Investigations on the optimization of the soluble production of heterologous proteins in *Escherichia coli*

Dissertation

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CONTENTS

Abbreviation

APS	ammonium persulfate
ATP	3´, 5´-adenosine triphosphate
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
CDS	centrifugal disc photosedimentation
DCU	digital control unit
DCW	dry cell weight
DDT	1, 4-dithiothreitol
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOT	dissolved oxygen tension
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESZ	electrical sensing zone
GSH	reduced glutathione
GSSG	oxidized glutathione
h	hour
HDF	defined medium
Hsp	heat shock protein
IBs	inclusion bodies
IgY^{++}	immunoglobin in egg yolk
IPTG	isopropyl-β-D-thiogalactopyranoside
Kcps	kilo count per second
kDa	kilo Dalton
L-Arg	L-arginine
LB	Luria-Bertani medium
mRNA	message ribonucleic acid
nd	not detected
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PCS	photon correlation spectroscopy
p-NPG	p-nitrophenyl-α-D-glucopyranoside
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rPA	tissue-type plasminogen activator variant
rpm	revolutions per minute
s	second
scFv	single-chain fragment antibody
SDS	sodium dodecyl sulphate
Sec	independent protein export
sHsp	small heat shock protein
TAT	twin-arginine translocation system
TBS	tris buffered saline
TF	trigger factor
tPA	tissue-type plasminogen activator

Tris	Tris-(hydroxymethyl)-amino methane
U	unit
v/v	volume per volume
Val-Leu-Lys-pNA	valine-leucine-lysine-para Nitroanilid
w/v	weight per volume
YE	yeast extract medium
%	procent
x *g*	earth's gravitational field

Nomenclature

C_{CO2in} :	inlet carbon dioxide concentation, (%)
C_{O2in} :	inlet oxygen concentation, (%)
C_{O2out} :	outlet oxygen concentation, (%)
C_{CO2out} :	outlet carbon dioxide concentation, (%)
μ_{set} :	set point of the specific growth rate, h^{-1}
CER:	volumetric carbon dioxide evolution rate, (mmol L^{-1} h^{-1})
d (H) :	hydrodynamic diameter
D :	translational diffusion coefficient
F:	feeding rate, (g h^{-1})
k :	Boltzmann´s constant
m_E :	maintenance coefficient, (g g^{-1} h^{-1})
Q:	outlet gas flow rate, (L h^{-1})
$q_{CO2:}$	specific carbon dioxide evolution rate, (mmol L^{-1} OD^{-1} h^{-1})
q_S :	specific substrate consumption rate, (g g^{-1} h^{-1})
S_0 :	substrate concentration in the feeding solution, (g g^{-1})
T :	absolute temperature, °C
t :	cultivation time, h
t_f :	time of feeding start, h
V:	volume at feeding start, L
V:	volume, L
V_f :	volume at feeding start, L
X:	cell density, g L^{-1}
X_f :	dry cell weight at the time of feeding start , (g L^{-1})
$Y_{X/S}$:	biomass yield coeffcient , (g g^{-1})
η :	viscosity

1. INTRODUCTION

Escherichia coli is an important microorganism for production of recombinant proteins applied in therapy and diagnosis. *E. coli* offers several advantages, including growth on inexpensive carbon sources, rapid biomass accumulation and simple process for scale-up. Thus, *E. coli* has been usually selected to obtain enough material for biochemical and structural studies and for the large-scale production of valuable recombinant proteins. However, overexpression of recombinant proteins often leads to an incorrect conformation, proteolytic degradation of proteins and formation of insoluble aggregates of nonnative proteins or inclusion bodies (IBs) (Baneyx and Mujacic, 2005). In some cases, formation of IBs can be desired and simplify some steps of purification. However, refolding of small proteins can be efficient, but not with large proteins as multiple disulfide bonds normally fail to fold in their correct conformations. Moreover, renaturation and refolding of IBs *in vitro* are complicated and costly. After refolding, low yields of soluble proteins are obtained due to protein aggregation resulted from interactions among hydrophobic regions of the proteins.

In order to obtain high yield of soluble heterologeous recombinant proteins from *E. coli*, other strategies have been applied, e.g. the use of different host strains, promoters, reduction of cultivation temperature, coexpression of chaperones, localization of the gene products in the periplasm and culture medium or other words: secrection of recombinant proteins into the periplasmic space and export of proteins to growth medium using different helper proteins and additives. These strategies are indicated in Table 1.

Table 1. Optimization of functional soluble protein expressed in *E.coli*

Parameter		**Cultivation method**	**Protein expression**	**Reference**
Localization				
		Batch, shake flask	Transglutaminase	Yokoyama et al., 1998
		Fed-batch	α-glucosidase, creatinine imino hydrolase	Lin, 2000
		Fed-batch, high cell density	hydrolase	Dresler et al., 2006
Additives				
	L-Arg , glutathione	Shake flask	native tissue-type plasminogen activator (rPA)	Schäffner et al., 2001
	glutathione	Shake flask	α -amylase/trypsin inhibitor	Wunderlich and Glockhuber, 1993
	glycine, Triton X-100	Shake flask	sFV/TGF- α	Yang et al., 1998
Temperature				
		Shake flask	human tumour necrosis factor-β	Mak et al., 1993
		Shake flask	β -lactamase	Baneyx et al., 1992
Coexpression				
	DsbA or DsbC	Fed-batch, high cell density	insulin-like growth factor (IGF)-I	Joly et al., 1998
	DsbC	Fed-batch	tissue-type plasminogen activator (tPA)	Qiu et al., 1998
	DsbC, DsbD	Shake flask	Human nerve growth factor β	Kurokawa et al., 2001
	FkpA	Shake flask	single chain antibody fragments scFv	Bothmann and Plückthun, 2000
	DegP	Batch	recombinant penicillin acylase	Lin et al., 2001; Pan et al., 2003
	DegP+FkpA	Shake flask	recombinant penicillin acylase	Wu et al., 2007
	DnaJ, Hsp25	Shake flask	native tissue-type plasminogen activator (rPA)	Schäffner et al., 2001

From the previous reports, although genetic optimization of production strains has been well investigated, few research on processes designed for secretory production was developed. Effects of environment conditions such as medium formulation and parameters of cultivation process for translocation of recombinant proteins to the periplasm and growth medium during fedbatch cultivations should be also taken into account.

The effect of feeding strategies during fed-batch cultivation on cytoplasmic protein production has been well documented (Curless et al., 1989; Fu et al., 1993; Hellmuth et al., 1994; Kim et al., 1996; Le Thanh and Hoffmann, 2005; Lee et al., 1996; Seo and Bailey, 1986; Turner et al., 1994). However, few studies investigate the impact of growth or feeding rates specifically for periplasmic production. Changes in substrate and growth rate during fedbatch cultivations affect the secretion of the product to the periplasm (Boström et al., 2005). Growth rate influences the membrane structure of *E. coli* and leakage of periplasmic proteins to the culture medium (Shokri et al., 2002; Shokri et al., 2003; Dresler et al., 2006). Pre-induction growth rate results in a sharp maximum of secretion efficiencies (Curless et al., 1994).

In this dissertation, the coexpression of certain helper proteins together with the target proteins, the optimal conditions for production of the soluble proteins in the three compartments (cytosol, periplasm and culture supernatant) were investigated. Also, in order to evaluate the expression of the protein in the cytosol of *E.coli*, a method (Dynamic Light Scattering) for quantitative measuring size particles of IBs was developed.

1.1 State of the art for soluble protein production from *Escherichia coli*

1.1.1 Coexpression of molecular chaperones for production of soluble protein

1.1.1.1 Functions of chaperones

In the cells, the chaperone systems can prevent misfolding of proteins by providing a proper evironment for nascent proteins to fold. Chaperones assist protein folding by blocking improper intermolecular interactions and hold the emerging part of the nascent chain until synthesis is complete (Stryer, 1996). Many chaperones in *E.coli* are able to

bind to unfolded polypeptides and prevent aggregation of proteins (Georgiou and Valax, 1996). The structure and function of the chaperone families are described in Table 2.

Table 2. Chaperone families: Structure and function (Mogk et al., 2002)

Chaperone family	Structure	ATP	Prokyryote	Eukaryote	Co-chaperone	Function
Hsp100	6-7-mer	+	ClpB			Disaggregation together with Hsp70
			ClpA			Proteolysis together with the ClpP protease
				Hsp104		thermotolerance disaggregation together with Hsp70
Hsp90	dimer	+	HtpG			tolerance to extreme heat shock
				Hsp90	Hop, p23, CDC37	stress tolerance control of folding and activity of steroid hormone receptors, protein kinases, etc.
Hsp70	monomer	+	DnaK		DnaJ, GrpE	de novo protein folding; prevention of aggregation of heat-denatured proteins; solubilization of protein aggregates together with ClpB; regulation of the heat shock response
				Hsp70, Hsc70	Hsp40, Bag1, Hip, Chip, Hop, HspBP1	de novo protein folding; prevention of aggregation of heat-denatured proteins; solubilization of protein aggregates together with Hsp104; regulation of the heat shock response; regulation of the activity of folded regulatory proteins (such as transcription factors and kinases)
Hsp60	14-mer, 16-mer	+	GroEL		GroES	de novo protein folding; prevention of aggregation of heat-denatured proteins
				CCT/TRiC	prefoldin	de novo folding of actin and tublin
sHsps	8-24-mer		IbpA, IbpB			prevention of aggregation of heat-denatured proteins binding to inclusion bodies
				Hsp25 (crystalline)		prevention of aggregation of heat-denatured proteins component of the lens of the vertebrate eye
trigger factor	monomer		trigger factor			ribosome-associated potential function in de novo protein folding
NAC	Hetero-dimer		NAC			ribosome-associated potential function in de novo protein folding
SecB	tetramer		SecB			protein secretion

1.1.1.2 Applications of heat shock proteins in Biotechnology

A number of publications demonstrated that by overproduction of molecular chaperones, misfolding and subsequent aggregation of heterologous proteins in *E. coli* can be reduced (de Marco and De Marco, 2004; Nishihara et al., 2000; Thomas et al., 1997; Tomoyasu et al., 2001). Simultaneous overproduction of several molecular chaperones and recombinant protein in the host cell can introduce sufficient amounts of chaperones inside the cell to increase de novo folding of the products and the yield of correctly folded proteins. Hence, coexpression of chaperones plays an important role in enhancement of soluble protein production.

In several cases, the amount of soluble protein was significantly increased upon the overproduction of ribosome-associated trigger factor (TF) and GroEL/GroES (Nishihara et al., 2000). Overproduction of the bi-chaperone system ClpB/DnaK could completely reverse the IB-like aggregates identified in *ΔrpoH* mutant cells after temperature upshift (Tomoyasu et al., 2001). Co-overproduction of ClpB/DnaK or IbpAB might help to increase the amount of native protein. The resolubilization of proteins from IBs was mediated by the disaggregating activity of ClpB/DnaK (Carrio and Villaverde, 2001).

Overproduction of molecular chaperones inside the host cell can be obtained by introducing extra copies of chaperones encoding genes into the host cells together with the target genes (Mogk et al., 2002). Cells can be transformed with several plasmids which encode chaperone or the target gene under control by a promoter to ensure appropriate co-expression of those genes (De Marco et al., 2004) (Fig. 1).

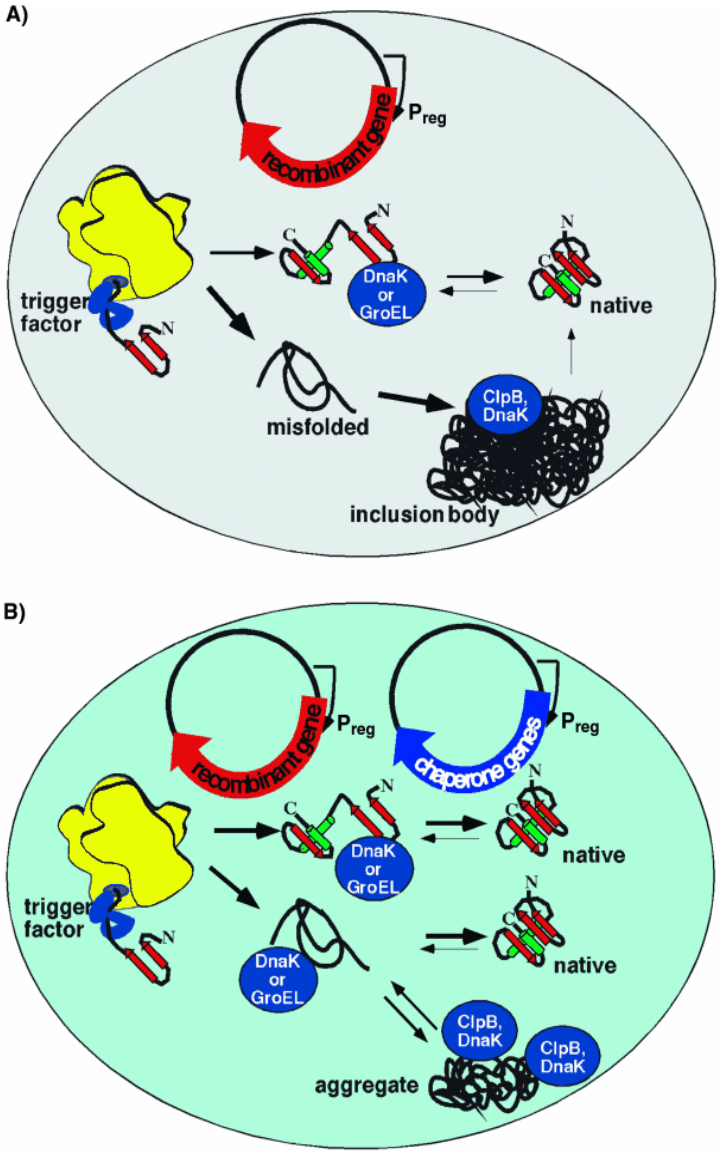

Fig. 1: Biotechnological production of recombinant protein without (A) and with (B) optimized amounts of chaperones. A) Without sufficient amounts of chaperones the recombinant protein is high aggregation and forms inclusion bodies. B) The controlled co-overproduction of molecular chaperones together with the target protein leads to increased levels of the properly folded recombinant protein.
P_{reg} is a regulatable promoter, for example, isopropyl-D-thiogalactopyranoside (IPTG) controlled. (Mogk et al., 2002).

However, the beneficial effect of chaperones may depend on the target proteins and on the level and mixture of chaperones provided. None of the individual chaperones could prevent misfolding of all proteins (Mogk et al., 2002). The success of chaperone co-overproduction depends on the relative affinities of the chaperone system to the folding

intermediates and the folding and aggregation kinetics of such species (Schlieker et al., 2002).

1.1.2 Protein secretion pathways in *Escherichia coli*

1.1.2.1 Pathways of disulfide bond formation in periplasm

In the cytoplasm of *E. coli*, disulfide bonds are rarely found due to the reducing environment of this compartment. In contrast, the periplasm contains a family of proteins that catalyze disulfide bond formation in newly translocated proteins. These proteins belong to the disulfide oxidoreductase "Dsb" family (DsbA, DsbB, DsbC, DsbD and DsbG) (Missiakas and Raina, 1997; Bessette et al., 1999; Messens and Collet, 2006). Most periplasmic Dsb proteins contain a thioredoxin fold and a catalytic CXXC motif. Depending on the function of the Dsb protein, the CXXC motif is kept in the reduced form (Dsb-$(SH)_2$; with a dithiol) or in the oxidized form (Dsb-S_2; with an intramolecular disulfide bond). The Dsb proteins are involved in two major pathways: an oxidation pathway (DsbA and DsbB) and an isomerization pathway (DsbC, DsbG, DsbD) (Messens and Collet, 2006).

The oxidative protein folding pathway

In the Dsb family, DsbA is the most important thiol oxidase in the periplasm. DsbA is a monomeric protein of 21.1 kDa. The *dsbA* gene contains a characteristic Cys30-Pro31-His32-Cys33 catalytic motif (Bardwell et al., 1991; Martin et al., 1993). *In vivo*, DsbA functions as an oxidant (Kishigami et al., 1995), whereas *in vitro* the protein acts as both an oxidant and a sulfide bond isomerase, depending on the redox state of the two cysteines in the protein. Oxidized DsbA containing the Cys30-Cys33 disulfide bond with a redox potential of -120 mV is a strong thiol oxidant which promotes disulfide bond formation rapidly *in vivo* and *in vitro* (Zapun et al., 1993; Åslund et al., 1997; Huber-Wunderlich and Glockshuber, 1998).

DsbA is released in the reduced form after transfer of its disulfide bond to a target protein. To function as a catalyst in a new oxidation cycle, DsbA needs to be reoxidized. This reoxidation is accomplished by the inner-membrane protein, DsbB. DsbB is a 20 kDa protein that is predicted to have four transmembrane helices and two periplasmic loops. Four conserved cysteines are arranged in the two loops: Cys41 and

Cys44 in the N-terminal loop and Cys104 and Cys130 in the C-terminal loop. After the reoxidation of DsbA, DsbB needs to pass the electrons it gained from DsbA to an acceptor. DsbB is a unique enzyme that has the ability to use the oxidizing power of membrane-bound quinones to generate disulfide bond de novo. DsbB can transfer electrons between quinines, and DsbA is extremely rapidly oxidized (Messens and Collet, 2006).

The protein disulfide bond izomerization pathway

In vivo, the disulfide bond formation catalysts regularly introduce non-native disulfides into proteins with more than two cysteins. In *E. coli*, the system rescueing misfolded proteins is composed of two periplasmic disulfide bond isomerases, DsbC and DsbG – a homolog to DsbC, and a membrane protein DsbD.

DsbC is a homodimeric protein with two 23.4 kDa subunits. Each subunit contains four conserved cysteine residues. Only two of them (Cys98 and Cys101), arranged in a CXXC motif, are essential for the oxidoreductase activity of the protein. DsbC promotes the rearrangement of non-native disulfide bond in a number of proteins e.g. bovine pancreatic trypsin inhibitor, RNase, tissue type plasminogen activator (tPA) and urokinase with multiple cysteine residues (Kadokura et al., 2003). Therefore, incorrect disulfide bonds in proteins can be successfully corrected by DsbC. Although DsbA and DsbC have some similarities in the redox properties and structures, the active site of DsbC is kept reduced in the periplasm, in contrast to DsbA (Kadokura et al., 2003; Messens and Collet, 2006).

A second protein disulfide isomerase, DsbG, is present in the periplasm. DsbG is a 25.7 kDa protein that forms a dimer and has two conserved cysteine residues, Cys109 and Cys112, which are present in a CXXC motif (Bessete, Cotto, Gilbert, Georgiou, 1999). DsbC and DsbG have a similar redox potential which is in the range between −126 mV and −129 mV). Like DsbC, DsbG is able to correct non-native disulfide bonds both *in vitro* and *in vivo*. However, the major difference between DsbG and DsbC is probably their specificity (Messens and Collet, 2006).

The disulfide isomerases, DsbC and DsbG have to be reduced in the oxidizing environment of the periplasm. The protein that is responsible for maintaining DsbC and

DsbG in the reduced state is the inner-membrane protein DsbD. DsbD is a 546 amino-acid protein and has three different domains: N-terminal periplasmic domain (DsbDα), a transmembrane domain with eight transmembrane segments (DsbDβ) and a C-terminal domain (DsbDγ). Each domain includes a pair of cysteines that are essential for protein activity (Stewart et al., 1999). The function of DsbD is to give electrons to the periplasmic protein disulfide isomerases DsbC and DsbG. *In vivo* and *in vitro* DsbD itself receives electrons from cytoplasmic thioredoxin. The mechanism by which DsbD transfers electrons across the membrane is still unclear. Experiments *in vivo* and *in vitro* show that electrons are transferred via disulfide bond exchange reactions between the three domains (Messens and Collet, 2006).

1.1.2.2 Translocation pathways for protein secretion

There are three pathways which are involved in the secretion of proteins via the cytoplasm of bacteria: the Sec-dependent, signal recognition particle (SRP) and Twin-arginine translocation (TAT) pathway.

Sec-dependent pathway

Generally, in *E. coli*, most of proteins synthesized in the cytoplasm as premature proteins are found in the outer membrane or periplasmic space. These premature proteins contain a signal sequence including a short 15-30 specific amino acid sequence e.g. PelB, OmpA, PhoA, endoxylanase, and StII that allows the proteins to be exported out of the cytoplasm via the Sec pathway. During transport of proteins out of the cytoplasm, the signal sequence is cleaved by signal peptidase to form the mature protein (Choi and Lee, 2004). The process that brings these proteins to the periplasm is called secretion.

Protein export through Sec pathway is post-translational. The SecYEG complex is necessary for proteins to be translocated in to the periplasmic space. Mature proteins must be in unfolded or partially unfolded conformation before targeting to the SecYEG pore (Georgiou and Segatori, 2005; Santini et al., 1998).

SRP pathway

In contrast to Sec dependent pathway, export of protein through SRP pathway is co-translational. Premature proteins are not exported to SRP pathway due to protein synthesis is arrested until the ribosome contacts with the secretion pore. In general, the SRP pathway was used for membrane proteins and could be useful for secretion of prone aggregated proteins in cytoplasm when exported via Sec pathway. This may avoid the accumulation of many preproteins inside the cytoplasm (Georgiou and Segatori, 2005).

TAT pathway

The TAT system is a Sec-independent pathway (Santini et al., 1998). Whereas preproteins that have not reached a native conformation can be secreted by the Sec pathway, the TAT system can directly transfer folded proteins into the periplasm by employing a particular signal peptide containing a twin-arginine sequence (Baneyx and Mujacic, 2004; Choi and Lee, 2004, de Leeuw et al., 2002, DeLisa et al., 2003; Stanley et al., 2000). However, TAT system has some drawbacks due to relatively slow protein transport as the translocation reachs maximum level after 10 minutes (Santini et al., 1998) and the secretion is rapidly saturated (Barrett et al., 2003).

1.1.3 Engineering of periplasmic protein folding pathway

The periplasm, an oxidative environment containing a disulfide bond formation machinary, is suitable for synthesis of proteins with multiple disulfide bonds. A number of studies on coproduction of Dsb proteins have been conducted to increase the yield of the recombinant proteins with multiple disulfide bonds. Either DsbA or DsbC can double the yield of periplasmic insulin-like growth factor (IGF)-I in *Escherichia coli* (Joly et al., 1998). DsbA is essential for folding of many proteins, i.e. BPTI, hirudin, α-lactalbumin, alkaline phosphatase, bovine ribonuclease A, human proinsulin (Winter et al., 2000). However, overexpression of DsbA could only increase yield of native heterologous proteins as reduced glutathione (Wunderlich and Glockshuber, 1993) or L-arginine (Winter et al., 2000) were added to the growth media.

Coexpression of DsbC is found to significantly increase the formation of active length tissue-type plasminogen activator (tPA) in *E.coli* (Qiu et al., 1998). Horseradish peroxidase (HRP) production was increased severalfold upon overexpression of at least disulfide-bonded isomerase DsbC. Maximum translocation of HRP to the periplasm probably require overexpression of all DsbABCD proteins, suggesting that Dsb proteins assist folding and translocation of HRP (Kurokawa et al., 2000). The soluble and functional single-chain Fv antibodies (scFv) yields is increased from about 50% to 90% as the fused protein DsbG-scFv was co-expressed simultaneously with DsbC under the same promoter (Zhang et al., 2002).

