 5 mg/ml) with 94 % activity (West et al. 1998; Tsumoto et al. 2003b; Vallejo and Rinas 2004).

Stepwise dialysis

The denaturant concentration is descending during stepwise dialysis. Refolding is started by dialysis against a buffer containing a high denaturant concentration (8 M urea or 6 M GdnCl) and thiol/disulphide exchange reagents, then with middle, and low concentrations of denaturants. The gradual removal of the denaturing reagent (GdmHCl) using stepwise dialysis with the introduction of an oxidizing reagent and L-arginine resulted in the highly efficient refolding of various denatured single-chain Fv fragments (scFvs) from inclusion bodies expressed in *E. coli* (Umetsu, et al. 2003). In contrast to the one-step dialysis, that the equilibrium is established at each denaturant concentration. An intermediate concentration of denaturant may allow the protein molecule to fluctuate and convert to the native structure. However, this does not work if the rate of misfolding or aggregation is faster than the rate of refolding. (Vallejo and Rinas. 2004; Tsumoto et al. 2003b).

Diafiltration

Diafiltration is the more practical membrane-based alternative to dialysis because the rate of the denaturant removal is not limited by diffusion. However, the protein accumulation on membrane may limit the diafiltration application. Despite that the diafiltration method is time-saving and efficient even at high concentrations of protein e.g., the refolding yields of lysozyme were 85 % and 63 % even at concentrations of 5 mg/ml and 10 mg/ml, respectively, after 11 hours dialfiltration (Varnerin et al. 1998; Yoshii et al. 2000).

Gel filtration

Buffer exchange for denaturant removal can also be carried out by using gel filtration. Most frequently, the denaturant-protein solution is injected into a column previously equilibrated with the refolding buffer. Desalting columns are used to separate proteins from denaturant, while protein-sizing columns are used to fractionate the protein species. In any case, gradual change in denaturant concentration occurs as in the one-step dialysis, but faster. If the unfolded or intermediate folded structure converts more slowly into the native state than into the misfolds or aggregates, there may not be enough time for the misfolds to exchange into the native structure in the descending concentration of the denaturant. Alternatively, a prolonged exposure to the intermediate denaturant concentration may cause protein aggregation or misfolding. The difference of gel filtration from dialysis or/and dilution refolding is the environment of a column matrix surrounding the proteins during folding. The column matrix reduces protein-protein interaction of folding intermediates, reduces intermolecular aggregation and thus improves the renaturation yield of the

denatured protein (Vallejo and Rinas 2004; Dong, et al. 2002; Singh and Panda 2005).

Protein refolding using urea gradient SEC on Superdex-75 was more efficient for recombinant human granulocyte colony-stimulating factor (rhG-CSF) compared to the refolding by dilution (Wang C. et al. 2008). An additional advantage of this chromatographic method is the purification during the refolding process.

1.2.5. Matrix-assisted protein refolding

A very efficient strategy to prevent protein aggregation is to minimize the risk of intermolecular interactions by adsorbing the denatured protein molecules onto a solid support. Since protein molecules are bound to resin, this procedure minimizes the aggregation of unfolded protein or folding intermediates.

IEC refolding

Ion exchange chromatography (IEC) process has been developed to improve protein refolding. Denatured proteins are reversibly bound to the ion-exchange column previously equilibrated with a strong denaturant (e.g., 8 M urea). The denaturant concentration in the column is gradually decreased leading to gradual refolding of the adsorbed protein molecules. After all the denaturant is washed out of the column, the refolded and still adsorbed protein molecules are released by the addition of salt or other additives to the refolding buffer (Li M., et al. 2004; Chen, Y. and Leong 2009).

Dual-gradient (urea concentration and pH) was used in the IEC process to refold denatured human lysozyme. The gradient of urea concentration minimized protein aggregation and enhanced proper refolding. The pH gradient helped the formation of disulphide bonds at alkaline conditions. Furthermore, refolding and purification of human lysozyme was achieved in one step (Li M. and Su 2002).

IMAC refolding

Immobilized metal ion affinity chromatography (IMAC) is an efficient procedure for simultaneous purification and refolding of proteins with a genetically engineered polyhistidine tags. Polyhistidine tags form high-affinity complexes with immobilized divalent metal ions even in the presence of high concentrations of chaotropic agents, thereby allowing isolation and refolding of the tagged protein. Thus, refolding and purification of the protein have become possible in one-step. In order to induce protein refolding, the denaturant concentration was gradually decreased and the elution of the target protein was

achieved by increasing the imidazole concentration or by using a decreasing pH-gradient (Swietnicki 2006; Li et al. 2004; Wang F., et al. 2005).

Thus, His-tagged tumor necrosis factor (His-TNF) expressed in inclusion body of *E. coli* was refolded on Ni^{2+}-Sepharose 6B column and refolding yield was equal more than 90 % (Xu, et al. 2000). However, IMAC refolding is mostly limited to proteins where the inclusion of a polyhistidine-tag does not interfere with the formation of the native protein configuration (Jungbauer, et al. 2004).

HIC refolding

Hydrophobic interaction chromatography (HIC) has also been successfully used for protein refolding with a concomitant removal of contaminating proteins during the refolding process. Unfolded proteins are applied to the HIC column at high salt concentrations and simultaneous refolded and eluted with a decreasing salt gradient (Vallejo and Rinas 2004).

Since, hydrophobic regions of the unfolded proteins are adsorbed to the hydrophobic matrix during HIC, the proteins are not prone to aggregation and native structure elements can be formed. During the migration through the column, the protein passes through several steps of adsorption and desorption, controlled by the salt concentration and hydrophobicity of the intermediate(s), resulting finally in the formation of the native structure (Li M., et al. 2004; Hwang, et al. 2010).

In order to enhance refolding yield of consensus interferon (rIFN-con), the unfolded protein was applied to the Poros ET 20 hydrophobic interaction column and eluted using a linear gradient of GdnCl. The refolding yield of IFN-con from inclusion bodies of *E.coli* was equal more than 80 % (Wang F., et al. 2006).

Chaperones, foldases and antibodies assisted refolding

The strategy of chaperone-assisted refolding aims to mimic the function of the natural GroEL-GroES chaperonin. The chaperonin GroEL posses an oligomeric double ring structure that together with the cochaperonin GroES, assists protein folding *in vivo*. GroEL apparently captures the non-native protein substrate by binding to exposed hydrophobic surfaces. This binding inhibits both folding and aggregation, and may promote unfolding of misfolded structures. In a second step, GroEL interacts with ATP and the protein substrate is released in the folded form (Wang J. D., et al. 1998; Swietnicki 2006). Based on this scheme, there are two methods of chaperone assited refolding. The first one in which the denatured protein is applied to the column containing immobilized chaperone-protein and refolded by chromatographic procedure (Dong X.-Y. et al. 1999 biotechnol. techniques). For example, denatured human interferon-γ was applied to a short Sephadex G-200 gel-filtration column to remove the

denaturant and then to a second column containing an immobilized GroEL chaperone fragment (amino acids 191–345). The chaperone column increased the refolding yield sixfold compared to refolding on SEC column (Gao, et al. 2003).

The second one emphasizes the ability of chemicals, 'artificial chaperones', such as cyclodextrins to prevent the aggregation of renatured proteins when denaturants and/or detergents are removed. In a typical scheme, the denatured protein is immobilized reversibly on a solid support and the denaturant is removed by washing with buffer containing cyclodextrin. The cyclodextrin is then removed by washing with another buffer and the properly folded protein is released from the column (Swietnicki 2006). In a variation of this strategy, Li et al. used an immobilized cyclodextrin column to adsorb the denatured Staphylococcus aureus elongation factor G in the presence of detergent. The detergent was removed by a simple wash and the protein was eluted with buffer containing cyclodextrin (Li J.-J., et al. 2006)

Antibodies have been shown to facilitate the refolding of the target protein antigen. The antibody recognizes the motif of a denatured antigen and facilitates the correct protein folding. If the antibody could retain or recover its binding to the antigen at reasonably high denaturant concentrations, the immobilized antibody would be a useful tool to assist in the refolding of the antigen, especially from an industrial point of view (Li M., et al. 2004).

1.2.6. Micelles and liposomes assisted protein refolding

Detergents and phospholipids at higher concentrations form micelles and liposomes as well as mixed micelle systems formed by phospholipids and detergents. The micelles and liposomes have been shown to promote protein refolding. Proteins are quite insoluble below CMC (critical micelle concentration), while highly soluble at or above CMC (Hanson and Rouan 1992; Bogdanov and Dowhan 1999). Zardeneta et al. showed that mixed micelle systems can prevent misfolding and/or aggregation and promote correct folding of protein. Since mixed micelle has both polar and nonpolar moieties, it can bind to various exposed interactive sites in either unfolded proteins or protein folding intermediates (Zardeneta and Horowitz 1994).

Reversed micelles, formed when an aqueous detergent solution is mixed with an organic solvent, can also facilitate protein refolding by avoiding aggregate formation. The denatured protein, once transferred to this solution, tries to avoid the organic phase, and, after reaching the hydrophilic core of the reversed micelle, can refold as a single molecule. However, the recovery of a refolded protein from these micellar structures is complicated (Vallejo and Rinas 2004).

1.2.7. High pressure refolding

An alternative disaggregation and refolding procedure that employs high hydrostatic pressure (HHP) has been described. HHP (up to 3 kbar) is used to solubilise proteins from insoluble aggregates including amorphous precipitates, amyloid fibrils, and inclusion bodies (IBs). Since aggregates are accumulated at atmospheric pressure (1 bar), proteins can reach their native state after gradual depressurization (St John, et al. 1999).

HHP (1–2 kbar) combined with low, nondenaturing concentration of denaturant, promoted disaggregation and refolding of denatured and aggregated recombinant human growth hormone (rhGH), lysozyme, β-lactamase from IBs (St John, et al. 1999, 2001, 2002). Intermolecular hydrophobic and electrostatic interactions between protein molecules within aggregates are disrupted in high pressure. In contrast, hydrogen bonds are not sensitive to HHP (protein monomers retain native-like secondary structure up to 5 kbar) due to the negligible volume change associated with breaking of these bonds. In order to facilitate disruption of hydrogen bonding between protein molecules within aggregates, the temperature can be raised on pressurized samples and/or non-denaturating concentrations of chaotropes such as GdnCl can be included in the protein solution. HHP also cannot break disulphide bonds that sometimes covalently cross-link protein aggregates. In these cases, redox-shuffling reagents are included in the pressurized solution to facilitate the breaking of intermolecular disulphide bonds and reshuffling of nonnative disulphide bonds into native ones. (St John, et al. 1999, 2001, 2002; Lee, et al. 2006, Randolph, et al 2002).

1.3. Improvement of refolding process

1.3.1. Competition between folding and aggregation

Correct folding into native structure *in vitro* or *in vivo* competes with unproductive side reactions, i.e., misfolding or aggregation. Therefore, both how fast the denaturant concentration is reduced and how long protein molecules are exposed to intermediate denaturant concentration determine the rate of folding and the degree of flexibility or solubility of folding intermediates (Rudolph and Lilie 1996; Mukhopadhyay A. 1997).

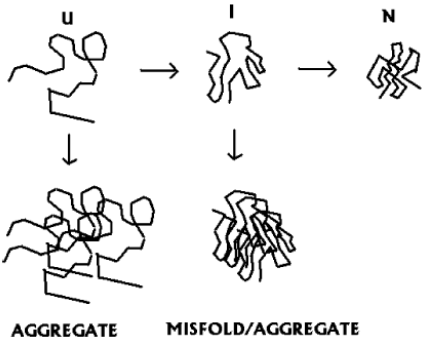

Fig. 2. Folding and aggregation during protein renaturation. U, I, and N correspond to the unfolded, intermediate, and native state of protein, respectively (Tsumoto, et al. 2003b)

Equilibrium unfolding studies have demonstrated the existence of an intermediate structure (I) at the interim concentrations of denaturant. Such intermediate structures are also formed during refolding. The intermediate structure is unstable, less soluble than unfolded protein and thus more susceptible to misfolding and aggregation. Therefore, it is important to promote correct folding of the intermediate into the native-stable structure (N) (Tsumoto, et al. 2003b).

Intermediates with hydrophobic patches exposed to the solvent play a crucial role in the partition between native and aggregated conformations. Partially unfolded states (folding intermediates) contain significant elements of a secondary structure, but little of the native tertiary structure and are much more prone to aggregation than both native and completely unfolded conformations. Thus, the 'molten globule' (intermediate) plays a major role in the kinetics of folding and aggregation (Rudolph and Lilie 1996; Tsumoto, et al. 2003b; Roberts 2007). Finally, folding and aggregation are fundamentally different as folding occurs through specific intramolecular interactions, whereas aggregation is nonspecific and occurs through intra- and intermolecular interactions.

Proteins are only marginally stable and are highly susceptible to degradation, both chemical and physical. Chemical degradation refers to modifications involving covalent bonds, such as deamidation, oxidation, and disulphide bond shuffling. Physical degradation includes protein unfolding, undesirable adsorption to surfaces, and aggregation. Thus, renaturation yield can be improved not only by optimising the folding conditions such as protein concentration, temperature, time, ionic strength, pH, various additives and, in the case of disulphide-bonded proteins, additives which promote direct disulphide

bond formation, but also changing intrinsic polypeptide properties (De Marco 2009).

Protein aggregation is generally protein concentration dependent due to an intermolecular interaction. Also, the size of protein aggregates may increase with increasing protein concentrations. It has been suggested that low protein concentrations (10–100 μg/ml) are more preferable for efficient renaturation. This, however, leads to large reaction volumes in order to produce quantities of renatured IB proteins (De Bernardez Clark 2001).

The refolding of recombinant pGH was strongly dependent on the concentration. Maximum monomer yield (~90 %) was obtained at protein concentrations of 0.05 mg/ml, and the decreasing yield was found with increasing protein concentration (Cardamone, et al. 1995).

Temperature is an important parameter that influences folding. In most cases, a higher refolding yield and less aggregation can be observed at lower temperatures. This decreases the folding speed and hydrophobic interaction, whereas high temperatures favor aggregation. The optimum refolding temperature must be determined experimentally for each target protein. In most cases refolding is performed at temperatures between 5 and 15 ºC (Mayer and Buchner 2004).

Xie et al. showed that another alternative for decreasing protein aggregation, during folding at relatively high protein concentrations, is to use the "temperature leap" tactic. Initially, the protein is allowed to refold at low temperatures, in order to minimize aggregation, and then the temperature is rapidly raised to promote fast folding. The results showed that refolding at ordinary (low) temperatures competes with hydrophobic aggregation which is strongly suppressed in the cold (Xie Y. and Wetlaufer 1996).

Based on the hypothesis that aggregation is caused by the interactions between hydrophobic patches of the partially folded polypeptide chains, it is possible to envision strategies to decrease aggregate formation during refolding. An examination of structural and amino acid sequence data can lead to the identification of hydrophobic patches within the protein molecule that could participate in intermolecular interactions. Aggregation – prone regions are blocked in the native state of globular proteins since their side chains are usually hidden in the interior of the protein hydrophobic core. This is the reason why globular proteins rarely aggregate from their native states. Mutations causing the disruption of such aggregation – prone regions may reduce aggregation. There are a number of protein systems in which it has been shown that point mutations may dramatically affect the aggregate formation (Chiti, et al. 2002, 2003; Taddei, et al. 2000; Fernandez-Escamilla, et al. 2004; Ishibashi, et al. 2007).

Chiti et al. showed that the intrinsic effect of specific mutations on the aggregation rate of intermediate structure of polypeptide (e.g., human muscle

acylphosohatase) can be correlated to a remarkable extent with changes in such protein properties as hydrophobicity, net charge and secondary structure (Chiti, et al. 2002, 2003).

The pH of the solution is also an important factor. All proteins have a characteristic pH range at which they can fold efficiently and reach their active state (Mayer and Buchner 2004). The pH has a strong influence on the aggregation rate. Proteins are often stable against aggregation over narrow pH ranges and may aggregate rapidly in solutions with pH outside these ranges. Solution pH determines the type (positive or negative) and total charge of the protein, thereby affecting electrostatic interactions. Since the number of charged groups on protein surface is increased by increasing the acidity or basicity of the solution, the increased charge repulsion within the protein destabilizes the folded protein conformation. Thus, the charge density of the folded protein is greater than of the unfolded protein. Therefore pH at both extremes decreases the protein conformational stability and solubility, resulting in a greater tendency for irreversible aggregation or precipitation (Fan K., et al. 2000; Parkinson, et al. 2000; Pace, et al. 1996).

For proteins containing disulphide bonds, an alkaline pH is necessary to allow the formation and reshuffling of disulphide bonds (Mayer and Buchner 2004). However, the solubilisation of IBs, protein refolding, storage or processing at high pH may not be applicable to many proteins. Because at alkaline pH some amino acids side chains may undergo a chemical modification (Violand, et al. 1990, 1992; Tou, et al. 2009). Deamidation appears to be most common degradation in proteins. Asn and Gln are two amino acids susceptible to deamidation in proteins and Asn is much more labile than Gln. Most of the deamidated asparagine forms iso-aspartate, which is not a natural amino acid and can potentially be immunogenic (Jenkins, et al. 2007, 2008). It was described that the deamidation of ribonuclease A (Asn→isoAsp at position 67) significantly decreases folding rate and alters the folding pathway. (Orru, et al. 2000). Also, it was identified that the principal site of deamidation for hGH is Asp149 during storage under alkaline conditions with a minor site of deamidation at Asn152 (Canova-Davis, et al. 1990; Becker, et al. 1988).

1.3.2. Disulphide bond formation during protein refolding

Disulphide bonds are one of the few posttranslational covalent modifications that occur during protein folding. The formation of disulphide bonds is one of the major rate-limiting steps in protein folding *in vivo*. Correct disulphide bond formation in proteins is required not only for folding, but also for stability and function. Failure to form the correct disulphide bonds is likely

to cause protein aggregation and subsequent degradation by cellular proteases *in vivo* (Mamathambika and Bardwell 2008).

The process that leads to the rearrangement of disulphide bonds within the protein is called disulphide reshuffling. The simplest method promoting disulphide bond formation *in vitro* is oxidation by molecular oxygen, catalysed by trace amounts of metal ions such as Cu^{2+} or Co^{2+}. But the rate and yield of this process is very low. In order to prevent coincidental oxidation of thiols by molecular oxygen, chelating reagents such as EDTA should be added to the buffer (Misawa and Kumagai 1999, De Bernardez Clark 1998).

For example, the oxidation of recombinant caprine and buffalo GHs was carried out at protein concentrations of 1.5 mg/ml in 6 M GdnCl, 50 mM Tris, pH 8.5 at room temperature in an open vessel with gentle stirring. The air-oxidation of thiol groups of proteins was catalysed by metal-ions which were available from the reagents used in the reaction (no metal-ions was externally added) and it was stopped by adding EDTA to a final concentration of 10 mM. The complete oxidation of proteins occurred within 36 hours by a simple air-oxidation process (Mukhopadhyay U.K. et al. 2002).

Low molecular weight thiols in a reduced and oxidised form are generally employed for protein disulphide bond formation in thiol-disulphide exchange reactions. This method usually results in much higher rates than those obtained through air oxidation and it has been the most widely applied oxidation method (Fig. 3) (Misawa and Kumagai 1999; Cardamone, et al. 1995).

The most commonly used oxido-shuffling reagents are reduced (GSH) and oxidized (GSSG) glutathiones. Other low molecular weight thiols such as cysteine/cystine, cysteamine/cystamine, or di-hydroxyethyl disulphide/2-mercaptoethanol are also used for disulphide bond formation of IB proteins (Mamathambika and Bardwell 2008).

Normally, a refolding buffer composed of a mixture of oxidized and reduced reagents is used during *in vitro* folding experiments. The addition of a mixture of the reduced (RS^-) (typically 1–3 mM of reduced thiol) and oxidized (RSSR) forms of low molecular weight thiols reagents to the refolding buffer usually provides the appropriate redox potential allowing the formation and reshuffling of disulphides (Fig. 3). Molar ratios of reduced and oxidized compounds varie from 2:1 to 10:1, respectively. These systems increase both the rate and yield of renaturation/reoxidation by facilitating rapid reshuffling of incorrect disulphide bonds (Rudolph and Lilie 1996; De Bernardez Clark 1998; Misawa and Kumagai 1999).

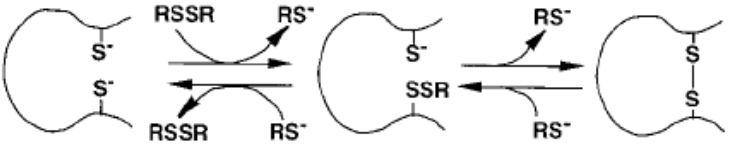

Fig. 3. Principle of 'oxido-shuffling' during disulphide bond formation (Misawa and Kumagai 1999)

The strategy employed to oxidize proteins during folding is the formation of mixed disulphides between an oxidized glutathione and a reduced protein before renaturation. The formation of mixed disulphides increases the solubility of the denatured protein. Disulphide bond formation is then promoted by adding catalytic amounts of a reducing agent in a renaturation step (De Bernardez Clark 1998; Misawa and Kumagai 1999).

Another method used to promote disulphide bond formation during renaturation is the reduction of an oxidized protein cysteine residues in the presence of strong denaturant. When the reducing and denaturing agents are removed, the protein is refolded in the presence of suitable buffers containing redox agents (Hevehan and De Bernardez Clark 1997).

One of the most important conditions that affect the oxidative folding is the pH of the refolding buffer. An alkaline pH is necessary to allow the formation and reshuffling of disulphide bonds. The pK_a of cysteines in denatured proteins is usually approximately 8.7. Therefore, rapid oxidative folding reactions tend to occur when the pH is above pK_a of cysteines, and oxidative folding becomes slower at solution pH values below the pK_a. Thus, the oxidative refolding of the proteins is usually performed around pH 8.0. The formation of disulphide bonds is thermodynamically coupled to the process of protein folding. The folded conformation stabilizes the disulphide bond to the same extent that the conformation is stabilized by the formation of disulphide bond (Mamathambika and Bardwell 2008).

The literature analysis revealed that the time required for complete protein renaturation can take a few minutes or days. For example, the time required for complete renaturation of arginine deaminase is 90 hours even at 15 ºC by the rapid dilution refolding method (Misawa, et al. 1994) while for human interleukin-4 − only 4 hours at room temperatures by the same refolding method (Van Kimmenade, et al. 1988). The renaturation of various growth hormones (GH) with high sequence similarity took from 16 to 24 hours (e.g., 24 hours at room temperature by dialysis for bovine GH (Langley, et al. 1987a, b), 24 hours at 4 ºC by dilution for salmon GH (Sugimoto and Yokoo 1991), 16 hours at 4 ºC by dialysis for porcine GH (Cardamone, et al. 1995)).

It has been emphasized that it is not possible to give a general recipe or a set of parameters optimal for any protein. Finding the best conditions for refolding of a specific protein can be complicated and laborious process (Mayer and Buchner 2004).

1.4. Effect of solution additives on protein aggregation

In this chapter I'll overview solution additives that affect protein stability and aggregation during refolding and heating processes.

Solution additives are mainly grouped into two classes, protein denaturants and stabilizers. One group includes GdnCl, strong ionic detergents, chaotropic salts as denaturants and the latter includes osmolytes (amino acids, amino acids derivatives, methylamines, carbohydrates (polyols and sugars)) as stabilizers. However, there are solution additives which are not placed in these two groups, e.g., amino acid arginine, some amino acids derivatives, cyclodextrins, polyamines, ionic liquids, amphiphilic polymers. They have marginal effects on the protein structure and stability, but appear capable of suppressing or disrupting protein-protein interactions, and are defined as aggregation suppressors (Hamada, et al. 2009, Tsumoto, et al. 2003b).

It has long been known that incubating protein solutions at high temperatures results in physical degradation (aggregation). It has been observed that aggregation starts at temperatures below the equilibrium melting temperature of the protein (Chi, et al. 2003). This observation suggests that aggregates are not formed from fully unfolded molecules. Thus, in some cases thermally induced denaturation may be reversible for some proteins. However, high temperatures usually lead to denaturation followed by irreversible aggregation. Kinetic analysis of the thermal aggregation of proteins has been classically described by the Lumry-Eyring model of protein aggregation (Hamada, et al. 2009; Baynes, et al. 2005; Chi, et al. 2003; Cromwell, et al. 2006):

$$N <=> TS -> A \quad (1)$$

$$A + A_m -> A_{m+1} \quad (2)$$

where N is the native protein, TS represents the transition state preceding the formation of an aggregation A, and A_m and A_{m+1} are aggregates of heat denatured state with the aggregate size of A_m and A_{m+1}. The model, shown above, involves a reversible conformational change of protein followed by irreversible assembly of the nonnative species to form aggregates. There are two

types of additives and mechanisms which can reduce protein aggregation. The first one is a protein stabilizer which stabilizes structure of a native protein and the latter is an aggregation suppressor which inhibits the protein-protein (intermolecular) interaction. Also, it has been observed that a number of proteins follow the first-order aggregation kinetics in the absence and presence of additives, suggesting that the rate-limiting step is unimolecular (e.g., a conformational change) (Weijers, et al. 2003; Shin, et al. 2002; Hamada, et al. 2009; Morris, et al. 2009).