Although eukaryotic proteins are oxidized complety when secreted to the periplasmic space of *E. coli* via Sec-system, they often fail to form the proper disulfide linkages. This is a major problem in the commercial expression of therapeutic active eukaryotic proteins. Many heterologous proteins containing multiple disulfide bonds synthesized in the *E. coli* periplasm often lead to protein aggregation as the incorrect thiol groups become linked even in the presence of the strong thiol oxidase DsbA and disulfide isomerase DsbC. Therefore, another strategy has been approached to optimize folding in the periplasm using folding catalysts and chaperones. Coproduction of the cell envelope chaperone - periplasmic peptidylprolyl *cis,trans*-isomerase (PPIase) FkpA improved the yield of a wide range of functional single chain antibody fragments scFv in the periplasm of *E. coli*, in some cases, 10-fold (Bothmann and Plückthun, 2000). The production of recombinant penicillin acylase (PAC) in the periplasm is significantly increased by the presence of the periplasmic heat-shock protein DegP (Lin et al., 2001; Pan et al., 2003) and by DegP and FkpA (Wu et al., 2006). Another cell envelope chaperone Skp enhances periplasmic scFv fragments (Bothmann and Plückthun, 1998; Hayhurst and Harris, 1999). Cosecretion of DnaJ and heat shock protein Hsp25 increased the yield of native tissue-type plasminogen activator (rPA) up to 170 and 120-fold, respectively (Schäffner et al., 2001). In the new report, yields of a functional anti-domoic acid single-chain fragment variable (scFv) antibody fragment from the *Escherichia coli* periplasm were increased by up to 100-fold upon co-production of *E. coli* DnaKJE molecular chaperones (Hu et al., 2006). Recently, by concomitant overexpression of DsbA, DsbC and the periplasmic peptidylprolyl *cis,trans*-isomerases with chaperone activity, FkpA and SurA improves the folding of recombinant proteins,

human plasma retinol- binding protein (RBP) and human dendritic cell membrane receptor DC-SIGN, in the periplasm of *E.coli* (Schlapschy et al., 2006). In recent report, native tPA and truncated tPA (vtPA) produced in fed-batch fermentation was enhanced by using the Twin-Arginine Translocation (TAT) system. By fusion of the Tat-dependent TorA signal peptides (ssTorA) with tPA or vtPA, both ssTorA-vtPA and ssTorA-tPA were secreted in the periplasm in active form at high levels without the assistance of DsbC (Kim et al., 2005).

Secretion of proteins into the *E. coli* provides several advantages over cytoplasmic production. First, the N-terminal amino acid sequence without the Met extention of the secreted product can be obtained after cleavage of the signal sequence by a specific signal protease. In addition, there are fewer proteases in periplasm than in cytoplasm and many of them have specific substrates. Also, most of secreted proteins contain essential disulfide bonds that are required for correct structure formation in the extracellular oxidizing environment (Pavlou and Reichert, 2004). In principle, in small scale, production of periplasmic proteins can also ease the purification due to fewer contaminating proteins in the periplasm. However, there also are some problems including: incomplete signal sequences, low or undetectable amounts of recombinant protein secretion despite correct signal sequences, inefficient export of protein due to its highly hydrophobic membrane, incorrect disulfide bond despite presence of Dsb enzyme resulting in formation of aggregate and IBs. Nevertheless, the problems can be solved using different promoters, signal sequences, and host strains under various culture conditions e.g. low temperature or manipulating periplasmic chaperones (Choi and Lee, 2004).

Production of periplasmic proteins with high yields obtained by high cell density fermentation using suitable expression systems has been well reported in the literature (Chen et al., 2004; Choi et al., 2000; Joly et al., 1998). By choice of suitable strain, growth conditions and fermentation parameters, it is reasonable to expect yields in the gram-per-litre range for a number of heterologous proteins, provided that these proteins are compatible with *E. coli* expression and folding machineries (Georgiou and Segatori, 2005). Although *E. coli* is well to grow high cell densitites, the limiting substrate supply during the fed-batch phase of high cell density cultivations and crowding can be a challenge to the vitality of the bacterial cell. A number of problems could be raised

during high cell density cultivation, for instance: decreased substrate uptake abilities, cell lysis, metabolic burden to the host cell resulting stress responses, leakage of recombinant proteins into environment (Rinas and Hoffmann, 2004).

1.1.4 Strategies for releasing recombinant proteins to the culture medium

Sometimes, periplasmic proteins (especially small proteins) can be released into the culture medium due to an increased permeability of outer membrane during long incubations. The “leakage” of the proteins from the periplasm to the culture medium is the result of compromsing integrity of the outer membrane. However, the precise mechanism responsible for the translocation of proteins over the outer membrane is not well understood and probably relates to the nature of proteins (Shokri et al., 2003). The leakage of periplasmic proteins to the culture medium should be distinguished from total cell lysis which must generally be avoided due to the release of intracellular proteins. Nevertheless, leakage of overproduced secreted proteins into the culture medium is sometimes associated with the loss of colony-forming ability or even followed by whole-cell lysis. These problems can be prevented by using weaker promoters, mutagenesis in the primary sequence of the overproduced protein targeted to the periplasm, or specific feeding protocols (Rinas and Hoffmann, 2004).

The major advantages of recombinant protein production in the culture medium compared to secretion into the periplasm include a simplification of downstream processing as outer-membrane disruption of *E.coli* cells is not required, less intensive proteolysis by periplasmic proteases (Choi and Lee, 2004). However, extracellular production also has some drawbacks due to release of unintended proteases and protein dilution. In terms of product recovery, no specific strategy was used to recover the protein from culture medium (Shokri et al., 2003). Therefore, this may be obstacles for high scale production of proteins.

Strategies for secretory production of recombinant proteins by *E.coli* are shown in Fig. 2.

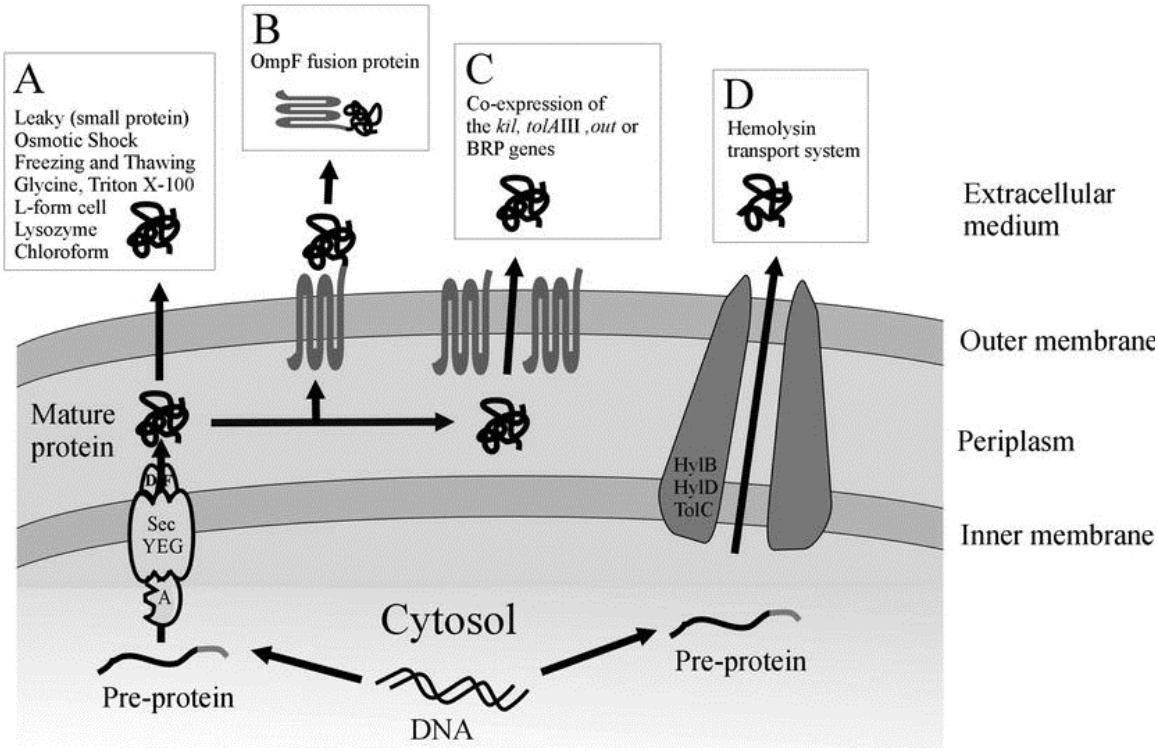

Fig. 2: Strategies for the extracellular production of recombinant proteins by *E. coli*. A Recombinant proteins can be excreted into the culture medium by treating cells with various agents or by using L-form cells. **B** Recombinant protein fused to outer-membrane protein F (OmpF) of *E. coli* can be excreted into the culture medium. **C** Proteins secreted into the *E. coli* periplasm can also be released into the culture medium by co-expression of *kil*, *out* genes, the gene encoding the third topological domain of the transmembrane protein TolA (TolAIII), or the bacteriocin-release protein gene. **D** The target protein fused to the C-terminal hemolysin secretion signal can be directly excreted into the culture medium through the hemolysin transport system (Choi and Lee, 2004)

There are a number of methods which have been used to enhance the export of recombinant proteins in culture supernatant.

For the first method, the mechanical, chemical and enzymatic treatments using ultrasonography, guanidine, Triton X-100, polyethylene glycol, lysozyme may break the outer membrane and cell wall of *E.coli*, therefore, affect the leakage of proteins into the

environment. However, this method is not applicable for production in large scale (Shokri et al., 2003).

Besides that, another method is to fuse the target protein to a carrier protein (hemolysin) which is normally secreted into the medium or to a protein (OmpF) expressed on the outer membrane (Fernandez et al., 2000; Jeong and Lee, 2002; Li et al., 2002; Nagahari et al., 1985).

Also, periplasmic proteins can be released into the culture medium by co-expression of the *kil* gene (Kleist et al., 2003; Miksch et al., 1997; 2002), or the gene coding for the third topological domain of the transmembrane protein TolAIII. Overexpression of TolAIII and a modified form of ß-lactamase with the OmpA signal sequence led to the release of most periplasmic proteins and 90% of the active enzyme into the culture medium (Wan and Baneyx, 1998).

Moreover, the extracellular production of recombinant proteins (Fu et al., 2003; Lin et al., 2001b; van der Wal et al., 1995) from *E.coli* can also be promoted by bacteriocin release protein (BRP) which is a small lipoprotein containing 28 amino acid residues. So far, the exact mechanism of BRP is not known. It may function as a precursor with a signal peptide and is secreted across the inner membrane and then is transported into the outer membrane. There, BRP activates the outer membrane phospholipase A leading to the formation of permeable zones in the cell envelope that proteins can go through and be exported into the medium (Luirink et al., 1986). By modification of the BRP protein, the growth inhibition, cell lysis or contaminating proteins which are accompanied with the wild type BRP can be avoided (van der Wal et al., 1998).

Another method is to use wall less cells or L-form cells for the extracellular production of recombinant proteins (Kujau et al., 1998; Rippmann et al., 1998). These L-forms are spherical cells derived by mutation and have been used to produce antibody fragments in a complex medium. However, this system is not suitable for industrial high cell density production without additional developments on stability and medium optimization (Shorki et al., 2003) due to the limited mechanical stability of the L-form cells.

1.1.5 Effects of cultivation conditions on the translocation of recombinant proteins to the culture medium

Although genetic optimization of production strains has been well investigated, few research on processes designed for secretory production was developed. There is some research investigating effects of environment conditions such as medium formulation and cultivation process parameters for export of recombinant proteins to the growth medium.

The compositions of the growth medium have a large impact on the function of the cell membrane. The presence of sodium chloride affects the outer membrane structure in the stationary phase of the cultivation. The divalent cations such as Mg^{2+} and Ca^{2+} are important for transport of compounds through the membrane. They can block the pores of the outer membrane leading a reduction of the transport of lysozyme and other molecules over the membrane (Shokri et al., 2003). Amino acids supplemented to the medium have an influence on the membrane structure. The leakage of human growth hormone is higher in a medium supplemented with casamino acids than in a minimal medium (Hsiung et al., 1989). The secretion of periplasmic protein cytochrome b5 and its discharge into the medium are stimulated by addition of glycine to the growth medium (Kaderbhai et al., 1997). Furthermore, an addition of 2% glycine or 1% Triton X-100 to the medium delayed formation of inclusion bodies in the periplasm and obviously promoted the production of extracellular FV fragment tumor necrosis factor alpha fusion protein (SFV/TNF-$^{\alpha}$) to 170 fold (Yang et al., 1998). It is believed that glycine can enlarge spheroidal morphology in *E. coli* and may break peptidoglycan cross-linkages and cell membrane integrity.

Changes of physical parameters e.g. temperature, pH, aeration and medium composition influence composition of the fatty acids in the cells of *E.coli* (Knivett and Cullen, 1965). Since these fatty acids play an important role in the function of the inner and outer cell membranes, these changes affect the translocation of proteins over the membrane (Arneborg et al., 1993). Temperature has a major effect especially on protein translocation. A reduction of temperature reduces the transport of proteins resulting in difficulty of leakage improvement due to the membrane changes from a liquid-crystalline bilayer to a more-ordered gel phase. Acidic or alkaline media increase or

decrease the amount of cyclopropane acids in the cells of *E.coli*, respectively. This probably influences growth rate during acetic acid formation. Partial pressure of carbon dioxide affecting membrane fluidity is also an important factor. High aeration conditions reduce the amount of carbon dioxide in the medium leading to structural changes of the membrane. This increases the amount of cyclic fatty acids and improves the rigidity of the membrane (Shokri et al., 2003).

Growth rate is also an important parameter in the cultivation process. The cell membrane becomes more rigid as the cultivation reached the stationary phase and the growth rate declined. Only at relatively low growth rates, a reduction in phosphatidylethanolamine is accompanied by an increase in phosphatidylserine.

There is a strong coupling between protein translocation and the amounts of lipid acids in the both membranes. The coupling is especially related to the amounts of acidic phospholipids and unsaturated fatty acids of the inner membrane. Increased amounts of phosphatidylglycerol enhance protein translocation. Therefore, cultivations under changing glucose limiting conditions can alter the structure of the both membranes causing protein leakage of periplasmic proteins to the culture medium (Shokri et al., 2002; Shokri et al., 2003; Dresler et al., 2006).

1.2 State of the art on the quantitative determination of IBs and other biological particles

1.2.1 Methods for detection of biological particle size

Production of soluble recombinant proteins from *E.coli* often faces up with formation of insoluble protein aggregates or IBs. For biological analysis, to obtain a quantitative evaluation of the formation level, particle size of IBs needs to be determined since the measurement of their solubility is inapplicable. The following techniques have been used so far for the charaterization of IBs including electron microscopy, centrifugal disc photosedimentation (CDS).

Electron microscopy has been used to measure IBs of *E.coli* (Bailey et al., 1995; Moriya et al., 1991). From the obtained micrographs, isolated IBs of Tumor Necrosis Factor-α from the cells were found to be relatively spherical which have an estimated size about

0.5 µm (Moriya et al., 1991). However, this method has some limitations due to a difficult estimation of size distributions from their micrographs and the unclear effect of the preparative procedure on the biological samples.

CDS has been used to determine the size distribution of IBs (Middelberg et al., 1990; Taylor et al., 1986; Thomas et al., 1991). However, this method is sentitive to particle extinction and baseline. Therefore, it is suitable for size of particles bigger than 0.4 µm (Middelberg et al., 1990). Moreover, due to unknown optical extinction coefficient of the IBs, this parameter was referred from the coefficients of polystyrene latex spheres since the extinction coefficient of the cell debris and IBs is proposed to be similar to that for polystyrene latex particles in water (Taylor et al., 1986; Thomas et al., 1991).

Size of IBs has been typically measured by electron microscopy and CDS indicated in Table 3.

Table 3. Methods for detemination of IB size

Method	**IB protein**	**Estimated IB size (µm)**	**Reference**
Electron microscopy	Human growth hormone	0.17	Bailey et al., 1995
	Tumor Necrosis Factor-α	0.5	Moriya et al., 1991
CDS	pGH	0.15-0.45	Middelberg et al., 1990
	γ-interferon prochymosin	0.81 0.17	Taylor et al., 1986
	Porcine somatotropin	0.4-0.5	Thomas et al., 1991

For measuring other biological particles e.g. cell debris electron microscopy (Bailey et al., 1995), CDS, photon correlation spectroscopy (PCS) or Dynamic Light Scattering (DLS) (Agerkvist and Enfors, 1990) and electrical sensing zone (ESZ) (Wong et al., 1997b) techniques have been applied. However, ESZ method employed to measure size

distribution of yeast and *Bacillus cereus* is inappropriate for measuring cell debris from *E.coli* which has sizes below 0.5 µm.

Besides that, many reports applying DLS for measuring size of other biorelated particles such as lipoprotein (O'Neal et al., 1998; Chou et al., 2004), amyloid fibrils (Gorman et al., 2003; Kim et al., 2004; Nichols et al., 2005; Shen et al., 1993), and soluble oligomer of β-amyloid (Meyer et al., 2004), small aggregates of malate dehydrogenase (Schlieker et al., 2004) have been published.

However, DLS is suitable for measuring spherical, monomodal particles (only one peak in size distribution of displayed graph) and reasonably narrow sample widths (typical polydispersities less than around 0.25). The technique has also some limitations if sample is non-spherical, has a board size distribution and requires a multimodal distribution analysis (Rawle, 1995). As abovementioned, IBs of isolated *E.coli* from the cells are relatively spherical, thus, DLS is applicable to measure size distributions of IBs. Since DLS is a rapid method, able to measure materials without damaging or altering them and requires a little sample preparation (Rawle, 1995), in this thesis, this technique was employed for measuring the size distributions of IBs.

1.2.2 Measurement of particle size distribution by Dynamic Light Scattering

The DLS technique relies on the random movement of particles due to collosion with the solvent molecules of the suspending medium (Brownian motion). The particle size is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation (Eq. 1) (Rawle, 1995).

$$d(H) = \frac{kT}{3\pi\eta D} \qquad (1)$$

Where:

d (H) : hydrodynamic diameter
D : translational diffusion coefficient
k : Boltzmann´s constant
T : absolute temperature
η : viscosity

Thus, the method is unsuitable for measuring small particles in a gas or if the motion of the particles is not random. Also, if the particles are too large and are sedimenting, then the technique is not applicable. If samples consist of particles which have different sizes, the scattered light from big particles will swamp the scattered light from the smaller ones (Rawle, 1995). This may lead to wrong results for the smaller particles to be obtained. Therefore, the necessary of preparative methods must take into account to get the meaningful data from DLS. In case particle sedimentation is occurring during measurement, a proper dispersion needs to be applied.

Another important factor should be carefully considered is sample concentration. At higher concentrations, size of the particles may depend on the sample concentration leading to two negative effects: multiple scattering and particle-particle interactions. Multiple scattering reduces the actual particle size due to the light is scattered by one particle and then this one is scattered by another particle before reaching the detector. Particle-particle interactions affect diffusion speed and thus the apparent particle size. To avoid these effects, samples must be routinely hightly diluted and measured by a conventional 90° detection angle DLS equipment. In this case, viscosity values of the diluted samples need to be correctly estimated. However, for many materials, such high dilution may change the particle size characteristics that lead obtained results not to be meaningful. To overcome this limitation, non-invasive back scatter (NIBS) technology has been applied in DLS to increase the concentration limits and sensitivity of the technique (Kaszuba et al., 2004).

To get realiable results from DLS measurement, the following factors must be accomplished: dust on the glassware must be removed, solvent must be clean and filtered, convection currents must be controlled since they will interfere with the Brownian motion, sedimentation of the particles needs to be prevented by proper stabilized dispersion, correct parameters of sample, e.g. refractive index, temperature and viscosity must be entered (Rawle, 1995).

1.3 Objectives

The aims of the present work were: first, to develop the optimal conditions for the production of soluble heterologous proteins from *E.coli* in different compartments: periplasm, culture supernatant and cytosol, and second, to apply a new method (DLS) for quantitative measuring size particles of IBs in order to evaluate the expression of the protein in the cytosol of *E.coli*. In this study, the two model proteins: tissue-type plasminogen activator variant (rPA) and α-glucosidase were used for investigation.

1. The influences of coexpression of the periplasmic chaperone protein and fermentation conditions on the localization of the target protein (rPA) in periplasm and culture supernatant were investigated.

Recombinant tissue-type plasminogen activator (tPA) with 17 disulfide bonds consisting of 5 domains: finger, 2 kringles, epidermal growth factor-like and protease from *E.coli* was produced in shake flask and bioreactor scale (Qiu et al., 1998). The production of the short form of tPA known as tissue-type plasminogen activator variant (rPA) consisting of Kringle-2 and protease domain (Kohnert et al., 1992) and containing 9 disulfide bonds was enhanced in the presence of low molecular additives including L-arginine, glutathione in LB medium in shake flask (Schäffner et al., 2001). Formation or re-arrangement of tPA is enhanced by coproduction of the two periplasmic chaperones: thiol-oxidase DsbA and the disulfide isomerase DsbC. Yield of the active tPA was significantly increased by coexpression of DsbC both in shake flask and in fed-batch cultivation. However, high level coexpression of DsbC was found to be very toxic as growth of the culture decreased dramatically within 3 to 4 h after induction (Qiu et al., 1998). Therefore, effects of co-production of DsbA, cultivation conditions (e.g. temperature, pH medium, culture media) and a presence of additives (L-arginine, redox shuffling systems) on production of rPA in the periplasm of recombinant *E. coli* and in the culture medium were investigated. Besides that, the influence of glycine and Triton X-100 which are added to the medium to enhance of native rPA was also studied.

Although production of native rPA is enhanced by addition of low molecular additives including L-arginine and glutathion, presence of arginine impairs *E. coli* growth in the media (Diez-Gonzalez and Karaibrahimoglu, 2004). Therefore, this could be a

challenge during fermentation using fed-batch strategies. The scale up of rPA production in fed-batch cultivation on defined medium was examined with respect to the adequate cultivation strategy in the presence of different additives to get high yield of native rPA in the periplasm and culture supernatant.

2. As α-glucosidase was expressed in the cytosol, a new technique (DLS) using the Zetasizer 3000 (Malvern, UK) was applied to determine the impact of production conditions on the size of IBs of α-glucosidase in cytolasm of *E.coli* and on the protein solubility.

Size distributions of IBs of α-glucosidase under various cultivation conditions such as: different media, temperature, batch and fed-batch cultivation at different temperature were analysed by DLS.

By overproduction of several molecular chaperones e.g. DnaK and ClpB in *E. coli*, effects of those chaperones on aggregated protein formation, resolubilization of α-glucosidase IBs and the relation of α-glucosidase activity to the size of the IBs were also studied.

2. MATERIAL AND METHODS

2.1 Strains and plasmids

The strains and plasmids used in these parts are described in the Table 4 below.

Table 4. List of the strains used in this work

Strains	Characteristics	Source or reference
BL21(DE3)		Invitrogen
MC4100	*araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301deoC1 ptsF25 rbsR*	Laboratory stock
MC4100 *ΔibpAB*		F. Banyeux
BB1553	MC4100 *dnaK:: cm*	B. Bukau

Table 5. List of the plasmids used in this work

Plasmids	Description	Source or reference
pET20b_rPA	*pelB* signal sequence	Schäffner et al., 2001
pUBS520_DsbA		J. Winter
pKK177GlucP1	pBR322 derivative encoding the α-glucosidase gene	Kopetzki et.al., 1989
pUBS520	pACYC177 derivative encoding the *dnaY* gene and *lacI* repressor gene	Brinkmann et al., 1989
pBB535	p15A *ori*, spectinomycin resistance, encoding *dnaJ*, *dnaK*	B. Bukau
pBB540	pSC101 *ori*, chloramphenicol resistance, encoding *clpB*, *grpE*	B. Bukau

The strain BL21(DE3) transformed with pET20b_rPA encoding a recombinant plasminogen activator and pUBS520_DsbA was used for the experiments for rPA production.

For inverstigation of impact of chaperones on the sizes of inclusion bodies of α-glucosidase from *Escherichia coli*, different strains were obtained after transformation of the host strains with the plasmids (Table 6).

Table 6. List of the strains after transformation

Host strain	plasmids	Strain after transformation
MC4100	pKK177_GlucP1	control
MC4100	pKK177_GlucP1; pUBS520	wild type
BB1553	pKK177_GlucP1; pUBS520	*dnaK* mutant
MC4100	pKK177GlucP1; pBB535; pBB540; pUBS520	Hsps co-expression

2.2. Molecular biological methods

2.2.1 Plasmid isolation

Plasmids were isolated from *E.coli* cells grown in 10 mL of LB medium overnight using the QIAprep Spin Miniprep kit (Quiagen, Hilden) following the protocol of the manufacturer.