On the other hand, the correct refolding from denaturant induced unfolded state competes with higher order (≥2) reaction of the intermolecular aggregation. Also, FT-IR spectra of lysozyme aggregates showed that the refolding-induced aggregates have a different structure from the heat-induced aggregates (Hamada, et al. 2008). Thus, the refolding reaction is governed by two competing reactions, i.e., an intramolecular reaction (folding) and an intermolecular reaction (aggregation):

$$U \rightarrow N \tag{3}$$

$$U + U \rightarrow A_2 \tag{4}$$

where U, N, and A_2 represent the unfolded state, the native state, and the aggregated state, respectively (Baynes and Trout 2004). There is a direct competition between proper protein refolding and aggregation. This simple model suggests that a fine balance between refolding and aggregation determines the refolding yield. Folding is a unimolecular reaction, while aggregation is a multimolecular reaction. During refolding reaction, the hydrophobic interaction drives the unfolded protein to isolate their hydrophobic patches from contacts with water when the denaturant concentration is reduced. The competition between inter-molecular and intra-molecular interactions is responsible for the decreased yield of a biologically active protein, in particular at high protein concentration. Thus, additives that prevent aggregation are conventionally used to assist protein refolding. And, additives that stabilize protein structure may accelerate refolding kinetics and enhance aggregation, leading to a reduced refolding yield (Baynes and Trout 2004; Baynes, et al. 2005; Jaenicke 1999).

1.4.1. Classification of solution additives

The denaturants (e.g. urea, GdnCl) at high concentrations completely disrupt both intra- and inter-molecular interactions of proteins, leading to denaturation (unfolding) and solubilisation. It is known, that aggregation is suppressed by protein denaturants during refolding process. The proteins that are

difficult to refold have been successfully refolded in the presence of non-denaturing concentrations of denaturants. On the other hand, GdnCl showed only little effect on the suppression of the heat-induced aggregation of lysozyme (Jaenicke, 1999; Kudou, et al. 2003; Hamada, et al. 2008).

Detergents (e.g. SDS) at CMC denature protein structure by binding to hydrophobic regions of the polypeptide and form detergent-protein complexes. The binding of the detergent to the protein suppress the aggregate formation. Refolding is achieved by lowering e.g. SDS concentration below CMC. However, SDS monomer can bind to the protein so tightly that dilution or dialysis does not completely dissociate SDS molecules. Therefore, artificial chaperones (e.g., cyclodextrin or cycloamylose) are used to adsorb SDS molecules. These hydrophobic cyclic sugars form strong complexes with detergents and cause their release from the protein allowing protein to complete its refolding (Hamada, et al. 2009; Desai, et al. 2006).

The effect of salts on protein stability is a set of ionic interactions on fully exposed surfaces, and fully or partially buried interior of proteins. Salts may stabilize, destabilize, or have no effect on protein stability depending on the type and concentration of salt, nature of ionic interactions, and charged residues in proteins (Wang W. 1999; Kohn, et al. 1997; Kita and Arakawa 2002).

The ability of salts to increase the surface tension of water is an indication of effects on protein stability (Table 1). Those additives, which raise the surface tension of water, increase the stability of the proteins against various stresses. Also, the solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. At low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Ammonium sulfate is widely used as a precipitant for protein purification and crystallization, as this salt prevents amorphous aggregation. However, this is not valid for the salting-in effect of chaotropic salts. This solubilizing influence is especially pronounced for small, electronegative anions such as the thiocyanate, perchlorate, iodide and to lesser extent for small cations (e.g. Li^{+}, Ca^{2+}, guanidinium). The order of effectiveness of such ions is codified in well-known Hofmeister series (Baldwin 1996; Moelbert, et al. 2004). Chaotropic salts are used to enhance protein solubility at low concentrations, but at higher concentrations they usually perturb macromolecular conformation of protein (Middaugh and Volkin 1992; Hirano, et al. 2007, 2008; Hamada, et al. 2009).

Table 1. Classification of solution additives (Arakawa, et al. 2007a)

	Kosmotropes	Chaotropes
Protein stability	Stabilizer	Destabilizer (or denaturant)
Protein solubility	Salting-out	Salting-in
Water structure	Maker	Breaker
Surface tension	Increase	Decrease

1.4.2. Osmolytes

Research over the past two decades has provided better understanding of the molecular biology of stress responses in different organisms. Organisms living under conditions of stress (e.g. high osmotic pressure, extreme temperatures and pressure) accumulate low molecular weight organic compounds known as osmolytes. Osmolytes are specific compounds: amino acids and their derivatives (e.g., glycine, proline, taurine and ectoine), carbohydrates (polyols (e.g., glycerol, inositols, sorbitol), sugars (e.g.,trehalose)), methylamines (e.g., N-trimethylamine oxide (TMAO), betaine), methylsulfonium (e.g., dimethylsulfonopropionate (DMSP)) and urea (Fig. 4). They are synthesized by microorganisms, plants, and animals in response to environmental stress and serve to protect proteins against denaturation. Except for urea, they are often called ‘compatible solutes’ based on the hypothesis that these solutes do not interact with macromolecules in a detrimental way, thus, they can be safely regulated with little impact on cellular functions. This is a contrast to inorganic ions, which at high concentrations typically bind and destabilize proteins and nucleic acids (Yancey 2005, 2001; Pradeep and Udgaonkar 2004; Kumar 2009; Milner, et al. 1987; Oganesyan, et al. 2007).

Most osmolytes are neutral (either zwitterionic or lacking charges) at physiological pH. Even though some bacterial and archaeal osmolytes are anionic, they are paired with K^+ to achieve neutrality. The ‘compatible solutions’ hypothesis suggests that organic osmolytes are interchangeable i.e. that the cell can be osmotically protected with a variety of compatible osmolytes (Yancey 2005, 2001).

All osmolytes have high viscosity indexes. Diamant et al. hypothesized that osmolyte viscosity possibly contribute to the osmolyte-mediated thermal protection and promotion of refolding. However, viscosity measurements in osmolyte solutions revealed that there is no direct correlation between osmolyte viscosity and the ability of the osmolyte to promote correct refolding of urea-unfolded malate dehydrogenase (MDH). In contrast, for all analysed osmolytes (betaine, glycerol, proline and trehalose), it has been indicated that viscosity is a major component in the mechanism of osmolyte-induced thermoprotection (Diamant, et al. 2001).

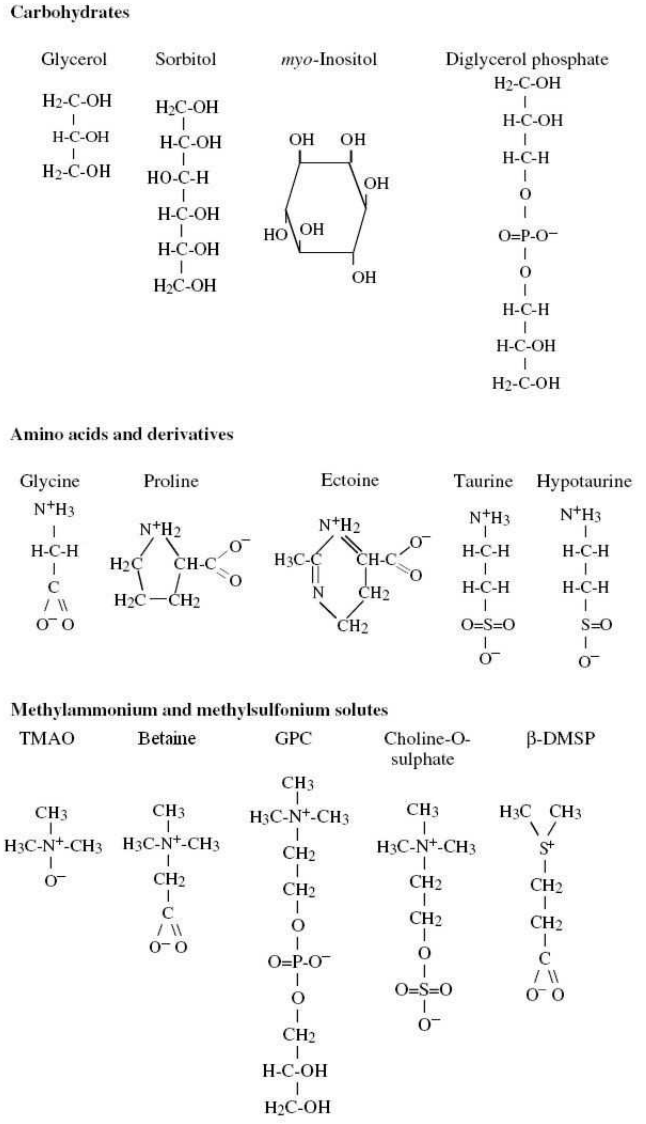

Fig. 4. Examples of organic osmolytes; TMAO, trimethylamine-N-oxide; GPC glycerophosphorylcholine; DMSP dimethylsulfonoproprionate (Yancey 2005)

Osmolytes are widespread in different kingdoms of life, except for urea which is used by relatively few types of animals. Betaine is found in every kingdom of life, taurine is widespread among marine animals and some mammalian organs. Carbohydrate osmolytes occur in archaea, fungi, algae, plants and mammalian kidneys, and possibly deep-sea invertebrates. Sugars and

polyols are usually dominant solutes in organisms that tolerate or avoid freezing, such as terrestrial plants, insects, amphibians and some polar fishes. Also, many organisms use mixtures of different osmolytes, e.g., the mammalian kidneys use the polyols myo-inositol and sorbitol, the methylamines glycerophosphorylcholine (GPC) and betaine, the amino acid taurine (the organ also has high concentration of urea as both a waste product and an osmotic agent that helps concentrate the urine) (Yancey 2005).

Protein folding, according to the thermodynamic description, is just a reequilibration between the unfolded and folded states under changed solvent conditions. Osmolytes promote protein folding into a native state and suppress aggregation that competes with protein folding, without making or breaking covalent bonds. Stabilizing osmolytes push the folding equilibrium toward native state (N), whereas denaturing osmolytes push the equilibrium toward unfolded state (U). Thus, the ability of osmolyte to stabilize protein under denaturing treatments may be attributed to unfavorable interaction between osmolyte and peptide backbone (the osmophobic effect). This results in transition of the protein into a more compact state (native state) characterized by lower free energy (Pradeep and Udgaonkar 2004; Street, et al. 2006).

Stabilizing osmolyte, such as TMAO, are preferentially excluded from the backbone of protein because water is more likely than TMAO to interact favorably with the backbone polar groups. Conversely, destabilizing osmolytes, such as urea, are preferentially accumulated near the backbone because they have a stronger propensity to interact with the backbone than water (Street, et al. 2006; Holthauzen, et al. 2010).

According to Timasheff, the main factor for the stabilizing effect of the osmolyte on protein is related to the fact that these osmolytes are preferentially excluded from the protein surface, i.e. they cause preferential hydration of the protein; this effect should favor a protein state with lower exposed surface, thus displacing the denaturation equilibrium, towards the N state (Timasheff 2002; Arakawa and Timasheff 1985; Nakakido, et al. 2008). However, the exact nature of interactions that govern the osmolyte-mediated stability of proteins is, therefore, not yet very clear. Overall, protein stability should depend upon a fine balance between favorable and unfavorable interactions of the native and the denatured protein states with the cosolvent molecules. The stabilizing effect would, thus, depend on the nature of both the proteins as well as the cosolvent molecules and generalization of the effect may not be possible (Kaushik and Bhat 2003).

Many organic molecules (osmolytes) have been identified which help to survive, tolerate and live under variaty abiotic stresses – drought, salinity, low and high temperatures, etc. Consequently, some of these effective solutes such as proline, betaine, glycine, trehalose, glycerol, amino acids derivatives etc. have

been used in biotechnology applications since they are natural compounds and can be safely used (Arakawa et al. 2007 b).

Proline

Proline is an important amino acid in terms of its biological functions and biotechnological applications. In response to osmotic stress, proline is accumulated in many bacterial and plant cells as an osmoprotectant. Proline is believed to serve multiple functions *in vitro* such as protein and membrane stabilization. The imino acid, proline, is biosynthesized from glutamate in the cytoplasm and the excess of proline is converted to glutamate. Proline as the compatible solute is remarkably high water soluble (>7 M) at room temperature. At high concentrations (>1.0 M) it behaves as a hydrotrope enhancing the solubility of hydrophobic compounds (Takagi 2008; Samuel, et al. 2000; Wang A. and Bolen 1996; Fisher 2006).

Proline at higher concentrations (>1.5 M) prevents protein aggregation during oxidative refolding of hen egg lysozyme, mitochondrial malate dehydrogenase (MDH), etc. Although proline almost completely prevented protein aggregation during refolding of hen egg lysozyme, the refolded protein sample showed only 30 % of native enzyme activity in the presence of proline and regained 75 % of the enzymatic activity after proline was removed by ultrafiltration. The observed suppression of aggregation was attributed to the formation of a higher order structure of proline at high concentrations (Samuel, et al. 2000, Kim, et al. 2006a).

Since the stabilization of proteins by the additives, e.g. amino acids, against heat-induced denaturation and subsequent aggregation is associated with the increased melting temperature of the proteins, proline above 0.2 M significantly increased the melting temperature (Tm) of keratinocyte growth factor (KGF), cytochrome-C and lysozyme (Arakawa et al. 2007b; Taneja and Ahmad 1994; Chen and Arakawa 1996).

Betaine

Osmolyte such as glycine betaine (N,N,N-trimethylglycine) (often referred to as betaine) can also act as a‘chemical chaperone’ by increasing the stability of native proteins and assisting refolding of unfolded proteins. Betaine is a universal compatible solute in various prokaryotes (e.g., in *E.coli*), animals, algae, and salt tolerant plants. The osmoprotectant, betaine, is accumulated in *E.coli* cells to molar concentrations. Betaine is uptaken from extracellular environment and/or certain strains can synthesize betaine from choline (Caldas, et al. 1999; Diamant, et al. 2001, 2003; Chebotareva 2007).

The use of osmolyte betaine may posses numerous advantages. For example, citrate synthase, was unfolded in the presence of 8 M urea, and

allowed to refold upon dilution of the denaturant, in the presence or absence of betaine. The refolding yield of citrate synthase increased from 9 % to 29 % in the presence of 300 mM of betaine (Caldas, et al. 1999). To address the thermoprotective nature of betaine *in vitro*, activity of mitochondrial malate dehydrogenase (MDH) under heat-denaturing conditions was examined. Thus, it was demonstrated that 1.5 M betaine fully protected native MDH over 40 minutes period at 44 ºC (Diamant, et al. 2001).

Glycine

Amino acid, glycine, influences the folding of proteins prone to aggregation. And its effect is very similar to betaine. Both glycine and betaine can serve as protective osmolytes and this may be the basis of their beneficial action.

It has been reported that glycine stimulates the synthesis of secretory cytochrome b5 of *Escherichia coli*, as well as its discharge into the medium. Extracellular amounts of cytochrome b5 increased with increasing concentrations of exogenous glycine and the duration of the culture period, in spite of the fact that increasing glycine in the medium progressively inhibited cell growth. For example, 1 % glycine in the growth medium reduced the bacterial growth by 50 %. Kaderbhai N. et al. concluded that supplementation of *E. coli* cultures with moderate amounts of glycine substantially stimulated the synthesis of exportable proteins and further enhanced their yield by discharging into the growth medium (Kaderbhai, et al. 1997).

Glycine has a variety applications in biotechnology:

- commonly used as bulking agent in a freeze-drying process,
- greatly increases the solubility of proteins at isoelectric pH,
- used in formulations of protein pharmaceuticals as an excipient.

Carbohydrates

Carbohydrates including sugars and polyols are essential for cell survival at temperatures below zero. Cryoprotectant such as glycerol, trehalose and sorbitol can be accumulated in the cell up to molar concentrations. *In vitro*, sugars and polyols have been found to be very effective in the stabilization of labile proteins during lyophilization and exposure to high temperatures in solutions (Kaushik and Bhat, et al. 2003; Kim, et al. 2006a).

Sugar, trehalose, acts directly during heat shock to stabilize proteins in the native state. Also, it has been observed that trehalose reduces the aggregation of proteins that have already been denatured. This function previously was attributed exclusively to heat shock proteins (Singer and Linquist 1998).

Since different organisms produce different compatible solutes (e.g., sugars and polyols), the attempts were made to find out whether the suppression of

protein aggregation is a universal characteristic of these compounds. For example. Rhodanese aggregation was suppressed in the presence of 0.5 M of sucrose, maltose or trehalose. Glucose and sorbitol had a smaller effect and glycerol or mannitol had little or no effect on rhodanese aggregation. Thus, authors concluded, that many of sugars and polyols, but not all can reduce aggregation of denatured proteins (Singer and Linquist 1998).

In all cases, the stabilizing effect of sugar or polyols depends on their concentrations. A concentration of 0.3 M (or 5 %) has been suggested to be the minimum one to achieve protein stabilization (Wang W. 1999; Miyawaki 2007). Also, Li et al. reported that sugars and polyols can protect proteins from chemical degradation. They studied the effect of sugars and polyols on the metal catalyzed oxidation of human relaxin and found that sugars and polyols can significantly inhibit the oxidative degradation (Li S., et al. 1996). Thus, sugars and polyols represent often used non-specific protein stabilizers and their stabilizing effect is widely demonstrated.

Proteins are very sensitive and critical compounds in biochemistry and molecular biology. Protein stabilization will continue to be a key issue in biotechnology. Neurodegenerative pathologies, including Alzheimer's, Huntington's, and Parkinson's diseases, are related to protein aggregation/misfolding. Various effects of osmolytes on proteins have become important to study because this may help scientists develop better therapeutic tools for the management of such diseases. Osmolytes, also enhance thermodynamic stability of proteins by providing natively folded conformations without perturbing other cellular processes. Under physiological conditions, the cellular compositions of osmolytes may vary significantly. This may lead to different protein folding pathways utilized in cells depending upon the intracellular environment. Proper understanding of the role of osmolytes in cell regulation should allow us to predict the action of osmolytes on macromolecular interactions in stressed and crowded environments typical of cellular conditions. (Pradeep and Udgaonkar 2004; Kumar 2009; Rosgen, et al. 2005).

1.5. Solution additives – aggregation suppressors

There are many solution additives that affect protein stability and aggregation during refolding, heating and freezing processes. Some additives stabilize native fold, while the others destabilize or denature the protein structure. They are mainly grouped into two classes, protein stabilizers and denaturants. However, there are some unique compounds, which do not belong in either of these classes. They have little effect on protein structure and stability, but they effectively suppress aggregation. This class of solution

additives is defined as aggregation suppressors. Aggregation suppressors encompass solution additives such as cyclodextrin, arginine and its derivatives, polyethylene glycol, etc. (Tsumoto, et al. 2003b; Hamada, et al. 2009).

1.5.1. L-arginine: an unique amino acid

L-arginine (S-2-amino-5-(guanidino) pentanoic acid) is the most basic of the proteinogenic amino acids, with a pI of about 10.8. It is commonly used in the form of its hydrochloride (L-ArgHCl), and has been widely employed as an additive for the *in vitro* refolding of recombinant proteins. The function of L-arginine (L-Arg) as an aggregation suppressor during *in vitro* protein refolding was first reported in a patent application by Rudolph and Fischer in 1990. L-ArgHCl was added to the renaturation mixture of recombinant full length tissue-type plasminogen activator (tPA) as a potential inhibitor of serine proteases. An increase in the refolding yield of recombinant tPA was observed. Authors came to the conclusion, after a more thorough investigation of the phenomenon, that something much more generally applicable than a specific protease inhibitor had been discovered (Rudolph and Fischer 1990).

Before its beneficial effect on *in vitro* refolding was discovered, L-arginine had been classified as “perturbing” osmolyte incompatible with enzyme activity and stability. This classification was based on the observation that L-arginine does not form a part of the evolutionary selected set of small organic molecules that balance the osmolality of the cytosol in most organisms (Yancey, et al. 1982). This does not seem surprising now, as high concentrations of L-arginine suppress protein-protein interactions, and are therefore hardly compatible with a working intracellular environment (Lange and Rudolph 2009; Tsumoto, et al. 2005).

After L-Arg had been used as a refolding enhancer in the patent application, it has been shown that arginine exhibits a variety of effects on proteins. The main effect of arginine is considered to be its ability to suppress aggregation of folding intermediates during thermal unfolding or refolding process. Biotechnology applications of arginine are gradually expanding, e.g. mild solubilization of proteins from IBs, milder elution of antibodies from affinity resins and improved separation and recovery of proteins in size exclusion, ion-exchange, affinity chromatography (Arakawa, et al. 2007a; Lange and Rudolph 2009).

1.5.2. Refolding of proteins in the presence of L-arginine

It has been observed that proteins tend to aggregate during *in vitro* refolding when the denaturant concentration is being decreased. To minimize aggregation and increase refolding yield is always the main target of protein refolding research. The balance between aggregates and correct refolding of proteins could

be modulated by various environmental factors, especially by the addition of small molecule additives. Among various folding additives, the amino acid, arginine, is the most popular. Arginine has been effective in improving refolding yields of many proteins, e.g., human plasminogen activator (Rupdolph and Fischer 1990), lysozyme (Hevehan and De Bernardez Clark 1997), consensus interferon (Liu, et al. 2007a), interleukin-21 (Asano, et al. 2002), immunoglobulin-folded proteins (Buchner and Rudolph 1991), neurotrophin-3 (Suenaga, et al. 1998) and many others. Final yields of proteins increase several times by adding L-arginine to the renaturation buffer, e.g., 0.75 M–1 M of L-arginine increased the final renaturation yield of lysozyme by 2-fold. Normally, L-ArgHCl at concentration of 0.5–1 M appears to always have positive impact on protein refolding (Hevehan and De Bernardez Clark 1997; Liu, et al. 2007a, b; Suenaga, et al. 1998). Thus, L-arginine has a very strong preventive effect against aggregation for various proteins with different molecular weights structutre and pIs.

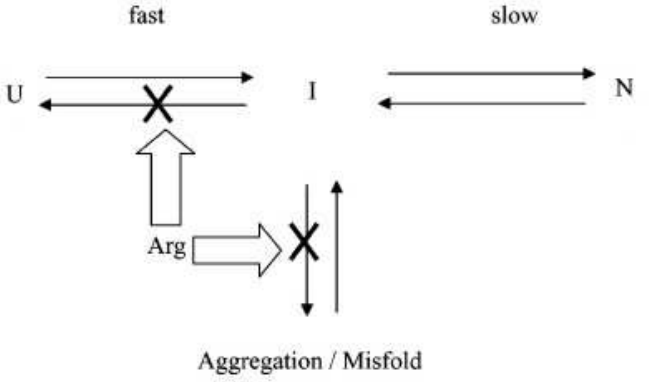

Fig. 5. Schematic representation of effect of arginine on refolding of proteins. U unfolded state; I folding intermediate; N (Tsumoto, et al. 2004)

As shown in Fig. 5, the effect of L-arginine on proteins refolding is considered to be its ability to suppress aggregation of folding intermediates, but not to enhance or facilitate folding reaction (Tsumoto, et al. 2004; Arakawa and Tsumoto 2003).

Although arginine contains a guanidino group, it even at higher concentrations has only a marginal effect on protein stability and is not protein denaturant. On the other hand this additive strongly enhances the solubility of folding intermediates. The increase in solubility of folding intermediates without significant destabilisation of the final native structure results in an improved refolding of proteins (Lilie, et al. 1998; Wang X. T.and Engel 2009). For example, proteins produced as inclusion bodies (IBs) are generally solubilized by using strong denaturants, which means that native protein must be renaturated

in a subsequent complicated refolding process. However, when a protein in IBs has a considerably native like structure, it should be possible to solubilize it under less strongly denaturing (milder) conditions. In order to achieve non-denaturing solubilization, an L-arginine solution was tested. This approach was successfully used to obtain the native structure of green fluorescent protein (GFP) and β2 microglobulin (β2m) as it was observed that these proteins have a native like secondary structure in the IBs. Since arginine does not denature proteins, the native proteins were observed in the aqueous arginine solutions used for solubilization. If arginine is unwanted in the final protein solutions, it can be simply removed by dialysis. (Tsumoto, et al. 2003a; Umetsu, et al. 2005).

Along with *in vitro* refolding, where L-arginine is very effective, Schaffner et al. showed that the addition of L-arginine and redox compound (glutathione (GSH)) to the culture medium increase the expression of recombinant disulphide-containing proteins in their active forms in the periplasm of *E. coli* (Fig.6). The periplasm is a compartment where oxidation of thiols can occur due to the activity of the disulphide oxidoreductase (Dsb) system. When incorrect disulphide bonds of proteins are formed (assisted by DsbA), Dsb system components (DsbC, DsbG) with disulphide isomerase functions convert incorrect disulphide bonds to correct ones. It has been suggested that arginine penetrates into the periplasmic space and suppresses aggregation, thus allowing DsbC to correct disulphide bonds of proteins in the periplasmic space (Schaffner, et al. 2001; Ejima, et al. 2005a).

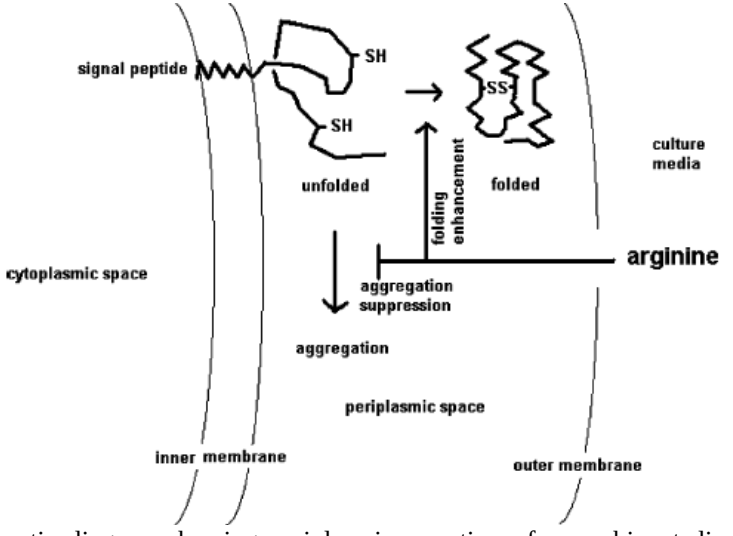

Fig. 6. Schematic diagram showing periplasmic secretion of recombinant disulphide-containing proteins by E.coli (Ejima, et al. 2005a)

Recently Liu et al. examined arginine effect on the refolding of consensus interferon (rIFN-con1). They observed that arginine was able to suppress the formation of insoluble precipitate during rIFN-con1 refolding, but not all soluble protein refolded on the productive pathway (Fig. 7). There were oligomers, dimmer, and monomers of partially refolded intermediates as well as a native structure of rIFN-con1 in the refolded samples in the presence of 0.5 M arginine (Fig. 7). Arginine suppressed precipitation (insoluble aggregates formation) and increased the soluble rIFN-con1 yield, but on the other side, it promoted the formation of oligomers. The refolding yield of rIFN-con1 increased with arginine concentration up to 0.5 M and decreased when its concentration exceeded 0.5 M. The authors came to the conclusion that the excessive arginine makes denatured species of protein too stable for refolding. Thus, denatured peptides with free cysteines have more possibilities to form intermolecular disulphide-bonds and the oligomers would form, which explains why the refolding yield inversely decreased when the concentration of arginine increased to a certain degree (Liu, 2007a; Chen, J. et al. 2008; Hevehan and De Bernardez Clark 1997).

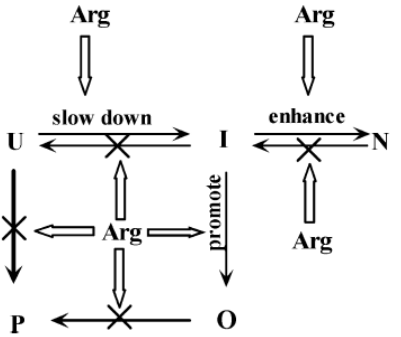

Fig. 7. Schematic description of arginine effects on rIFN-con1 refolding process. U: unfolded rIFN-con1; I: intermediate; N: native structure; O: soluble oligomers; P: precipitate (Liu, et al. 2007a)

Taken together that arginine may slow down protein conformational movement and promote the formation of intermolecular disulphide-bonded oligomers (Fig. 8), two-stage or stepwise dialysis folding strategy was proposed (Umetsu, et al. 2003; Liu, et al. 2007b; Kumagai, et al. 2010).

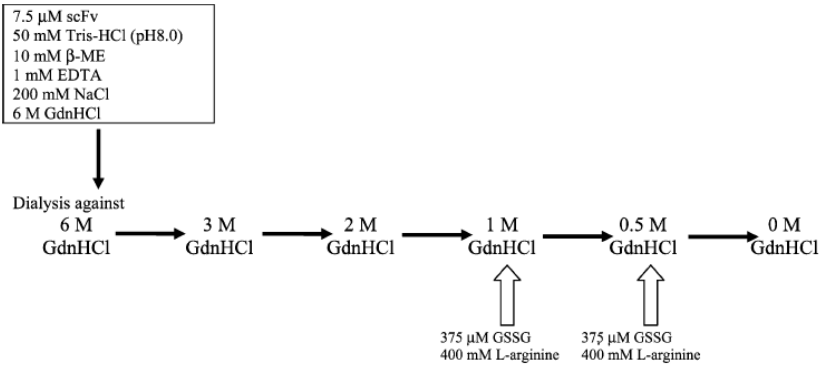

Fig. 8. The stepwise dialysis system. Inclusion bodies of scFv were solubilized by denaturant, and then the denaturant was gradually removed by stepwise dialysis against decreasing concentrations of denaturant. At appropriate stages, e.g., at the 1 M GdnHCl stage, additives such as arginine and GSSG were added to the dialysis solution (Umetsu, et al. 2003)

The role of arginine was examined in a stepwise dialysis refolding of single chain antibody (scFv) with reduced cysteinyl residues. The system is based on the gradual removal of denaturing reagent. Addition of L-arginine and oxidized glutathione (GSSG) to the refolding solution at appropriate stages appeared to interrupt disulphide bond formation and to maintain the solubility of partly folded polypeptides that have free thiol groups. Thus, controlled coupling of the effects of L-arginine and oxidized glutathione (GSSG) led to complete refolding of functional scFv in high yield (Umetsu, et al. 2003).

1.5.3. L-arginine effect on heat induced aggregation

Shiraki et al reported that L-arginine is the most effective suppressor for heat-induced aggregation among natural amino acids (Shiraki, et al. 2002). Heat-induced protein aggregation leads to irreversible protein inactivation. However, the addition of arginine can prevent heat-induced aggregation and inactivation of proteins. The amount of aggregates during heating of lysozyme (0.2 mg/ml) at 98 ºC for 30 min gradually decreased with increasing concentration of L-Arg (Kudou, et al. 2003; Baynes, et al. 2005).

Since arginine does not stabilize proteins, in contrast to other structure stabilizing amino acids, it only slightly affects the melting temperatures (T_m) of proteins. The melting temperatures of ribonuclease and lysozyme change little below 0.5 M arginine and slightly decrease above 0.5 M, e.g., T_m of ribonuclease at 0 M L-Arg is 63 ºC, whereas at 0.5 M L-Arg – 62 ºC and at 2 M L-Arg – 60 ºC (Arakawa and Tsumoto 2003).

A comparison of the effects of D-arginine with natural enantiomer L-arginine showed that both substances have the same efficiency, indicating that effects of arginine are not based on stereospecific interactions between arginine and protein. For example. Lysozyme (at concentration of 0.2 mg/ml) was heated at 98 ºC for 30 min in the presence of D-Arg or L-Arg at concentrations greater than 200 mM. The amount of soluble protein after heat treatment was equal in both cases. Thus, the preventive effect of D-Arg was identical to that of L-Arg (Shiraki, et al. 2002).

Recently it has been demonstrated that some arginine derivatives act even more effectively than arginine. Arginine ethyl ester (L-ArgEE·2HCl) and L-arginine amide (L-ArgAd·2HCl) were found to be even more effective in suppressing the aggregation of lysozyme than L-ArgHCl (Shiraki, et al. 2004, 2005; Hamada and Shiraki 2007; Matsuoka, et al. 2007). Thus, the authors suggested that the molecular mechanism of L-ArgEE action in preventing heat-induced aggregation of lysozyme is different from that of Arg. The isoelectric point (pI) of lysozyme is ~11. The net charge of protein at studied pH 6.5 is positive. Therefore, Shiraki et al. proposed that the hydrophobic end of the ethyl group of L-ArgEE interacts with the hydrophobic patch of the unfolded polypeptide, leading to enhanced electrostatic repulsion between aggregation-prone unfolded or partially unfolded lysozyme molecules. In the case of L-ArgAd the authors suggested that it interacts with folding intermediates of lysozyme and neutralizes local negative charges leading to enhanced electrostatic repulsion between aggregation-prone species. Thus, L-ArgAd and L-ArgEE bound to the unfolded lysozyme increase protein positive net charge and effectively prevent the intermolecular interaction among aggregation prone molecules (Shiraki, et al. 2004, 2005; Hamada and Shiraki 2007).

1.5.4. L-arginine as an effective additive in chromatography

Amino acids have rarely been used to facilitate purification or to stabilize proteins during purification. However, L-ArgHCl has been successfully exploited in improved chromatographic protocols on a series of separation matrices for protein purification, e.g., for gel permeation (GPC) (Ejima, et al. 2005b), ion-exchange (IEC) (Arakawa, et al. 2007c; Langenhof, et al. 2005), hydrophobic interaction (HIC) (Tsumoto, et al. 2007), affinity chromatography (Abe, et al. 2009; Ejima, et al. 2005c).

A major problem in gel permeation chromatography (GPC) is non-specific binding of applied proteins to the column matrix (stationary phase). Such non-specific binding occurs more frequently for aggregated proteins. Arginine at 0.2–0.75 M is highly effective in suppressing such non-specific interaction. For a series of proteins including mouse monoclonal antibody, recombinant human

activin, interleukin-6, basic fibroblast growth factor, and interferon-γ, improved separation of the proteins and their soluble oligomers from GPC silica- or polymer-based columns was observed, when L-ArgHCl was added to the eluent buffer (Ejima, et al. 2005b).

Arginine is ionic above ~pH 4 and hence is not always suitable as an additive for ion exchange chromatography (IEC). The addition at high concentrations may interfere with binding of proteins to the IEC resin. Despite of that, the addition of arginine (0.2 M) in the loading sample of interleukin-6 (IL-6) on cation exchange columns suppressed aggregation and resulted in greater recovery. (Arakawa, et al. 2007c).

Due to strong hydrophobic binding of some proteins under salting-out conditions, the elution from HIC resins is complicated or even impossible by reducing the salt concentrations. Despite of that, activin was eluted from the butyl-sepharose column (HIC) in the presence of arginine with reasonable yield. In addition to arginine, the arginine derivatives were also examined in HIC. Acetyl-arginine, in which the amino group of arginine is acetylated, has no net charge at neutral pH. Agmantine, in which the carboxyl group is replaced by the proton, has two positive charges at neutral pH. Despite the differences, both arginine derivatives enhanced elution of activin from butyl-sepharose, similarly to arginine (Arakawa, et al. 2007a, c; Tsumoto, et al. 2007).

Affinity chromatography is based on specific interactions between column ligands and proteins. However, high affinity is often dissadventageous for elution. The elution is mores efficient at lower pH, which alters conformation of antibodies or promotes aggregation. Ejima D. et al. demonstrated that antibodies (mAb) can be eluted more efficiently at higher pH (above 4) from Protein-A column when arginine is included in the elution solvent (Ejima, et al. 2005c).

In all these cases, the beneficial effect of L-ArgHCl was attributed to its ability to suppress protein aggregation as well as non-specific protein-surface interactions, and to favorably influence the solvation of exposed hydrophobic surface area of purified proteins.

1.5.5. A proposed mechanism of L-arginine action

The precise molecular mechanism of arginine action remains still not fully understood (Lange and Rudolph 2009; Li J., et al. 2010). Arginine is more effective at higher concentrations. A requirement for arginine at high concentrations indicates that the interactions between the amino acid and proteins are weak. The effects of the weakly interacting additive (arginine) on protein solubility, stability and aggregation have been explained from different approaches: i.e., the effects of the additive on water structure (surface tension,

ion hydration) and the interaction of additive with amino acids (amino acids solubility) and proteins (preferential interaction).

Literature analysis provided in the section 1.4.1. shows that protein stabilizers increase the surface tension of water, while destabilizers – decrease. Since arginine is classified as an aggregation suppressor, it is expected to decrease, or at least not to increase the surface tension of water. However, it has been observed that arginine increases the surface tension of water, which means that this amino acid should stabilize protein and enhance protein-protein interactions. In contrast to structure stabilizing amino acids, arginine weakens the protein-protein interactions and has no stabilizing effects on proteins. These observations suggest that the surface tension effect cannot explain the effects of arginine as a suppressor of protein aggregation. The same is true for more solutes, which do not follow the surface tension mechanism. For example, both GdnCl (denaturant) and $MgCl_2$ (salting-in salt), increase the surface tension of water, but they are not protein stabilizers. So, their effect on protein must be due to factors other than the surface tension effect. Also, the increase of the surface tension was observed to be linearly dependent on additive concentration. Whereas arginine at higher concentrations loses the ability to increase the surface tension of water, e.g., the surface tension increment (surface tension vs. molal additive concentration) at 0.7 M and 1.5 M arginine concentrations is 1.71 and 1, respectively and it cann't be defined as the protein stabilizer. There is no clear explanation to this concentration dependence and is considered that it is due to self-association of arginine as such association has been observed for proline (Arakawa, et al. 2007b; Tsumoto, et al. 2004, 2005; Schneider and Trout 2009; Samuel, et al. 1997).

Therefore, recently, Das et al. proposed a mechanism based on the hydrophobic interaction. It was suggested that molecular clusters of arginine in aqueous solutions display a hydrophobic surface by the alignment of its three methylene groups. The hydrophobic surfaces on the proteins interact with the hydrophobic surface presented by the arginine clusters. The covering of hydrophobic surfaces inhibits the protein-protein interaction. It is also may explain why other amino acids fail to inhibit the protein aggregation (Das, et al. 2007; Lange and Rudolph 2009; Reddy, et al. 2005).

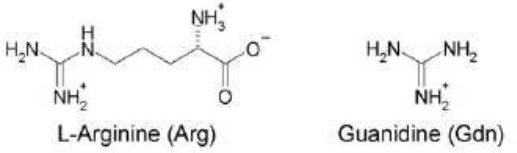

Fig. 9. Chemical structures of L-Arginine and Guanidine (Hamada, et al. 2007)

Amino acid, arginine, has a unique side chain, the guanidinium group (Fig. 9). The importance of the guanidinium group has been suggested to be a factor in increasing protein solubility (Ghosh, et al. 2009; Xie Q., et al. 2004). It has been observed that L-ArgHCl increases the solubility of most amino acids, with the exception of L-valine and L-isoleucine. On the other hand, the denaturant, GdnCl, also, favorably interacts with most amino acids side chains and the interaction pattern with amino acids is very similar between arginine and GdnCl. However, the interaction of arginine with aromatic amino acids (e.g., Trp and Tyr) appears more favorably than with other amino acids and it is stronger than with GdnCl (Arakawa, et al. 2007b). It has been suggested that arginine interacts with tryptophan and perhaps tyrosine residues through its guanidinium group. Structural analyses of proteins have shown that the guanidinium group of arginine is oriented parallel to the side chains of aromatic amino acids. Thus, if guanidinium group of arginine may interact with aromatic side chains via cation-π electron interaction, this interaction may play a crucial role in suppressing aggregation of folding intermediates or disaggregating proteins from complexes (Tsumoto, et al. 2005; Ishibashi,. et al. 2005).

However, the interaction of arginine with the proteins is greatly different from GdnCl as it has been observed from the preferential interaction measurements. The preferential interaction is a measure of weak interactions between protein molecules and co-solutes or water molecules. Preferential interaction measurements are carried out by dialysis equilibrium, vapor pressure equilibrium, or other thermodynamic techniques (Arakawa and Timasheff 1985). The denaturation of proteins by urea or GdnCl, especially at high concentrations, is attributed to their binding to the proteins and the stabilization of proteins by polyols, sugars, certain amino acids and salts – preferential exclusion from the protein surface. The amino acid, arginine, at higher concentrations, is preferentially excluded from protein surface and would indicate of protein stabilization, but not denaturation. Also, L-Arg binding to proteins is a concentration- and protein-dependent process. And this is one more difference to denaturants (e.g., urea or GdnCl) (Lange and Rudolph 2009; Tsumoto, et al. 2004; Timasheff 2002; Ishibashi, et al. 2005; Li J., et al. 2010).

Although great progress has been made over the years in understanding of arginine effects on the state of proteins in solution, none of the described theoretical approaches provides a full explanation (Lange and Rudolph 2009; Tsumoto, et al. 2004; Timasheff 2002, Ishibashi, et al. 2005).

1.5.6. Cyclodextrins (CDs) as agregation suppressors

Cyclodextrin, PEG (polyethylene glycol), proline and L-arginine were reported to suppress aggregation by preventing the association of refolding intermediates or unfolded species through hydrophobic or ionic interactions (Liu, et. al 2007a).

Cyclodextrins (CDs) are cyclic oligosaccharides composed of α-(1→4)-linked α-D-glucosyl residues. The most common types of cyclodextrin are α-, β- and γ-cyclodextrin, which consist of 6, 7 and 8 glucosyl units, respectively. The more glucose units there are in the circle of cyclodextrin, the larger cavity is (Fig. 10, Table 2) (Szejtli and Osa 1996).

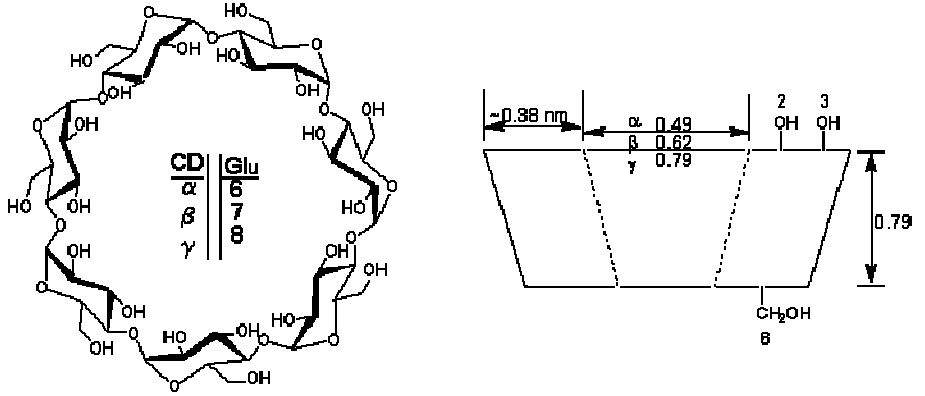

Fig. 10. Cyclodextrin chemical structure (Challa, et al. 2005)

The structure of CD is a cylinder shaped with hydrophilic rims outside where OH-6 is on the narrow rim and OH-2 and OH-3 are on the wide rim. The interior of cyclodextrin (H-3, H-5, and H-6 hydrogens and O-4 ether oxygens) forms a hydrophobic cavity. The polar groups of exterior help cyclodextrin to dissolve in water, whereas the central nonpolar cavity enables it to interact with the hydrophobic molecules of appropriate dimensions (Karuppiah and Sharma 1995).

Cyclodextrin is water soluble, non-toxic, stable and biocompatible. However natural cyclodextrins have rather limited solubility in water (Table 2), and various derivatives have been synthesized to improve their solubility. The physicochemical properties of CDs, including their complexation ability, may be greatly affected by the type, number, and position of the substituents on the parent CD molecule. Since the number of reactive hydroxyls per mole of glucopyranose unit is 3, the maximum numbers of substituents possible for α-, β-, and γ-CDs are 18, 21, and 24, respectively.

Table 2. Some characteristics of α-, β-, and γ-cyclodextrin (Challa, et al. 2005)

Type of CD	Cavity Diameter, nm	Molecular weight, g/mol	Solubility, (g/100 ml)
α-CD	0.49	972	14.5
β-CD	0.62	1135	1.85
γ-CD	0.79	1297	23.2

Cyclodextrins as well as their chemically modified variants have been widely used in pharmaceutics as drug carriers. For example, hydrophilic CDs such as branched β-CDs (glucosyl, maltosyl forms), 2-hydroxypropyl-β-CD (HP-β-CD) and sulfobutyl ether β-CD (SBE-β-CD) have been evaluated as a new class of parenteral drug carriers, because they are highly hydrophilic and have less hemolytic activity than the parent and other hydrophilic CDs (Tavornvipas, et al. 2006).

1.5.7. Refolding of proteins in the presence of cyclodextrins (CDs)

The properties of cyclodextrin also make it an ideal additive to prevent proteins from aggregation during refolding or other stresses. The origin of this has been suggested to be formation of inclusion complexes i.e., a specific interaction between cyclodextrin cavity and hydrophobic (i.e. aliphatic and aromatic) amino acids (Yazdanparast, et al. 2005). For example, the extent of aggregation of the α-amylase during refolding process was reduced by almost 90% in the presence of 100mM α-CD. However, the addition of free hydrophobic amino acids (Phe, Ile, Trp, Leu) to the refolding buffer reduced recovery of active α-amylase considerably, due their competition for hydrophobic interaction with α-CD. Also, it has been emphasized that these hydrophobic amino acids, in the absence of α-CD, do not have any significant effects on the aggregation suppression of the denatured α-amylase during refolding (Khodarahmi, et al. 2004).

NMR studies by Otzen et al. showed that the hydrophobic interior of the CD cavity enables cyclodextrin to interact with different amino acids side chains such as Phe, Trp, Tyr (hydrophobic-aromatic), Ile, Leu (hydrophobic-aliphatic) and Asp, Glu (protonated aspartic and glutamic acids). Linear aliphatic chain from, e.g., Ile allow a snug fit in the narrow α-CD cavity, but the binding affinities of aliphatic amino acids are several fold lower than aromatic amino acids to the β-CD. Since various cyclodextrins (α-, β-, γ-) have the different cavity dimensions, it has been determined that the cavity of β-CD with a diameter of ~6.4 Å at H-3 and ~6.0 Å at H-5 allows for a snug fit of a benzene

ring as diameter of a benzene ring ranges from 5.2 to 6.0 Å. In contrast, α- and γ-CD with the cavity diameter (~5.2 Å at H-3 and ~4.7 Å at H-5) and (~8.3 Å at H-3 and ~7.8 Å at H-5) are too small and too large for a benzene ring, respectively. Thus, aromatic amino acids are expected to form weaker interactions with α- and γ-CD than with β-CD (Fig. 11) (Otzen, et al. 2002; Aachmann, et al. 2003; Wimmer, et al. 2002).

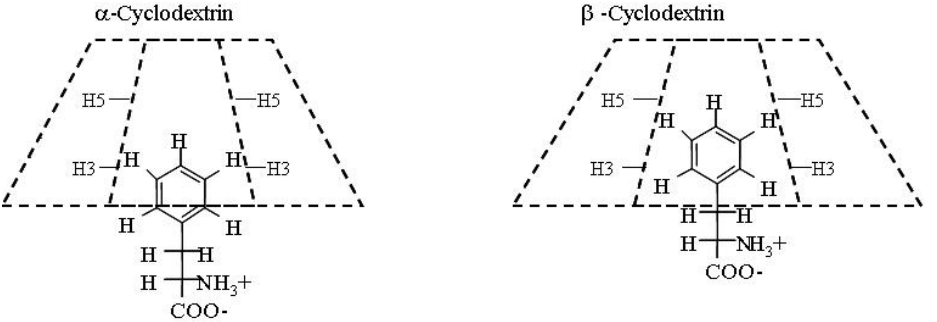

Fig. 11. Phenylalanine interaction pattern with α- and β-cyclodextrin (Larsen, 2006)

Since, it is believed that the binding of the ‘‘host’’ cyclodextrin derivatives with the aromatic side chains of the ‘‘guest’’ proteins is crucial for its ability to assist protein refolding. It has been proposed that the ability of cyclodextrins to bind with protein aromatic residues contributes to their effects on protein refolding. However, the ability of various cyclodextrin and their derivatives to prevent protein aggregation varied significantly (Table 3) (Kim, et al. 2006b).

Table 3. Effects of different cyclodextrins on protein aggregation (Kim, et al. 2006b)

Proteins	Effects of CDs	Residues (n)	Aromatic residues (m)	m/n	Reference
Carbonic anhydrase	α>β>γ	252	10 F + 8 Y + 4 W	0.087	Karrupiah, et al. 1995
Phosphofructo-kinase-1	α; γ=0	322	8 F + 5 Y	0.04	Bar, et al. 2000
Phosphatidylinositol phosphate kinase	β>α, γ	202	12 F + 8 Y + 2 W	0.109	Davis, et al. 2004
Amyloid β-peptide	β; α, γ=0	17	2 F	0.118	Qin, et al. 2002
Human growth hormone (hGH)	β>α, γ	191	1 F + 8 Y + 13 W	0.115	Otzen, et al. 2002
Aminoacylase	β>γ>α	782	46 F + 18 Y + 16 W	0.102	Kim, et al. 2006a

Kim S. H. et al. suggested that various effects of cyclodextrins on different proteins in literature may be associated with the numbers of aromatic residues of these proteins and their locations in proteins. Although only limited numbers of proteins were studied, a preliminary hypothesis was proposed. Thus, β-cyclodextrins were the most efficient of the cyclodextrin derivatives when the proteins contained a high content of aromatic residues (>0.1), whereas α-cyclodextrins were the best when the proteins contained a low content of aromatic residues (<0.1) (Kim, et al. 2006b). Also, the effects of CDs were found to be dependent on both cyclodextrin and protein concentrations. This might be one of the reasons why different effects of cyclodextrins were observed for different proteins (Desai, et al. 2006).