2.2.2 Transformation of *E.coli* cells

2.2.2.1 Preparation of electrocompetent cells

LB medium were inoculated with 1 % of the overnight preculture and the culture was incubated at 37°C on the rotary shaker at 140 rpm. At an OD_{600} of 0.5- 0.6, the culture

was chilled on ice for 30min, centrifuged at 4000 x *g* and 4°C for 10 min. After removing supernatant, cell pellets were washed with decreasing volumes of ice cold 10% (v/v) glycerol for 3 times. The volume of glycerol of 3 time washing was 100%, 50% and 10% of the culture volume. Aferwards, the washed cells were resuspended in 1mL of 10% (v/v) glycerol. Aliquots of 40 µL were distributed into sterilized Eppendorf tubes, frozen in liquid nitrogen and stored at –80°C.

2.2.2.2 Transformation via electroporation

The electrophration process was conducted by Gen Pulser II (Bio-Rad, München). About 1-10 ng of plamid was mixed with a 40µL aliquot of the electrocompetent cells and transferred into a ready cold cuvette (0.2 cm gap). The electroporator was set at 25 µF, 200 Ω and a voltage of 2.5 kV. Immediately following pulsing, 1 mL of LB medium was added to the mixture to promote growth of viable cells. The solution was incubated at 37°C with moderate shaking at 650 rpm on an Eppendorf thermomixer for one hour. Afterwards, 100 µL of the cell solution was directly plated on LB agar plate supplemented with appropriate antibiotics. The rest of cell suspension was spun at 5000 rpm for 1 min, 750 µL of supernatant was discarded. The cell pellets were resuspended and plated on LB agar plates with the same antibiotics. The plates were incubated overnight at 37°C.

2.3. Cultivation media and conditions

2.3.1 Cultivation media

2.3.1.1 Complex medium

The Luria-Bertani (LB) medium consists of the following components in distilled water:

- Pepton from casein 10.0 g/L
- Yeast extract 5.0 g/L
- NaCl 5.0 g/L

2.3.1.2 Defined medium

The composition of the defined medium (Korz et al., 1995) is given below:

• Glucose	10	g/L
• $MgSO_4$	1.2	g/L
• KH_2PO_4	13.3	g/L
• $(NH_4)_2HPO_4$	4	g/L
• Citric acid	1.7	g/L
• Ferric (III) citrate	100.8	mg/L
• $CoCl_2$*6 H_2O	2.5	mg/L
• $MnCl_2$* 4 H_2O	15	mg/L
• $CuCl_2$* 2 H_2O	1.5	mg/L
• H_3BO_3	3	mg/L
• Na_2MoO_4*2 H_2O	2.1	mg/L
• $Zn(CH_3COO)_2$*2 H_2O	33.8	mg/L
• EDTA	14.1	mg/L

Stock solutions of appropriate antibiotics were sterilized by filtration and aseptically supplemented to LB agar, LB broth and defined medium after sterilization. Final concentrations of the antibiotics in the medium were: 100 µg/mL ampicilline, 35 µg/mL kanamycine, 50 µg/mL chloramphenicol and 50 µg/mL spectomycin.

2.3.2 Cultivation conditions

2.3.2.1 Shake flask experiments

Shake flask experiments were conducted with some different procedures:

For periplasmic and extracellular production, 10 mL LB medium or defined medium with the appropriate antibiotics were inoculated with 1% over night preculture and incubated at 30°C for 6 h to reach an OD_{600} of 0.6. After induction, temperature was reduced to 24°C. 10 mL of 2 times concentrated LB or HDF medium were added to 10

mL of culture. At the same time, 10 mL additive stock solution (consitsting L- arginine, reduced and oxidized glutathione) was also added to reach final concentrations of 0.4M L- arginine. Glutathione was calculated to get final concentrations of reduced and oxidized glutathione at 0, 2.5 and 5 mM. Induction of rPA was obtained by addition of 0.4 mM of isopropyl- β-D-thiogalactopyranoside (IPTG). Specific and volumetric rPA activites were analyzed after 22 h of induction.

For cytoplasmic production of inclusion bodies, 10 mL of LB medium supplemented with appropriate antibiotics was inoculated with a single colony. The culture was incubated overnight on a rotary shaker at 30°C, 140 rpm. For the main culture, LB or HDF medium with the same antibiotics was inoculated with 1% (v/v) of inoculum of the overnight preculture. The main culture was incubated at 37°C, 160 rpm to an OD_{600} of 0.5-0.6. Synthesis of α-glucosidase was induced by addition of 1mM IPTG. Temperature was shifted to the temperatures indicated in the result section.

2.3.2.2 Fed-batch cultivation

The first preculture on 10 mL LB medium supplemented with the appropriate antibiotics in a 100 mL shake flask was inoculated with a single colony from LB agar plate and incubated on a rotary shaker at 30°C, 140 rpm for 10 h. The second preculture in the defined medium was inoculated with 1% (v/v) of the first preculture and incubated for 14 h at 30°C, 140 rpm.

Initial concentrations of glucose used for batch phase were 5 or 30 g/L. The glucose and $MgSO_4$ solution are separately sterilized at 121°C for 30 minutes and aseptically added into the bioreactor after the other ingredients were sterilized inside the bioreactor. pH value of the medium (without glucose and $MgSO_4$) was adjusted to 6.3 with 5N NaOH before adding glucose and $MgSO_4$ into the bioreactor. After sterilization, pH of the whole medium was controlled by 25% (w/v) NH_4OH.

The feeding solution consists of 875 g/L glucose, 20 g/L $MgSO_4$ and trace element solution which were autoclaved and mixed together.

Trace element solution consists of:

- Feric(III)citrate 40 mg/L
- $CoCl_2$*6 H_2O 4 mg/L
- $MnCl_2$* 4 H_2O 23.5 mg/L
- $CuCl_2$* 2 H_2O 2.3 mg/L
- H_3BO_3 4.7 mg/L
- Na_2MoO_4 *2 H_2O 4 mg/L
- $Zn(CH_3COO)_2$ *2 H_2O 16 mg/L
- EDTA 13 mg/L

Cultivations starting at 6 L defined medium were carried out in a Biostat C 10 (BBI B. Braun Biotech International, Melsungen, Germany) with 5% of inoculum. The pH was constantly controlled at pH 6.8 by addition of aqueous ammonia and the dissolved oxygen concentration (DO) was maintained at 40% of air saturation. The cultivation strategy is described in the scheme (Fig.3). The cultivation temperature was kept at T_{growth} after inoculation and was reduced to T_{prod} at the time of induction (t_{ind}) and remained constantly before harvesting culture medium (t_{end}). In case of rPA production, 1.5 L of a stock solution of additives consisting L-arginine, reduced and oxidized glutathion was added immediately before induction. Induction of the recombinant protein was conducted by addition of IPTG.

In the fed-batch cultivation, feeding was started after glucose (S_0) was depleted as indicated by a sharp increase of DO and decrease of stirrer speed. The feeding solution was added into the bioreactor by a peristaltic pump (type 101 U/R, Watson Marlow Ltd, Rommerskirchen). Exponential feed profiles controlled by the DCU were used to keep constant specific growth rates (μ_{set}) as given in the result chapter.

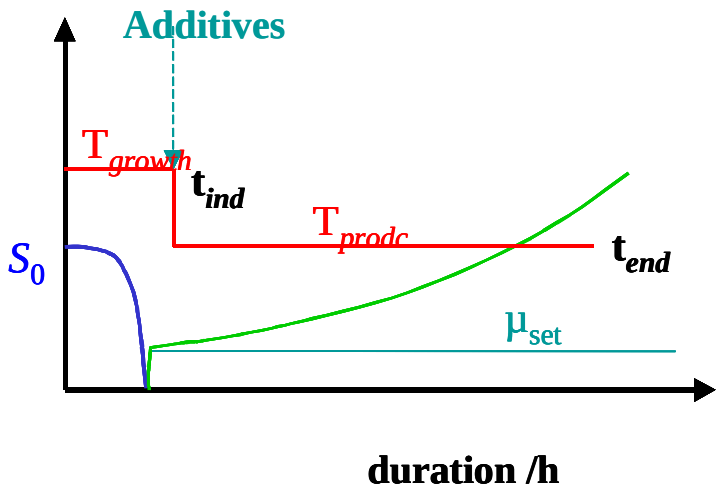

Fig. 3: Scheme for fed-batch cultivation. S_0: initial concentration of substrate. μ_{set}: specific growth rate. T_{growth} : cultivation temperature before induction. $\mathbf{t_{ind}}$: time of induction. T_{prod} : temperature after induction. t_{end} : time at the end of the cultivation. The volumetric feed rate was calculated from the Eq.2:

$$F = \frac{1}{S_0} \cdot qs \cdot X \cdot V = \frac{1}{S_0} \cdot \left(\frac{\mu_{set}}{Y_{X/S}} + m_E \right) \cdot X_f \cdot V_f \cdot \exp[\mu_{set} \cdot (t - t_f)] \qquad (2)$$

Where

F: feeding rate, (g h^{-1})

S_0 : substrate concentration in the feeding solution, (g g^{-1})

q_S : specific substrate consumption rate, (g g^{-1} h^{-1})

X: cell density, g L^{-1}

V: volume, L

μ_{set} : set point of the specific growth rate, h^{-1}

$Y_{X/S}$: biomass yield coeffcient , (g g^{-1})

m_E : maintenance coefficient, (g g^{-1} h^{-1})

X_f : dry cell weight at the time of feeding start , (g L^{-1})

V_f : volume at feeding start, L

t : cultivation time, h

t_f : time of feeding start, h

The outlet oxygen and carbon dioxide concentrations, C_{O2out} and C_{CO2out} , were continuously measured by Uras14 and Magnos16 appliances (BBI, Melsungen). The

volumetric carbon dioxide evolution rate (CER) and the specific carbon dioxide evolution rate (q_{CO2}) were calculated according to Eq. 3 and Eq. 4.

$$CER = \frac{Q}{22.4L \cdot V} \cdot \left(C_{CO2\,out} \cdot \frac{1 - C_{O2\,in} - C_{CO2\,in}}{1 - C_{O2\,out} - C_{CO2\,out}} - C_{CO2\,in} \right) \quad (3)$$

$$q_{CO2} = CER / OD_{600} \quad (4)$$

Where:

CER:	volumetric carbon dioxide evolution rate, (mmol L^{-1} h^{-1})
Q:	outlet gas flow rate, (L h^{-1})
V:	volume at feeding start, L
C_{O2out} :	outlet oxygen concentation, (%)
C_{CO2out} :	outlet carbon dioxide concentation, (%)
C_{O2in} :	inlet oxygen concentation, (%)
C_{CO2in} :	inlet carbon dioxide concentation, (%)
q_{CO2} :	specific carbon dioxide evolution rate, (mmol L^{-1} OD^{-1} h^{-1})
OD_{600} :	optical density at the wavelength of 600 nm

2.4. Cell disruption methods

2.4.1 Ultrasonication

Cell pellets corresponding to the sample volume of V_{sample}= 3/OD_{600} mL were resuspended in 580 µL of 10 mM potassium phosphate buffer pH 7 with 1mM EDTA. 20 µL of lysozyme (concentration of 1 g/L) was added and the cell suspension was incubated for 30 minutes at 4°C before sonication with the sonicator (UP 200S Ultraschallprozessor, Dr.Hielscher GmbH, Teltow) for 20 seconds at 4°C, amplitude 50%, and cycle 0.5 s^{-1}. By centrifugation at 13000 rpm for 20 min at 4°C, insoluble and soluble fractions were separated.

2.4.2 High pressure homogenization

For the experiments which needed large amount of inclusion bodies (IBs), cells were disrupted by a high pressure homogenizer. 2 g of wet cell biomass was resuspended in 40 mL of Tris-HCl pH 7, 1 mM EDTA using the homogenizer Ultraturrax T25 (IKA-Labortechnik, Staufen). After adding 3 mg of lysozyme, the cell suspension was incubated on ice for 30 min. Then the cells were disrupted by high pressure homogenization in the homogenizer Gaulin Micron Lab 40 (APV Homogenizer GmbH, Lübeck) for 3 to 5 passages. For digesting DNA, the cell lysate was incubated at room temperature for 30 min after adding 100 µg of DNAse and 0.1 mM of $MgCl_2$. 20 mL of 60 mM EDTA, 6% Triton X-100 and 1.5 M NaCl pH 7 was added to the cell lysate and the cell suspension was further incubated for 30 min. The pellet was obtained after spinning the suspension at 31000 × *g* at 4°C for 10 min. Afterwards, the pellet was resuspended in 16 mL of 0.1 M Tris-HCl pH 7, 20 mM EDTA by the Ultraturrax followed by centrifugation. After repeating this step for at least 2 times, the IB pellet was immediately used or stored at –20°C up to some weeks.

2.5 Analytical methods

2.5.1 Cell density

Growth of cultures was monitored by measuring the optical density at a wavelength of 600 nm by a spectrophotometer (Ultrospec 3000, Pharmacia, Sweden). Sodium chroride 0.9% (w/v) NaCl was used as a reference. Samples were diluted to keep absorbance values below 0.6.

2.5.2 Dry cell weight

For the determination of dry cell weight (DCW), 1 mL of the culture supernatant was added in triplicate to pre-weighed Eppendorf tubes and centrifuged for 4 min at 13000 rpm and 4°C. After a subsequent washing step with 0.9% (w/v) NaCl, cell pellets were dried until constant mass at 60°C for at least 4 days.

2.5.3 Glucose concentration

Glucose concentration was determined by the hexokinase/glucose-6-phosphate dehydrogenase method (Kit No. 139106, Boehringer Mannheim GmbH, Mannheim). Culture supernant after centrifugation at 13000 rpm at 4°C for 5 min was heat at 80°C for 20 min to deactivate enzymes. Samples were diluted with distilled water to have glucose concentration in the range of 1- 100 mg/L. Each sample was added in quadruplicate onto a 96 well-microtiter plate. Absorbance absorption values of samples before and after adding hexokinase/glucose-6-phosphate dehydrogenase were measured by the ELISA reader (TECAN, Sunrise Remote, Austria). Glucose concentrations were calculated according to a standard curve established from glucose standard solutions.

2.5.4 Glucosidase activity assay

The soluble cell fraction obtained from 2.4.1 was used for α-glucosidase activity assay. The activity assay is based on absorbance kinetics at 405 nm resulting enzymatic cleavage of *p*-nitropenyl- α- D-glucopyranoside (pNPG) by α-glucosidase. The kinetics of absorption was measured on the ELISA reader TECAN, Sunrise Remote, Austria at room temperature using pNPG (Sigma) as a substrate. Activity of α-glucosidase was calculated according to a standard curve established from different α-glucosidase standard concentrations.
The reaction volume in each well of an Elisa plate was 250 µL including 25 µL sample, 200 µL phosphate buffer and 25 µL pNPG (stock solution of 20 mM).

2.5.5 Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels was carried out according to standard techniques. Soluble protein samples from a culture volume of $3/OD_{600}$ mL were resuspended in 100 µL of the loading buffer consisted of 0.125 M Tris HCl pH 6.8, 20 % (v/v) glycerol, 4% (v/v) SDS, 5% (v/v) 2-mecarptoethanol and 0.02 % (v/v) bromphenolblue. Insoluble proteins were first totally suspended in 50 µL of 10mM potassium phosphate buffer, pH 7, 1 mM EDTA before adding 50 µL of the loading buffer. After incubation at 95°C for 10 min,

the samples were loaded on gels and run at 140 V for about 60 min in the running buffer (0.025 M Tris, 0.192 M glycine and 0.1%(w/v) SDS).

The low molecular weight protein marker for electrophoresis (Amersham Biosciences, Freiburg) from 14.4 to 94 kDa served as a standard molecular weight.

For staining, SDS gels were soaked in Coomassive blue solution (see below) for at least 2 hours.

- Coomassive Brilliant Blue G250 (Roth) 1% (w/v)
- Methanol 40% (v/v)
- Acetic acid 10% (v/v)
- Distilled water 50% (v/v)

Afterwards, the gels were destained using destaining solution containing 40% (v/v) methanol and 10% (v/v) in water until protein bands became visible and clear.

2.5.6 Extraction of periplasmic fraction

For periplasmic extraction, cell pellets corresponding to the sample volume of V_{sample}= $5/OD_{600}$ mL were obtained by centrifugation at 13000 rpm, 4°C for 20 min. The pellets were washed with 1 mL of 0.9% (v/v) NaCl and then incubated in the extraction buffer consisting of 150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA and 1 mg/mL polymyxine-B sulfate for 1 h at 5°C under shaking at 1200 rpm. Afterwards, cells were separated from the periplasmic fraction by centrifugation at 13000 rpm at 4°C for 20 min.

2.5.7 Activity assay for rPA

Determination of native rPA on microplate was conducted according to the modified protocol with purified rPA as a standard (Schäffner et al., 2001). The periplasmic fractions were diluted with 75 mM Tris buffer pH 7.5 to have rPA concentrations less than 2 ng/mL. For rPA activity measurement, 30 µg/mL of plasminogen and 48 µg/mL of plasminogen stimulator (Chromogenix, Italy) were incubated with diluted rPA

samples (periplasmic fractions). Kinetics of absorbance at 405 nm on the ELISA reader at room temperature was measured after adding 275 µg/mL of a chromogenic substrate Val-Leu-Lys-pNA (Bachem, Switzerland). The slope of the absorbance kinetics curve against the square of the reaction time was determined and converted into µg/L after calibration using pure rPA.

2.5.8 Protein concentration

2.5.8.1 Bradford assay

Protein concentrations were determined according to the protocol by Bio-Rad Laboratories (München) for protein concentrations less than 25 µg/mL. 800 µL of sample diluted in 10 mM potassium phosphate buffer, pH 7, 1 mM EDTA and mixed with 200 µL of dye reagent concentrate (Bio-Rad). After 5 min of incubation, absorbance at 595 nm was measured versus blank. Bovine serum albumin (BSA) from Bio-Rad was used as a protein standard.

2.5.8.2 Western Blot

For Western Blot, antibodies were dissolved in TBS buffer consisting 10 mM Tris pH 7.4, 0.154 M NaCl and 0.05% glycerol and 1% skim milk powder. Proteins were separated by SDS-PAGE at 100 V, 30 mA per one gel and blotted to a polyvinyliden fluoride (PVDF) membrane at 140 V, 1mA per cm^2 of a blot for 2 h. Then the blot was incubated overnight with 5% skim milk in the TBS buffer at 4°C. After rinsing 3 times with the same buffer, the blot was incubated by 2.5 µg/mL (w/v) of an anti-rPA antibody from hen eggs, which was purified by affinity chromatography (Davids Biotech, Regensburg, Germany) for 1h at room temperature. Washing step was followed by 50 mL of the buffer for 5 min and repeated 6 times. Afterwards, 0.2 µg/mL (w/v) of an AffiniPure donkey anti-Chicken IgY^{++} conjugated with alkaline phosphatase (Dianova GmbH, Hamburg, Germany) was added and the blot was further incubated for 1 h at room temperature. The washing step was pepeated the same procedure as after incubation with the first antibody. For detection of alkaline phosphatase, 0.125 mL of Lumi-Phos ^{TM} WB (Perbio Science, Bonn, Germany) per cm^2 of the blot was applied. The blot was incubated for 3 min under subdued light. The blot was exposed to X-ray

film for 2 min. Images of the protein was developed in development solution for 20 s and then fixed in a fixation solution for 10 s.

2.5.9 Separation of IBs by sucrose density gradient

Inclusion bodies in the cell lysate were isolated by sucrose density gradient. Step gradients consisting of 35, 40, 45, 50, 55, 60 % of sucrose in 3 mM EDTA, pH 7.6 were prepared by pipette. 0.5 mL of decreasing sucrose concentrations was carefully layered in a centrifugation tube. Afterwards, 0.5 mL of IB suspension was added on the top of the step sucrose gradients. After centrifugation on the swing-out rotor ultracentrifuge SW55 – Ti (Beckman Coulter L8- 60M) at 15000 rpm, at 4°C for 2 hours, different fractions were carefully collected by a pipette. The fractions were dissolved with 3 mM EDTA, pH 7.6 for size determination by Dynamic Light Scattering (DLS).

2.5.10 Detection of size of inclusion bodies by Zetasizer 3000

Buffers and sucrose solutions used for experiments were filtered through 0.22 µm filter to remove dust. Refrection index of IBs suspensions in 3mM EDTA, pH 7.6 was measured by the refractometer (ATAGO, serie NAR-1T, Japan). Sucrose concentrations of the samples used for measurement were calculated from measured refraction index values by the Handbook of chemistry and physics (Weast et al., 1974). Relative viscosity values of the sucrose in the samples were determined according to the standard curve of relative viscosity of different sucrose concentrations (Fig. 4).

A **B**

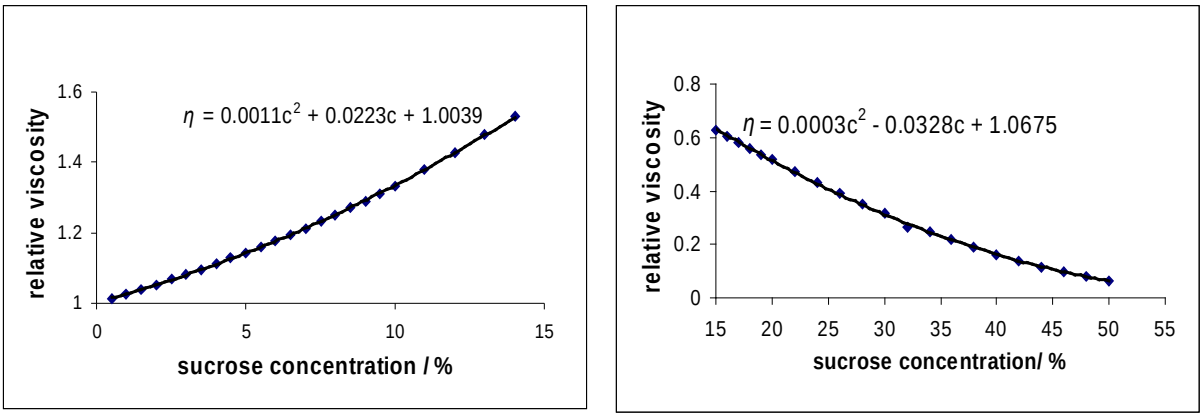

Fig. 4: Standard curve of relative viscosity of sucrose at low (A) and high (B) concentrations.

Viscosity of the samples was calculated from the relative viscosity of the sample and viscosity of water at 25°C indicated at Eq. 5.

$$\eta_{\text{sample}} = \eta * \eta_{\text{water}} \qquad (5)$$

Where:

$\eta_{\text{sample:}}$ viscosity of the sample used for DLS measurement

η: relative viscosity at 25°C

η_{water}: absolute viscosity of water at 25°C ($\eta_0 = 0.8904$)

The refraction index and viscosity values of each sample are the necessary parameters which need to be entered of each measurement of IBs size distributions by DLS. 1 to 2 mL of samples was transferred into a 10 mm square quartz sizing cell which was placed in a sizing cell holder of the Zetasizer 3000, Malvern, UK. The instrument Zetasizer 3000 uses a 10 mW helium-neon laser at a wavelength of 633 nm. Laser light is scattered and detected at an angle of 90° by a photon detector. The intensity fluctuations of the scattered light are converted into electrical pulses that are transferred to a digital correlator. The software of the instrument generates particle size from the correlator function. Results are expressed as the Z-average - the size mean using the cumulants analysis according to ISO 13321 International method. For the study, all measurements were performed at 25°C, at automatic analysis mode in triplicate.

Concentration of sample is an important parameter for measurement of particle size by DLS. At high concentrations, the size reported may depend on the sample concentration due to multiple scattering and particle interactions (Rawle, 1995). Hence, protein concentrations of the samples used for DLS measurement need to be analysed in order to see at which concentrations, the apparent size is independent of concentrations. For measurement of an unkonwn sample, appropriate concentrations must be determined by plotting sample concentrations against particle size, suggested by the Application note (Malvern, 2005). The correct measurement region will be shown by the plot as a plateau where the size is independent of concentration.