Substitutions of the hydroxy groups on the CD ring such as glucose, maltose, methyl, acetyl or hydroxypropyl, have been reported, also influence the suppression of protein aggregation as well as CD solubility and the accessibility of the cavity. In some cases, the presence of appropriate substituents on the CD ring is more important than the cavity size during protein refolding. For example, under identical experimental conditions, α-CD is more effective in suppressing aggregation of lysozyme than γ-CD; however, the acetyl-γ-CD is more effective than acetyl-α-CD. These results suggest that the ring substituents may hinder the accessibility of hydrophobic amino acid side chains of the protein to the CD cavity (Sharma and Sharma 2001; Desai, et al. 2006).

Another successful use of CDs in protein refolding is as a secondary reagent to remove detergent. Rozema D. and Gellman S. H. showed that unfolded proteins form detergent-protein complexes when renatured in buffers containing detergents such as cetyltrimethylammonium bromide (CTAB) or sodium dodecyl sulfate (SDS). The binding of detergent to the protein folding intermediates suppresses aggregate formation. When the protein-detergent micelles are exposed to CDs, the detergents form strong complexes with the CD molecules, causing their release from the protein. Thus, allowing protein to complete its refolding. This method has been claimed to mimic the GroEL-GroES chaperonin action *in vivo* and has been named ‘artificial chaperone-assisted refolding’ (Rozema and Gellman 1996a, b; Daugherty, et al. 1998; Kim, et al. 2007; Yazdanparast, et al. 2005). Since residual detergent concentrations may interfere with downstream purification processes, their removal from the refolded protein is required. The use of immobilized CDs or CD polymer beads as well as affinity columns makes this process more convenient (Mannen, et al. 2001; Oganesyan, et al. 2005).

1.5.8. Effect of cyclodextrins (CDs) on heat induced aggregation

Since CD is one of the additives used for the solubilization or stabilization of proteins, it effect on thermal unfolding of proteins was studied, too. Differential scanning calorimetry (DSC) studies revealed that cyclodextrins reduces the thermal stability of proteins as T_m of proteins decrease with increasing cyclodextrin concentration (Cooper 1992; Yamamoto, et al. 2006). For example, the T_m value of lysozyme in the presence of cycldextrin decreased in the order of γ-<α-<β-CDs. Branched cyclodextrin, maltosyl-β-CD, decreased T_m value about 5 °C and maltose increased T_m value about 6 °C. So, authors came to the conclusion that the binding of branched β-CD to the exposed hydrophobic side chains during the heating destabilizes the native conformations of lysozyme by shifting the equilibrium in favor of the unfolded state (Tavornvipas, et al. 2006). Alternatively, the CD interactions with groups on oligomeric folded proteins can lead to a dissociation of these protein aggregates, especially if the complexation occurs at sites in a protein-protein interface. Thus, CD interaction with unfolded proteins may enhance the solubility of a denatured protein by hiding the exposed hydrophobic residues, thereby assisting the refolding of the polypeptide (Lovatt, et al. 1996). For example, the amount of aggregates during heating of basic fibroblast growth factor (bFGF) at 60 °C for 10 min decreased with addition of CDs thereby CDs could prevent the heat-induced aggregation and inactivation of protein (Tavornvipas, et al. 2006).

The literature overview on cyclodextrins revealed that both the cavity size and the environment around the two rims have important impact on cyclodextrin assisted refolding of protein.

1.6. Growth hormones

Growth hormone (GH), also known as somatotropin, is a polypeptide hormone that is synthesized by the somatotropic cells of the anterior pituitary. It plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates and lipids (Moller and Jorgensen 2009). Human growth hormone (hGH) is currently used for the treatment of pediatric hypopituitary dwarfism and in children suffering from low levels of hGH (Filikov, et al. 2002). Animal growth hormones (GHs) are legally used in several countries (e.g., United States and Australia) (Sillence 2004): bovine growth hormone (bGH) to stimulate milk production in dairy cows (Tou, et al. 2009; Byatt, et al. 1997), porcine growth hormone (pGH) to improve swine growth rate

and to reduce fat deposition (Verstegen, et al. 1991), equine GH (eGH) to improve nitrogen balance in aged horses (Borromeo, et al. 2005).

The advent of recombinant DNA technology has allowed the production of large quantities of biologically active GHs. The heterologous high-level expression of GHs in *E. coli* results in the hormone depositing inside the cell as inclusion bodies, so different protocols have been proposed to recover the active proteins (Langley, et al. 1987a, b; Puri and Cardamone 1992). Methionyl-pGH (Met-pGH) is produced recombinantly. Hormone is 191-amino acid single-chain polypeptide with a molecular weight of approximately 22 kDa (Fan, et al. 2000; Bewley and Li 1984). The three-dimensional structure of a genetically engineered variant of porcine growth hormone, has been determined at 2.8 Å resolution, using single crystal x-ray diffraction techniques (Fig. 12) (Abdel-Meguid, et al. 1987).

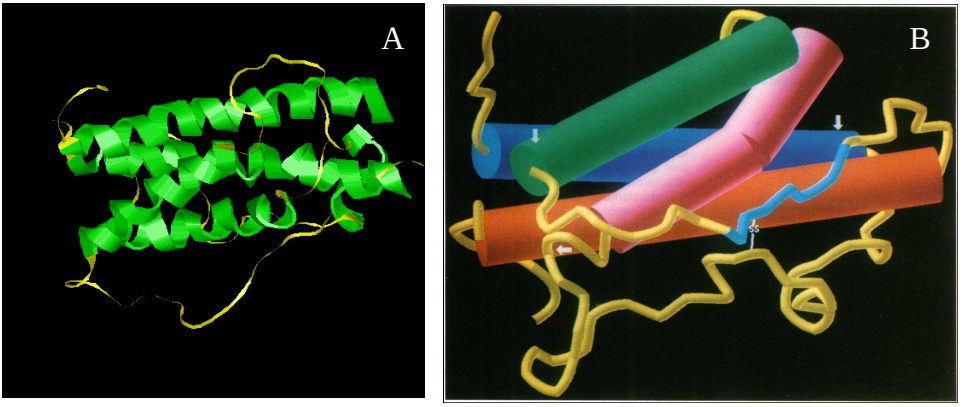

Fig. 12. The three-dimensional structure of human growth hormone (hGH) (A) (Chantalat, et al. 1995) and a genetically engineered variant of porcine growth hormone (pGH) (B) (Abdel-Meguid, et al. 1987), determined by X-ray crystallography at 2.5 angstrom resolution

The pGH structure consists of four helices, two of these helices contain ~20 amino acid residues each, the other two contain ~30 amino acid residues each. Thus, 54 % of the amino acid residues of the protein can be accounted by these four helices. The helices are arranged in a tightly packed anti-parallel helix bundle. The amino acid sequence of pGH contains four cysteine residues at positions 53, 164, 181, and 189. Residues 53 and 164 form one disulphide bridge, whereas residues 181 and 189 form the other (Abdel-Meguid, et al. 1987). The tertiary structures of human growth hormone (hGH) (De Vos, et al. 1992; Chantalat, et al. 1995) and bovine growth hormone (bGH) (Carlacci, et al.1991) are very similar to that of porcine growth hormone (pGH) (Abdel-

Meguid, et al. 1987). The primary amino acid sequence of pGH shares 68 % and 91 % identity with human and bovine growth hormones, respectively (Parkinson, et al. 2000).

Table 4. Comparison of amino acid sequences of mink GH (mGH), pig GH (pGH), rat GH (rGH), bovine GH (bGH) and human GH (hGH). The numbering is that of mature mGH. Gaps (–) were introduced to optimize the amino acid alignment (Harada, et al. 1990).

Growth hormones	Amino acid sequences	
mink GH	-FPAMPLSSLFANAVLRAQHLHQLAADTYKDFERAYIPEGQRYSI-QNAQ	48
pig GH	- E -	
rat GH	- E -	
bovine GH	A S G F E T - T	
human GH	- TI R D M HR F QE E KE K FL P	
mink GH	AAFCFSETIPAPTGKDEAQQRSDMELLRFSLLLIQSWLGPVQFLSRVFTH	98
pig GH	V	
rat GH	E T I R	
bovine GH	V N K L I L	
human GH	TSL S T SNRE T K NL I E RS A	
mink GH	SLVFGTSD-RVYEKLKDLEEGIQALMRELEDGSPRAGPILKQTYDGFDTN	47
pig GH	- Q	
rat GH	M - Q I Q A	
bovine GH	- L T Q	
human GH	J A SN DL T GR T Q F S	
mink GH	LRSDDALLKNYGLLSCFKKDLHKAETYLRVMKCRRFVESSCAF	190
pig GH		
rat GH	M A	
bovine GH	M R T G A	
human GH	SHN Y R MD V F IVQ S- G G	

There is some very limited information on mink growth hormone (mGH). The mGH gene was cloned and sequenced (Harada, et al. 1990; Shoji, et al. 1990). The resulting protein contained four cysteine residues and potentially two disulphide bridges that might be formed based on the high level of sequence homology with pGH and other GHs (Table 4). The mGH shares 96 %, 90 %, 88 %, and 66 % amino acid homology with the known mammalian sequences porcine, rat, bovine and human (Harada, et al. 1990). Recently structure homology modeling, FT-IR studies revealed that recombinant mGH is an all-helical protein with the same secondary and 3D structure as pGH (Borromeo, et. al. 2008).

2

Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals used in this study were of the highest quality available.

2.1.2. *E. coli* strains

E. coli BL21 (DE3)
E. coli BL21 (DE3):
- pET21a+/pGH
- pET21a+/mGH

E. coli RRI pET21a+
E. coli JM109

2.1.3. Buffers

Cell resuspension buffer: 0.1 M Tris-HCl (pH 7.2 at 25 °C), 5 mM EDTA, 1 mM PMSF.
IBs washing buffer: 0.1 M Tris-HCl (pH 7.2 at 25 °C), 2 M urea, 1 M NaCl, 5 mM EDTA, 1 mM PMSF.
Denaturation buffer: 0.1 M Tris-HCl (pH 8.0 at 25 °C), 8 M urea, 20 mM reduced glutathione.
Refolding buffer: 0.1 M Tris-HCl (pH 8 at 25 °C), 6 mM oxidized glutathione and/or various concentrations of solution additives.
TE buffer: 10 mM Tris-HCl (pH 7.4 at 25 °C), 1 mM EDTA.

2.1.4. DNA

pUC 57/T Plasmid Vector was obtained from Fermentas UAB (Vilnius, Lithuania).

Oligonucleotides:
5’ GTT TAA CTT TAA GAA GGA G 3’
5’ GGC CCG AGG CCC GAC TGG ATG 3’
5’ GGT GGT GCT CGA GTG CGG C 3’

All non-modified oligodeoxynucleotides were purchased from Metabion (Martinsried, Germany).

2.1.5. Enzymes

DNA polymerases Taq and Pfu, T4 DNA ligase and all REases used for DNA manipulations were obtained from Fermentas UAB (Vilnius, Lithuania).

2.2. Methods

2.2.1. Microorganism cultivation

E. coli strain BL21(DE3) harboring the plasmid pET21a+/pGH_wt or pET21a+/mGH_wt was cultivated in a batch fermentation process. The bacteria were grown at 37 °C in the LB medium supplemented with ampicillin (~70 μg/ml). Protein expression was induced at OD_{600}=2.0 by adding into the fermentation medium IPTG solution to the final concentration of 1 mM. Cells were cultivated for 200 min after induction and then harvested by centrifugation

at 5,000×g for 15 min. Both recombinant pGH and mGH were expressed as insoluble proteins and accumulated as inclusion bodies.

2.2.2. Isolation and solubilization of rmGH or rpGH inclusion bodies

Frozen biomass was homogenized in cell resuspension buffer (0.1 M Tris-HCl buffer pH 7.2, containing 5 mM EDTA) at ratio equal to 1g/10ml, and the cells were sonicated 5 min on ice using a Sonics Vibracell VCX 750. PMSF to a final concentration of 1 mM was added just before the sonication. The suspension of disrupted cells was centrifuged at 13,000 g for 25 min at 4 °C. The collected pellets of inclusion bodies from 1 g biomass were washed with an equal volume of water, then resuspended in 10 ml of washing solution (2M urea, 1 M NaCl, 5 mM EDTA, 1 mM PMSF in 0.1 M Tris-HCl buffer pH 8.0) and centrifuged at 13,000g for 25 min at 4 °C. Subsequently, the pellets were washed with water. Purified inclusion bodies were solubilized in 10 ml of 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione with gentle stirring at 4 °C for 30 min, and then the solution was centrifuged at 13,000 g for 25 min at 4 °C.

2.2.3. Refolding of rmGH or rpGH in the absence of solution additives

To refold rpGH or rmGH, the solubilized protein (the concentration of both rmGH and rpGH was 4.3–4.9 mg/ml) in denaturation buffer (0.1 M Tris-HCl, 8 M urea and 20 mM reduced glutathione (pH 8.0)) was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione in order to reduce urea concentration to 3 M. The renaturation reaction was carried out for several hours at 4 °C with gentle stirring. Finally, the solution was centrifuged at 13,000g for 25 min at 4 °C in order to remove insoluble aggregates. Then volume of soluble protein was measured and protein concentration was determined spectrophotometrically at 595 nm by the Bradford assay (Bradford, 1976).

2.2.4. Influence of various solution additives on the aggregation of rmGH or rpGH in the refolding process

To refold rpGH or rmGH, the solubilized protein (the concentration of both rmGH and rpGH was 4.3–4.9 mg/ml) in denaturation buffer (0.1 M Tris-HCl, 8 M urea and 20 mM reduced glutathione (pH 8.0)) was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione and various concentrations of additive in order to reduce urea concentration to 3 M. Then the

transmittance at 600 nm was immediately registered. Subsequently, the renaturation samples containing various concentrations of additive were kept at 4 °C with gentle stirring, and the changes of transmittance were registered during the period of 44 hours.

2.2.5. Influence of L-Arg, L-ArgEE, HP-β-CD and Me-β-CD concentrations on the renaturation yield of both rmGH and rpGH

The solubilized protein (the concentration of both rmGH and rpGH was 4.3–4.9 mg/ml) in denaturation buffer was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione and various concentrations of L-Arg, L-ArgEE, HP-β-CD and Me-β-CD in order to reduce urea concentration to 3 M. The renaturation reaction was carried at 4 °C with gentle stirring. 1 ml of the refolded samples was withdrawn after 20, 28 or 44 hours of refolding at 4 °C and centrifuged at 13,000 g for 25 min at 4 °C. Finally, chaotropic agent, thiol-compounds and solution additives were removed by size exclusion chromatography using a Sephadex G-25 gel filtration column (1.0 x 9.5 cm), equilibrated with 0.025 M Tris-HCl buffer pH 8.0 at a flow rate of 0.3 ml/min. The eluent was monitored at 280 nm. Fractions containing protein were pooled, and subsequently a 200 µl aliquot of the solution was analyzed by RP-HPLC or SEC on Superdex 75 HR 10/30 column previously equilibrated with 0.025 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl at a flow rate of 0.5 ml/min. Superdex 75 HR 10/30 column was previously calibrated using protein standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotripsinogen A (25 kDa) and cytochrome c (12.3 kDa). The absorbance was monitored at 280 and 215 nm, using a Waters 2487 Dual λ absorbance detector.

2.2.6. RP-HPLC

Renaturation samples of both proteins rmGH and rpGH were analyzed by RP-HPLC using reversed-phase column Protein C4 (250 x 4.6 mm, Vydac); solvent A, 0.1 % trifluoracetic acid in water; solvent B, 0.1 % trifluoracetic acid in acetonitrile (HPLC gradient grade). The column was initially equilibrated with 9 % B at a flow rate of 1 ml/min. After injection, a linear gradient of 9 % B to 40 % B for 3 min, 40 % B to 48 % B for 10 min, 48 % B to 57 % B for 40 min, 57 % B to 67 % B for 10 min, 67 % B to 81 % B for 1 min was applied. The absorbance was monitored at 280 and 215 nm, using a Waters 2487 Dual λ absorbance detector. Data analysis was performed using Waters Breeze chromatography software.

2.2.7. Refolding and purification of rmGH or rpGH

The solution of solubilized rpGH or rmGH in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione in order to reduce urea concentration to 3 M. Arginine was added to the dilution buffer to a final concentration of 1 M in the renaturation mixture. Since L-Arginine monohydrochloride (L-ArgHCl) was used, prior to adding oxidized glutathione, pH of dilution solution was adjusted to pH 8.0. The renaturation reaction was carried out for 20 h at 4 °C with gentle stirring. Finally, the solution was centrifuged at 13,000g for 25 min at 4 °C and chaotropic agent, thiol-compounds and arginine were removed by size exclusion chromatography using a Sephadex G-25 gel filtration column (2.5 x 80.5 cm), equilibrated with 0.025 M Tris-HCl buffer pH 8.0 at a flow rate of 1.5 ml/min or by dialyzing overnight against the 0.025 M Tris-HCl buffer pH 8.0 at 4 °C. Fractions containing rpGH or rmGH after SEC were pooled and loaded on Q-Sepharose column (2.5 x 12 cm) previously equilibrated with 0.025 M Tris-HCl buffer pH 8.0 at a flow rate of 2 ml/min. Unbound proteins were washed out with the same buffer, and bound proteins were eluted with a linear salt gradient as follows: 1 bed volume of 0 M NaCl to 0.15 M NaCl, 2 bed volumes of 0.15 M NaCl to 0.3 M NaCl and 2 bed volumes of 0.3 M NaCl to 1 M NaCl in 0.025 M Tris-HCl buffer pH 8.0. The peak which appeared in the range of 0.3 M–1 M of NaCl and contained pGH or rmGH was pooled. The salt concentration was adjusted approximately to 2 M, and the protein solution was applied on Phenyl-Sepharose column (1.5 x 13.5 cm) previously equilibrated with 0.025 M Tris-HCl buffer pH 8.0 containing 2 M NaCl at a flow rate of 1 ml/min. Unbound proteins were washed out with the same buffer, and bound proteins were eluted with 10 bed volumes of linear NaCl gradient (0.8 M–0.2 M) in 0.025 M Tris-HCl buffer pH 8.0. Fractions containing rpGH or rmGH were pooled and stored at -20 °C.

2.2.8. Determination of Protein Concentration

The protein concentration was determined spectrophotometrically at 595 nm by the Bradford assay (Bradford, 1976). Bovine serum albumin was used as a standard protein.

2.2.9. Denaturing (SDS) polyacrylamide gel electrophoresis of proteins

SDS-PAGE under nonreducing and reducing conditions was carried out according to the method of Laemmli (Laemmli, 1970). The acrylamide concentration of the resolving gel was 15 %. Proteins were stained with

Coomassie Brilliant Blue R-250 dye. Digital images of the gels were scanned, and the percentage of both rpGH and rmGH was estimated by densitometric analysis using the UN-SCAN-IT software (Silk Scientific, Inc., USA).

2.2.10. Non-denaturing electrophoresis through agarose

Separation of DNA fragments and plasmid DNA were performed in 0.8 – 1.2 % agarose gels in the ethidium bromide-free TAE electrophoretic buffer containing 40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA (pH 8.5). DNA samples were mixed with 1/5 volume of Loading dye solution and electrophoresed at 5 V/cm until the bromphenol blue dye migrated approximately 2/3 of gel length. Gels were stained with 0.5 μg/ml ethidium bromide and visualized under UV light.

2.2.11. Cloning and mutagenesis of rmGH

E. coli strain BL21 (DE3) carrying the pET21a+_wt_mGH, *E. coli* strain JM 109 and cloning vector of pUC 57/T were used for the site directed mutagenesis of mGH_W86G.

Site-directed mutagenesis of mGH mutant was performed by the two-step 'megaprimer' method. The plasmid pET21a+_ mGH_wt was used as a template for PCR method using first-downstream or upstream primer 5'GTTTAACTTTAAGAAGGAG3' and second-downstream or upstream primer 5'GGCCCGAGGCCCGACTGGATG3' containing the Trp86Gly mutation of the wt_mGH gene. The reaction contained: 1x PCR buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs, 1 μM of primers, 0.2 ng pET21a+ (template DNA), 2.5 U Pfu proofreading DNA polymerase (Fermentas UAB, Vilnius, Lithuania) per 100μl of reaction mixture. The PCR program was as follows: 94 °C for 4 minutes, 30 cycles of 94 °C for 30 seconds, 48 °C for 40 seconds, and 72 °C for 40 seconds followed by incubation at 72 °C for 5 minutes. The PCR product 293bp long (M primer) were purified by the gel extraction method (Fermentas UAB, Vilnius, Lithuania) and used in a second round of mutagenesis with third-upstream or downstream primer 5'GGTGGTGCTCGAGTGCGGC3'.

The reaction contained: 1x PCR buffer, 20 mM $(NH4)_2SO_4$, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.1 μM of M primer, 0.8 μM of 3 primer, 0.2 ng pET21a+_wt_mGH (template DNA), 1.25U Pfu proofreading DNA polymerase and 3U Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania) per 50μl of reaction mixture. The PCR program was as follows: 95 °C for 5 minutes, 30 cycles of 95 °C for 60 seconds, 65 °C for 75 seconds, and 72 ºC for 60 seconds followed by incubation at 72 ºC for 10 minutes. The PCR product ~700bp long was purified by the gel extraction method (Fermentas UAB, Vilnius, Lithuania).

The resultant product was ligated into pUC57/T and transformed to the competent *E.coli* JM109 cells by the $CaCl_2$ method (Sambrook, et al. 2001). The plasmid DNA was isolated by the alkaline lysis procedure (Sambrook, et al. 2001) and purified using gel extraction kit (Fermentas UAB, Vilnius, Lithuania). Both strands of the entire region were sequenced to confirm that the designed amino acid change (Trp86Gly) was the only change in the DNA sequence. Sequencing of the entire gene of the mutant confirmed that only the designed mutations had been introduced. Sequencing was performed at IBT Center for DNA sequencing (Vilnius, Lithuania).

The mGH mutant gene was cleaved by NdeI/HindIII and ligated into pET21a+ that had been cleaved with the same enzymes. Competent *E. coli* BL21 (DE3) cells were prepared and transformed with pET21a$^+$_mutant_mGH by the $CaCl_2$ method (Sambrook, et al. 2001).

2.2.12. Expression of rmGH_W86G

For the refolding studies of rmGH_W86G, *E. coli* BL21 (DE3) stock culture containing the plasmid pET21a$^+$/mGH_W86G was cultured overnight at 37 °C in LB medium in the presence of ampicillin 50 μg/ml. The overnight culture was used to inoculate 500 ml of the fresh LB medium (1/25 (v/v)), bacteria was grown to the mid-log phase (absorbance at 600 nm ~ 0.7–0.9), and induced with 1 mM IPTG and further cultured for another 2 h and 30 minutes at 37 °C, 200 rpm. The wild type strain was grown in parallel under the same conditions as *E. coli* BL21 (DE3) containing the plasmid pET21a+/mGH_W86G. Both recombinant rmGH_W86G and rmGH_wt were expressed as insoluble proteins and accumulated as inclusion bodies. Expression yield of rmGH_W86G and rmGH_wt was determined by densitometric scanning of denaturing polyacrilamide gels using the UN-SCAN-IT software (Silk Scientific, Inc., USA). Finally, the refolding studies were performed as described above (see sections 2.2.2. and 2.2.4.).

2.2.13. Fluorescence measurements

Intrinsic fluorescence was measured using a LS55 fluorescence spectrometer (Perkin-Elmer, USA). The fluorescence emission spectra of purified GHs in 0.025 M K-Na phosphate buffer pH 7.5 were measured between 300 and 450 nm with an excitation at 295 nm. Excitation and emission slit widths were equal to 5.0 nm. At least two scans were averaged for each spectrum. All fluorescence spectra were corrected by subtracting the control buffer or buffer with various concentrations of arginine spectra. Temperature scans were performed using a cuvette holder connected to an external constant

temperature circulator/bath (F25, Julabo). The series of spectra were taken with a protein concentration 0.13 mg/ml in the absence and presence of arginine. The quartz-cuvette with an optical pathlength of 1 cm was used.

2.2.14. *In vitro* bioassay for rmGH and rpGH

For determination biological activity of both rmGH and rpGH *in vitro*, mouse myeloid cell line (FDC-P1), transfected with the full length ovine growth hormone receptor (oGHR) was used. The *in vitro* bioassay for growth hormones was done by Vitaliano Borromeo and Camillo Secchi in University of Milan (Italy) by stimulation of oGHR-FDC-P1 cells using the MTT-formazan dye assay (Mosmann 1983). This assay monitors the stimulation of both metabolic and mitogenic activity in these cells.

2.2.15. Bioinformatics analysis of both recombinant mink and porcine growth hormones

The 3D structure of mink growth hormone was predicted using HHpred server (http://toolkit.tuebingen.mpg.de/hhpred) alignments based on the pairwise comparison of profile Hidden Markov Models (HMMs) (Soding, 2005; Soding, et al. 2005) and the MODELLER software (http://toolkit.tuebingen.mpg.de/modeller) (Eswar, et al. 2006; Marti-Renom, et al. 2000). The modeled structure data of mink growth hormone was saved in PDB file and visualized with RasWin Molecular Graphics program. Distances between atoms were determined in the 5 Å range.

Multiple sequence alignment of vertebrate growth hormones sequences was performed with Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) in order to identify the conserved sequence regions.

Theoretical pI, molecular weight and extinction coefficient for rmGH and rpGH were determined with the ProtParam tool (http://expasy.org/tools/protparam.html).

2.2.16. Influence of L-Arginine on rmGH thermal aggregation

The solution containing 0.25 mg/ml of highly purified rmGH or rpGH in the presence or absence of 50 or 100 mM L-Arg in 0.025 M K-Na phosphate buffer pH 7.5 was incubated at 70 °C for 5 h. The samples were withdrawn every 30 min and cooled on ice for 1 h. Finally, the solutions were centrifuged at 13,000 g for 25 min at 4 °C in order to remove insoluble aggregates and protein concentration of both protein solutions was determined spectrophotometrically at 280 nm using the extinction coefficient of 15930 M^{-1} cm^{-1}. The results are

presented as mean±SD for three experiments. The rate constants of aggregation were determined by fitting data to single exponential equations.

2.2.17. Influence of various cyclodextrins on rmGH thermal aggregation

The solution containing 0.2 mg/ml of highly purified rmGH and 5, 15, 30 or 45 mM of HP-β-CD or Me-β-CD or 30 mM of Ac-β-CD or γ-CD in 0.025 M K-Na phosphate buffer pH 7.5 was incubated at 60 °C for 5 h, and the transmittance at 600 nm was registered every 30 min. The results are presented as mean±SD for three experiments.

2.2.18. Circular dichroism measurements

Circular dichroism data were recorded using Jasco J-815 spectropolarimeter equipped with a Jasco PTC-423S/15 temperature control system. Purified rpGH and rmGH were dialyzed against 0.025 M K-Na phosphate buffer pH 7.5 and filtrated using 0.22 μm membrane filter. The protein concentration of both protein solutions was determined spectrophotometrically at 280 nm using the extinction coefficient of 15930 M^{-1} cm^{-1}. Melting curves were recorded at 220 nm using the quartz cell of 1 cm light path length. The final protein concentration in the sample volume of 2.5 ml was 0.06 mg/ml, and the concentration of HP-β-CD ranged from 18 to 180 mM. The temperature of samples was measured directly by the external sensor of temperature control system and raised linearly at a constant rate of 1°C/min.

3

Results and discussion

Overexpression of cloned genes in bacteria often results in insoluble refractile body (IB) formation. IBs must then be solubilized and refolded in order to obtain biologically active protein conformation. The main arising problem during refolding that protein folding competes with it misfolding and aggregation. Thus, it is necessary to establish efficient refolding protocol. In many cases refolding strategies have been improved by the use of low molecular additives as folding enhancers or aggregation suppressors. The purpose of this study was to establish an efficient refolding strategy for two model proteins, mink and porcine growth hormones, in the presence of low molecular weight additives.

3.1. Optimization of refolding process of recombinant mink and porcine GHs

E.coli strain BL21 (DE3) harboring pET21a+/mGH or pET21a+/pGH expression vectors were used for recombinant mGH or pGH production. The gene products are 191 amino acid length polypeptides which sequences refer to

P19795 of mGH and P01248 of pGH in Swiss-Prot database (residues 27–216 plus methionine at the first position of the N-end of proteins). The mGH sequence differs from pGH for three amino acids, as shown in the multiple sequence alignment (Fig. 13).

```
mGH MFPAMPLSSLFANAVLRAQHLHQLAADTYKDFERAYIPEGQRYSIQNAQAAFCFSETIPA 60
pGH MFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYSIQNAQAAFCFSETIPA 60
    ******************************:***************************

mGH PTGKDEAQQRSDMELLRFSLLLIQSWLGPVQFLSRVFTNSLVFGTSDRVYEKLKDLEEGI 120
pGH PTGKDEAQQRSDVELLRFSLLLIQSWLGPVQFLSRVFTNSLVFGTSDRVYEKLKDLEEGI 120
    ************:*********************************************

mGH QALMRELEDGSPRAGPILKQTYDKFDTNLRSDDALLKNYGLLSCFKKDLHKAETYLRVMK 180
pGH QALMRELEDGSPRAGQILKQTYDKFDTNLRSDDALLKNYGLLSCFKKDLHKAETYLRVMK 180
    ************** ******************************************

mGH CRRFVESSCAF 191
pGH CRRFVESSCAF 191
    ***********
```

Fig. 13. Multiple sequence alignment of rmGH and rpGH. The alignment was performed by ClustalW

Vertebrate growth hormone family is highly conserved (Bewly and Li 1975; Nicoll, et al. 1986), the similarity of mGH and pGH sequences is 98 %. Also, pGH shares 91 % and 68 % of the primary sequence identity with bGH (bovine) and hGH (human), respectively (Bastiras, et al. 1992).

The pH was a significant factor during our refolding experiments. It is known that the pH for refolding must be quite far from the protein isoelectric point (pI). The computed pI for sequences of both mink and porcine were 6.83. Thus, the total proteins charge was only slightly negative in the renaturation mixture pH 8.0.

The recombinant GHs were accumulated in *E. coli* as insoluble inclusion bodies. The inclusion bodies were released from *E. coli* by sonication and centrifugally collected. The over-expressed GHs comprised ~80 % of proteins in the insoluble *E. coli* fraction after several washing and centrifugation steps, as judged by SDS-PAGE (data not shown). The inclusion bodies were solubilized in 8 M urea under reducing conditions. The renaturation of denatured-reduced growth hormones was initiated by the drop-wise dilution of proteins with folding buffer pH 8.0 containing oxidized glutathione. The final concentration of urea in the refolding mixture of GHs was reduced to 3 M as it was previously reported that this concentration was the most suitable for naturation of rpGH (Bentle, et al. 1987). Although this concentration of urea was low enough for efficient GHs refolding, but was not high enough to maintain solubility and flexibility of folding intermediates. Thus, the protein precipitation in the refolding mixture at

high GHs concentrations (~1.5–2 mg/ml) and pH 8.0 in the presence of a glutathione pair ((GSH)/(GSSG) ratio equal to 2/1) at a final concentration 11.3 mM was immediately visible during the dilution process.

The renaturation of both proteins rmGH and rpGH by solubilization-dilution protocol under the same condtions and at pH 9.0 was performed in our laboratory previously (Baranauskaite, et al. 2005; Sereikaite, et al. 2007). However, the renaturation of proteins at lower pH is always more preferable, because at alkaline pH some amino acids side chains may undergo a chemical modification. Residues of Asn are very labile and susceptible to deamidation under alkaline conditions in growth hormones. It was described that Asn99 in pGH readily forms isoAsp99 and the rate of reaction increases with increasing pH (Violand, et al. 1990). The principal site of deamidation for hGH stored under alkaline conditions is Asp149, with a minor site at Asn152 (Canova-Davis, et al. 1990; Becker, et al. 1988). Therefore, an attempt was made to reduce pH to 8.0 in the renaturation buffer.

A decrease in the pH is also known to cause an increase in the hydrophobicity of proteins (Kaushik and Bhat 2003), whereas hydrophobic interaction and disulphide-covalent binding are two main forces producing aggregates in the process of protein refolding. It has been observed that rmGH as well as rpGH precipitated during dilution in the presence of the suitable redox system and at pH 8.0. Since little or no aggregation-precipitation has been observed under the same conditions, but at pH 9.0, this suggests that a decrease in pH resulted in an increased association of folding intermediates or unfolded species due to the more hydrophobic nature of both growth hormones at lower pH.

The aggregation-precipitation is intermolecular phenomenon and it is protein concentration dependent process (De Bernardez Clark 1998). In order to minimize the aggregation, attempts were made to reduce high rpGH concentration (~1.5–2.0 mg/ml) in the renaturation mixture (pH 8.0). The recovery of soluble protein was only 12–14 % at high rpGH concentration and increased to 24 % and 28 % when protein concentration was reduced to 0.8 mg/ml and 0.4 mg/ml, respectively. Thus, the recovery of soluble protein only slightly increased with decreasing the final protein concentration in the refolding mixture. However, the renaturation of protein at high concentrations is always more preferable as this does not require large renaturation volumes and allows to produce a certain quantity of renatured protein (De Bernardez Clark 2001). To achieve this and prevent the precipitation of GHs, various small molecules such as saccharose, glycerol, lysine, betaine, glycine, proline, cyclodextrins and arginine known as additives assisting in refolding process were tested (Tsumoto, et al.2003b; Arakawa, et al. 2007a; Khodarahmi and Yazdanparast 2004; Samuel, et al. 1997; Misawa and Kumagai 1999; Baynes and Trout 2004).

3.2. Refolding of both rmGH and rpGH at pH 8.0 and in the presence low molecular weight additives

3.2.1. Influence of additives on the refolding process of both rmGH and rpGH

The addition of small molecules (additives) to the refolding mixture is often essential since low concentration of urea is not enough to maintain solubility and flexibility of folding intermediates. As shown in Fig. 14, the insoluble aggregates were accumulated immediately and the transmittance at 600 nm decreased within the first few minutes when the reduced-denatured proteins were diluted with refolding buffer pH 8.0 in the absence of additives. Among these studied additives, only arginine showed the obvious effect on aggregation suppression for both rmGH and rpGH. The percentage transmittance was close to 100 % when arginine concentration was above 0.75 M in the refolding mixture, the insoluble aggregates almost disappeared. Also, the soluble pGH recovery reached 100 % in the presence of 0.75 M arginine even at high concentration of protein in the renaturation mixture. This suggests that arginine could suppress the precipitation of GHs during renaturation as it did for many other proteins (Reddy, et al. 2005; Tsumoto, et al. 2004; Umetsu, et al. 2005; Baynes, et al. 2005).

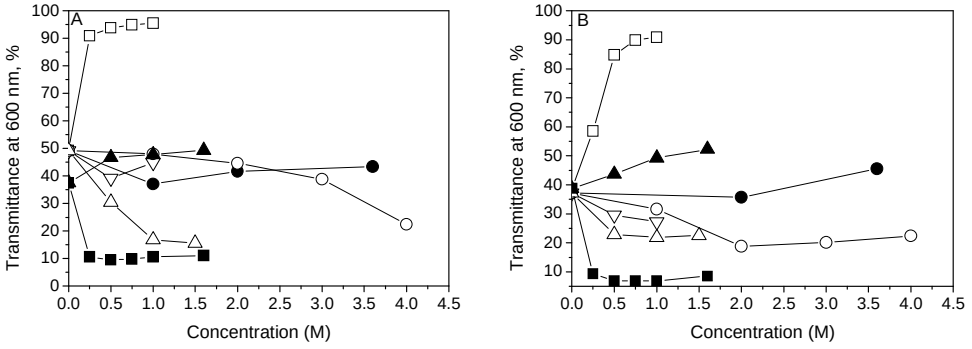

Fig. 14. Effect of additives on rmGH (A) and rpGH (B) aggregation in the refolding process by solubilization-dilution protocol. A volume of 1.5 ml of solubilized protein (the concentration of both rmGH and rpGH was 3.5-4.3 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione and various concentrations of additive (arginine (□), lysine (▲), proline (●), glycerol (○), saccharose (▽), glycine (△), betaine (■)) in order to reduce urea concentration to 3 M. The percentage transmittance at 600 nm was immediately registered.

A slight positive influence of lysine was observed. The transmittance of both rpGH and rmGH solutions at high (1.5 M) concentration of lysine increased by almost 10 % comparing with the one without the additive. Proline did not suppress the aggregation of both rpGH and rmGH (Fig. 14 A and B). On the contrary, it was found that proline prevents aggregation during *in vitro* refolding of hen egg-white lysozyme (Samuel, et al. 2000), arginine kinase (Xia, et al. 2007), creatine kinase (Meng, at al. 2001), bovine carbonic anhydrase (Kumar, et al. 1998), aminoacylase (Kim, et al. 2006a). Despite of those references it seems that the ability of proline to prevent aggregation during the refolding process differs for various proteins. Glycerol and sucrose was also found to be the effective suppressors of aminoacylase aggregation in concentration dependent manner (Kim, et al. 2006a). However, those osmolytes had no positive effect during the refolding of recombinant mink and porcine growth hormones. The aggregation of both rmGH and rpGH during refolding in the presence of glycerol and sucrose confirms once more that the choice of additive is a trial and error approach. The additives, which work well for some proteins, could be unhelpful in the refolding of other ones (De Bernardez Clark 1998).

3.2.2. Influence of different arginine concentrations on the renaturation yield of both rmGH and rpGH

Different concentrations of arginine were tested for the ability to inhibit aggregation in renaturation mixtures.

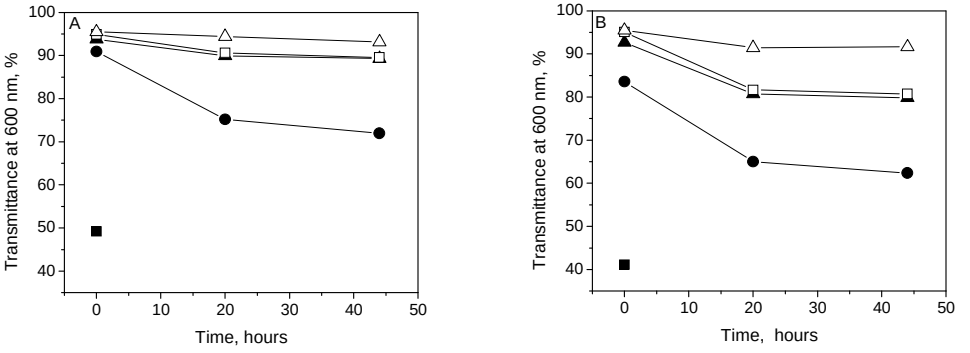

Fig. 15. Time dependence of arginine effect rmGH (A) and pGH (B) aggregation in the refolding process by solubilization-dilution protocol. A volume of 1.5 ml of solubilized rmGH (4.3 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione and various concentrations of arginine in order to reduce urea concentration to 3 M. The final concentrations of arginine were equal to 0 M (■), 0.25 M (●), 0.5 M (▲), 0.75 M (□), 1 M (△). The renaturation reaction was carried out at 4 ^{o}C with gentle stirring.

As shown in Fig. 15, arginine concentration of 1 M was effective not only at the initial moment of dilution, but also for a long time during the refolding process of both rpGH and rmGH. The percentage transmittance at 600 nm was close to 100 % in the presence of 1 M of arginine even after 44 h.

The influence of different arginine concentrations on the renaturation yield of growth hormones was also examined. Both rmGH and rpGH were refolded at the high protein concentration of 1.7–2.5 mg/ml and in the presence of glutathione pair, respectively. Renaturation mixtures were analyzed by RP-HPLC method. The typical chromatogram is presented in Fig. 16.

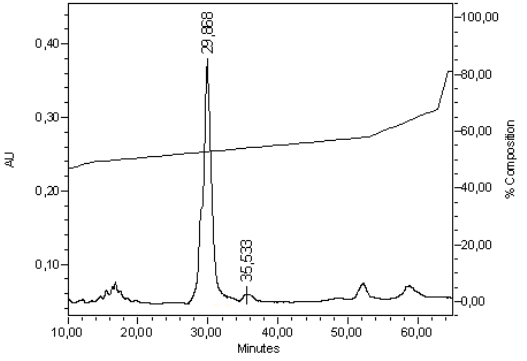

Fig. 16. RP-HPLC analysis of pGH refolded in the presence of 0.75 M arginine for 20 h at 4 °C. (GSH)/(GSSG) ratio and the final total glutathione concentration were 2/1 and 11.3 mM, respectively. Reversed-phase column Protein C4 (250 × 4.6 mm, Vydac) was used; solvent A, 0.1 % trifluoracetic acid in water; solvent B, 0.1 % trifluoracetic acid in acetonitrile (HPLC gradient grade). The column was initially equilibrated with 9 % B at a flow rate of 1 ml/min. After injection, a linear gradient of 9 % B to 41 % B for 3 min, 41 % B to 49 % B for 10 min, 49 % B to 58 % B for 40 min, 58 % B to 68 % B for 10 min, 68 % B to 81 % B for 1 min was applied. The absorbance was monitored at 215 nm.

The main peak of chromatogram corresponds to the native oxidized form of the protein (Fig. 16). The reduced form of mink and porcine growth hormones eluted 5.42±0.2 and 5.49±0.22 minutes later (mean±SD for nine RP-HPLC experiments is given), respectively. The retention time of oxidized and reduced forms of both recombinant growth hormones was verified. The purified proteins were reduced with DTT and analyzed by RP-HPLC. The molar ratio of reduced and oxidized glutathione (2/1) was considered to be optimal allowing the formation of correct and reshuffling of incorrect disulphide bonds of both growth hormones (Baranauskaite, et al. 2005; Sereikaite, et. al. 2007).

The oxidation of recombinant mink and porcine was completed after 20 hours as monitored by RP-HPLC. The ratio of reduced and oxidized forms of growth hormones did not change over the extended periods of refolding (over 20 hours). RP-HPLC analysis shows that the renaturation yield is equal to 50–60 % for both rpGH and rmGH and approximately is the same in the presence of 0.5, 0.75 or 1 M of arginine after 20 hours refolding at 4 ºC (Fig. 17 A and B). However, the percentage transmittance at 600 nm was higher in the presence of 1 M of arginine (Fig. 15). The prolongation of renaturation duration did not change the renaturation yield. Therefore, the duration of 20 h and arginine concentration of 1 M were chosen for further rmGH and rpGH renaturation at pH 8.0.

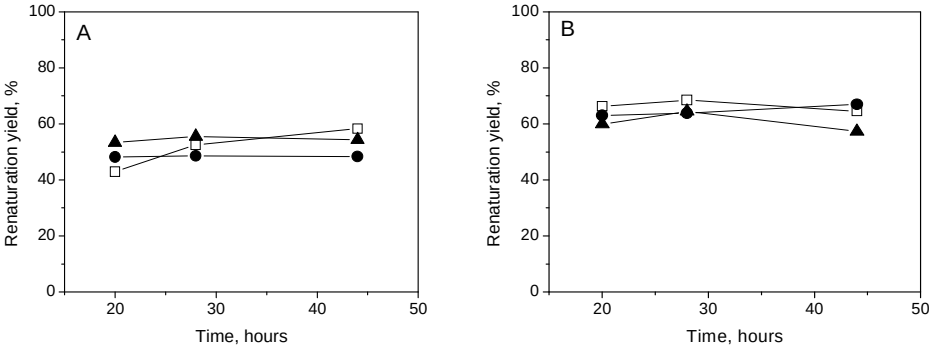

Fig. 17. Renaturation yield of rmGH (A) and pGH (B) as the percentage of all IB proteins as judged by RP-HPLC at different arginine concentrations. A volume of 4.5 ml of solubilized protein (the concentration of both rmGH and pGH was 4.5–6.6 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione in order to reduce urea concentration to 3 M. Arginine was added to the dilution buffer to a final concentration of 0.5 M (□), 0.75 M (●) or 1 M (▲) in the renaturation mixture. The renaturation reaction was carried out at 4 ^{o}C with gentle stirring.

Liu Y. D. and coworkers observed that in the refolding process of recombinant consensus interferon, arginine suppressed the protein precipitation, but could not eliminate formation of soluble oligomeric species. Moreover, the amount of the oligomers increased with the increase in arginine concentrations (Liu, et al. 2007a). The renaturation mixtures of both rmGH and rpGH were analyzed by size exclusion chromatography in the presence of four different arginine concentrations (Fig. 18 and Table 5) using a Superdex 75HR 10/30 column, previously calibrated with the protein standards (Fig. 28).

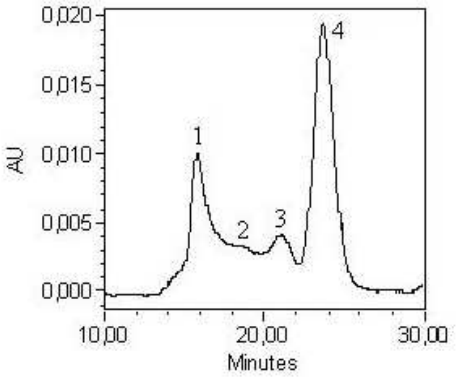

Fig. 18. SEC analysis of rmGH refolded in the presence of 0.5 M arginine using Superdex 75 HR 10/30 column previously equilibrated with 0.025 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl at a flow rate of 0.5 ml/min. The column was eluted with the same buffer, and the eluent was monitored at 215 nm. Peaks 1 and 2, higher oligomers; peak 3, dimer; peak 4, monomer.

Table 5. SEC analysis of renaturation mixtures in the presence of different arginine concentrations.

Arginine conc., M	rmGH			rpGH		
	Monomer	Dimer	Higher oligomers	Monomer	Dimer	Higher oligomers
0.25	59	10	31	63	8	29
0.5	60	12	28	–	–	–
0.75	61	14	25	65	9	26
1.0	58	13	29	60	8	32

[a] The amount of different proteins forms as the percentage of relative peak area eluted from Superdex 75 HR 10/30 column

Dimers and higher oligomers were found in the renaturation mixtures of both rmGH and rpGH as well as described in the reference mentioned above. However, contrary to consensus interferon the percentage of monomer, dimer and higher oligomers of growth hormones did not considerably change with the increase in arginine concentration. It seems that it depends on the nature of protein, and the testing of a large number of different proteins could lead up to some conclusions. Moreover, the quite similar amount of GH's monomeric form was detected by SEC and RP-HPLC methods suggesting that they were primarily covalent in nature. Thus, these results indicate that arginine can suppress aggregates produced by hydrophobic interaction, not covalent binding.

It is important to emphasize that in the absence of arginine it would have been impossible to refold both rmGH and rpGH at the high protein concentration and pH 8.0, due to the high level of protein precipitation. Moreover, experimental data demonstrate that arginine becomes effective only at high concentrations (Figs. 14 and 15) similar to other folding additives. Thus, the mode of L-ArgHCl action on protein aggregation is more like due to weak intermolecular interactions rather than strong binding.

3.2.3. Influence of ArgEE on the aggregation of rmGH in the refolding process

Among the nondenaturing reagents, arginine is the most widely used additive for increasing refolding yields by decreasing aggregation. However, recently it was reported that arginine analogs such as L-arginine ethylester (ArgEE), L-argininamide (ArgAd) increased the refolding yield of a hen egg lysozyme (Shiraki, et al. 2004), bovine carbonic anhydrase (Hamada and Shiraki 2007) more effectively than arginine. ArgEE compared to Arg lacks the carboxy group due to its esterification (Fig. 19). Therefore the pKa of amino groups of ArgEE and Arg differs and equals 7.4 and 9.2, respectively.

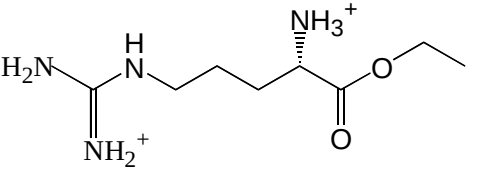

Fig. 19. Chemical structure of L-arginine ethylester (ArgEE)

Arginine ethylester has been tested in order to find out if L-ArgEE prevents aggregation and increases refolding yield of rmGH more effectively than L-Arg. The rmGH was refolded in the presence of a glutathione pair at a final concentration of 11.3 mM, at the high protein concentration (1.7 mg/ml) and various concentrations of arginine ethylester (ArgEE), respectively. The percentage transmittance of rmGH refolding mixtures in the presence of various concentrations of ArgEE was almost the same as with arginine for mink growth hormone (Fig. 20). There was no visible precipitation in the refolding mixture containing 1 M of ArgEE. The final 1 M concentration of ArgEE was the most effective not only at the initial moment of dilution, but also for a long time during the refolding process of rmGH (Fig. 20). However, in the presence of

0.75 M and 0.5 M of ArgEE, the percentage transmittance at 600 nm from the initial moment of dilution decreased approximately 10 % during the refolding of the protein.