Initially, sizes of commercially available polystyrene standard latex beads (Sigma) of known diameter (100 and 310 nm) were determined by the instrument under the operating conditions described above. The typical size distribution curves of the latex beads (Sigma) were shown in the Fig. 5A, B. Z-average sizes of standard latex beads with sizes of 100 nm and 310 nm after measuring were 107.9 ± 0.3 and 349 ± 8.4 nm, respectively. The results obtained matched the diameters indicated by the manufacturer.

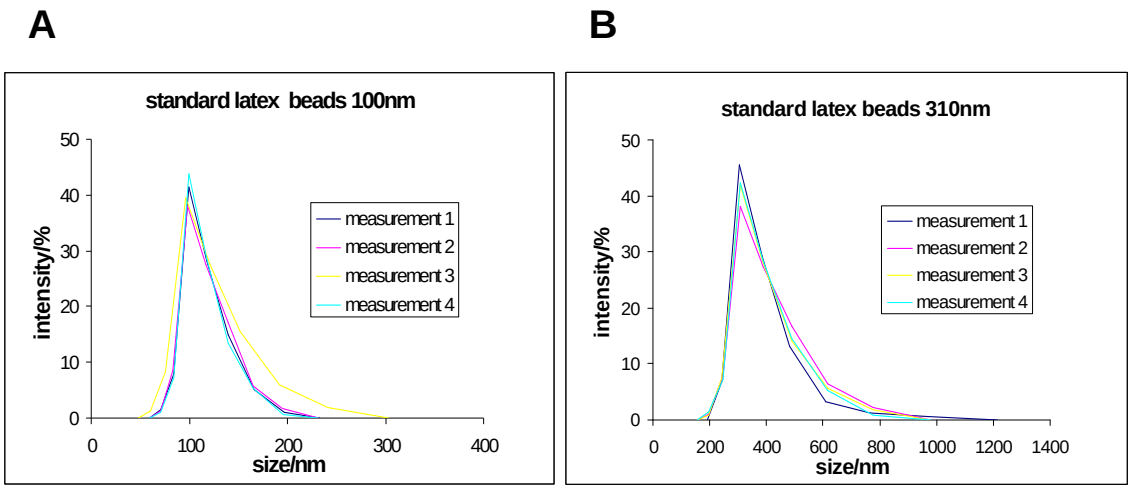

Fig. 5: Size distribution of the latex standard beads. . Z-average sizes of standard latex beads with sizes of 100nm and 310 nm were detected by the Zetasizer 3000, Malvern, UK.

3. RESULTS

3.1 Cultivation strategies for secretion and export of native rPA from *Escherichia coli*

3.1.1 Effect of DsbA co-production and the additive on culture growth and rPA activities

Recombinant plasminogen activator (Kohnert et al., 1992) was synthesized and directly secreted to the periplasm of *Escherichia coli* (Schäffner et al., 2001). Production of rPA was examined in shake flasks on complex medium including 0.4 M arginine to enhance folding into the active conformation (Schäffner et al., 2001). The thiol-oxidase DsbA was coproduced together with rPA.

To evaluate effects of the low molecular additives on rPA activities, the DsbA co-production strain was grown in shake flask in LB medium with initial pH of 6.8 at 30°C to an OD_{600} of 0.6. Stock solution was added to adjust a L-arginine concentration of 0.4 M, reduced and oxidized glutathione (GSH, GSSG) of 0 mM, 2.5 mM, 5 mM each, and an IPTG concentration of 0.4 mM, resulting in 3fold dilution of the culture. The cultures were further incubated at 24°C for 22 h.

Growth of the cultures after adding the additives with different concentrations of glutathiones was nearly similar and reached an OD_{600} of about 4.8- 4.9.

The accumulation of rPA after induction was slow since very low activity of rPA could be determined after 5h of induction (data not shown). Therefore, samples were taken to analyse activities at 22 h of induction. The specific activity of rPA was highest as the cultures were supplemented with 2.5 mM reduced and oxidized glutathione (GSH, GSSG) each, and reached about 0.22 µg/L/OD1. At other glutathione concentrations (ratios of GSH/GSSH are 5/0 and 0/5), the rPA specific activity was 0.14- and 0.18 µg/L/OD1, respectively. Thus, the redox system of reduced and oxidized glutathione had a little but reproducible influence on the growth of the culture and rPA activity. An equimolar mixture with 2.5 mM each of reduced and oxidized glutathione was beneficial for accumulation of active rPA in the periplasm (Fig. 6).

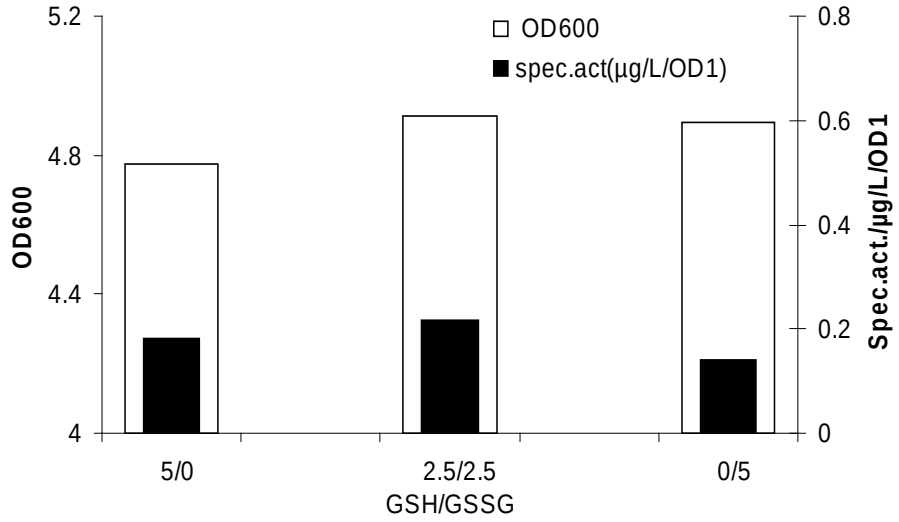

Fig. 6: Impact of co-production of modified disulfide isomerase and redox conditions of the medium on growth and production. The DsbA coproduction strain was grown on LB medium, pH 6.8 at 30°C. At OD_{600} about 0.6, the culture was inducted with IPTG=0.5 mM and grown at 24°C. The additives composed of 0.4M L-arginine, reduced and oxidized glutathione (GSH/GSSG) with ratios: 5/0; 2.5/2.5; 0/5 (mM/mM) were added at the induction. Samples were taken after 22 h of induction.

In order to test effects of DsbA co-production and the components of the additives on enhancement of disulfide bond formation, folding activity of native rPA and the growth of the bacteria on shake flask, the DsbA co-production strain and control strain (without DsbA) were incubated in shake flask in LB medium with initial pH of 6.8, at 30°C to an OD_{600} of 0.6. Afterwards, stock solution was added to adjust L-Arg concentration of 0.4 M, 2.5 mM each of reduced and oxidized glutathione and an IPTG concentration of 0.4mM. The cultures were further incubated at 24°C for 22 h. Samples were taken at 22h after induction for analysis of rPA activities in the periplasm and culture supernatant.

The results showed that highest periplasmic specific, periplasmic volumetric and supernatant activities of rPA were obtained as the DsbA co-production strain was supplemented with 0.4 M L-Arg, 2.5 mM each of reduced and oxidized glutathione (Fig. 7).

In case of DsbA co-production, periplasmic specific and supernatant volumetric activities of the cultures added with L-Arg and redox were 1.69 and 10.83 fold, respectively, higher than those of the cultures were supplemented with L-Arg. Similarly, as the cultures were supplemented with only redox system, the periplasmic specific and supernatant volumetric activities were 5.35 and 17.16 fold, respectively, lower than those of the cultures were added with full components. Low molecular additives showed a very obvious effect as the periplasmic specific activity of the cultures were added with L-Arg and redox system was 13.75 fold higher than that of the cultures without additive supplemetation. From these results, it is assumed that L-Arg has stronger effect on the production of active rPA than redox system (Fig. 7).

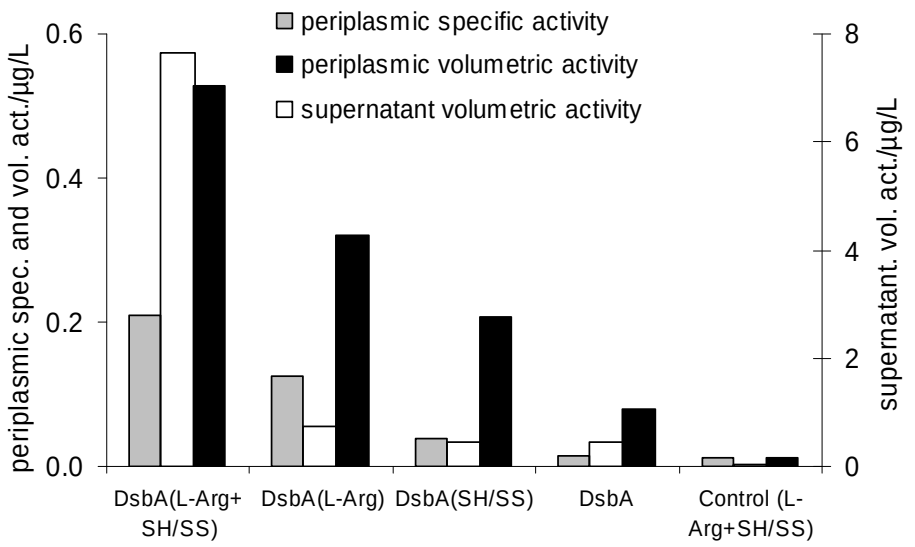

Fig. 7: Impact of DsbA co-production and components of additives on rPA production. The DsbA coproduction strain was grown on LB medium, pH 6.8 at 30°C. At OD_{600} about 0.6, the cultures were inducted with IPTG=0.5 mM and grown at 24°C. Additives composed of 0.4M L-arginine; 2.5 mM SS; 2.5 mM SH were added at the induction. Samples were taken after 22 h of induction.

Interestingly, as also can be seen from Fig. 7, although the control strain was supplemented with L-arginine and redox system, rPA yields from periplasm and culture medium of the control strain were 6.6 and 12.6 times lower than those of the DsbA co-production strain without addtion of the additives. Hence, co-production of DsbA had a

noticeable impact on the secretion of native rPA in the periplasm and especially on the export of the active protein to the culture supernatant.

Therefore, co-production of DsbA and low molecular additive were essential for accumulation of active rPA either in the periplasm or in the culture supernatant.

Also, to investigate the influence of the components of the additives on cell growth, the experiments were conducted at the same procedure as described in the obove-mentioned part (indicated in Fig. 7). During the first 8h of induction, samples were taken every two hours and at 24h of induction for optical density measurement.

During cultivation, growth of the cultures supplemented with full additives and with only L-Arg was slower than that of the cultures supplemented with only redox system or without additives. At the end of the cultivation, OD_{600} of the cultures in the presence of L-Arg reached approximately 4.0 compared to OD_{600} of about 5.0 obtained from the culture without adding L-Arg. This indicates that the growth was impaired during production due to the addition of L-Arg, whereas glutathione or production of rPA without additives did not interfere with culture growth (Fig. 8).

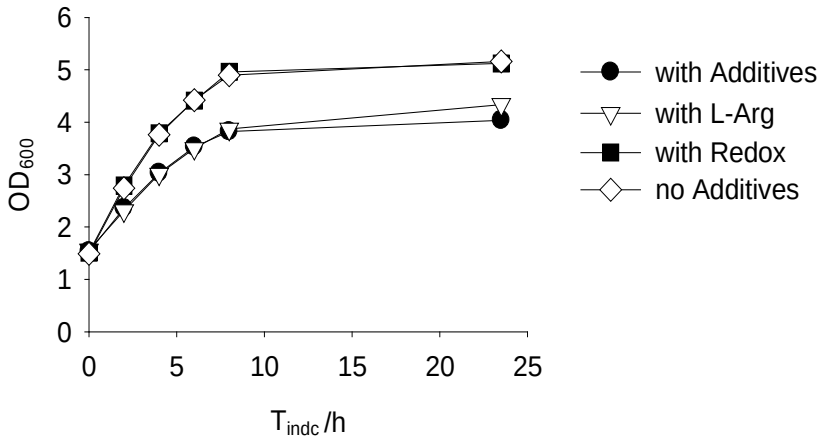

Fig. 8: Impact of components of additives on cell growth. The DsbA co-production strain was grown on LB medium, pH 6.8 at 30°C. At OD_{600} about 0.6, the culture was inducted with IPTG=0.5 mM and grown at 24°C. Additives composed of 0.4M L-arginine; 2.5 mM SS; 2.5 mM SH were added at the induction.

3.1.2 Effect of pH medium, cultivation temperatures and media on culture growth and rPA activities

3.1.2.1 Effect of starting pH of cultivation medium on culture growth and rPA activities

Thiolate anions are necessary for formation and rearrangement of disulfide bonds in the cells. Therefore, the reaction rate depends on the pH value (Ruddock et al., 1996). To study effect of the pH of the culture medium during induction of rPA, the DsbA co-production strain was grown in shake flask in LB medium. pH of the medium was adjusted to 4.8, 5.8, 6.8, 7.8 by HCl 10% and NaOH 10%.

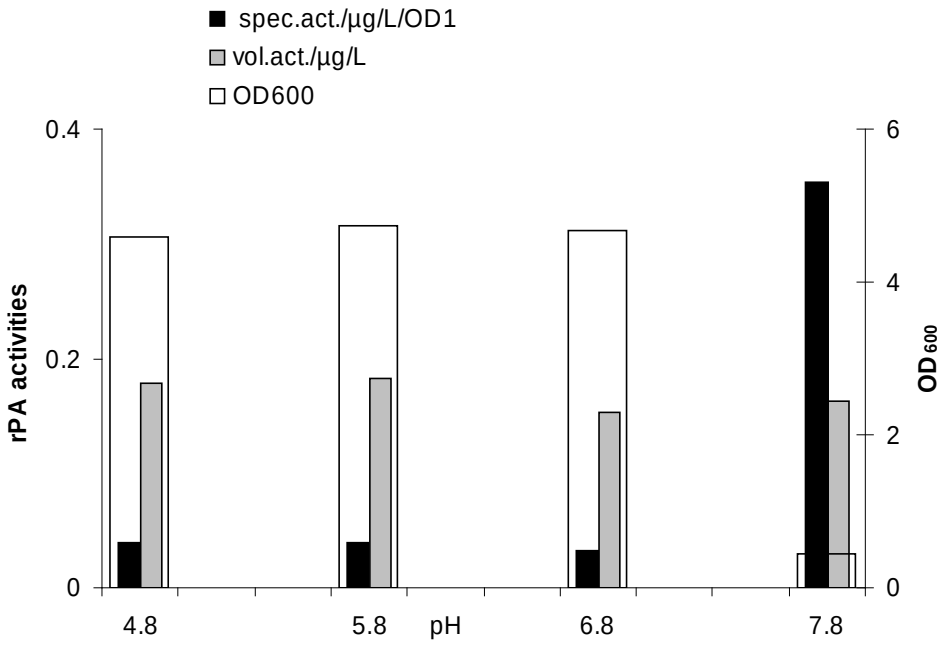

Fig. 9: Impact of pH medium during induction on growth of the cultures and rPA production. The DsbA co-production strain was grown in shake flask in LB medium at 30°C. pH medium was adjusted to 4.8, 5.8, 6.8, 7.8 by HCl 10% and NaOH 10%. At OD_{600} about 0.6, the culture was inducted with IPTG=0.5 mM and grown at 24°C. Additives composed of 0.4 M L-arginine, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione were added at the induction. Samples were taken after 22 h of induction.

When recombinant rPA was produced in the medium at pH values of 4.8-6.8, the rPA specific activity was roughly similar. Concominant to the specific activity, OD_{600} of the culture were nearly the same and reached around 5 after 22h of induction. But at pH of 7.8, yield of the specific rPA was increased about 10fold. However, in such a basic medium, growth was severely impaired. Very low OD_{600} of the culture of only about 0.44, 10 times lower than at other pH values was observed. Consequently, the volumetric rPA yield was enhanced only marginally (Fig. 9).

3.1.2.2 Effect of cultivation media on culture growth and rPA activities

From the previous results, a significant increase of the specific activity was found as culture incubated in LB medium with starting pH of 7.8. Thus, in this study, the DsbA co-production strain was grown in shake flask in LB, HDF and yeast extract medium at 30°C. Initial pH of the medium was adjusted to 7.8 by NaOH 10%.

Highest and lowest specific activity of rPA was obtained as the cultures were grown in LB and yeast extract medium, respectively. In HDF medium yield of periplasmic rPA was approximately half of yield obtained in LB medium. Growth of the cultures on yeast extract was severely impaired leading to an extremely low yield of volumetric rPA. Therefore, yeast extract medium was not suitable for rPA production due to low activities and improper cell growth (Fig. 10).

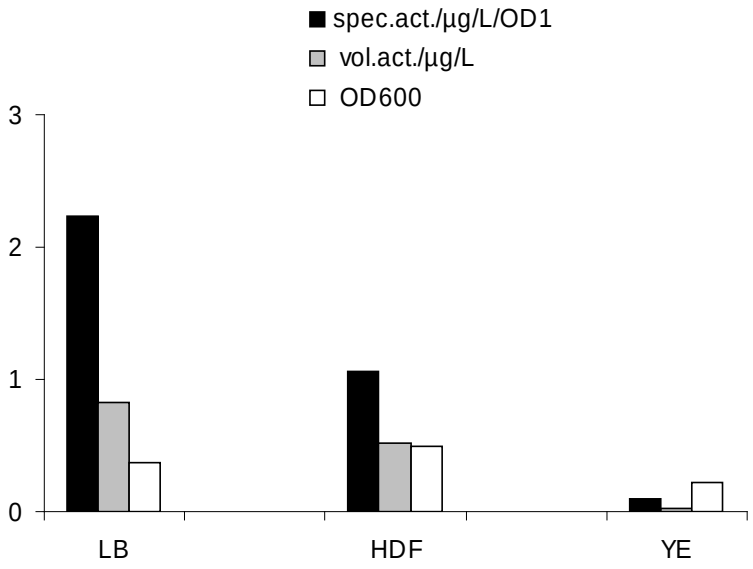

Fig. 10: Impact of different cultivation medium on growth of the cultures and rPA production. The DsbA coproduction strain was grown in shake flask, in LB, defined (HDF) and yeast extract (YE) medium, at pH 7.8 and 30°C. At OD_{600} about 0.6, the culture was inducted with IPTG=0.5 mM and grown at 24°C. Additives composed 0.4 M L-arginine, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathionem were added at the induction. Samples were taken after 22 h of induction.

3.1.2.3 Effect of cultivation temperature on culture growth and rPA activities

Lower yield of native rPA was obtained as culture was grown in LB medium and induced at 37°C compared at 24° and 30°C (Schäffner, 2000). Therefore, the DsbA co-production strain was grown on LB medium only at 30°C and 24°C at pH adjusted of 7.8. Temperature after induction was kept at 24°C for 22h.

As culture grown at 30°C, higher specific activity than at 24°C was observed. At this temperature, due to lower OD_{600}, volumetric activity was roughly lower than at 24°C. However, cultivation of the cultures at 24°C consumed more time to get an appropriate OD_{600} value to start induction. Therefore, cultivation at 30°C was more beneficial for cell growth and accumulation of native rPA in the periplasm (Fig. 11).

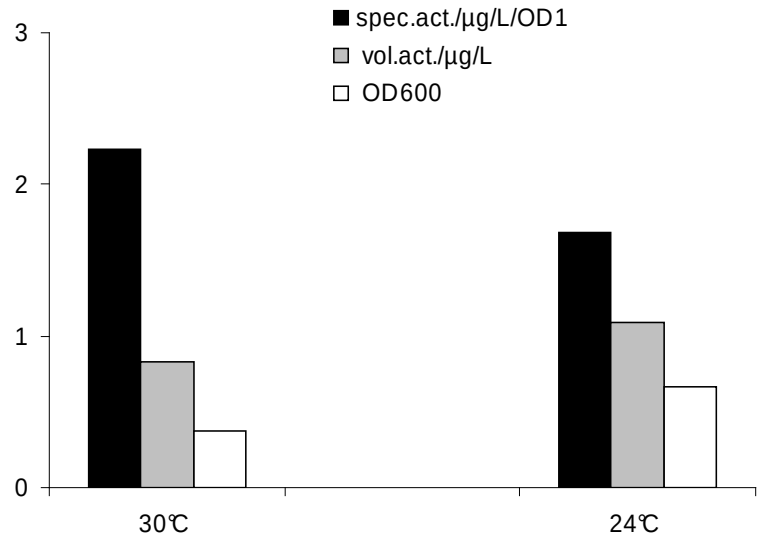

Fig. 11: Impact of cultivation temperature on growth of the cultures and rPA production. The DsbA coproduction strain was grown on LBmedium, pH 7.8 at 24°C and 30°C. At OD_{600} about 0.6, the culture was inducted with IPTG=0.5 mM and grown at 24°C. Additives composed 0.4 M L-arginine, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione were added at the induction. Samples were taken after 22 h of induction.

Therefore, for scale-up to fed-batch cultivation, experiments were performed in HDF medium at pH value of 6.8, temperature before and after induction was 30°C and 24°C, respectively.

3.1.3 Fed-batch cultivation strategy for rPA production

3.1.3.1 Effect of additives on rPA activity in fed-batch cultivation

Fed-batch cultivations for rPA production were performed on defined glucose mineral salt medium. After a short batch phase with an initial glucose concentration of 5 g/L, the glucose feeding was started to maintain a specific growth rate of μ_{set} = 0.12 h^{-1}. In order to investigate effect of additives on enhancement of native rPA production, when the OD_{600} exceeded 20, a solution of 1.5 L HDF medium consisted of L-Arg and glutathione was added to give final concentrations of 0.4 M L-Arg and 2.5 mM of each reduced and oxidized glutathione, resulting in 5/4 dilution of the culture. The solution also composed only HDF medium for the experiments using no additives, or only 0.4 M L-Arg in HDF medium for the experiments using only L-Arg. After adding the solution,

temperature was reduced from 30 to 24°C. IPTG was added to 0.5mM for induction of recombinant gene expression.

Samples were taken after 40h of induction. rPA activities in the periplasm were analysed according to the section 2.5.7. For the analysis of rPA activity in the culture supernatant, after removing cells from the samples by centrifugation at 13000rpm/ 3min/ 4°C, supernantant was diluted by 75 mM Tris buffer pH 7.5 and followed the protocol in 2.5.7.