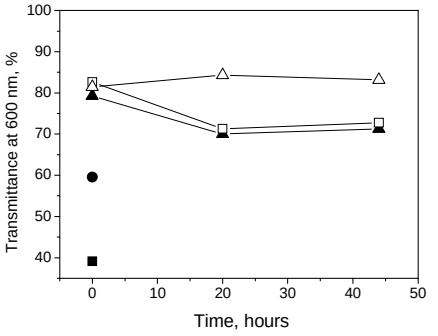

Fig. 20. Time dependence of arginine ethylester effect on rmGH aggregation in the refolding process by the solubilization-dilution protocol. A volume of 1.5 ml of solubilized rmGH (4.6 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM of oxidized glutathione and various concentrations of arginine ethylester (ArgEE) in order to reduce urea concentration to 3 M. The final concentrations of ArgEE were equal to 0 M (■), 0.25 M (●), 0.5 M (▲), 0.75 M (□), 1 M (△). The renaturation reaction was carried out at 4 ^{o}C with gentle stirring.

The yield of rmGH renaturation was determined by RP-HPLC. The typical chromatogram is presented (Fig. 21 A). RP-HPLC analysis shows that the renaturation yield is equal approximately 65–70 % for rmGH in the presence of 0.5, 0.75 or 1 M of arginine ethylester after 20 hours and it is approximately 10% higher than in the presence of arginine (Fig. 21 B). The prolongation of renaturation duration for more than 20 hours in the presence of 0.75 M and 1 M ArgEE did not change the renaturation yield of rmGH, substiantally. However, the renaturation yield considerably decreased after 44 hours of renaturation when ArgEE concentration was 0.5 M (Fig. 21 B). Since the insoluble aggregates almost disappeared in the presence of 1 M of ArgEE as well as with Arg, this concentration of ArgEE was determined as the most effective one for rmGH renaturation at pH 8.0. These results indicate that arginine ethylester prevents protein misfolding-aggregation and promotes mink growth hormone renaturation more effectively than arginine.

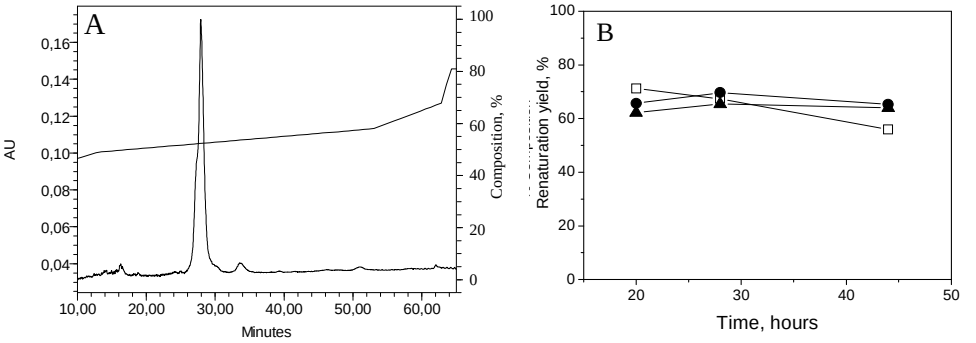

Fig. 21. RP-HPLC analysis of rmGH refolded in the presence of 0.75 M arginine ethylester for 28 h at 4 °C (A). Renaturation yield of rmGH as the percentage value of all IB proteins judged by RP-HPLC at different arginine ethylester concentrations (B). A volume of 1.5 ml of the solubilized protein (the concentration of rmGH was 4.6 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M of urea and 20 mM of reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM of oxidized glutathione in order to reduce urea concentration to 3 M. Arginine ethylester was added to the final concentration of 0.5 M (□), 0.75 M (▲) or 1 M (●) in the renaturation mixture. The renaturation reaction was carried out at 4 °C with gentle stirring.

The refolding process is dependent on the charged state of both the protein and the additive. Thus, Hamada and Shiraki suggested that interaction between hydrophobic end of the ethyl group of ArgEE's and the hydrophobic core of the unfolded protein enhanced electrostatic repulsion between the aggregation-prone species. (Hamada and Shiraki, 2007).

3.2.4. Influence of CD on the aggregation of both rmGH and rpGH in the refolding process

To suppress rmGH and rpGH aggregation during the renaturation process by solubilization-dilution protocol, another group of additives – various cyclodextrins differing in ring size (α-CD, HP-β-CD instead of β-CD due to higher solubility and γ-CD) and ring substituents (Me-β-CD, Ac-β-CD and HP-γ-CD) was tested. Turbidity experiments showed that at the initial moment of dilution the derivatives of β-CD were the most effective assistants in suppressing the aggregation of both rmGH and rpGH (Fig. 22 A and B).

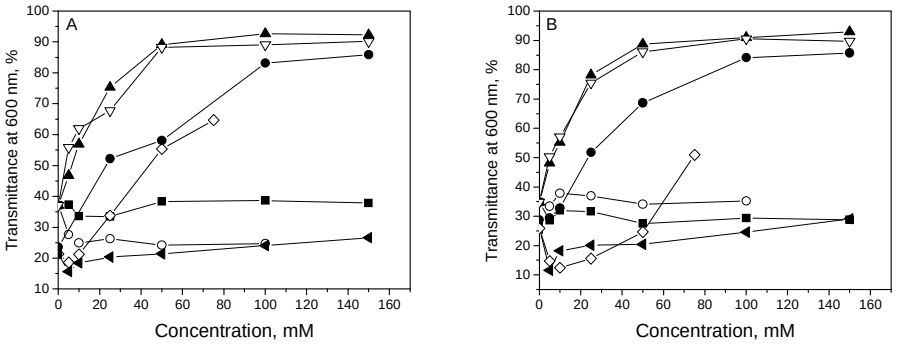

Fig. 22. Effect of cyclodextrins on rmGH (A) and rpGH (B) aggregation in the refolding process by solubilization-dilution protocol. A volume of 1.5ml of solubilized protein (the concentration of both rmGH and rpGH was 4.3–4.9 mg/ml) in 0.1M Tris-HCl buffer pH 8.0 containing 8M urea and 20mM reduced glutathione was diluted with 0.1M Tris-HCl buffer pH 8.0 containing 6mM oxidized glutathione and various concentrations of additive (Me-β-CD (▲); Ac-β-CD (▽); HP-β-CD (n=0.58–0.73) (●); α-CD (◊); HP-β-CD (n=0.28–0.57) (■); γ-CD (○); HP-γ-CD (◀)) in order to reduce urea concentration to 3 M. The percentage transmittance at 600 nm was immediately registered.

Moreover, it was determined that the degree of molar substitution per anhydro glucose unit is very important for HP-β-CD (2-hydroxypropyl-β-cyclodextrin) action. Its ability to suppress the aggregation obviously increases for the molecules with a higher degree of substitution (Fig. 22 A and B). The effect of the β-CD derivatives was tested as a function of time. Only the highest concentrations of Me-β-CD (methyl-β-cyclodextrin) and HP-β-CD suppressed the aggregation of both proteins for extended periods (over 20 h). As an example, the results of the turbidity experiments for rmGH are presented in Fig. 23 A and B. The derivative of β-cyclodextrin, Ac-β-CD (acetyl-β-cyclodextrin), suppressed the aggregation of both rmGH and rpGH only at the initial moment of dilution. Even in the presence of 100 mM or 150 mM of Ac-β-CD the transmittance of the solution decreased approximately 50 % over 4 hours (Fig. 23 C).

Glucose, 1-O-methylglucose or linear oligosaccharides, dextrin from corn, in the concentration range from 0.25 to 1M or from 0.25 % to 1%, respectively, had no positive effect on the aggregation suppression of both proteins. It seems that the cavity of CD and its diameter are the key-factors in the aggregation suppression of both rmGH and rpGH. In spite of the substituents, only β-CD, but not α- or γ-CD influenced in the positive way the renaturation process of both rmGH and rpGH. Previously, NMR studies have shown that the β-cyclodextrin

cavity forms inclusion complexes especially with the aromatic amino acid residues on the protein surface (Otzen, et al. 2002; Aachmann, et al. 2003). However, several proteins such as carbonic anhydrase (Karuppiah and Sharma 1995; Sharma and Sharma 2001), phosphofructokinase-1 (Bar, et al. 2000), α-amylase (Khodarahmi and Yazdanparast 2004), lysozyme (Desai, et al. 2006), alkaline phosphatase (Khodagholi and Yazdanparast 2008) have been reported to be renatured in the presence of α-CD, not β-CD. The cyclodextrins with a larger cavity were less effective or not tested. Only for the renaturation of aminoacylase the best aggregation suppression was found using HP-β-CD (Kim, et al. 2006b).

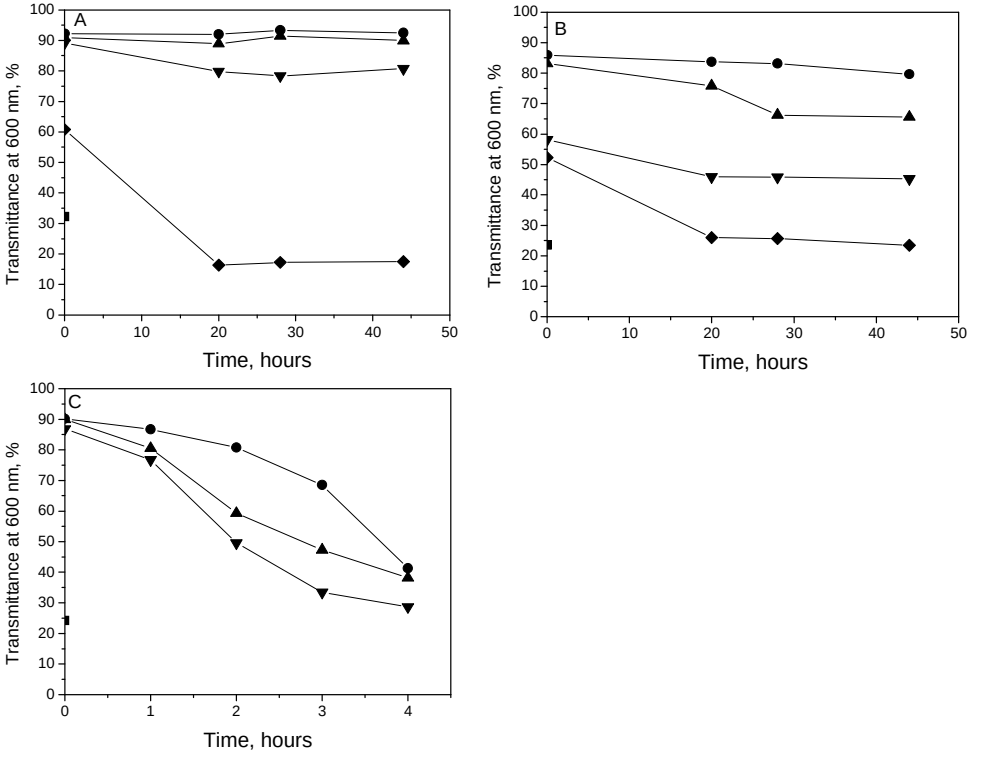

Fig. 23. Time dependence of Me-β-CD (A), HP-β-CD (B) and Ac-β-CD (C) effect on rmGH aggregation in the refolding process by solubilization-dilution protocol. A volume of 4.5 ml of solubilized rmGH (4.3–4.9 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione and various concentration of cyclodextrins in order to reduce urea concentration to 3 M. The final concentrations of CD in the renaturation mixture were equal to 0 mM (■), 25 mM (♦), 50 mM (▼), 100 mM (▲) or 150 mM (●). The renaturation reaction was carried out at 4 ºC with gentle stirring.

It seems that predicting the efficiency of cyclodextrins as aggregation suppressors is complicated, and that successful prevention of aggregation depends on both the protein and the cyclodextrin. This may be supported by the fact that the stability of various cyclodextrin-aromatic acid complexes varies. For example, the interaction character of α- and β-CD with Tyr is similar, but β-CD has almost 10-times higher affinity for Trp than α-CD (Matsuyama, et al. 1987). Kim et al. found out that the intrinsic fluorescence of free Trp is affected by HP-β-CD, but not by HP-α-CD or HP-γ-CD (Kim, et al. 2006b). Moreover, competetive experiments show that the interaction of denatured α-amylase with α-CD was canceled more effectively in the presence of Phe as a competitor than of Trp (Khodarahmi and Yazdanparast 2004). Therefore, the efficacy of CD in the renaturation process is highly dependent on which aromatic acid residues of the denatured protein and what cyclodextrin take/s part in the complex formation. On the other hand, a significant influence of the exterior of the cyclodextrin as well as the neighboring amino acids of the interaction site cannot be excluded while strengthening or weakening the formation of the inclusion complex. Both rpGH and rmGH share approximately 67–68 % sequence identity with human growth hormone (hGH). In spite of high level sequence homology, a weak inhibiting effect on the aggregation of hGH was observed for HP-β-CD. On the contrary, α-CD significantly suppressed the aggregation of hGH (Tavornvipas, et al. 2004), but it was ineffective for rpGH and rmGH.

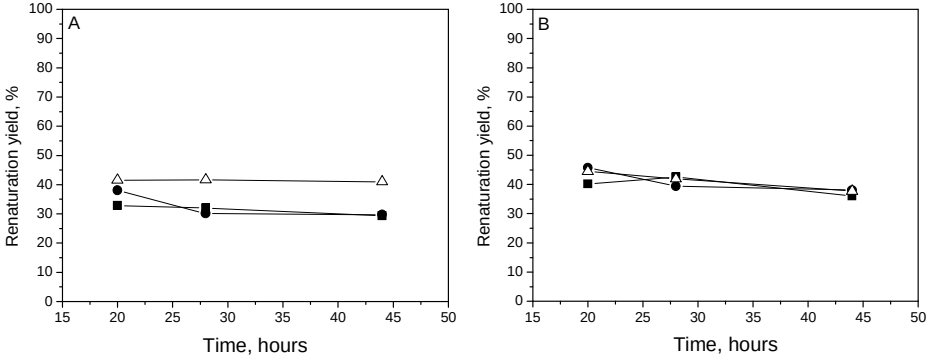

Fig. 24. Renaturation yield of rpGH (A) and rmGH (B) as the percentage of all inclusion bodies proteins as judged by RP-HPLC in the presence of different Me-β-CD concentrations. A volume of 4.5 ml of solubilized protein (the concentration of both rmGH and rpGH was 4.3–4.9 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione in order to reduce urea concentration to 3 M. Me-β-CD was added to the dilution buffer to a final concentration of 50 mM (△), 100 mM (●) or 150 mM (■) in the renaturation mixture. The renaturation reaction was carried out 4 ^{o}C with gentle stirring.

On the basis of the turbidity experiments, the HP-β-CD concentrations of 100 and 150 mM and the Me-β-CD concentrations of 50, 100 and 150mM were chosen for further examination of the cyclodextrins influence on the renaturation yield of both rmGH and rpGH. Renaturation mixtures were analyzed by RP-HPLC, and the growth hormones, which had been refolded in the presence of arginine as an aggregation suppressor and which had been biologically active proteins, were used as a standard for establishing the retention time.

For both rpGH and rmGH, the renaturation yield in the presence of Me-β-CD was approximately equal to 40–45 % after 20 h and did not change with prolongation of the renaturation time (Fig. 24 A and B). The lower concentration of Me-β-CD (50mM) was slightly more favorable for pGH renaturation (Fig. 24 A). For both proteins, similar renaturation yields were found in the presence of HP-β-CD (Fig. 25 A and B).

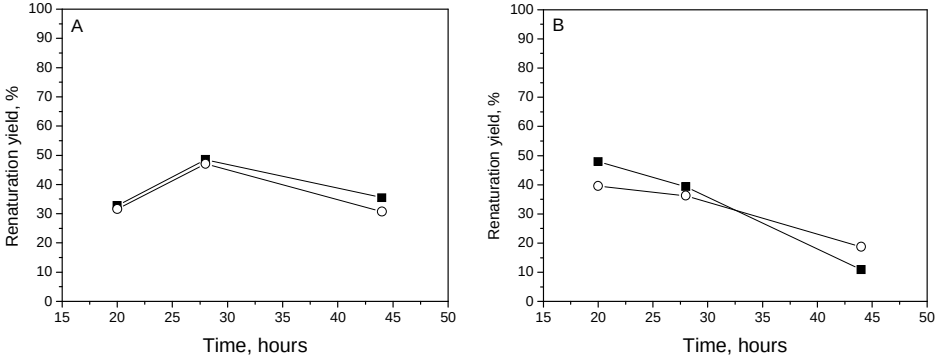

Fig. 25. Renaturation yield of rpGH (A) and rmGH (B) as the percentage of all inclusion bodies proteins as judged by RP-HPLC in the presence of different HP-β-CD concentrations. A volume of 4.5 ml of solubilized protein (the concentration of both rmGH and rpGH was 4.3–4.9 mg/ml) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione in order to reduce urea concentration to 3 M. HP-β-CD was added to the dilution buffer to a final concentration of 100 mM (○) or 150 mM (■) in the renaturation mixture. The renaturation reaction was carried out 4 oC with gentle stirring.

In spite of high level of rpGH and rmGH sequence homology of 98 %, some differences in the renaturation behavior between rpGH and rmGH in the presence of HP-β-CD were observed. For rmGH, the renaturation yield was approximately equal to 40 % and 50 % after 20 h in the presence of 100 and 150

mM, respectively, and dramatically decreased with prolonged renaturation time. On the contrary, the renaturation yield of pGH was slightly higher after 28 h compared to the renaturation time of 20 h in the presence of both concentrations of HP-β-CD.

Therefore, the effect of both Me-β-CD and HP-β-CD was significant. In the absence of the cyclodextrins it would have been impossible to refold both rmGH and pGH at the high protein concentration and pH 8.0, due to the high level of protein precipitation.

3.3. Purification of both rmGH and rpGH

3.3.1. Improving the yield of soluble GHs after refolding

Arginine at higher concentration successfully suppressed the aggregation of recombinant growth hormones during refolding by solubilization-dilution protocol in the presence of 3 M urea and glutathione pair ((GSH)/(GSSG) molar ratio is 2/1) at a final concentration of 11.3 mM. On the basis of experiments described above, the arginine concentration of 1 M was used for both rmGH and rpGH refolding for 20 h at 4 ºC and pH 8.0. After refolding, the low molecular mass compounds (3 M urea, glutathione pair and 1 M arginine) were removed by the size exclusion chromatography on Sephadex G-25, for subsequent rmGH as well as pGH purification. The data of the chromatographic cycle from 4 g of biomass are presented in Table 7. It is evident that a significant amount of both proteins was lost during gel filtration. The recovery of both rpGH and rmGH after size exclusion chromatography on Sephadex G-25 column, equilibrated with 25 mM Tris-HCl buffer at pH 8.0, was 40–55 % of the total protein (Table 7). Most likely, the misfolded species precipitated in the gel filtration column during the removal of arginine (De Bernardez Clark, et al. 1999). In gel filtration chromatography, the denaturant, thiol reagents and arginine enter the pores of the matrice, whereas proteins are exposed to the purification buffer. Such rapid procedure for buffer exchange possibly caused the precipitation of GHs misfolded species in the column. Therefore, another technique, dialysis, was tested to transfer proteins to conditions allowing the recombinant mink and porcine growth hormones to finish the formation of the native structure. Since dialysis is based on the diffusion of smaller molecules and ions through membranes, it can provide slower buffer exchange conditions than gel filtration chromatography. Also, in contrast to the gel filtration chromatography, the change from refolding buffer to the purification buffer during the dialysis occurs more gradually. Thus, the proteins passed through different denaturant, thiol

reagents and arginine concentrations that should help to avoid proteins precipitation.

Table 6. The results of two-stage refolding from 2 g of biomass

Stage	Amount of protein, mg[a]	
	rpGH	rmGH
Solubilizing of inclusion bodies	96±4[b]	87±8[b]
Refolding at pH 8.0	90±2.5	86±11
Dialysis against 25 mM Tris-HCl pH 8.0	46±2	55±5

[a]Amount of protein is given after each stage.
[b]Standard deviations for three experiments are presented.

However, it was determined that the removal of arginine, denaturing and thiol reagents by overnight dialysis at 4 ºC against 25 mM Tris-HCl buffer pH 8.0 (purification buffer), resulted in the precipitation of both rpGH and rmGH as well as by gel filtration (Table 6).

It has been reported that arginine does not facilitate refolding, but suppresses aggregation by enhancing solubility of aggregation-prone molecules (Arakawa and Tsumoto 2003; Taneja and Ahmad 1994). Thus, possibly the misfolded species form stable structures which complete their folding pathway due to the formation of the insoluble aggregates when arginine is removed.

3.3.2. Both rmGH and rpGH purification

Since slow removal of arginine, thiol reagents and urea by dialysis did not improve the amount of soluble protein, the size exclusion chromatography method was chosen. The analysis of both proteins by SDS-PAGE (Fig. 26, lane 2 and 4) revealed the presence of high molecular weight aggregates after desalting on Sephadex G-25. Also, higher molecular weight bands were observed under non-reducing conditions indicating that disulphide-bonded aggregates were formed. The major part of aggregates formed during the renaturation process was removed by the subsequent chromatography on Q-Sepharose. After this step both proteins seemed to be pure by SDS-PAGE under reducing conditions (Fig. 26 lane 5). However, SDS-PAGE under non-reducing conditions revealed some impurities which seem to be mainly comprised of a dimeric form of growth hormones. The purity of pGH as judged by SDS-PAGE under nonreducing conditions was 88±3 % (mean±SD for three experiments is given). A similar result was obtained for rmGH.

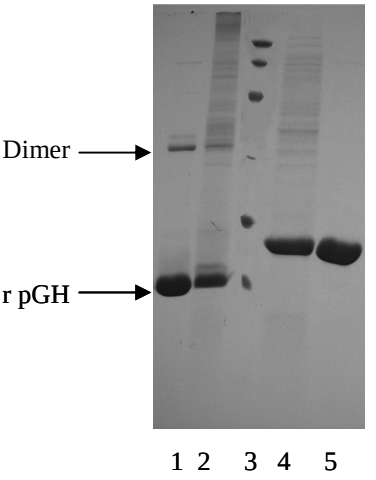

Fig. 26. SDS-PAGE (15 %) of recombinant pGH at various steps of purification. 5 μg and 10 μg of protein were loaded under nonreducing and reducing conditions lanes 1, 2 and 4, 5 respectively. Lane 3: protein MW markers (117, 90, 49, 35, 26, 19 kDa); lane 2 and 4: samples of pGH after gel filtration under nonreducing and reducing conditions, respectively; lanes 1 and 5: samples of pGH after Q-Sepharose under nonreducing and reducing conditions, respectively.

To prepare highly purified proteins, hydrophobic interaction chromatography on Phenyl-Sepharose was used. This stage of purification was able to remove dimeric form of growth hormones (Fig. 27 A). Despite the loss of some protein amount during the gel filtration stage, the overall yield of the purified refolded growth hormones at pH 8.0 was equal approximately to 10 % of all IBs solubilized proteins and was similar to the usual one for recombinant protein from inclusion bodies of *E. coli* (Table 7).

Table 7. The results of chromatographic cycle from 4 g of biomass

Stage	Amount of protein, mg[a]	
	rpGH	rmGH
Solubilizing of inclusion bodies	178±27b	187±25b
Refolding at pH 8.0	177±15	184±23
Gel filtration on Sephadex G-25	97±20	77±20
Ion exchange chromatography on Q-Sepharose	25±1.5	20±4
Hydrophobic chromatography on Phenyl-Sepharose	18±5	17±6

[a]Amount of protein is given after each stage.
[b]Standard deviations for at least three chromatographic cycles are presented.

The final purity of proteins was checked by SDS-PAGE and additionally by RP-HPLC. The results of pGH analysis are presented as an example in Fig. 27 A and B. The purity of both recombinant mink and porcine growth hormones were ≥95 % as judged by RP-HPLC.