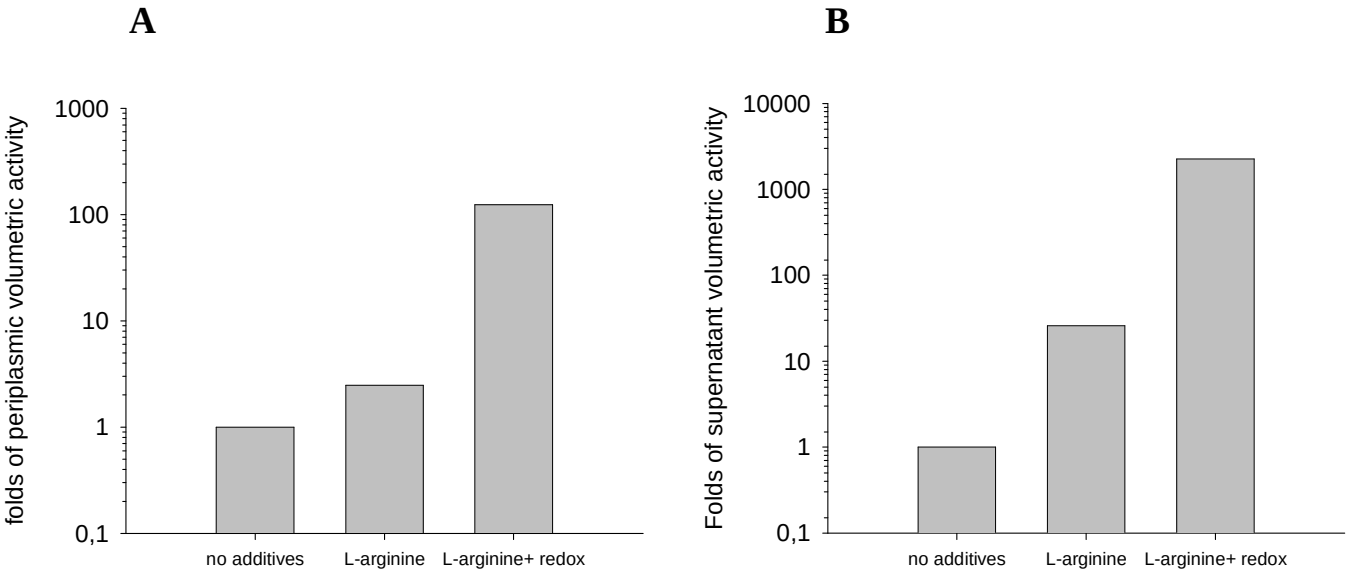

Fig. 12: Impact of additives on rPA activities in fed-batch cultivation. Cultivation of DsbA coproduction strain was conducted on HDF medium at 30°C. Batch phase started with initial glucose of 5 g/L. Culture was induced at 24°C, 0.5 mM IPTG after 1 h of feeding started with μ_{set}=0.12 h^{-1}. A solution of 0.4 M L-Arg, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione in 1.5 L HDF medium was added at the induction. Yields of rPA obtained in the periplasm (**A**) and in the culture supernatant (**B**) are given relative to yields from the reference experiment (without addition of additives).

Periplasmic activity of the culture added with L-Arg and redox system, and only with L-Arg increased more than 123 and 2.4fold, respectively, than that of the culture without any additive supplementation (Fig. 12A). Whereas, higher yields of native rPA in culture supernatant as the cultures were added with the additives were obtained, indicating that nearly 25 and 2250 fold increased compared to the yields of the experiments using only L-Arg and no addivtives, respectively (Fig. 12B).

Also, redox system has a remarkable influence on the yields of native rPA obtained from the periplasm and culture medium. Compared to the batch without addition of redox system (only L-Arg), yields of native rPA in the periplasm and culture supernatant of the batches with addition of L-arginine and redox system increased about 50 and 87 fold, respectively.

Therefore, addtition of L-Arg and redox system could enhance the release of native rPA into culture medium. In contrast to the result from shake flask experiment, redox system played an important role on the secretion of rPA.

3.1.3.2 Effect of growth rates on the yield of the periplasmic rPA

The influence of growth rate during fedbatch cultivations on the secretion of the product to the periplasm was previously reported. At feed rate of 0.5 h^{-1}, production of the 38kDa-product was enhanced by the translocation of the product into the periplasm. (Boström et al., 2005). In this study, after an initial glucose concentration of 5g/L was depleted, feeding rates were chosen at 0.06, 0.12 and 0.2 h^{-1}. Induction was made during fed-batch phase 1h after feeding started. OD_{600}, rPA activities in the periplasm were analysed and shown in Fig. 13.

At the low feeding rate of 0.06 h^{-1}, after 40h of induction, nearly twofold lower OD_{600} than at feeding rate of 0.12 h^{-1} was obtained (Fig. 13 A). Optical density of the culture at the feeding rate of 0.12 h^{-1} was roughly higher than at feeding rate of 0.06 h^{-1}. Similar pattern of the volumetric activity was observed as the optical density pattern due to specific rPA activity at the different feeding rates was nearly similar (Fig. 13 B, C). Hence, feeding rates aiming at specific growth rates between 0.06 and 0.2 h^{-1} did not influence the specific rPA activity profiles.

rPA accumulation in the periplasm and culture supernatant was slow within 5-10 h and 20h after induction, respectively. However, a steady increase of rPA activity for another 40 h was occured (Fig. 13B, C). After 40h of induction, rPA specific and volumetric activities reached approximately 5-7 µg/L/OD_{600} and 300-500 µg/L, respectively. Therefore, rPA synthesis should be induced early to prolong production.

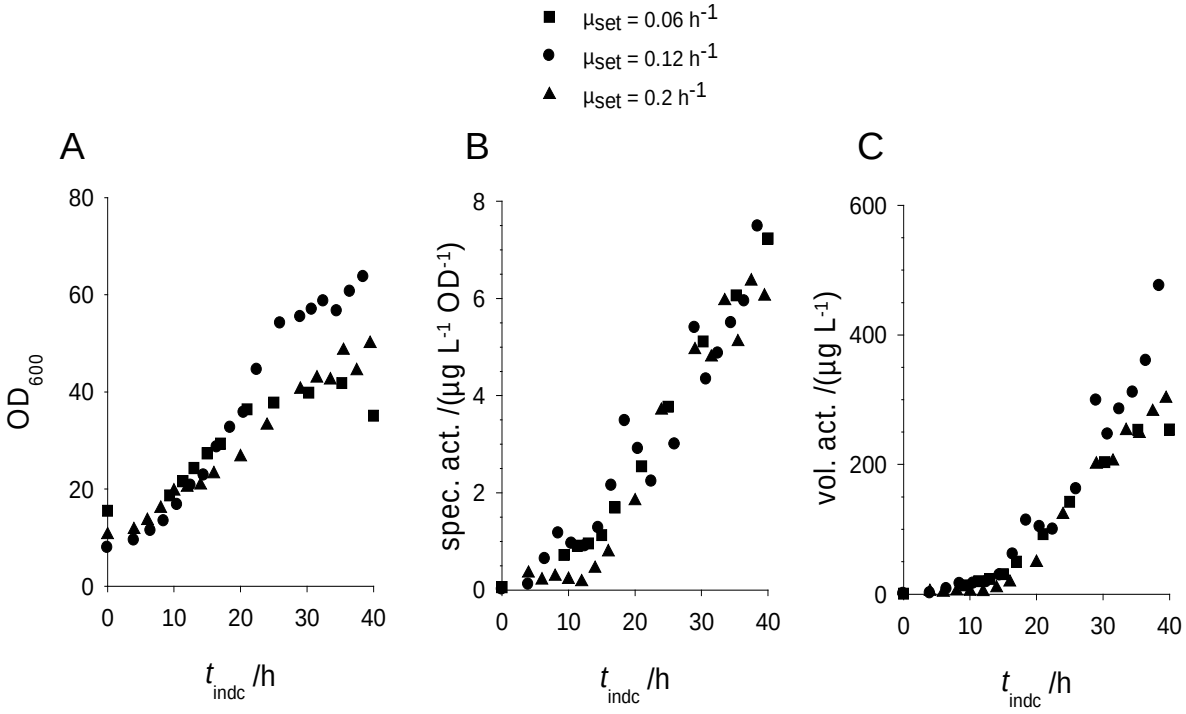

Fig. 13: Impact of growth rates on cell growth and yields of the secreted protein in the periplasm. Cultivation of DsbA co-production strain was conducted on HDF medium at 30°C. Batch phase started with initial glucose of 5 g/L. Culture was induced at 24°C, 0.5 mM IPTG after 1 h of feeding started with different μ_{set} of 0.06, 0.12 and 0.2 h^{-1}. A composition of 0.4 M L-arginine, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione was added at the induction.

3.1.3.3 Effect of specific growth rates and the time of induction on the secretion and export of the native rPA

Some research on the impact of growth rate on the membrane structure of *E. coli* and leakage of periplasmic proteins to the culture medium was communicated (Shokri et al., 2002; Shokri et al., 2003; Dresler et al., 2006). Pre-induction growth rate results in a sharp maximum of secretion efficiencies (Curless et al., 1994). In order to test effect of grow rates and the induction time on yields of active rPA in the periplasm and in the culture supernatant, cultivations of DsbA co-production strain were conducted on HDF medium at 30°C. Culture was induced at 24°C with 0.5 mM IPTG after 1 h of feeding started with different μ_{set} of 0.06 and 0.12 h^{-1} (fed-batch induction) and after 7 h of inoculation (prolonged batch induction). Batch phase started with initial glucose of 30 g/L. A composition of 0.4 M L-arginine, an equimolar mixture with 2.5 mM each of

reduced and oxidized glutathione was added at the induction. Schemes for fed-batch induction and prolonged batch induction are shown in Fig. 14.

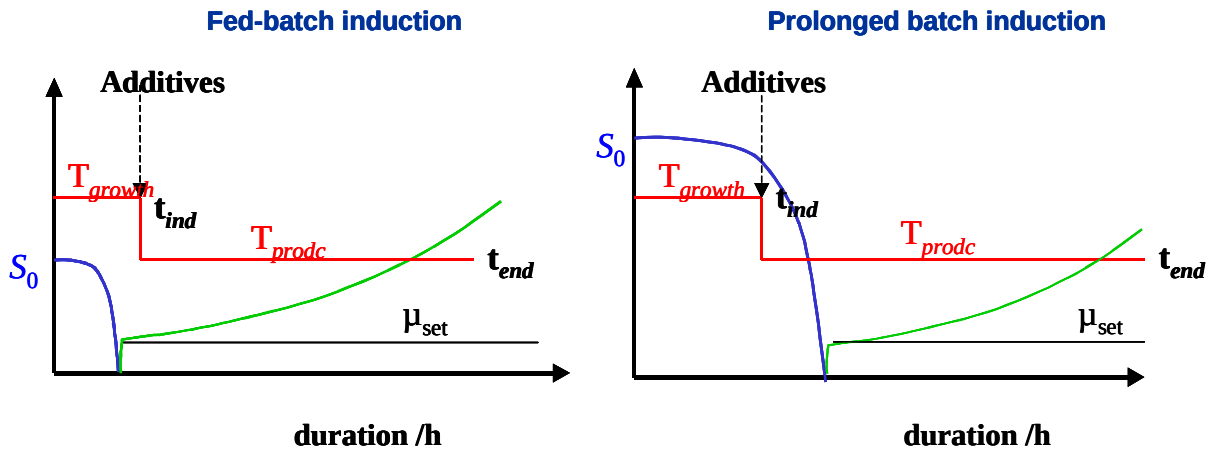

Fig. 14: Schemes for fed-batch induction and prolonged batch induction cultivation. S_0: initial concentration of substrate. μ_{set}: specific growth rate is kept constant. T_{growth} : cultivation temperature before induction. $\mathbf{t_{ind}}$: time of induction. T_{prod} : temperature after induction. t_{end} : time at the end of the cultivation.

3.1.3.3.1 Induction of rPA production during prolonged batch phase and fed-batch phase at feeding rate μ_{set}= 0.06 h^{-1}

rPA productions in a prolonged batch phase and fed-batch phase with a higher initial glucose concentration of 30 g/L were evaluated. For the production of rPA prolonged batch induction, the culture was induced at an OD_{600} of 8 during the batch phase. Whereas, for the fed-batch induction, after 1h of auto- feeding of glucose started, the culture was induced at an OD about 44.

After addition of L-Arg, glucose consumption in prolonged batch cultures was slower than in fed-batch cultures resulting in 12 h production under glucose excess conditions. However, no glucose residue in the prolonged batch cultures was detected after starting feeding, whereas after approximately 30h of feeding, high glucose accumulation was observed in the fed-batch cultures (Fig. 15B).

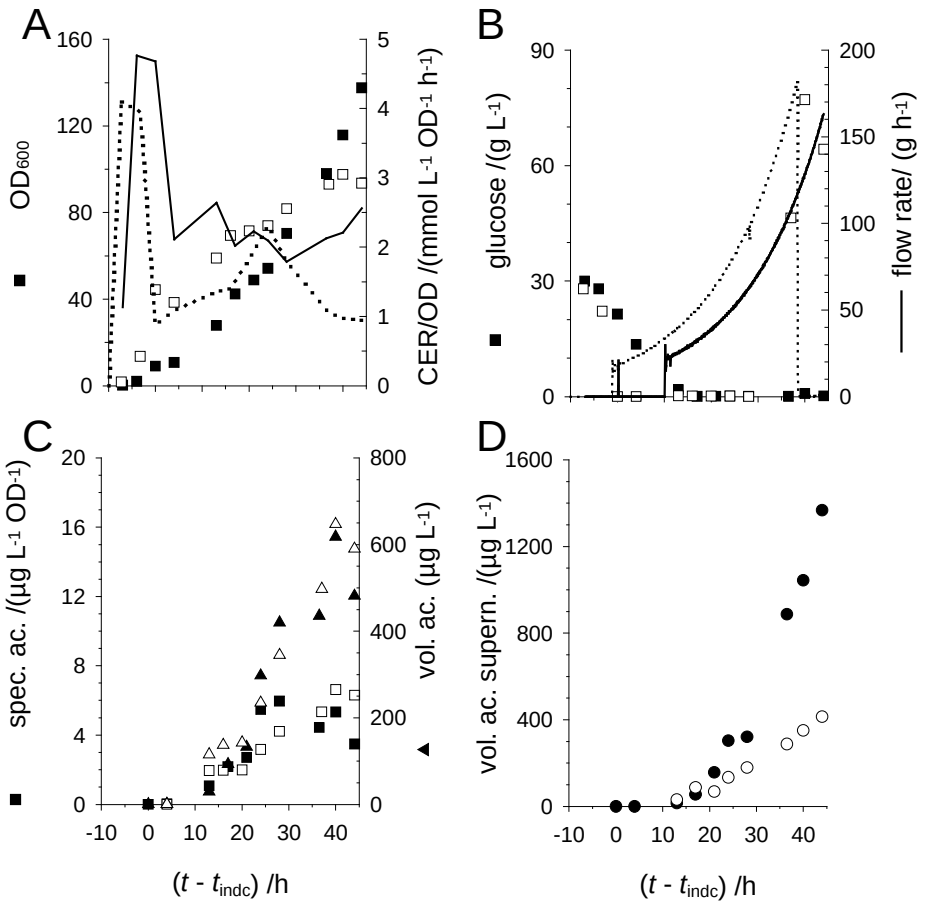

Fig. 15: Comparison of induction of rPA production during prolonged batch phase and fed-batch phase, μ_{set}= 0.06 h^{-1}. Induction took place at an OD about 8 during prolonged batch phase (solid symbol, solid lines) and at an OD about 44 and during fed-batch phase (open symbols, dashed lines), 1h after feeding started.

After glucose depletion, glucose feeding with a rate of μ_{set} = 0.06 h^{-1} was started, and growth continued for another 40 h, resulting in a final OD_{600} exceeding 130 and 90 as the culture were induced during prolonged batch and fed-batch phase, respectively (Fig. 15A). The induction conducted during batch phase remained constant specific CER of 2 mmol/OD_{600}/h, whereas the specific CER declined after 25 h of induction as the culture was induced during fed-batch phase (Fig. 15A).

During the first 12h after induction, little rPA specific activity was obtained from the prolonged batch and fed-batch cultivation (Fig. 15C). The specific rPA in the both cases reached a maximum of about 6 µg/L/OD_{600}. But the activity decreased subsequently from the 28 h after induction when the induction was taken place during the prolonged

batch phase. Whereas, the activity remained increasing in the case the induction was conducted during fed-batch phase (Fig. 15C). However, periplasmic volumetric activity in the both cultivation strategies increased gradually and reached approximately 600 µg/L after 40 h of induction. Interestingly, in comparison to the cultivation with induction during the fed-batch phase, nearly 4 times of native rPA was transferred to the culture supernatant, corresponding to 1300 µg/L, as the induction started during batch phase (Fig. 15D).

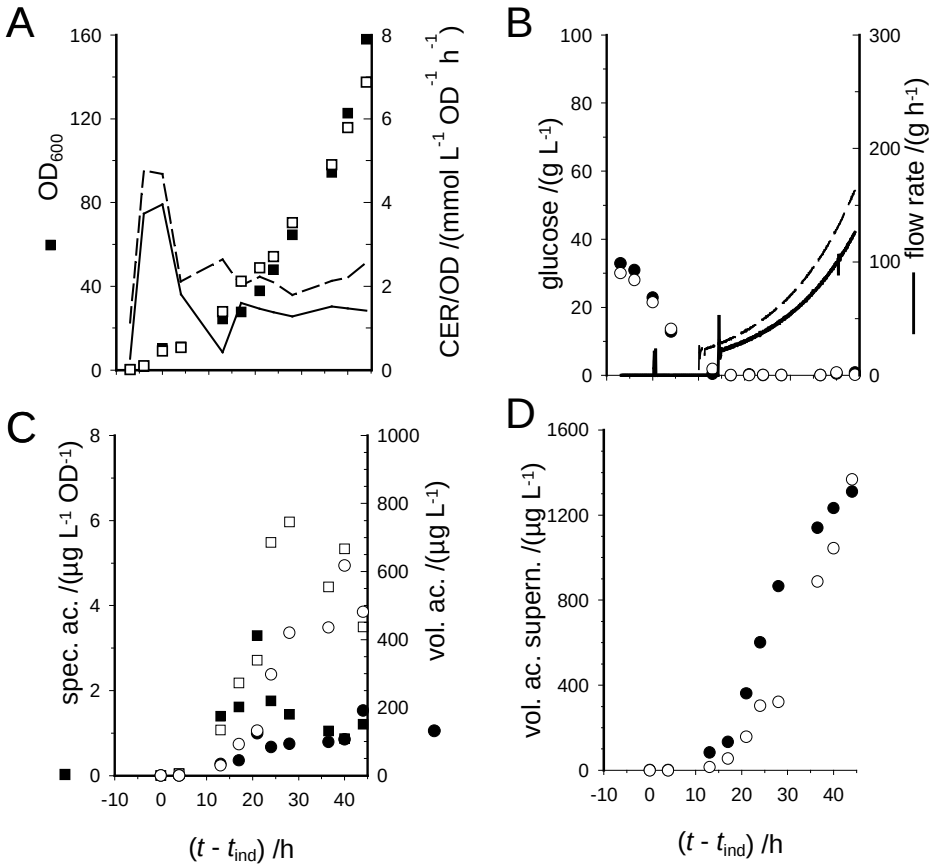

Fig. 16: Effect of glucose starvation on rPA production. Batch phase started with an initial glucose concentration of 30 g/L, induction took place at an OD about 8. Glucose was fed with μ_{set}= 0.06 h^{-1} starting immediately after deletion of glucose (open symbols, dashed lines) or after a starvation period of 3h (solid symbol, solid lines). (A) OD_{600} (square) and CER/OD (lines), (B) glucose feeding rate (lines) and glucose concentration (symbols), (C) specific (squares) and volumetric (triangles) rPA activities in the periplasm and (D) volumetric rPA activity in the culture supernatant are shown. Time is given relative to the time of induction.

To investigate whether glucose depletion could affect the product released to the culture medium, prolonged-batch-induction cultivations in which the glucose feeding was started with a delay of 3 hours were conducted (Fig. 16).

Growth of the cultures in the both cases was similar as after 40h of induction resulting in OD_{600} of about 120. The batch which feeding started immidiately after glucose deletion specific CER maintained about 2 mmol/OD_{600}/h, whereas the specific CER of the batch with a delayed feeding was little lower, around of 1.5 mmol/OD_{600}/h (Fig. 16 A).

After 40h of induction, specific and volumetric activities in the periplasm of the batch with delayed feeding strategy was nearly 5 times lower than those of the control batch. However, volumetric activity of about 1300 µg/L in the supernatant in the both cases was obtained. Thus, the delayed feeding scheme only resulted in low periplasmic, whereas the volumetric activity in the culture supernatant was in the same range as with immediate feeding start (Fig. 16C, D). In other experiments, the feeding was started before glucose depletion to maintain unlimited conditions, the rPA activity in both, periplasm and culture supernatant were low (data not shown). Therefore, induction during batch phase followed immediately by slow glucose feeding maximized the overall production of native rPA in the periplasm and culture supernatant.

3.1.3.3.2 Induction of rPA production during prolonged batch phase and fed-batch phase at feeding rate μ_{set}= 0.12 h^{-1}

To investigate whether at higher feeding rate could influence the accumulation of rPA in the periplasm and the culture medium, cultivation with induction at prolonged batch and fed-batch phase at feeding rate of 0.12 h^{-1} were conducted.

There was no different in growth pattern of the culture in the batch with induction in fed-batch and prolonged batch phase. After 40h of induction, OD_{600} of the both cases reached approximately 120 (Fig. 17A). Only after 2h of glucose depletion, accumulation of glucose in the cultivations induced during fed-batch and prolonged batch phase increased steadily and reached about 50 and 90 g/L, respectively (Fig. 17 B).

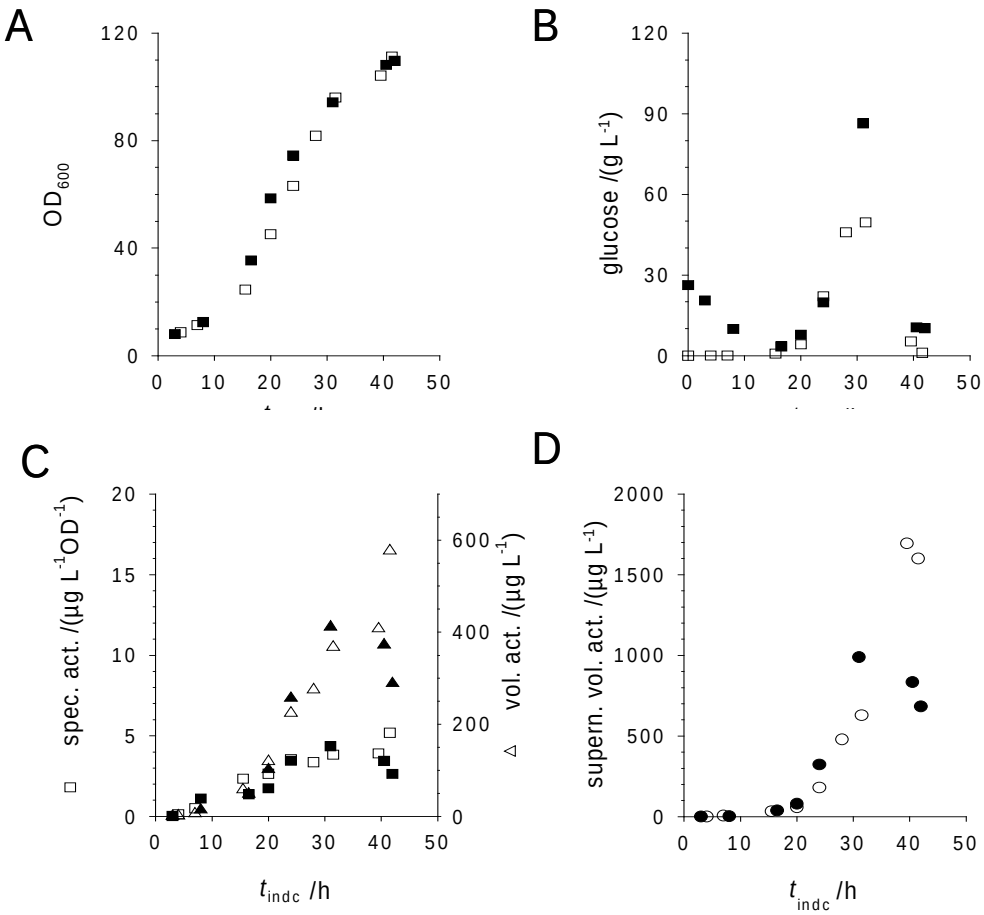

Fig. 17: Comparison of induction of rPA production during prolonged batch phase and fed-batch phase, μ_{set}= 0.12 h^{-1}. Induction took place during prolonged batch phase (solid symbol, solid lines) and during fed-batch phase, 1h after feeding started (open symbols, dashed lines).