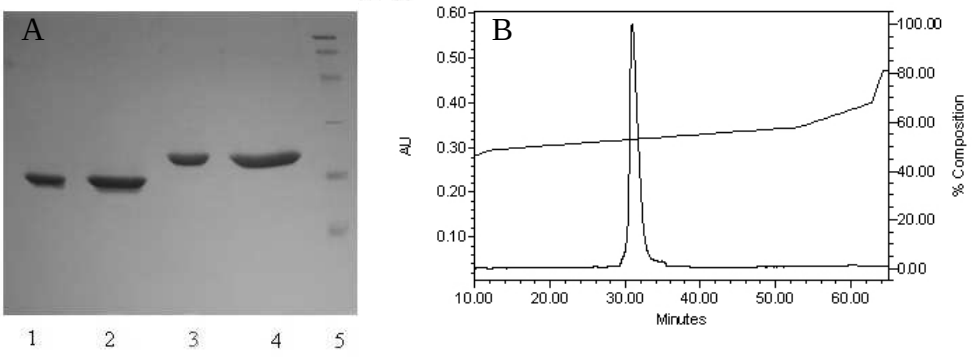

Fig. 27. A. SDS-PAGE (15 %) of highly purified recombinant pGH. 5 μg and 10 μg of protein were loaded under nonreducing and reducing conditions lanes 1, 3 and 2, 4, respectively. Lane 5: protein MW markers (94, 67, 43, 30, 20.1, 14 kDa) B. RP-HPLC analysis of highly purified pGH using reversed phase column Protein C4 (250×4.6 mm, Vydac). Solvent A, 0.1% trifluoracetic acid in water; solvent B, 0.1% trifluoracetic acid in acetonitrile. The column was initially equilibrated with 9% B at a flow rate of 1 ml/min. After injection, a linear gradient of 9 % B to 41 % B for 3 min, 41 % B to 49 % B for 10 min, 49 % B to 58 % B for 40 min, 58 % B to 68 % B for 10 min, 68 % B to 81% B for 1 min was applied. The absorbance was monitored at 215 nm.

The highly purified growth hormones (previously refolded at pH 8.0 in the presence 1M of arginine) were also analyzed on size-exclusion chromatography using Superdex 75 HR 10/30 column. Both growth hormones showed single sharp peak. No other peaks were observed demonstrating that no contaminants occurred in the final preparation of GHs. As shown in Fig. 28 B, the pGH sample had retention time equal to 24.27 min which was calculated from three chromatographic experiments. The calculated molecular mass was equal to 20.1 kDa.

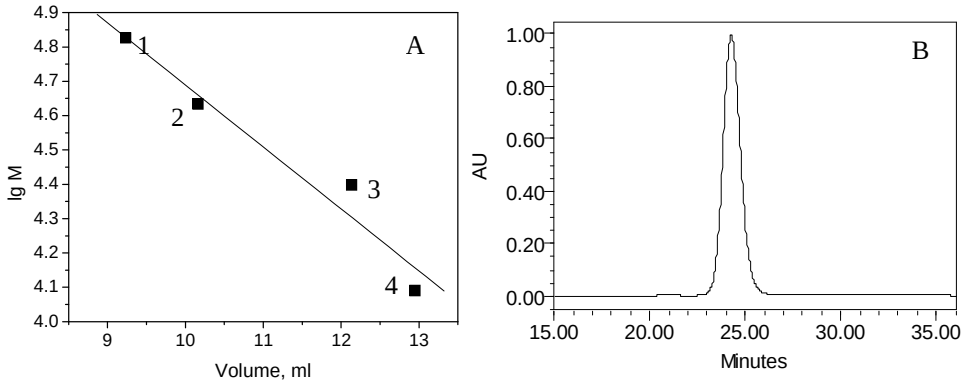

Fig. 28. A. Calibration curve for SEC analysis. Superdex 75 HR 10/30 column was equilibrated with 0.025 M Tris–HCl buffer pH 8.0 containing 0.5 M NaCl at a flow rate of 0.5 ml/min and calibrated using protein standards: bovine serum albumin (1), 67 kDa; ovalbumin (2), 43 kDa; chymotrypsinogen A (3), 25 kDa and cytochrome c (4), 12.3 kDa. The coefficient of the correlation was equal to 0.977. B. SEC analysis of highly purified pGH refolded at pH 8.0 using Superdex 75 HR 10/ 30 column previously equilibrated with 0.025 M Tris–HCl buffer pH 8.0 containing 0.5 M NaCl at a flow rate of 0.5 ml/min. The column was eluted with the same buffer, and the eluent was monitored at 215 nm.

These results let us expect that growth hormones were correctly refolded at pH 8.0 and could have been biologically active.

3.3.3. Biological activity of both rmGH and rpGH

Indeed, further experiments showed that a highly purified recombinant pGH and mGH, which were refolded at pH 8.0 in the presence of arginine, possessed the biological activity. The biological activity of proteins was determined by measuring the ability to stimulate the metabolic and mitogenic activity of mouse myeloid cell line, FDC-P1, stably transfected with the full-length ovine GH receptor (oGHR) using MTT-formazan dye assay. Since the oGHR-FDC-P1 cells express the oGH receptor, the bovine GH (bGH) was used as a positive species-specific control in the bioassay (bGH differs from oGH for two amino acids).

The comparison of the affinity of oGHR for the highly homologous bovine GH with its affinity for mink and porcine GHs indicated a higher affinity for the ruminant GH than for mink or porcine GHs. Despite that the purified recombinant mGH and pGH were highly biologically active and had equal efficacy (i.e., same maximal response and parallel S-curves), but lower potency

(i.e., higher dose needed to cause 50 % (EC50) of the maximal response) of recombinant bovine GH in the oGHR-FDC-P1 cell bioassay (data not shown). The same efficacies of the bovine, mink and porcine GHs indicate that rmGH as well as rpGH are full agonist of the oGHR, causing the receptor to change to an active conformation. Thus, the EC50 (ng/ml) values for rmGH and rpGH were 42.1±3.2 and 38.2±1.8 (mean±SD of triplicate experiments), respectively (Borromeo, et al. 2008; Sereikaite, et al. 2007).

3.4. Preventing aggregate formation by site directed mutagenesis

3.4.1. Effect of the mutation on the aggregation process of rmGH

The mechanism of aggregation suppression by arginine is still not clearly understood. Arginine has the guanidinium group as denaturing agent guanidine hydrochloride (GdnCl). Srinivas et al. and Tsumoto et al. suggested that interactions between the guanidinium group of arginine and tryptophan side chains might be responsible for suppression of protein aggregation by arginine (Tsumoto et al. 2004; Srinivas, et al. 2003). Since arginine interacts with the proteins differently from guanidine hydrochloride (see section 1.5.5.), it was made an attempt to find out the possible interaction mechanism between the unfolded protein and arginine.

Since the interaction among folding intermediates with exposed hydrophobic patches is the major cause of aggregation (De Bernardez Clark 1998), the aggregate formation could be prevented by introducing amino acid mutations that disrupt such hydrophobic patches. Previously, this site directed mutagenesis method was tested by Knappik and Pluckthun (Knappik and Pluckthun 1995) and Nieba and co-workers (Nieba, et al. 1997), who demonstrated by *in vivo* and *in vitro* folding experiments that mutations on exposed hydrophobic residues, in order to disrupt hydrophobic patches of a protein, resulted in decreased aggregation.