Periplasmic specific activity declined and reached about 2.5 µg/L/OD_{600} after 30h of induction as the cultures were induced during prolonged batch phase. In contrast to the cultivation with induction at the prolonged batch phase, periplasmic activity increased gradually and reached approximately 4 µg/L/OD_{600} after induction of 30h. About 400 µg/ L of volumetric activity in the periplasm of the both cases were obtained (Fig. 17 C).

At higher growth rate of 0.12 h^{-1}, after 40h of induction, volumetric activities in the culture medium of the cultivations with the induction at the prolonged batch phase and fed-batch phase reached about 800, 1600 µg/L, respectively. Nearly two times of native rPA was accumulated in the culture supernatant as culture was induced during fed-batch

phase compared to induction at prolonged batch phase. In contrast to low feeding rate of 0.06 h^{-1}, induction of rPA during prolonged batch phase at higher feeding rate of 0.12 h^{-1}did not enhance the yield of rPA in the culture medium (Fig.17 D).

As can be seen from Fig. 18, in case cultures were induced at the batch phase, leakage of the rPA into the culture supernatant was enhanced nearly 2 times at feeding rate of 0.06 h^{-1} compared to at feeding rate of 0.12 h^{-1} (Fig. 18 A). But in contrast to prolonged batch induction, at the feeding rate of 0.06 h^{-1}, yield of rPA in the supernatant was about 4 times lower than at the feeding rate of 0.12 h^{-1} (Fig. 18 B). Therefore, feeding rates probably have a remarkable influence on the leakage of native rPA into the culture medium.

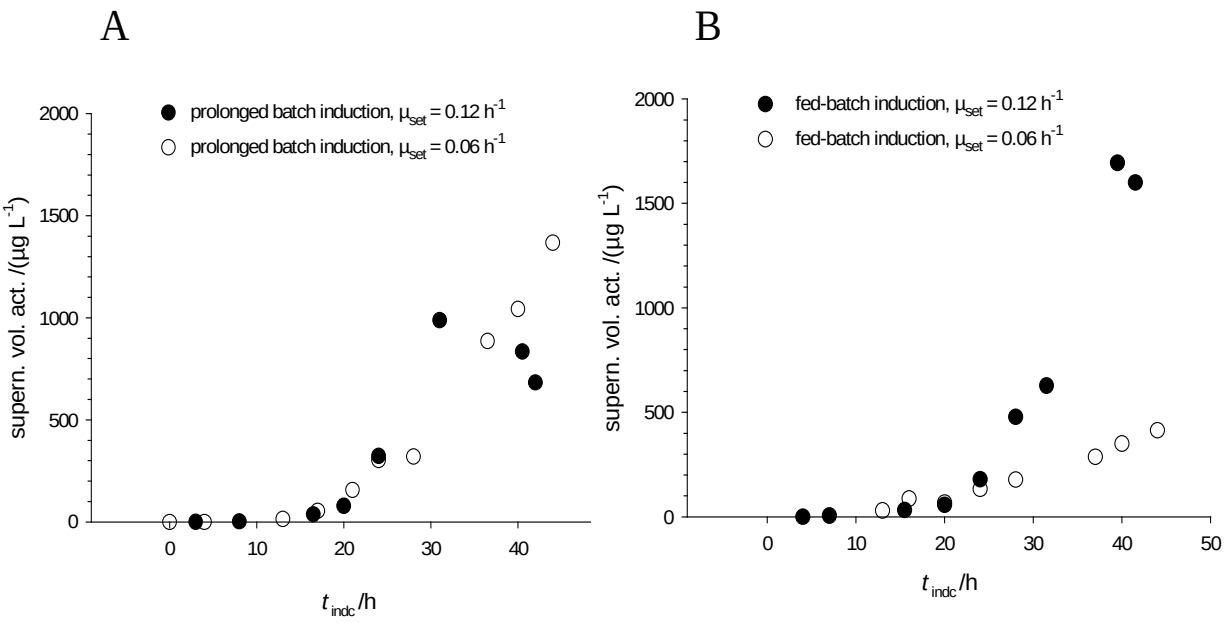

Fig. 18: Comparison of the prolonged batch, fed-batch induction and feeding rates on rPA activity in culture medium. Volumetric activity in the supernatant of the batch with induction at prolonged batch phase (A), and induction at fed-batch phase (B). Close and open symbols indicate feeding rate at 0.12 h^{-1} and 0.06 h^{-1} , respectively.

3.1.3.4 Localization of rPA during the production process

To understand the transfer of the product into the periplasm and culture supernatant, cellular fractions were analyzed by Western Blot. Samples were taken at different time

after induction during prolonged batch cultivation at growth rate of 0.06 h^{-1}. By ultrasonication, soluble fractions were separated from insoluble fractions. Periplasmic fractions were extracted from cell pellets by polymyxine-B sulfate according to the section 2.5.6.

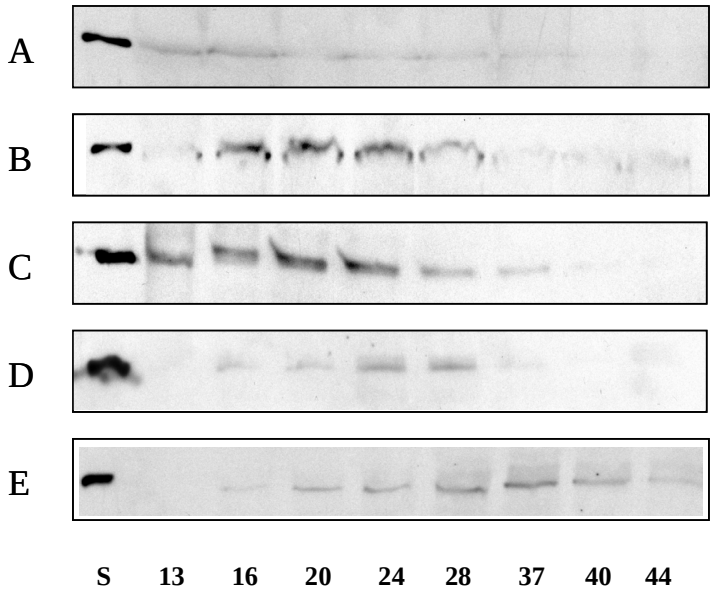

Fig. 19: Localization of rPA during the production process. Western blot of (A) samples of soluble protein fraction, (B) samples of insoluble protein fraction, (C) cell pellets remaining after extraction and, (D) periplasmic extract, (E) culture supernatant after 13, 16, 20, 24, 28, 37, 40, 44 hours of induction. Lane S: purified rPA standard.

The product was detected in the soluble fractions from early time after induction (Fig. 19A), but could not extracted from the periplasm (Fig. 19D); instead, it remained in the cells (Fig. 19C). From 16 to 24h after induction, the product was detected in the insoluble fraction (Fig. 19B) indicating aggregation. Afterwards, the product could be extracted (Fig. 19D) and was released into the culture supernatant, where it reached maximum levels from 37 h after induction (Fig. 19E).

In addition, as can be seen from Fig.19, rPA have the same size in all fractions, indicating cleavage of the signal sequence and proper protein export. Thus, the aggregates probably were located in the periplasm. After 24h of induction, the accumulation of the product was decreased in the insoluble fractions, cell pellets and periplasm while an increase of native rPA was found in the culture supernatant

indicating a translocation of the active protein from peiplasm into the medium (Nguyen et al., 2006).

- **Total protein of the culture supernatant**

In order to understand the leakage of rPA in the supernatant during fed-batch cultivation could be a consequence of cell lysis or protein translocation. Samples taken from the prolonged batch and fed-batch cultivations were analysed for total protein in the supernatant and dry cell biomass.

3 g/ L (corresponding to the 2% of the protein in the biomass) of total protein concentration was obtained in the supernatant after batch induction, whereas protein concentration above 6 g/ L (corresponding to the 4% of the protein in the biomass) were reached with induction during the fed-batch phase. Therefore, the overall protein concentration in the supernatant of the batch-induced culture was two times lower than in the fed-batch-induced culture, showing no indication of extensive cell lysis (Fig. 20).

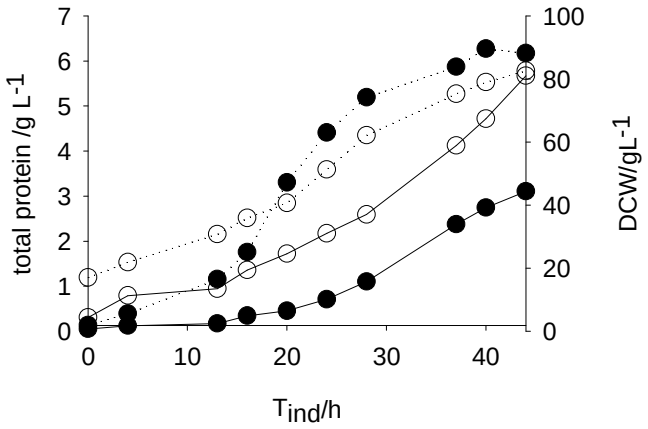

Fig. 20: Total protein in the culture supernatant and dry cell weight. Cultivation of DsbA co-production strain was conducted on HDF medium at 30°C. Culture was induced at 24°C with 0.5 mM IPTG after 1 h of feeding started with μ_{set} of 0.06 h^{-1} (fed-batch induction) or at an OD_{600} about 8 (prolonged batch induction). A composition of 0.4 M L-arginine, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione was added at the induction. Black circles indicate total protein and open circles indicate DCW of fed-batch induction (solid lines) and of fed-batch induction (dash lines).

3.1.4 Impact of other additives e.g. glycine and Triton X-100 on the secretion and export of native rPA

Addition of 2% glycine and 1% Triton X-100 enhanced more than 170fold of native extracellular FV fragment tumor necrosis factor alpha fusion protein (SFV/TNF-$^{\alpha}$) excreted into the culture medium (Yang et al., 1998). To investigate effect of glycine and Triton X-100 on yields of rPA in the periplasm and culture supernatant, shake flask experiments were conducted like the experiments using only L-Arg and glutathione as the additives. However, due to growth was inhibited as cultures were induced at OD_{600} about 0.6 in the presence of glycine and Triton X-100, induction was conducted at an OD_{600} about 4. rPA activities in the periplasm, culture supernatant and OD of the cultures were analysed and indicated in Fig. 21, 22.

rPA activities in the periplasm and culture supernatant were proportional to concentrations of of glycine and Triton X-100. In the presence of lower concentrations of these chemicals, lower yields of native rPA were obtained. At 0.4M L-Arg and an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione, rPA in the periplasm and culture medium reached highest yields of 2.6 and 100 µg/ L, respectively. At concentrations of 0.25 % glycine and 0.125 % Triton X-100, rPA concentrations in the periplasm and culture medium of only 0.46 and 11.75 µg/L, respectively, were obtained although L-Arg and glutathione were present (Fig. 21).

Interestingly, the absence of L-Arg or glutathione in the additives played an important role in the accumulation of rPA. Although with the addition of 2 % glycine and 1 % Triton, no active rPA was detected in the periplasm and the activity in the supernatant was about 150fold lower compared to experiments with addition of both L-Arg and glutathione (Fig. 21). With the presence of L-arginine, redox system, addition of 2 % glycine and 1 % Triton X-100 to the culture after induction, rPA activities increased not only in the periplasm but also in the culture medium.

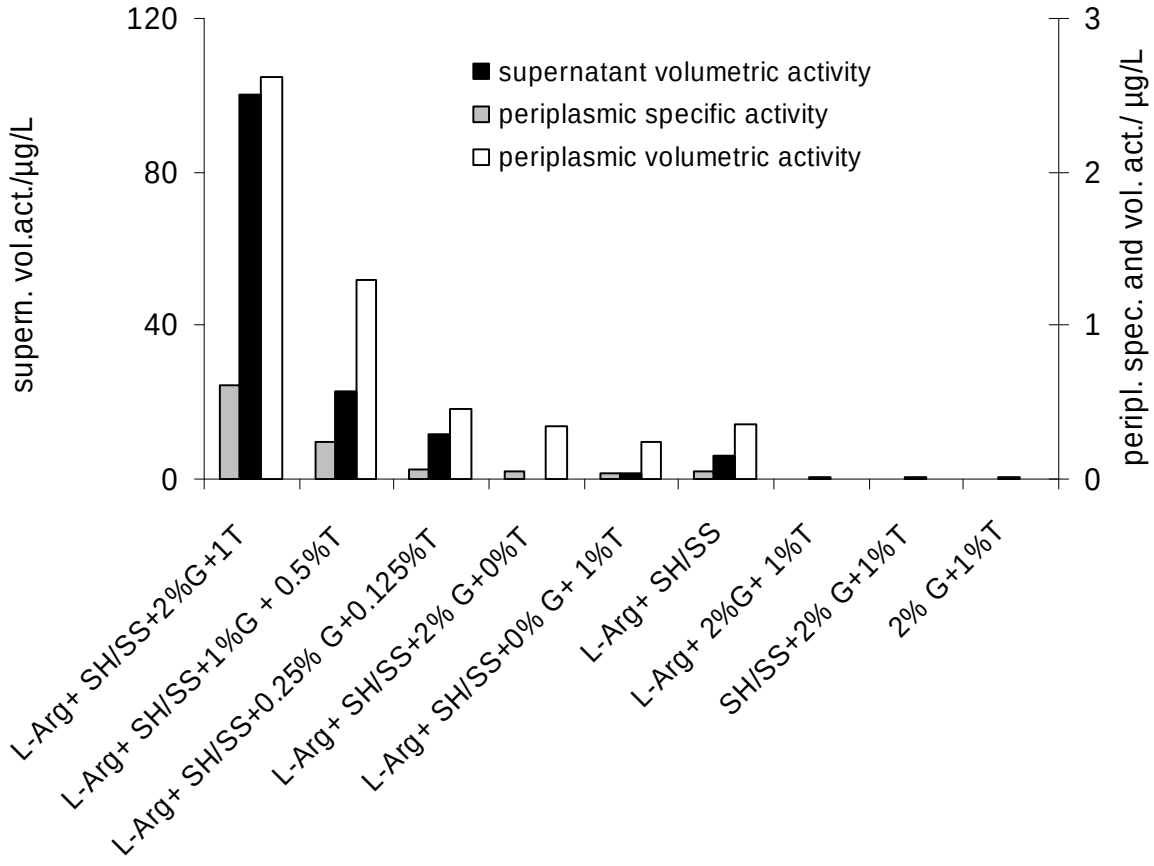

Fig.21: Effect of glycine and Triton X-100 on rPA activities. The DsbA co-production strain was grown in shake flasks, in LB medium at pH 6.8 at 30°C. Induction was conducted at an OD_{600} about 4 with 0.5 mM IPTG at 24°C. A mixture composed of 0.4M L-Arg, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione, and different concentrations of glycine and Triton X-100 were added at the induction. Samples were taken after 22 h of induction.

Similarity to the activities, growth of the cultures depended on concentrations of additive components. In case the additives consist of L-Arg and glutathione, when concentrations increased from 1 to 2 % (glycine) and 0.5 to 1 % (Triton X-100), OD_{600} of the cultures declined from 5 to 4, respectively, equivalent of growth inhibition of 20%. However, growth was extremely inhibited as the additives contain only 2% glycine and 1% Triton X-100 but no L-Arg and/or glutathione. In this case, growth of *E.coli* decreased approximately up to 96% (Fig. 22). Thus, the presence of L-arginine and redox system is necessary for cell growth.

Attempt to scale-up the production of rPA in bioreactor with the presence of glycine and Triton X-100 was made but the results were unsuccessful due to uncontrolled overfoam after adding Triton X-100 into the culture medium.

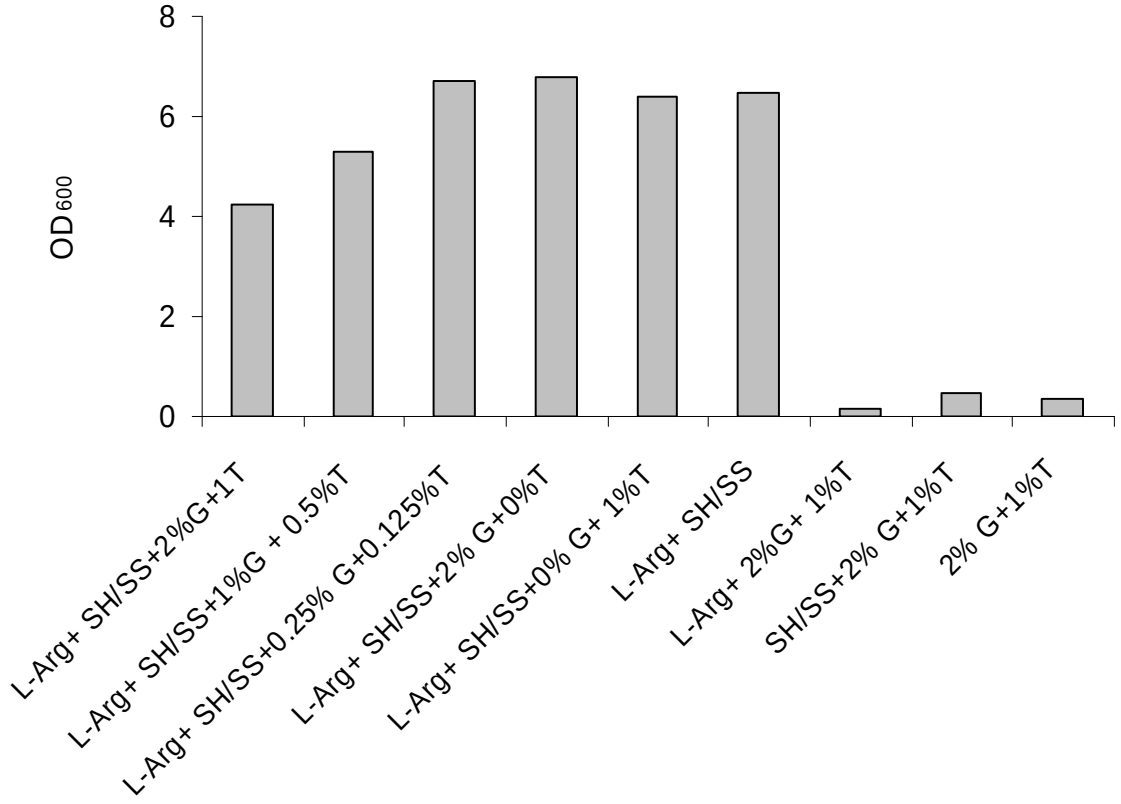

Fig.22: Effect of glycine and Triton X-100 on cell growth. The DsbA co-production strain was grown in shake flasks, in LB medium at pH 6.8 at 30°C. Induction was conducted an OD_{600} about 4 with 0.5 mM IPTG at 24°C. A mixture composed of 0.4M L-Arg, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione, and different concentrations of glycine and Triton X-100 were added at the induction. Samples were taken after 22 h of induction.

3.2 Impact of chaperones on the sizes of inclusion bodies of α-glucosidase from *Escherichia coli*

3.2.1 Effects of measurement conditions by DLS on sizes of the IBs

3.2.1.1 Effect of protein concentrations on IB size

A sample containing IBs which were isolated from sucrose gradient method was dissolved and diluted with 1mL of EDTA 3mM, pH 7.6 as a dilution factor of 1. Afterwards, the IB suspension (with dilution 1) was routinely diluted with the same solution to get higher dilution factors. Sizes of IBs in the sample at different dilutions were measured by Zetasizer 3000. The relation among parameters which were used for measurements, protein concentrations and mean size of IBs was shown in the Table 7 and Fig. 23

In general, Z-average sizes of IBs increased as samples were highely diluted corresponding to lower protein concentrations. However, at the protein concentrations between 0.05 to 0.08 mg/mL, the curve reached a plateau where IB sizes are nearly similar, indicating IBs size is independent of protein concentration. At higher protein concentrations (dilution 1), size of IBs was much larger than at lower concentrations. In contrast, at higher dilutions (from factors of 10) corresponding to concentrations lower than 0.04 mg/mL, sizes of IBs decreased dramatically. Nevertheless, the results of IB sizes obtained from high diluted samples were unrealiable due to low count rates (lower than 10 Kcps) according to the Technical note of the instrument (Malvern, 2005). Therefore, an actual mean size of IBs could be estimated approximately between 632 and 641 nm.

Table 7. Relation of parameters and size of IBs measured by Zetasizer 3000.

Dilution factor	Refraction Index (20°C)[a]	Viscosity (25°C)[b]	Count rate (Kcps)[c]	Total protein concentration (mg/mL)[d]	Mean size of IBs/ nm
1	1.3355	0.939	39.5 ± 0.2	0.135	733.5 ± 25.2
2	1.335	0.932	32.5 ± 0.3	0.084	641.8 ± 6.8
3	1.3345	0.925	25 ± 0.6	0.063	637.9 ± 4
4	1.334	0.916	22.7 ± 0.1	0.05	632.7 ± 2.8
5	1.3338	0.914	21.1 ± 0.1	0.042	614 ± 3.3
10	1.333	0.901	9.6	0.033	568.8 ± 4.4
20	1.333	0.901	2.8 ± 0.1	0.028	319.5 ± 9.4

[a] calculated according to Handbook of Chemistry and Physics (Weast et al., 1974)
[b] calculated according to the Eq.5
[c] obtained after each measurement by Zetasizer 3000
[d] obtained from Bradford assay

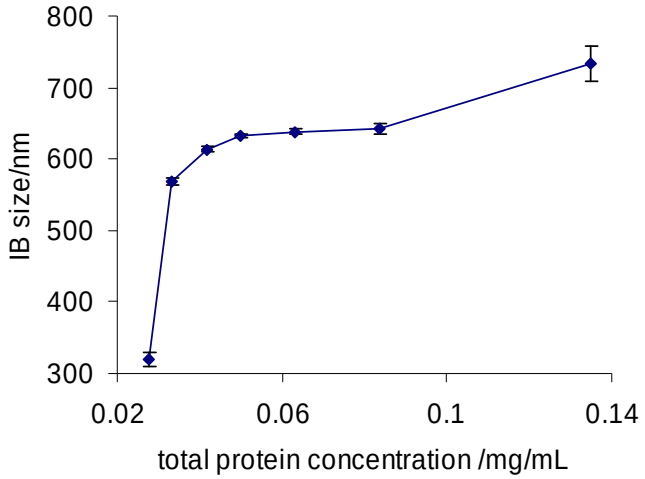

Fig. 23: Influence of protein concentration on IB size. Expression of α-glucosidase from the wild type strain after induction at 37°C for 4h by 1mM of IPTG. IBs isolated by sucrose gradient at 15000 rpm/ 2h/ 4°C. A sample containing IBs was dissolved and routinely diluted with EDTA 3mM, pH 7.6 at different dilution factors. Size distributions of IBs were measured by Zetasizer 3000 at 25°C.

3.2.1.2 Effect of cell disruption methods on IB size

Conditions and methods for cell disruption can affect the release inclusion body and size of cell debris (Wong et al., 1997a; van Hee et al., 2004).Therefore, experiments to investigate sizes of IBs obtained from cell disruption by sonicator and homogenizer were conducted.

Cells were disrupted using the homogenizer (Gaulin) and sonicator to compare the size of inclusion bodies produced from the *dnaK* mutant and Hsps co-expression strain. Cells were disrupted 2 and 5 times by the Gaulin, and 2 times at 4°C for 20 seconds, amplitude 50% and cycle 0.5 s^- by sonicator. Z-average sizes of IBs from the two strains were measued by Zetasizer 3000.

Results show that Z-average sizes of the inclusion bodies from the *dnaK* mutant and Hsps co-expression strain are approximately of 600 and 300 nm, respectively. Sizes of IBs were similar as cells were disrupted either by homogenizer at two and five passes or by sonicator (Fig. 24). It is assumed that sizes of IBs released from cell disruption probably are independent of disruption methods.

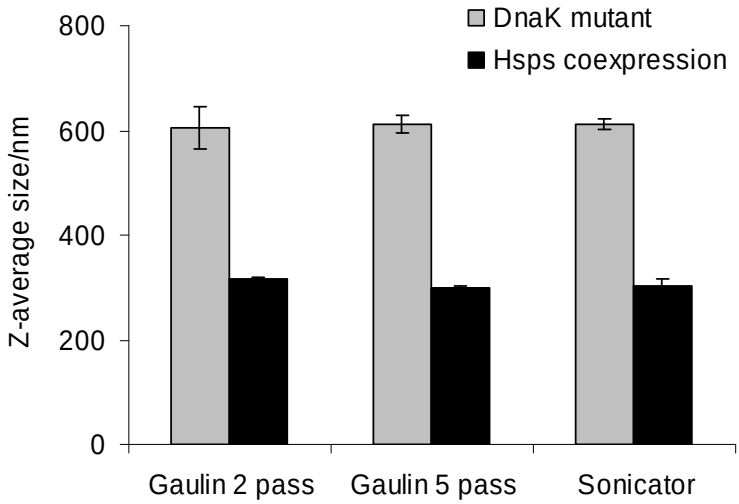

Fig. 24: Effect of cell disruption methods on IB size. The first, second and third group bars indicate z-average size of the IBs of the *dnaK* mutant and Hsps co expression strains released as the cells were disrupted 2 and 5 times by the Gaulin, and by sonicator.

3.2.2 Effects of cultivation conditions on sizes of the IBs

3.2.2.1 Impact of fermentation conditions on formation of IBs

Production of α- glucosidase from the wild type strain was conducted in bioreactor in HDF medium. Batch phase started with 5 g/L of glucose at 37°C. When glucose was depleted, in case of the fed-batch cultivation, feeding solution was fed to keep growth rate at μ_{set} of 0.12 h^{-1}, tempature was shifted to 30°C for 5h. After 1 h of feeding, induction was made with IPTG 1 mM. For batch cultivation, after the initial glucose was consumed, the protein was induced with IPTG 1mM and temperature was maintained the same at 37°C for 5h. As a control experiment, cultivation of the non-producing strain (host strain) was conducted in shake flask in LB medium, at 37°C. Cells were harvested when OD_{600} reach approximately 0.6.

IBs of the wild type strain from batch and fed-batch cultivations, and cellular proteins from the host strain incubated on shake flask were obtained as described in 2.4.2. Cells dissolved in the solution (0.1M Tris-HCl buffer, pH 7 and EDTA 1mM) with a ratio (1:5) were disrupted by the homogenizer. By sucrose gradient gradient method, IBs and other cellular components were separated from different fractions. By Zetasizer 3000, Z-average size of those components from different fractions was shown in the Table 8. Also, insoluble protein fractions of those samples after cell disruption were used for SDS-PAGE (Fig. 25).

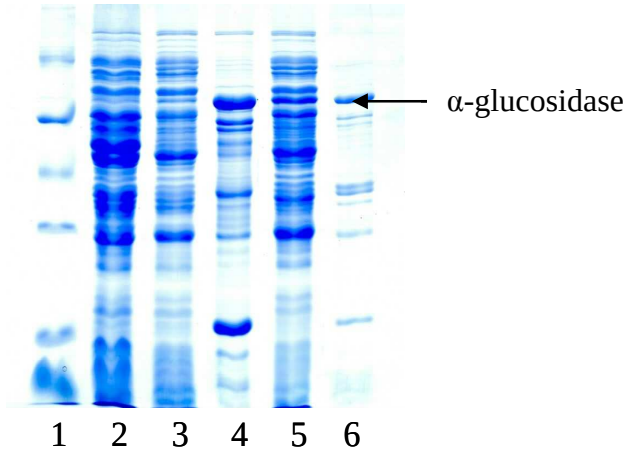

Fig. 25: SDS-PAGE pattern of the samples from bioreactor cultivation. Lane 1: marker, lane 2: host strain on shake flask, lane 3: soluble fraction of the batch induced at 37°C, lane 4: insoluble fraction of the batch induced at 37°C, lane 5: soluble fraction of the fed-batch induced at 30°C, lane 6: insoluble fraction of the fed-batch induced at 30°C.

Different mean sizes in different fractions of the strains were obtained. In general, larger sizes of the aggregated protein are distributed in lower fractions of the producing strain incubated on bioreactor. Most of cellular proteins from the host strains are located in the higher fractions (fraction 1 to 4) and in the lowest fraction which is close to bottom of the tube. In both cases, largest sizes of cellular protein particles are found in the sediment fractions (Table 8). Insoluble α-glucosidase from the batch cultivation with temperature induced at 37°C was formed much more than that of fed batch cultivation induced at 30°C as indicated in the lane 4 and 6 of Fig. 25 and Table 8.

After sucrose gradient separation, IBs were supposed to be settled as a sediment due to higher density than other cellular components (Middelberg, 2002; Wong et al., 1997b). Table 8 shows that sizes of particles in the sediment fractions from the producing strain indicate sizes of IBs and correspond to the formation levels of the insoluble protein in the SDS-PAGE pattern (Fig. 25).

Table 8. Z-average sizes of IBs in the different fractions.

Sample		fraction 1	fraction 2	fraction 3	fraction 4	fraction 5	fraction 6	fraction 7	sediment
Non producing strain	mean size(nm)	**144.6**	**146.9**	**131.2**	**101.9**	nd	nd	nd	**155.3**
	deviation +/-	7.6	2.7	7.3	22.1	-	-	-	2
batch 37°C	mean size(nm)	**127.9**	**128.6**	**369.3**	**459.4**	**496.4**	**514.7**	**389.2**	**823.8**
	deviation +/-	0.7	1.7	22.2	23.9	40.0	43.8	46.2	5.1
fedbatch 30°C	mean size(nm)	**131.2**	**216.3**	**211.4**	**232.8**	**191.1**	**259.1**	nd	**345.4**
	deviation +/-	0.6	2.44	3.8	18.6	1.9	21.8	-	7.4

3.2.2.2 Effect of medium on size of IBs after induction

In order to investigate effects of media for growing the *E.coli* strains on IB size, the wild type strain was grown on LB and HDF. Samples were taken before and after 4h of induction for measurement of IBs size by Zetasizer 3000.

Before induction, sizes of cellular protein produced by the wild type strain grown on LB and HDF medium were approximately 280 and 180 nm, respectively. Z-average sizes of IBs of the culture grown in LB and HDF medium were of about 600 and 700 nm, respectively. The differences of the particle sizes indicate that cultivation media has a little effect on the formation of IBs (Fig. 26). Due to the simplicity for preparation, LB medium was chosen for the following shake flask experiments.

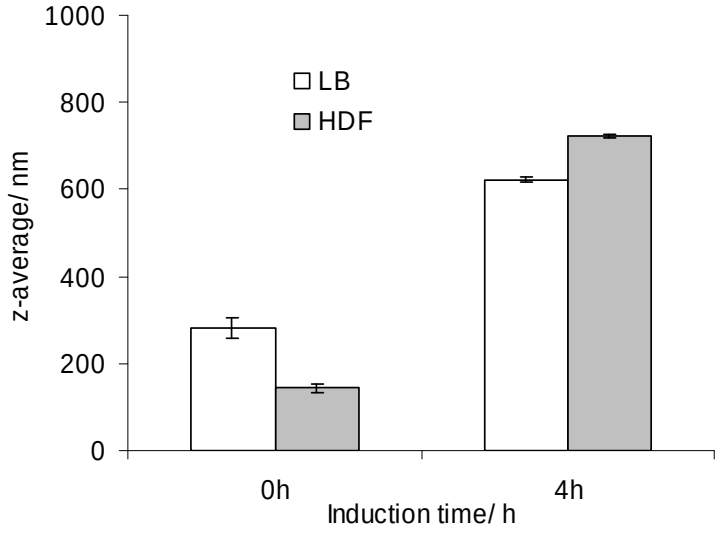

Fig. 26: Z-average sizes of inclusion bodies of the wild type strain grown in LB and HDF medium. The wild type strain was grown on LB and HDF medium in shake flask at 37°C. Expression of α-glucosidase was induced with IPTG 1mM at OD_{600} of about 0.6. Temperature was kept at 37°C for 4h after induction. Samples were taken before and after 4h of induction. Cells were disrupted 3 times by homogenizer. After isolation by sucrose gradient at 15000 rpm/ 2h/ 4°C, IBs in the sediment fraction was collected, diluted with EDTA 3mM, pH 7.6. Z-average size of IBs were analysed by the Zetasizer 3000 at 25°C.

3.2.2.3 Effect of temperature on size of IBs after induction

To examine the impact of temperature on IB size, the host strain MC4100 and wild type strain were grown at 37° C. Samples were taken before and after 4h of induction.

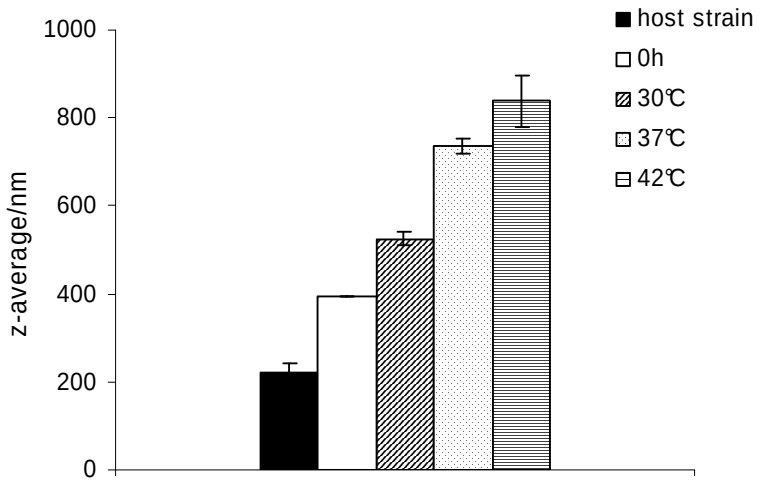

Fig. 27: Effect of temperature after induction on aggregated protein size. The wild type strain was grown on LB medium, in shake flask at 37°C. Expression of α-glucosidase was induced with IPTG 1mM at OD_{600} of about 0.6. Temperature was shifted to 30°, 37°and 42°C for 4h after induction. Samples were taken before and after 4h of induction. Cells were disrupted 3 times by homogenizer. After isolation by sucrose gradient at 15000 rpm/ 2h/ 4°C, IBs in the sediment fraction was collected, diluted with EDTA 3mM, pH 7.6. Z-average size of IBs were obtained by the Zetasizer 3000 at 25°C.The black, white, diagonal and horizontal pattern column indicate the aggregated protein sizes of the host strain, wild type strain before shifting temperature, after 4h at 30°C, 37° and 42°C, respectively.

As can be seen in the Fig.27, at higher temperature after induction, larger IB sizes were obtained. As induced at 42°C, z-average size of the IBs exceeds 800 nm, nearly 2fold larger than that induced at 30°C. This result shows temperature after induction has strong impact on the size of inclusion bodies.

3.2.3 Impact of chaperones on formation of insoluble and soluble α-glucosidase

The wild type, control, Hsps co-production and *dnaK* mutant strains were incubated in shake flask in LB medium. All strains show the strong effect of temperature on α-glucosidase activity. Lower specific activity was obtained at higher temperatures, also

growth is favoured by higher temperatures. The activities are similar for all strains except for the *dnaK* mutant (Fig. 28). With lower activities, higher amounts of α-glucosidase were found in the insoluble cell fraction. That is not a problem of synthesis, but of folding versus aggregation. While DnaK is clearly necessary for proper folding (as seen from low activity and severed aggregation already at low temperature), overproduction of DnaK+ClpB did not improve accumulation of activity (Fig. 28, 29). Similar results were reported with the highest specific α-glucosidase activity in the soluble fractions of the wild type strain at 24°, and the activities decreased as the culture induced at higher temperatures (LeThanh, 2005).

High expression of DnaK and ClpB both in soluble and insoluble fractions of the Hsps coexpression strain but little accumulation of these chaperones from other strains could be visualized by SDS-PAGE (Fig. 29, 30).

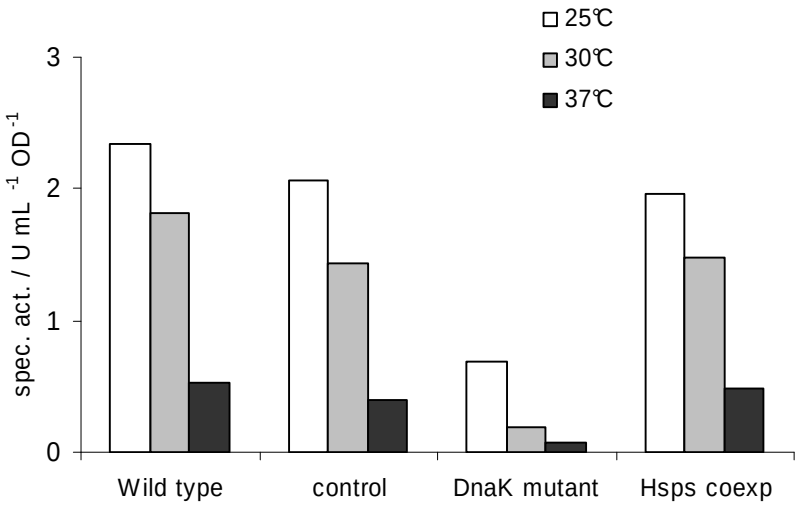

Fig. 28: Comparison of specific α-glucosidase activities in the soluble protein fractions from the wild type, control, Hsps co-expression and *dnaK* mutant strains at different temperatures after induction. The strains were grown on LB medium in shake flask at 37°C. Expression of α-glucosidase was induced with IPTG 1mM at OD_{600} of about 0.6. Temperature was kept at 37°C for 4h after induction. Samples were taken after 4h of induction. Cells were disrupted 3 times by homogenizer. After isolation by sucrose gradient at 15000 rpm/ 2h/ 4°C, IBs in the sediment fraction was collected, diluted with EDTA 3mM, pH 7.6. Z-average size of IBs were analysed by the Zetasizer 3000 at 25°C.

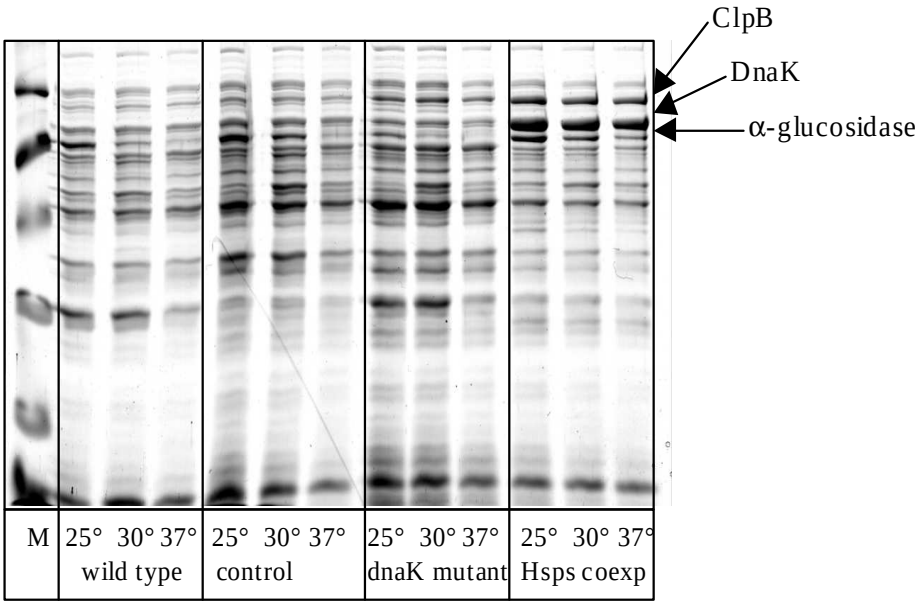

Fig. 29: SDS-PAGE pattern of soluble protein fractions from the wild type, control, *dnaK* mutant and Hsps co expression strains at different temperatures after induction.

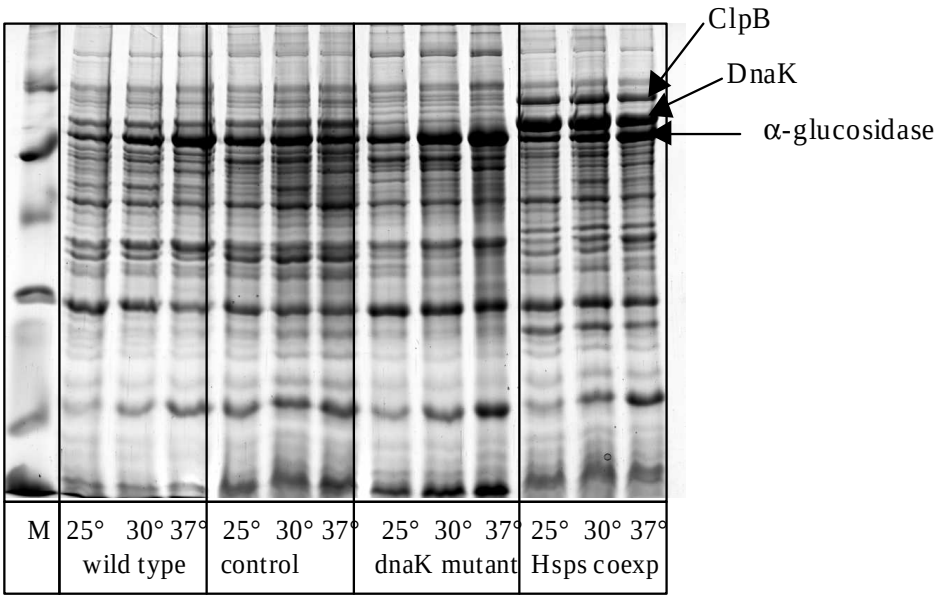

Fig. 30: SDS-PAGE pattern of insoluble protein fractions from the wild type, control, *dnaK* mutant and Hsps co expression strains at different temperatures after induction.

In general, z-average sizes of IBs decreased from the *dnaK* mutant to the wild type, control and Hsps co-expression strains at different temperature after induction. Moreover, larger IB size was observed, lower activity in the soluble fractions was obtained. In otherwords, IBs sizes are inversely proportional to α-glucosidase activity of the strains (Fig. 31).

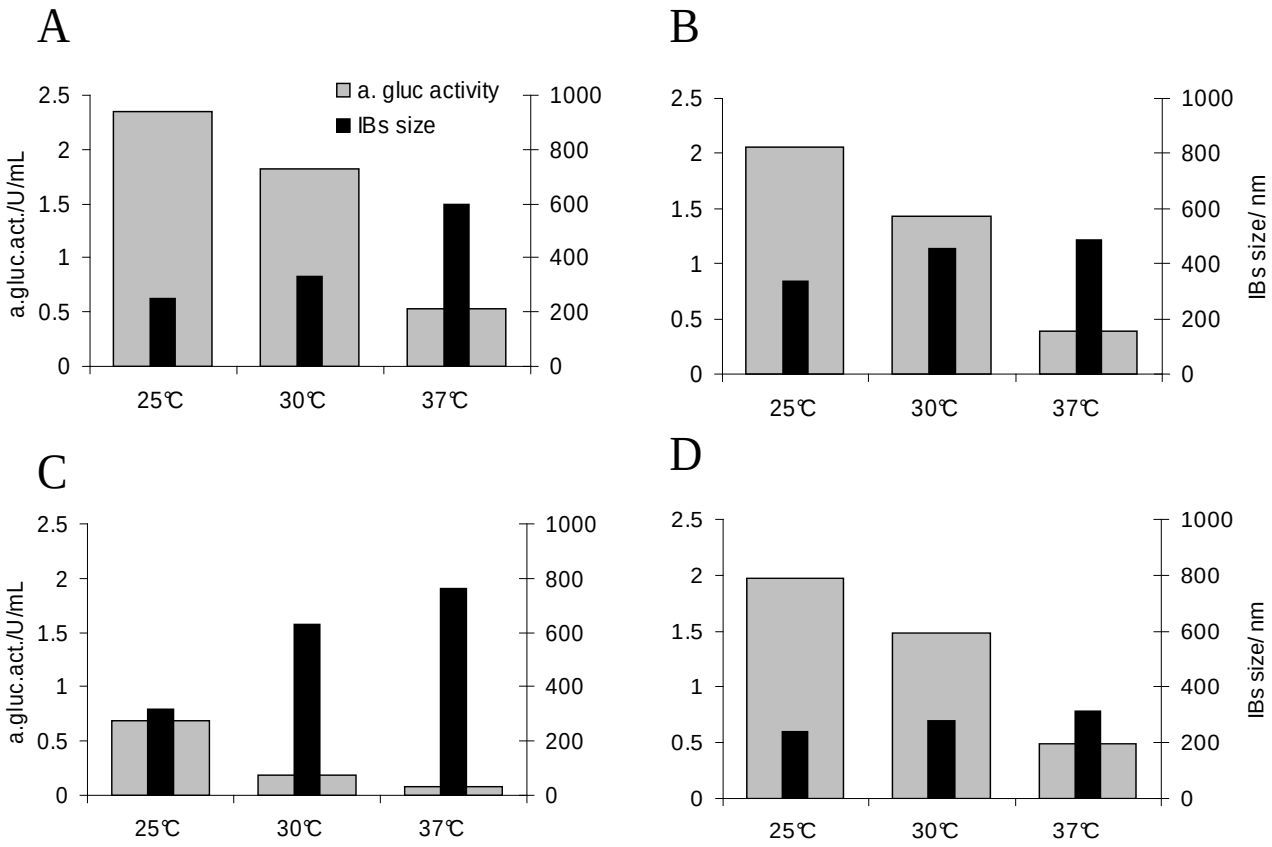

Fig. 31: Relation of size of inclusion bodies and α-glucosidase activity of the wild type (A), control (B), *dnaK* mutant (C) and Hsps co-expression (D) strains at different temperatures after induction. The strains were grown on LB medium in shake flask at 37°C. Expression of α-glucosidase was induced with IPTG 1mM at OD_{600} of about 0.6. Temperature was kept at 37°C for 4h after induction. Samples were taken after 4h of induction. Cells were disrupted 3 times by homogenizer. After isolation by sucrose gradient at 15000 rpm/ 2h/ 4°C, IBs in the sediment fraction was collected, diluted with EDTA 3mM, pH 7.6. Z-average size of IBs were analysed by the Zetasizer 3000 at 25°C.

3.2.4 Effect of co-production of the chaperones on dissolution of the inclusion bodies

IBs of the control, *dnaK* mutant and Hsps coexpression strains were resuspended and incubated in different urea concentrations from 2 to 8M. After incubation, the pellets (remnant of IBs) were separated from soluble fractions by centrifugation and determined for protein concentrations.

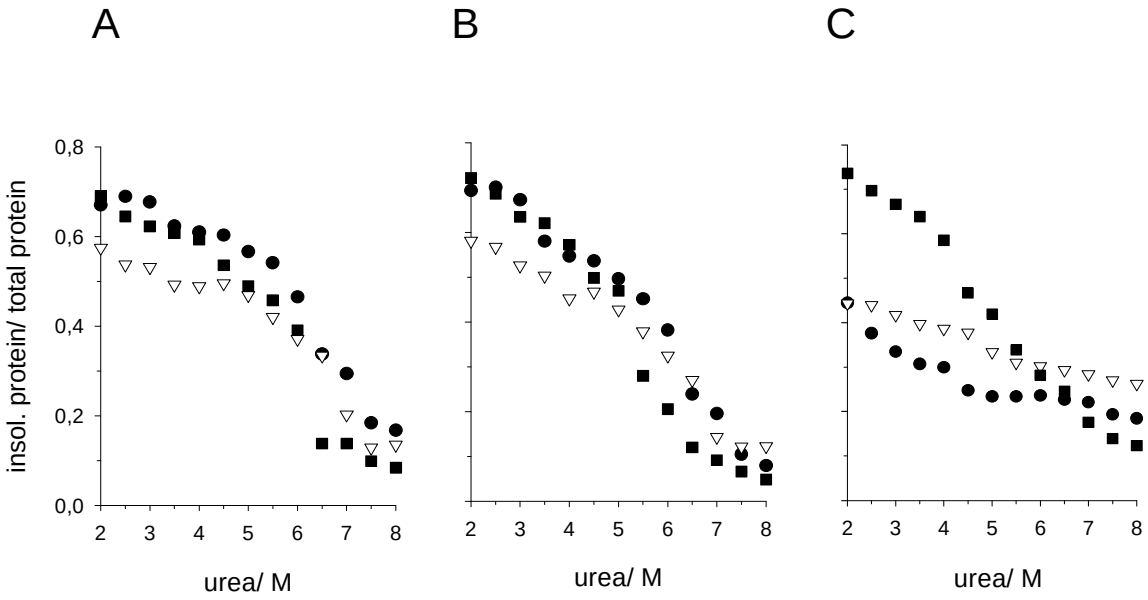

Fig. 32: Fraction of remnant aggregates after incubation of inclusion bodies with urea. (A) control, (B) *dnaK* mutant and (C) Hsps co-expression strain induced at 25°C (circle), 30°C (triangle) and 37°C (square). The control, *dnaK*⁻ and Hsps co-production strains were incubated on LB medium at 37°C. When OD_{600} reached 0.6, cultures were induced with 1 mM IPTG and subsequently incubated at 25°, 30° and 37°C for 4 hours. IBs after isolating were resuspended in different concentrations of urea. IBs after isolating were resuspended in different concentrations of urea (2 to 8 M). The IB suspension was incubated at 4°C for 2h. The soluble fractions and pellets were separated by centrifugation at 13000rpm / 45min/ 4°C. The pellets were dissolved in 150µL of Urea 8 M. Protein concentrations of resuspended pellets were analysed by Bradford assay.