Both porcine and mink growth hormones are very beneficial proteins as they posses single tryptophan (Trp) residue at position 86 of the amino acid sequence. As shown in Fig. 29, the amino acid tryptophan (Trp86) is conserved in all vertebrate GHs.

```
horse      FPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYS-IQNAQAAFCFSETIPA 59
mink       FPAMPLSSLFANAVLRAQHLHQLAADTYKDFERAYIPEGQRYS-IQNAQAAFCFSETIPA 59
fox        FPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYS-IQNAQAAFCFSETIPA 59
porcine    FPAMPLSSLFANAVLRAQHLHQLAADTYKEFERAYIPEGQRYS-IQNAQAAFCFSETIPA 59
chicken    FPAMPLSNLFANAVLRAQHLHLLAAETYKEFERTYIPEDQRYT-NKNSQAAFCYSETIPA 59
bovine     FPAMSLSGLFANAVLRAQHLHQLAADTFKEFERTYIPEGQRYS-IQNTQVAFCFSETIPA 59
human      FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT 60
           **::.** ** **:***::** ** :*:::**.:***: *:*:  :*.*.::*:**:**:
           -----HHHHHHHHHHHHHHHHHHHHHHHHHHHH------------------------

horse     PTGKDEAQQRSDMELLRFSLLLIQSWLGPVQLLSRVFTNSLVFGTSDR-VYEKLRDLEEG 118
mink      PTGKDEAQQRSDMELLRFSLLLIQSWLGPVQFLSRVFTNSLVFGTSDR-VYEKLKDLEEG 118
fox       PTGKDEAQQRSDVELLRFSLVLIQSWLGPLQFLSRVFTNSLVFGTSDR-VYEKLKDLEEG 118
porcine   PTGKDEAQQRSDVELLRFSLLLIQSWLGPVQFLSRVFTNSLVFGTSDR-VYEKLKDLEEG 118
chicken   PTGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDR-VFEKLKDLEEG 118
bovine    PTGKNEAQQKSDLELLRISLLLIQSWLGPLQFLSRVFTNSLVFGTSDR-VYEKLKDLEEG 118
human     PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEG 120
          *:.::::**:*::****:**:****** *:* *  **:*.**:*:**  *:: *:*****
          ------EE----HHHHHHHHHHHHHHHHHHHHHHHH---------HHHHHHHHHHHHHHH

horse     IQALMRELEDGSPRAGQILKQTYDKFDTNLRSDDALLKNYGLLSCFKKDLHKAETYLRVM 178
mink      IQALMRELEDGSPRAGPILKQTYDKFDTNLRSDDALLKNYGLLSCFKKDLHKAETYLRVM 178
fox       IQALMRELEDGSPRAGQILKQTYDKFDTNLRSDDALLKNYGLLSCFKKDLHKAETYLRVM 178
porcine   IQALMRELEDGSPRAGQILKQTYDKFDTNLRSDDALLKNYGLLSCFKKDLHKAETYLRVM 178
chicken   IQALMRELEDRSPRGPQLLRPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 178
bovine    ILALMRELEDGTPRAGQILKQTYDKFDTNMRSDDALLKNYGLLSCFRKDLHKTETYLRVM 178
human     IQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIV 180
          * :** .*** :**   ::: **.*** : :.:********** **:**:.*.**:*:::
          HHHHHHHH------------------------HHHHHH—HHHHHHHHHHHHHHHHHHHH

horse           KCRRFVESSCAF 190
mink            KCRRFVESSCAF 190
fox             KCRRFVESSCAF 190
porcine         KCRRFVESSCAF 190
chicken         KCRRFGESNCTI 190
bovine          KCRRFGEASCAF 190
human           QCR-SVEGSCGF 191
                :**   *..* :
                H-- --------
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Fig. 29. CLUSTAL W alignment of a set of vertebrate growth hormone sequences. The alignment displays the following symbols denoting residues that are identical or similar: "*" means that the residues in that column are identical in all sequences in the alignment; ":" means that conserved substitutions have been observed; "." means that semi-conserved substitutions are observed. The single residue of tryptophan (W86) in all GHs sequences is boxed and shown in grey, respectively. The secondary structure assignment of hGH, as determined by X-ray crystallography (PDB ID 1HGU), is shown below the sequences (letters and symbols represent extended (E), helical (H) and other (-) types of secondary structure, respectively).

Due to a high degree of GHs sequence homology, it is likely that all growth hormones possess four-α-helix bundle motifs and similar connective topologies. The results of crystallographic studies on pGH (Abdel-Meguid, et al. 1987) and hGH (De Vos, et al. 1992), FT-IR analysis and structure homology modeling on

rmGH (Borromeo, et al. 2008), theoretical studies on bGH (Carlacci, et al. 1991) confirmed this point of view. Thus, hydrophobic Trp of GHs is located in the second helix and in a hydrophobic pocket formed by the hydrophobic faces of the four-α-helix bundle. Also, it was suggested that protein residues which are critical for the structure and function, are expected to be conserved throughout evolution and often are found in the hydrophobic core and/or protein-protein or protein-ligand binding sites (Schueler-Furman and Baker 2003). These observations and literature data suggested that Trp in both mink and porcine growth hormones might be responsible for protein-protein interaction and subsequent GHs precipitation during the refolding process at pH 8.0.

In 2007 paper Arakawa T. et al. determined solubility of amino acids in an aqueous arginine solution. The results revealed that the interaction between arginine and a majority of amino acids is favorable i.e. 1 M of arginine increases the solubility of amino acids. The solubility of tryptophan in 1 M arginine is considerably increased while that of glycine is increased only slightly (Arakawa, et al. 2007b). In order to find out if the interaction between tryptophan and L-arginine play a crucial role in aggregation suppression during refolding of GHs, we have replaced Trp86 with Gly within recombinant mink growth hormone by site-directed mutagenesis method.

Site-directed mutagenesis of recombinant mGH was performed by the two-step megaprimer method. The plasmid pET21a+_mGH_wt was used as a template for PCR with a primer containing the appropriate mispair and the second primer upstream or downstream of the mGH gene. The purified PCR product, a megaprimer, containing a 2-base mismatch in its central region was used in the second round of mutagenesis with the third primer downstream or upstream of mGH. The resultant product was inserted into the cloning vector pUC 57/T and *E. coli* strain JM 109. Sequencing of the mutant mink growth hormone gene confirmed that only the designed mutation (Trp86Gly) was introduced. The mGH gene carrying the Trp86Gly mutation was cleaved by the NdeI and HindIII and ligated back to the pET21a+ expression vector via the same restriction sites. The host *E. coli* strain BL21 (DE3) cells were transformed with the pET21a+_mGH_mutant and used for the expression of the mutant mGH (W86G_mGH). *E. coli* BL21 (DE3) cells harboring the plasmid pET21a+_mGH_wt were grown in parallel. The target protein was induced with 1 mM IPTG at OD600=0.9, since pET21a+ contains the lac promoter. The W86G protein has the same electrophoretic properties as wild type mGH: W86G mGH after induction appears as a wide band at the same position as wt_mGH in SDS-PAGE gel (Fig. 30 A). The expression level of mutant mGH and wt_mGH was estimated by SDS-PAGE. It was shown previously that maximum expression level of wt_mGH during the fed batch fermentation was at 120 min after induction (Sereikaite, et al. 2007).

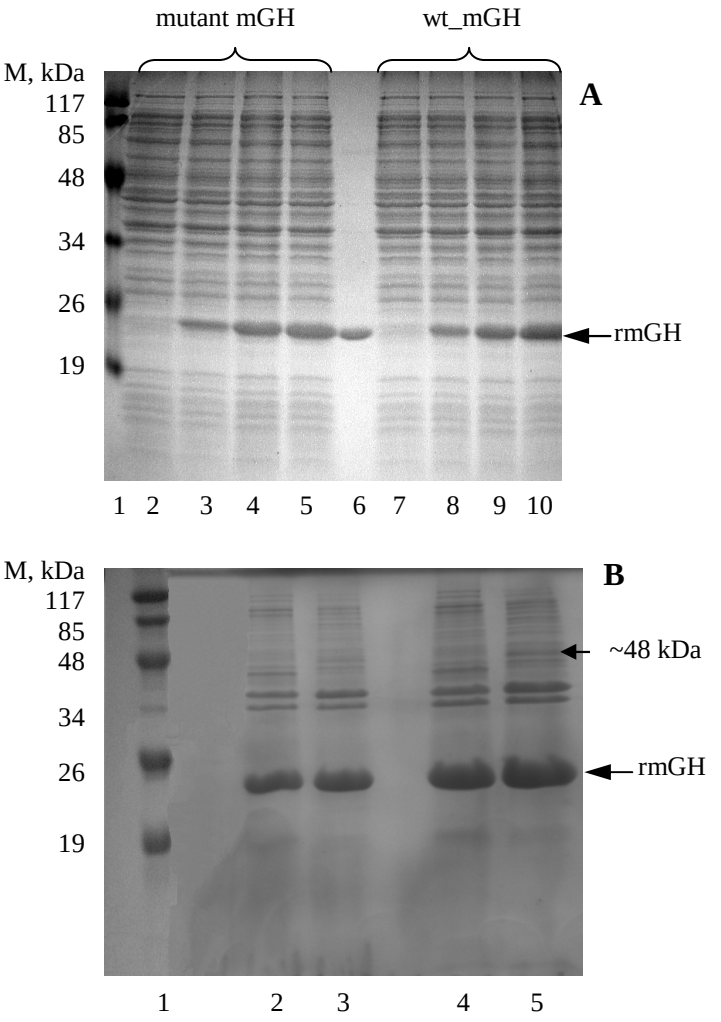

Fig. 30. A. SDS-PAGE (15 %) analysis of recombinant wt_mGH and W86G_mGH expression in *E. coli* BL21 (DE3) harboring pET21a+/wt_mGH or W86G_mGH. Lane 1: protein MW markers in kDa; lane 6: highly purified mGH; lanes 2 and 7: uninduced cells; 3–5 and 8–10 lanes: cells at 80, 120 and 160 min after induction with 1mM IPTG respectively. B. SDS-PAGE (15 %) analysis of washed IBs pellets. Lane 1: protein MW markers in kDa; lane 2 and 4: solubilized IB proteins of wt_mGH; lanes 3 and 5: solubilized IB proteins of W86G_mGH.

However, in order to increase the cell density during the fermentation, cell growth was prolonged to 160 min after induction. The expression levels of mink growth hormone at 120 min and 160 min after induction were found to remain relatively constant and represented 28 % and 32 % of total cellular proteins for W86G_mGH and wt_mGH, respectively. Since recombinant GHs were expressed as inclusion bodies in *E. coli*, the inclusion bodies of mutant mGH and wt_mGH were isolated and washed as described above. The inclusion bodies were found to contain the most of the monomeric mutant mGH and wt_mGH representing 59 % and 62 % of the total proteins in the inclusion bodies, respectively, as judged by SDS-PAGE.

Despite a very similar expression level of mutant mGH and wt_mGH, a comparison of IBs proteins revealed differences in their composition. As shown by SDS-PAGE (Fig. 30 B, lane 3 and 5) the formation of a protein (~48 kDa) in inclusion bodies of W86G_mGH was observed. Ventura S. and Villaverde A. suggested that the effect of sequence changes on the aggregation propensity in *E. coli* is not easy to predict (Ventura 2005; Ventura and Villaverde 2006). Thus, it is possible that Trp substitution with Gly resulted in an oligomer with higher molecular weight in *E. coli* inclusion body. Since the target protein in inclusion bodies can be partially structured, the conserved hydrophobic Trp may be involved in the network of interactions that possibly stabilize folding intermediates of wt_mGH in IBs. Therefore the replacement of Trp by Gly leads to subtle changes in structure of W86G_mGH by altering the ionic and hydrophobic interactions. However in order to find out what kind of an oligomer it could be, a more detailed investigation is required.

The washed inclusion bodies (see above) containing mostly recombinant W86G_mGH and wt_mGH were used for subsequent solubilization and refolding studies. Successful refolding of wt_mGH by solubilization dilution protocol at high protein concentration and pH 8.0 in the presence of a glutathione pair and 1 M of arginine was described above (section 3.2.). The influence of arginine on the refolding of W86G_mGH was examined. No aggregation was observed when the reduced-denatured W86G_mGH was diluted with refolding buffer containing 1 M arginine (Fig. 31 A, 1). On the contrary, the precipitation of W86G_mGH in the refolding mixture was immediately visible during the dilution with refolding buffer pH 8.0 lacking arginine (Fig. 31 A, 2) and the resultant protein solution became highly turbid (with transmittance equal to 37 %). The percentage transmittance of W86G_mGH refolding mixture was close to 100 % in the presence of 1 M of arginine as well as wt_mGH (Fig. 31 B). Thus, arginine prevented W86G_mGH aggregation-precipitation not only at the initial moment of dilution, but also for the hole of a 20-hour-long during the refolding process.

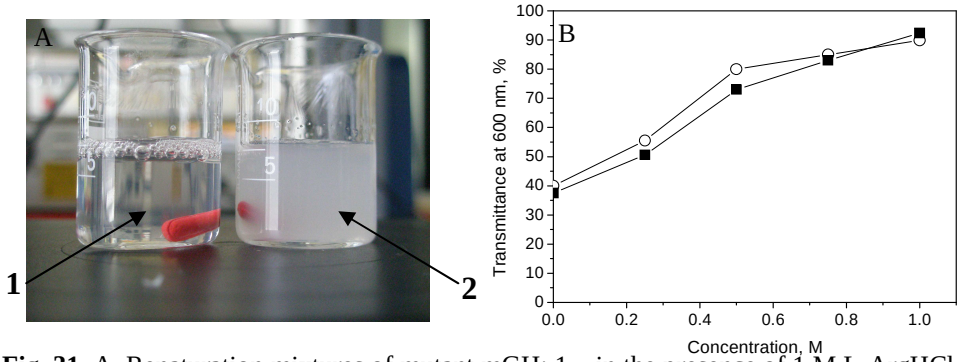

Fig. 31. A. Renaturation mixtures of mutant mGH: 1 – in the presence of 1 M L-ArgHCl and 2 – in the absence of 1 M L-ArgHCl. B. Effect of various L-arginine concentrations on the aggregation of mutant mGH (■) and wt_mGH (○) in the refolding process by solubilization-dilution protocol. A volume of 1.9 ml of solubilized protein (the concentration of mutant mGH and wt_mGH were 3.2 mg/ml and 3.1 mg/ml, respectively) in 0.1 M Tris-HCl buffer pH 8.0 containing 8 M urea and 20 mM reduced glutathione was diluted with 0.1 M Tris-HCl buffer pH 8.0 containing 6 mM oxidized glutathione and various concentrations of arginine in order to reduce urea concentration to 3 M. The percentage transmittance at 600 nm was immediately registered.

The literature overview provided in the section 1.5.2. revealed that arginine was used in many refolding systems to suppress protein aggregation such as interferon-c (Liu, et al. 2007a), interleukin-21 (Asano, et al. 2002) and fish growth hormone (Tsai, et al. 1995; Chang, et al. 2002). However the proteins refolded in the presence of arginine, all possessed at least one tryptophan in their amino acid sequences. Thus, the ability of arginine to prevent W86G_mGH aggregation during the refolding process which does not possess hydrophobic, aromatic amino acid tryptophan per polypeptide chain, suggests that not only the interaction between the guanidinium group of arginine and tryptophan side chain of protein is responsible for the aggregation suppression. Besides, this observation has confirmed the theory that protein aggregation is a set of different interactions and it is specific for each protein.

3.4.2. Effect of tryptophan mutation on the structure of rmGH

Mutational study of mink growth hormone revealed that Trp replacement by Gly did not prevent arginine from suppressing the protein aggregation during refolding process by solubilization-dilution protocol. Thus, this mutation should help to assess the extent to which the tryptophan side chain interactions formed by the native and mutated residue influence the protein refolding in the presence or absence of arginine.

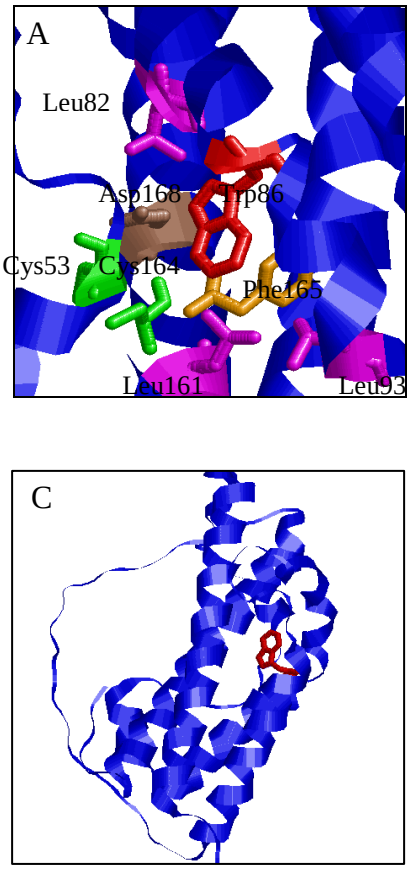

Fig. 32. The helical structures of mink growth hormone. Residues that are less than 5 Å distance from tryptophan atoms of wt_mGH (A) and W86G_mGH (B) are labeled and their side chains are shown. The side chain of the single tryptophan in the hydrophobic core of protein is shown in red (C). The figures were generated using Rasmol Version 2.7.

Tryptophan contain large rigid aromatic group on the side chain and is one of the biggest amino acids. Also, it is hydrophobic and tends to orient towards the interior of the folded protein molecule. Glycine is the smallest amino acid, rotates easily, and adds more conformational flexibility to the protein chain. This means that glycine can reside in parts of protein structures defined as tight turns (Betts and Russell 2003) and the Trp replacement with Gly could have an impact on secondary structure elements. Despite that Trp residue mutated in this study was a part of the helical structure and hydrophobic core of the protein, the Trp

substitution with Gly was not designed to increase or decrease the second α-helix propensity of the polypeptide chain in the mutated region.

According to the bioinformatic analysis (to a resolution of 5 Å) of the wt_mGH and W86G_mGH 3D structures, the mutation most affects the Trp 86 interactions with Leu 82, Leu 93, Leu 161, Cys 164, Phe 165 and Asp 168 (Fig. 32 A and B). Since the amino acids: Ser85 and Leu 87 are covalently linked via peptide bonds to the Trp 86, they were discarded from the list of amino acids interacting with tryptophan and/or glycine. It's not surprising that the single tryptophan replacement with glycine results in loss of stacking interaction with phenylalanine. Moreover, hydrophobic cluster formed between helices 2 and 3 from Leu 82, Leu 93, Trp 86 and Leu 161, Cys 164, Phe 165, respectively, was disrupted after mutation.

Interesting that negatively charged aspartate (Asp 168) is involved in tryptophan environment whereas it possibly may pair with a positively charged amino acid such as arginine creating protein stabilizing hydrogen bonds. Therefore we cannot rule out the possibility that other residues located in the hydrophobic core and structural elements also interact with arginine and contribute to the protein aggregation suppression during protein refolding.

3.5. Effect of solution additives on heat induced aggregation of both rmGH and rpGH

Proteins tend to form inactive aggregates at high temperatures. To reduce protein aggregation many types of additives have been developed. Moreover, it is important that an additive for the prevention of heat-induced aggregation does not destabilize the native protein structure. Thus, we examined the effect of additives which were effective in suppressing GHs aggregation during refolding, on heat induced aggregation of growth hormones. Also, we tried to evaluate the difference between the effects of the solution additives on the different aggregation processes i.e. refolding and heating. Since the additives which work well as aggregation suppressors during refolding process, might be unhelpful for heat-induced aggregation (Hamada, et al. 2008).

3.5.1. Effect of arginine on heat induced aggregation of both rmGH and rpGH

The amino acid, L-arginine, is more frequently used as a solution additive to prevent protein aggregation in the process of refolding. However, it has been also reported that L-arginine effectively suppresses heat induced aggregation of lysozyme (Kudou, et al. 2003; Baynes, et al. 2005), bovine serum albumin

(BSA) (Ghosh, et al. 2009), alcohol dehydrogenase (ADH) and insulin (Lyutova, et al. 2007). Thus, we tested L-arginine ability to prevent heat induced aggregation of both mink and porcine growth hormones.

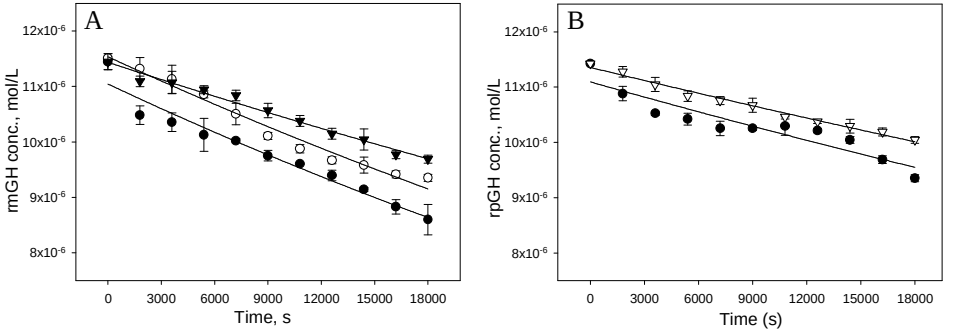

Fig. 33. Influence of arginine on rmGH (A) and rpGH (B) thermal aggregation. The solution containing 0.25 mg/ml of rmGH or rpGH and 0 mM (●), 50 mM (○), 100 mM (▼,▽) of arginine in 25 mM phosphate buffer pH 7.5 was incubated at 70 ºC for 5 h, and the concentration of soluble protein was determined by measuring absorbance at 280 nm. The continuous lines show the theoretical curves fitted to the data points with single exponential equations.

Aggregation of highly purified growth hormones were induced by incubating the proteins at 70 °C and the soluble protein concentration were determined at 280 nm. As shown Fig. 33 the soluble GHs concentration decreased due to the formation of insoluble aggregates. In the absence of arginine the GHs concentration steeply decreased after first 30 minutes (i.e. 1800 s) of heating at 70 °C especially it was seen on rmGH aggregation profile (Fig. 33 A). The addition of 50 mM and 100 mM of arginine decelerated insoluble aggregates formation of both mink and porcine growth hormones. Fig. 33 A and B demonstrates that experimental data points were well fitted to the single-exponential equation, as the heat induced aggregation of both mink and porcine growth hormones (at concentration 0.25 mg/ml) follows first-order kinetics. Thus, the rate limiting stage of aggregation is GHs denaturation proceeding as a monomolecular irreversible reaction N→D (where N and D are the native and denatured states of the protein molecule) when aggregation of proteins follows the first-order kinetics. However the kinetic mechanism of aggregation can be changed with the variation of the protein concentration (Kurganov, et al. 2002) indicating that high protein concentration accelerates aggregation which is consequence of intermolecular interaction. Since the rate of aggregation is dependent on protein concentration, the kinetics of aggregation of both rpGH

and rmGH is pseudo-first order, as reported previously (Kurganov 2002; Shiraki, et al. 2004; Wang K. and Kurganov 2003).

The rate constants of heat induced aggregation of both growth hormones in the absence or presence of arginine are listed in Table 8. The aggregation rates of rmGH and rpGH were equal to 1.4×10^{-5} s^{-1} and 0.83×10^{-5} s^{-1}, respectively, in the absence of arginine. While the concentrations of both rmGH and rpGH were equal at the initial moment of heating, the rmGH was more susceptible to heat induced aggregation than pGH. In the presence of 50 mM of arginine, the aggregation rate of rmGH only slightly decreased compared with that in the absence of additive. However, a further increase of arginine concentration (100 mM) decreased the extent and rate of heat induced aggregation to 0.9×10^{-5} s^{-1} and 0.69×10^{-5} s^{-1} for rmGH and rpGH, respectively.

Table 8. Effect of arginine on the aggregation rate constant of rpGH and rmGH.

Arginine conc., mM	Aggregation rate constant $\times 10^{-5}$, s^{-1}	
	rmGH	rpGH
0	1.4±0.1[a]	0.83±0.1
50	1.29±0.06	–
100	0.9±0.04	0.69±0.03

[a] Standard deviations for three experiments are presented.

To have a deeper insight into the suppression process of temperature induced aggregates formation, we performed intrinsic fluorescence measurements of both proteins solutions in the presence and in the absence of arginine. It is known that high temperature decreases the protein conformational stability and solubility, resulting in a greater tendency for irreversible aggregation (Baynes, et al. 2005; Kendrick, et al. 1998; Duy and Fitter 2006). During the conformational changes the maximum of the fluorescence emission spectrum (λ_{max}) usually undergoes a red shift. Fig. 34 A shows that fluorescence emission spectra of mGH has maximum at 335 nm with an excitation at 295 nm of the single tryptophan. This fluorescence emission maximum indicates that the single tryptophan is buried in the apolar protein interior at the initial moment of heating. However, the temperature induces a significant fluorescence intensity increase (2.6-fold) accompanied by a red shift of 6-8 nm after 30 minutes of heating (Fig. 34 A). Usually, the red shift accompanied by simultaneous increase or decrease in fluorescence intensity depends on tryptophan replacement to the more or less polar environment (Szabo 2000). Thus, the change of fluorescence emission spectrum suggests that single tryptophan is exposed to the solvent due to the conformational changes of rmGH during heating at 70 ºC in the absence of arginine (Fig. 34 A). The subsequent decrease of fluorescence intensity during

heating for 60, 90 and 120 minutes might be related not only to the conformational changes of protein molecule, but also due to the formation of insoluble aggregates.

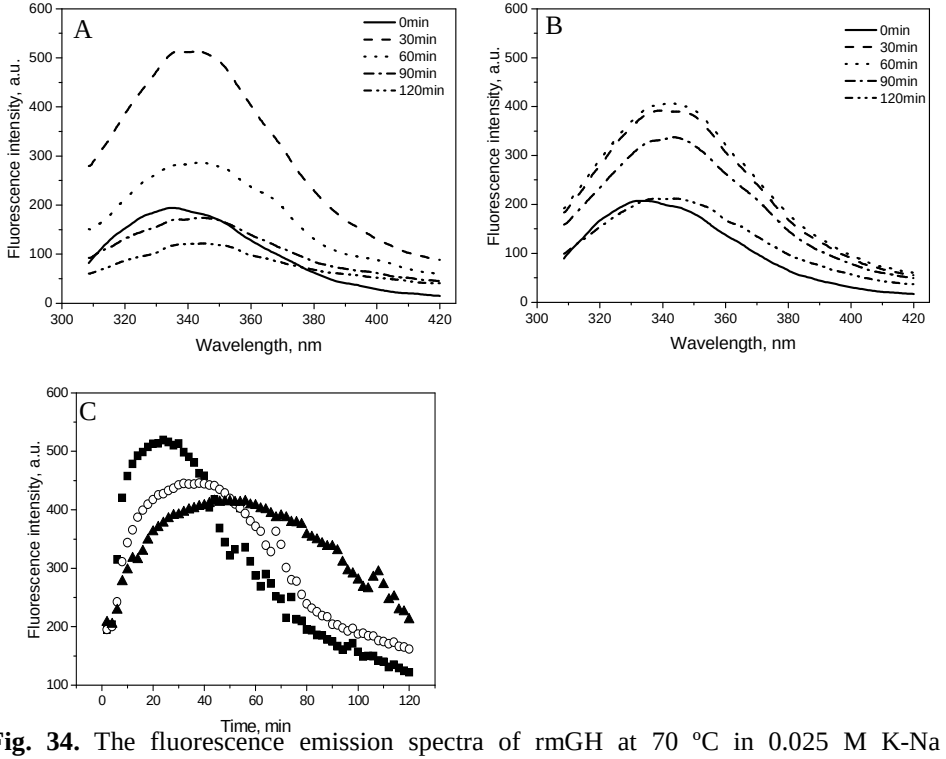

Fig. 34. The fluorescence emission spectra of rmGH at 70 ºC in 0.025 M K-Na phosphate buffer pH 7.5 were measured between 308 and 420 nm with an excitation at 295 nm. Excitation and emission slit were equal to 5.0 nm. The series of spectra were taken with a protein concentration 0.13 mg/ml in the absence of arginine (A) and in the presence of 25 mM arginine (B). Time dependence of arginine effect on rmGH fluorescence intensity during heating at 70 ºC (C). The final concentrations of arginine were equal to 0 M (■), 5 mM (○), 25 mM (▲).

The fluorescence emission spectra of rmGH in the absence and in the presence of 25 mM arginine were almost identical with no appreciable change in the λ_{max} value at the initial moment of heating (Fig. 34 B.). However, arginine was unable to prevent conformational changes of rmGH during heating at 70 ºC as the spectra shifted (6–8 nm) to the longer wavelengths. Despite that the increase in fluorescence intensity was lower and changed little in the presence of arginine for the hole of a 1.5-hour-long during heating. Moreover, Fig. 34 C reveals that the fluorescence intensity of rmGH decreases with increasing

arginine concentration upon the thermal unfolding. The similar results were obtained for pGH.

The fact that fluorescence signal (λmax) of both growth hormones shifted to the longer wavelengths during heating at 70 ºC in the presence or absence of arginine suggested that arginine did not stabilize protein structure, but reduced aggregation of unfolded species. This observation confirmed theory that arginine is not protein stabilizer, but suppresses protein aggregation during different stresses (Ishibashi, et al. 2005).

The amino acid L-arginine was effective in suppressing or disrupting protein-protein interactions of mink and porcine growth hormones during refolding and heating processes.

3.5.2. Effect of cyclodextrins on heat induced aggregation of both rmGH and rpGH

Cyclodextrins and their derivatives, due their unique structure, have been widely used as artificial chaperones for the proper protein folding in biotechnology as well as drug carriers of poorly soluble drugs in pharmaceuticals (Sharma and Sharma 2001; Khodarahmi, et al. 2004; Barzegar, et al. 2008). In this chapter we investigated effects of cyclodextrins on temperature-induced unfolding and subsequent aggregation of mink and porcine growth hormones.

Turbidity experiments showed that HP-β-CD and Me-β-CD were able to suppress temperature-induced aggregation of both growth hormones as well as during refolding (Fig. 35). This ability of cyclodextrins is a concentration dependent process as the amount of insoluble aggregates decreased with increasing HP-β-CD and Me-β-CD concentration. It should be noticed that γ-CD and Ac-β-CD, which had no positive effect in the renaturation process, successfully suppressed the formation of rmGH aggregates at 60 ºC. The effect of γ-CD and Ac-β-CD at concentration 30 mM on protein aggregation suppression was only slightly lower compared to HP-β-CD or Me-β-CD (Fig. 35 C). In the case of temperature-induced protein aggregation, the ability of cyclodextrins to form inclusion complexes with aromatic or exposed hydrophobic amino acids of the partially unfolded or native protein is no less important than in the refolding process. However, for the complex formation with native or partially unfolded protein, not only the cavity, but also the exterior of cyclodextrin may be of great importance. Thus, the set of various forces such as hydrogen bonding, electrostatic or van der Waals interaction may be involved in the exterior binding and be depended on the cyclodextrin used.

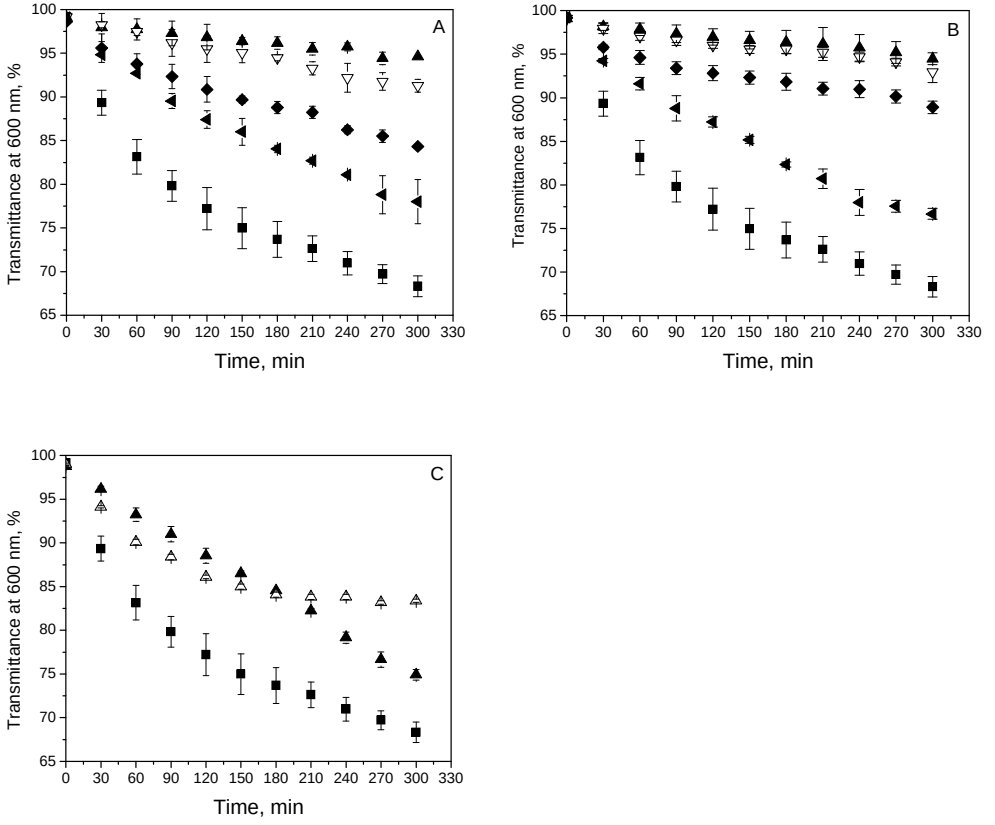

Fig. 35. Influence of various cyclodextrins on rmGH thermal aggregation. The solution containing 0.2 mg/ml of rmGH and 0 mM (■) 5 mM (◀), 15 mM (♦), 30 mM (▽) or 45 mM (▲) of HP-β-CD (A) or Me-β-CD (B) or 30 mM of Ac-β-CD (C, ▲) or 30 mM of γ-CD (C, △) in 25 mM phosphate buffer pH 7.5 was incubated at 60 ºC for 5 h, and the percentage transmittance was registered at 600 nm.

To have a deeper insight into the suppression process of temperature-related aggregates formation, we performed circular dichroism measurements of both proteins solutions in the presence and in the absence of HP-β-CD. rpGH and rmGH in an aqueous solution demonstrated negative circular dichroism bands in a wavelength region between 245 and 200 nm with two maxima at 220 and 208 nm. The absolute value of ellipticity at 220nm was used to monitor thermal unfolding of both rpGH and rmGH. However, the unfolding of both rpGH and rmGH is an irreversible process as protein solutions develop turbidity, and the absolute value of ellipticity does not increase after cooling of the protein solutions. Moreover, unfolding and aggregation occurs simultaneously. The high

tension voltage applied to the photomultiplier tube of detector increased suddenly when the absolute value of ellipticity decreased sharply due to protein unfolding. The variations in high tension voltage may occure as a result of the changes in light scattering, which can be explained by the formation of large aggregates of thermally unfolded protein (Benjwal, et al. 2006).We determined that the temperature at which the high tension voltage increased was only 1 ºC higher as compared with the temperature at which the absolute value of ellipticity decreased sharply (Fig. 36).

A mid-temperature of denaturation is widely used to characterize the effect of various factors on the protein stability. However, if the protein unfolding and aggregation occur simultaneously equilibrium thermodynamics cannot be applied to the analysis of such transition (Benjwal, et al. 2006). Onset temperatures of melting and aggregation are better parameters, when the protein unfolding is concomitant with its aggregation (Arakawa, et al. 2006). The effect of HP-β-CD on the onset temperatures of melting and aggregation of both rpGH and rmGH during the thermal unfolding are summarized in Table 9.

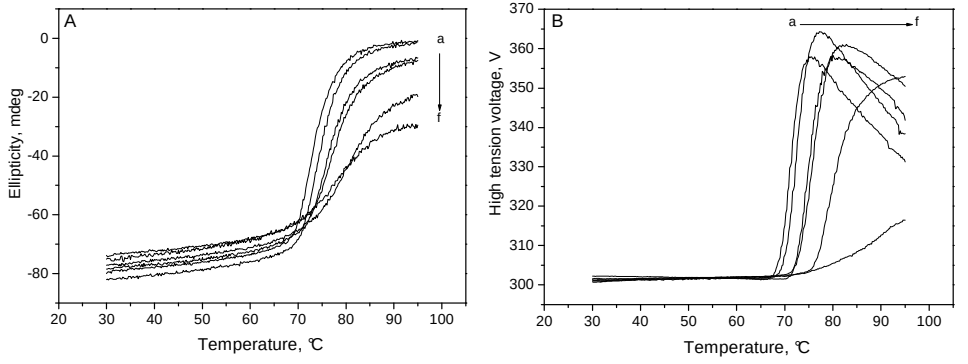

Fig. 36. Circular dichroism measurements of thermal unfolding and aggregation of pGH in the absence and in the presence of various concentrations of HP-β-CD (a, 0 mM; b, 18 mM; c, 36 mM; d, 60 mM; e, 120 mM; f, 180 mM). The changes in the ellipticity recorded at 220 nm report on the α-helical protein unfolding (A), and the high tension voltage curves recorded at the same wavelength report on the increase in the particle size due to the aggregation of unfolded protein (B).

The onset temperatures of melting and aggregation for both proteins increased by 1–2 ºC in the presence of low (18 mM) HP-β-CD concentration suggesting that HP-β-CD increases the melting temperature of both rpGH and rmGH. A further increase of the concentration of additive has different effects on the onset temperature of the protein unfolding and aggregation. While the

onset temperature of melting does not change in the presence of high (180 mM) or low (18 mM) HP-β-CD concentration, the onset temperature of turbidity development increases with the increase of the additive concentration indicating that HP-β-CD postpones the aggregation of unfolded protein. For example, in the presence of 120mM HP-β-CD, pGH unfolding begins at 57 ºC with the high tension voltage value remaining unvarying untill 74 ºC indicating that the unfolded protein is not aggregated in this temperature range (Table 9, Fig. 36).

Table 9. Effect of HP-β-CD on the onset temperature of rpGH and rmGH unfolding and aggregation

HP-β-CD conc., mM	Onset temperature of melting, °C[a]		Onset temperature of aggregation, °C	
	rpGH	rmGH	rpGH	rmGH
0	55.5	56.0	66.0	64.5
18	57.0	56.5	68.0	66.5
36	57.0	–	70.5	–
60	57.0	–	70.5	–
120	57.0	–	74.0	–
150	–	57.0	–	73.0
180	57.0	56.5	75.0	73.0

[a] determined as a point where the melting curve slightly deviates from the baseline drawn through the pre-transition region.

In the absence of HP-β-CD the turbidity of both rpGH and rmGH develops at temperature almost 10 °C lower than in the presence of additive. Moreover, HP-β-CD makes the unfolding of both rpGH and rmGH partially reversible while the degree of reversibility is an additive concentration-dependent process. Although the onset of aggregation temperature is postponed in the presence of HP-β-CD, the high tension voltage from 300 V increases 55–60 V in the presence or absence of HP-β-CD, and a sample solution remains turbid. However, in the presence of the highest concentration tested (180mM) the circular dichroism signal after the thermal scan and subsequent cooling down to 25 °C almost returned to the preheating value for rpGH and rmGH. During the thermal scan the high tension voltage increased only by 15 V for pGH or 23 V for rmGH solutions (the onset temperatures of aggregation were 75 ºC and 73 ºC for rpGH and rmGH, respectively), and the proteins solutions contained only a very small amount of aggregates after the completion of the scan. The magnitude of the decrease in the absolute ellipticity values during the thermal unfolding is also proportional to the HP-β-CD concentration (Fig. 36 A).

In conclusion, cyclodextrins are potential additives for suppression of aggregation of proteins in renaturation and thermal denaturation processes, as it was shown with two model proteins. Also, the cyclodextrins are capable of preventing storage related denaturation of proteins and thus can be used to ensure safe storage of the same proteins.

General conclusions

1. Refolding studies of recombinant mink and porcine growth hormones revealed that:

1.1 Renaturation of rmGH and rpGH at high protein concentration (~1.5–2 mg/ml) and pH 8.0 by solubilization-dilution protocol was impossible, due to the high level of protein aggregation and precipitation. The soluble protein recovery was only 12–14 % and it increased only slightly when the concentration of the protein in the refolding mixture was reduced.

1.2 The amount of precipitation in the refolding mixture (pH 8.0) of rGHs decreased evidently in the presence of L-Arg, L-ArgEE, Me-β-CD and HP-β-CD. The recovery of soluble protein reached almost 100% after refolding in their presence.

1.3 The refolding yield of both correctly folded rmGH and rpGH in the presence of L-Arg was 50–60 % as determined by RP-HPLC analysis. It was ~10 % lower in the presence of HP-β-CD and Me-β-CD, but ~10 % was higher in the presence of L-ArgEE.

2. The evaluation of the L-Arg effect on the refolding process of rGHs demonstrated that arginine effectively suppressed GHs precipitation, but could not eliminate the formation of soluble disulphide-bonded oligomers produced during the refolding process.

3. The efficiency of various cyclodextrins during refolding of both rmGH and rpGH was not only dependent on their cavity size, but also on the substituents and their molar substitution degree on the ring of cyclodextrin. The derivatives of β-cyclodextrins (Me-β-CD and HP-β-CD) were the most effective derivatives in suppressing the aggregation of both rmGH and rpGH during refolding. The ability of HP-β-CD to suppress the aggregation of GHs increased with a higher degree of substitution on the ring of cyclodextrin.

4. Both rmGH and rpGH refolded by solubilization-dilution protocol in the presence of L-Arginine were purified with the overall yield equal to ~10% of all solubilized IBs proteins. The purity of both growth hormones was ≥95 % as determined by RP-HPLC. The biological activity of refolded and purified GHs was confirmed by *in vitro* bioassay.

5. Refolding of W86G_rmGH which did not possess hydrophobic, aromatic amino acid tryptophan per polypeptide chain, by solubilization-dilution protocol in the presence of L-Arginine was demonstrated for the first time. The ability of L-Arginine to prevent W86G_rmGH aggregation during the refolding process suggested that L-Arginine effect on protein aggregation was a set of weak interactions between protein and amino acid.

6. Thermal stability studies on both rmGH and rpGH demonstrated that L-Arginine did not stabilize the protein structure, but reduced the aggregation of unfolded species during thermal unfolding.

7. The evaluation of the cyclodextrins effect on the thermal aggregation process of rGHs demonstrated that the derivative of β-cyclodextrin (Ac-β-CD) and γ-CD had no positive effect on the refolding process of GHs, but those derivatives successfully suppressed the formation of rmGH aggregates during thermal unfolding. The thermal stability studies revealed that HP-β-cyclodextrin did not increase thermal stability of rGH, but it postponed the aggregation of unfolded protein as determined by circular dichroism spectroscopy.

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STUDIES OF THE REFOLDING
PROCESSES OF RECOMBINANT GROWTH
HORMONES

Doctoral Dissertation

Technological Sciences,
Chemical Engineering (05T), Biotechnology (T490)

Eglė STRAINIENĖ

REKOMBINANTINIŲ AUGIMO HORMONŲ
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