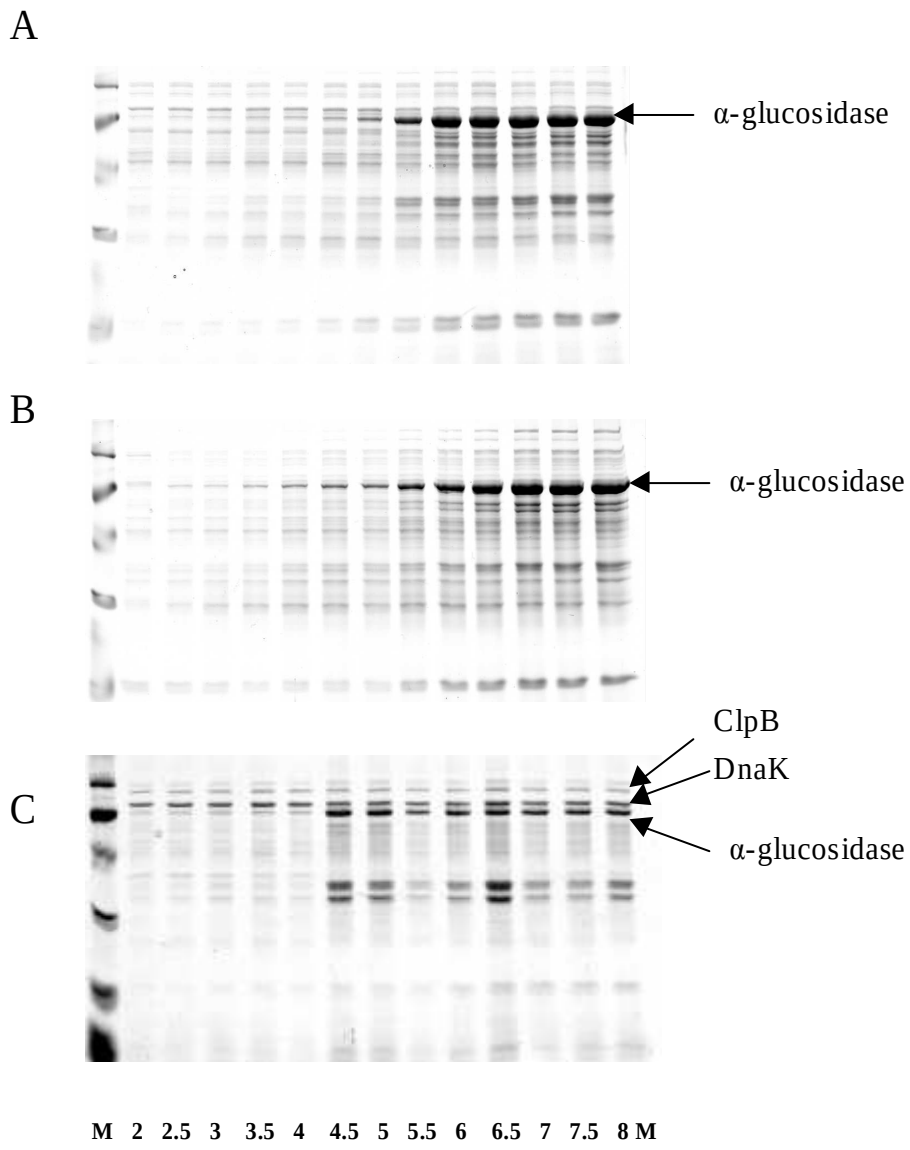

Fig. 33: Association of DnaK/ClpB with α-glucosidase in the soluble fractions after incubation of IBs with increasing concentrations of urea. SDS PAGE of soluble protein fractions after extracting IBs of the control (A), *dnaK* mutant (B) and Hsps co production (C) strain with different urea concentrations after 4h incubation at 4°C. The strains were incubated on LB medium at 37°C. When OD_{600} reached 0.6, cultures were induced with 1 mM IPTG and subsequently incubated at 37°C for 4 hours. IBs after isolating were resuspended in different concentrations of urea (2 to 8 M). The IB suspension was incubated at 4°C for 2h. The soluble fractions and pellets were separated by centrifugation at 13000rpm / 45min/ 4°C. The soluble fractions were dissolved in 150µL of Urea 8 M and loaded onto SDS PAGE.

As can be seen from Fig. 32, the Y-axis describes ratios of protein concentration of the insoluble fractions to total cell proteins indicating levels of the dissolutions of IBs. After incubation at 4°C for 2h, dissolution of IBs of the control and *dnaK* mutant strains which were induced at 25°, 30° and 37°C was partial at urea concentrations from 2 to 4M. At urea concentrations in the range of 4 to 6M, more IBs were solubilized and the dissolution reached stationary levels at urea concentrations from 6 to 8M.

Unlikely to the control and *dnaK* mutant strain, IBs of the DnaK/ClpB co-production strain induced at 25° and 30°C was only partially resolubilized even at high urea concentration, whereas more IBs of the culture induced at 37°C were dissolved than at lower temperatures (Fig. 32 C) indicating more solubilization of IBs at high temperature. High accumulation of DnaK and ClpB was detected on the SDS PAGE of the soluble fraction. The levels of those heat shock proteins were nearly constant and independent of urea concentrations (Fig. 33). Therefore, it could be assumed that there is a strong association between DnaK/ClpB and the IBs containing α-glucosidase. The co-production of DnaK/ClpB probably resulted in lower resolubilization of the IBs at high concentrations of urea when the cultures were induced at 25°C and 30°C than at higher temperature (37°C).

- To investigate the relation of chaperones (DnaK/ClpB) and size of the remnant of IBs, IBs of the control, *dnaK*$^-$ and Hsps co-production strains which were induced at 37°C for 4 hours were incubated with the increasing concentrations of urea (1- 8M). The remnant of IBs was dissolved and diluted with 3mM EDTA pH7.6. Z-averge size of IB remnant was measured by Zetasizer 3000.

After incubation with urea at concentrations in the range of 0 to 5 M, remnant of IBs obtained from the Hsps co-production strain has similar mean sizes which are about 300 nm. However, size of the Hsps co-production strain could not be detected in case of the sample incubated with higher urea concentrations. In constrast to the Hsps co-production strain, sizes of IB remnant of the control and *dnaK* mutant strain decreased from approximately 420 nm to 300 nm and 611 nm to 333 nm, respectively, as samples were incubated with urea at concentrations from 0 to 3 M. But, in case IBs were treated with urea at the concentrations from 4 M, the IBs were almost extracted (Fig. 34).

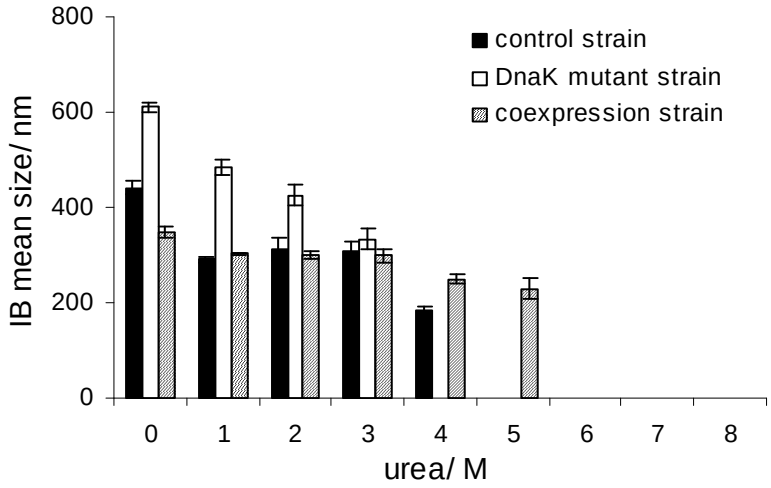

Fig. 34: Effect of Hsps on size of remnant of IBs after extracting IBs with different urea concentrations. The control, *dnaK*⁻ and Hsps co-production strains were incubated on LB medium at 37°C. When OD_{600} reached 0.6, cultures were induced with 1 mM IPTG and subsequently incubated at 37°C for 4 hours. IBs after isolating were resuspended in different concentrations of urea. IBs after isolating were resuspended in different concentrations of urea (2 to 8 M). The IB suspension was incubated at 4°C for 2h. The soluble fractions and pellets were separated by centrifugation at 13000rpm / 45min/ 4°C. The pellets were dissolved and diluted with 3mM EDTA pH7.6 . Size of remnant of IBs was measured by Zetasizer 3000 at 25°C.

4. DISCUSSION

4.1 Cultivation strategy for secretion and export of rPA from *Escherichia coli*

4.1.1 Effect of DsbA co-production, the additive and conditions on culture growth and rPA activities

In order to enhance soluble protein production, there are different approaches including overexpression of disulfide oxidoreductase “Dsb” family, co-production of molecular chaperones or other means as mentioned in details in the section 1.1.2. Production of full-length tPA by coproduction of the isomerase DsbC was enhanced more than overproduction of DsbA but high level of DsbC was found to be harmful to the cell as the growth of the culture stopped within 3-4h after induction (Qiu et al., 1998). Therefore, coproduction of a DsbA C33S mutant (obtained from labor stock), in which Cysteine 33 in the active site of DsbA (codon TGC) was changed to serine (codon AGC), with rPA was evaluated in order to compare its efficiency with DsbA wildtype coproduction. Similar results of rPA activities with overproduction of the wildtype and mutant DsbA, with slightly higher rPA activities with the wildtype DsbA were obtained (data not shown). Hence, coproduction of wildtype-DsbA was used for experiments in shake flask and bioreactor scale in this study.

Although DsbA was overexpressed, yields of native secreted heterologous proteins could only be increased by addition of glutathione (Wunderlich and Glockshuber, 1993) or L-arginine (Winter et al., 2000) to the growth media. This was also proved in this thesis, although DsbA was co-producted, without addition of L-Arg and glutathione, yields of secreted rPA was much lower than the cultures were supplemented with these additives. However, co-production of DsbA had a remarkable influence on the secretion of native rPA in the periplasm and especially on the export of the active protein to the culture supernatant. Addition of L-Arg and glutathione did not help the control strain to produce as much native rPA as the DsbA co-production strain, although the additives was added to the culture in this case (Fig. 7).

L-arginine is proposed not to facilitate refolding, but probably suppresses aggregation of the proteins during refolding (Arakawa and Tsumoto, 2003). Glutathione affects the

redox state of the periplasm therefore, influences correct disulfide bond formation (Georgiou and Valax, 1996). Accumulation of rPA was increased as the medium was supplemented with 0.4M L-Arg, and equimolar mixture with 2.5 mM each of reduced and oxidized glutathione (Fig. 7). For the fed-batch fermentation, addtition of L-Arg and redox system could enhance high release of native rPA into culture medium. Yields of native rPA in the periplasm and in the culture medium as the culture added with L-Arg and redox system increased more than 123 and 2250 fold, respectively, than that of the culture without any additive supplementation (Fig. 12). In comparison to the result from shake flask experiment, in bioreactor scale, redox system had higher effects on the yields of native rPA obtained from the periplasm and culture medium. Yields of native rPA in the periplasm and culture supernatant of the batches with addition of L-arginine and redox system increased about 50 and 87 fold, respectively, compared to the batch without addition of redox system (only with addition of L-Arg) (Fig. 12).

Glycine and Triton X-100 supposed to be able to alter morphology leading the disruption of cell membrane integrity of *E.coli*. Glycine has stimulatory effects on production and release of protein. Besides, glycine can induce bacteriolysis which enhances the leakage of proteins into culture medium (Yang, 1998).The export of proteins from *E.coli* into culture media was enhanced by glycine (Fujiyama et al., 1995; Ikura, 1989; Yang et al., 1998). By adding 1% Triton X-100, the secretion of fusion protein SFV/TNF-$^{\alpha}$ was increased 38fold. However, Triton X-100 is only appropriate for small protein like SFV/TNF-$^{\alpha}$ (43 kDa) whereas larger protein β-galactosidase (118 kDa) was not released to the medium (Yang, 1998). Thus, rPA protein which has molecular weight about 40 kDa can be released into the culture medium as the cultures were treated with this detergent. Similar to the results reported by Yang et al., 1998, adding 2% Glycine and 1% Triton X-100 improved yields of rPA in the periplasm and culture supernatant. However, this could only be obtained as L-Arg and glutathione were also added (Fig. 21). However, this is not clear about association effects of these substances on the levels of secreted proteins.

Growth of the cultures was affected by the additives. As the cultures were added with L-Arg, growth rate was impaired more than with glutathione (Fig. 8). Whereas glycine

at low concentrations in the range of 6-12 mM improved the gowth of *E.coli* (Han et al., 2002), at higher concentrations from 0.05 to 1.33 M, up to 80% of bacteria growth can be inhibited (Hammes et al., 1973). In this study, results showed that glycine and Triton X-100 also impaired cell growth. When concentrations of these chemicals increased from 1 to 2 % (glycine) and 0.5 to 1 % (Triton X-100) growth was decreased about 20%. Although L-Arg and glutathione impaired the growth of cultures, approximately 96% of growth was extremely inhibited as the additives contain no L-Arg and/or glutathione (Fig. 22). Thus, L-arginine and redox system seem to be benificial for cell growth in this case.

4.1.2 Cultivation strategies for the production of rPA in the periplasm and culture medium

In this study, the production of rPA was optimized in fed-batch cultures of recombinant *E. coli* and discussed in detail below (Nguyen et al., 2006).

In the presence of L-arginine and glutathione and with overproduction of DsbA, a slow but steady product accumulation and leakage of rPA to the culture supernatant were observed. Early after induction, rPA accumulated as inclusion bodies in the periplasm and continued to keep a major portion of rPA during cultivation.

In the thesis, the different feeding strategies of glucose in the presence of L-arginine and redox system that inhibits glucose uptake, were investigated. After addition of L-arginine and induction, glucose uptake was impaired and this led to accumulation of glucose in the medium. Consequently, the specific CER decreased afterwards. As a common phenomenon, high glucose concentrations result in formation of acetate, which may inhibit culture growth or production. Changes in the respiration rate can be occured during recombinant protein production, e.g. glucose uptake was impaired during production of α-glucosidase (Neubauer et al., 2003) or protein degradation was accelerated after stress responses (Schmidt et al., 1999; Hoffmann et al., 2001; Hoffmann et al., 2004). However, here, the inhibition of the respiratory activity had no remarkable relation with recombinant protein production but glucose accumulation in

the culture medium was observed. Thus, the specific CER was found to be a useful parameter to detect problems related to high glucose concentrations.

Attempt to avoid glucose accumulation by slowing down the glucose feeding was not appropriate. With a feeding rate that maintained glucose limited conditions for most of the production phase, cell growth and therefore the volumetric product yield were considerably reduced compared to faster feeding (Fig. 13). On the other hand, glucose accumulation to inhibitory levels could be prevented by temporary suspension of the feeding, however, this application did not help an increase of rPA yields (data not shown). Consequently, glucose feeding rates in the range of 0.06-0.2 h^{-1} had little influence on the specific rPA activity profiles, in contrast to the strong impact of the feeding rate on cytoplasmic folding of a recombinant protein (Curless et al., 1989; Fu et al., 1993; Hellmuth et al., 1996; Le Thanh and Hoffmann, 2005; Seo and Bailey 1986; Turner et al., 1994). Avoiding glucose accumulation by slow feeding was inappropriate, as growth or product accumulation, respectively, were affected. However, fed-batch cultures induced during limited feeding followed by gradual glucose accumulation were highly efficient, both in terms of specific and volumetric activities compared to shake flask cultivation.

Interestingly, at low feeding rate of 0.06 h^{-1}, there was a significant difference between cultures induced during fed-batch phase followed by gradual glucose accumulation and a culture induced during a prolonged batch phase. Cultures induced during a prolonged batch phase followed by limiting glucose feeding (or even followed by 3 h glucose starvation before glucose feeding) secreted large amounts of active rPA (Fig. 16). While the product concentration in the periplasm decreased after reaching a maximum or remained permanently at low level, the final volumetric activities in the culture supernatant and in the periplasm reached about 4 times as high as the activities obtained in the cultures induced during fed-batch phase. However, induction of rPA during prolonged batch phase at higher feeding rate of 0.12 h^{-1} did not enhance the yield of rPA in the culture medium. Nearly 2fold of native rPA was accumulated in the culture supernatant as culture was induced during fed-batch phase compared to induction at prolonged batch phase. (Fig.17 D).

The presence of IBs in the periplasm is found to interfere with proper folding of proteins. Thus, release of proteins into the supernatant may enhance protein folding from the negative influence of the IBs. By overproduction of a periplasmic protease (Lin et al., 2001; Pan et al., 2001) or release of the product into the supernatant using the *ompA* signal sequence (Mansoroi et al., 2001), yields of the active products were increased.

Enhancing release of the product in batch cultures compared to fed-batch cultures was also reported using a hydrolase fused to the *ompA* signal sequence and production at increased temperature (Dresler et al., 2006). Consequently, yields of the product in fed-batch cultivation were twofold (specific activity) and tenfold (volumetric activity) higher than in batch cultivation. In addition, the release of the protein was enhanced during fast growth (Dresler et al., 2006). In this study, the induction of rPA in a prolonged batch phase was increased only at the low growth rate (0.06 h^{-1}). By this strategy, high volumetric rPA yields and efficient release to the culture medium were both obtained.

The production kinetics was found to be relatively slow but steady product accumulation and leakage of rPA to the culture medium were observed. The product accumulation in the insoluble cell fraction with a similar kinetics as in the cell pellets remaining after osmotic shock indicates that the product mainly accumulated in an aggregated form. As rPA has the same size in all fractions, indicating cleavage of the signal sequence and proper export, the aggregates seemed to be located in the periplasm (Fig. 19).

If the release of the product would be limited due to low level of protein export to the periplasm, enhanced release after induction during prolonged batch phase might be a result of the cell lysis. However, the total protein concentration in the supernatant of the bach-induced culture was two times lower than in the fed-batch-induced culture indicating no extensive cell lysis (Fig. 19). Similarly, proteins found in the culture supernatant of the batch and fed-batch cultivations have been previously shown to be released from the periplasm (Dresler et al., 2006; Rinas and Hoffmann, 2004).

Moreover, from another report, cell lysis as a cause of enhanced product release in batch cultures has been positively excluded (Dresler et al., 2006).

Factors e.g. cultivation at low temperature and use of the *pelB* signal sequence enhanced the release of proteins to the culture medium (Georgiou and Segatori, 2005). In this study, these factors were also applied as the *E. coli* strain carrying the plasmid pET20b(+)_rPA consisting the *pelB* signal sequence was cultivated at 24°C after induction. A prolonged cultivation to the stationary phase or an interruption of the glucose feeding could stimulate protein leakage from the periplasm to the culture supernatant (Miksch et al., 2002). Besides, a remarkable release of product after glucose depletion in batch cultures as well as a large accumulation of the product in the periplasm and also in the cytoplasm during fed-batch cultivation were observed (Dresler et al., 2006). Nevertheless, a starvation between glucose depletion and feeding start did not increase the release of rPA in the process (Fig. 16D).

Although a fast production under unlimited conditions (batch phase) is stressful to the cells leading to low levels of active proteins by cytoplasmic production (Hoffmann et al., 2004; Lethanh and Hoffmann, 2005), the way to release protein from the periplasm under prolonged batch phase followed by limited feeding at the low feeding rate seems to be the appropriate process for production of active rPA. In addition, if high binding affinity of the protein would be possible, the release of the product to the culture medium may make extraction procedures become unnecessary. This probably facilitates downstream processing by using the expanded bed chromatography (Anspach et al., 1999).

4.2 Impact of chaperones on the sizes of inclusion bodies of α-glucosidase

At high concentrations, size of the particles may depend on the sample concentration leading to a reduction of the actual particle size due to higher viscosity value (Kaszuba et al., 2004). This is proved by the Eq. 1, size of paricles is inversely proportional to viscosity of the solutions. Thus, when visosity increases, *d* (H)- hydrodynamic diameter of the particles will be declined. Moreover, another parameter which has remarkable influence on *d* (H) is the diffusion coefficient (*D*). If the particles are suspended in a

solution of higher viscosity, e.g., ethylene glycol, treacle, their movement will be slowed down (Rawle, 1995) resulting in lower the diffusion coefficient. Consequently, the hydrodynamic diameter of the particles will be increased. Nevertheless, as can be observed from Fig 23, Table 7, Z-average of α-glucosidase IBs increased as samples were highly diluted corresponding to lower protein concentrations. Here, although viscosity of the diluted samples slightly declined at higher dilution, Z-average size of IBs was decreased.

The release of inclusion body can be influenced by some parameters e.g. homogenization pressure, number of homogenization and biomass concentration (van Hee et al., 2004). Cell disruption conditions have some effects on product release, cell debris size (Kula et al., 1990; Siddiqi et al., 1996; Wong et al., 1997a). Increasing the number of homogenizer passes leads to smaller cell debris size while unaltered inclusion body size (Wong et al., 1997a). Similarity to this result, sizes of IBs also did not change and were independent of the disruption methods as the cells were disrupted either by homogenizer at two and five passes or by sonicator (Fig. 24).

With the density gradient separation, IBs of *E.coli* with density of 1.3-1.4 g mL^{-1} can be separated from cell debris (1.085 g mL^{-1}), lighter outer membrane vesicles (1.224 g mL^{-1}) and denser ribosomes (1.5 g mL^{-1}) (Middelberg, 2002; Wong et al., 1997b). Thus, here, it could be assumed that after separation by sucrose gradient at high centrifugation speed, most of α-glucosidase IBs and ribosomes sedimented on the lowest part of centrifugation tubes where the density of sucrose 55% is 1.26 g mL^{-1}. Although density of the ribosome is higher than IBs, size of an *E.coli* ribosome is approximately of 20 nm (Stryer, 1996), that is much smaller than size of IB leading to no effect on measurement of size distributions of IBs by DLS.

The physical association of IbpAB with IBs of the recombinant human renin (Allen et al., 1992) and α -glucosidase (Le, 2005) after incubation of the purified IBs with increasing concentrations of urea was investigated. The results revealed that a strong association between ibpAB and human renin inclusion bodies but no tight relation of α-glucosidase and the IbpAB were observed. In this thesis, there was a strong association between DnaK/ClpB and the IBs containing α-glucosidase. High accumulation of DnaK

and ClpB was detected on the SDS gel of the soluble fraction. The expression levels of those heat shock proteins were nearly constant and independent of urea concentrations. However, ibpAB was not visualized by SDS-PAGE due to their low accumulation (Fig. 33). Higher resolubilization of the IBs under higher urea concentrations was observed in the presence of DnaK/ClpB when the cultures were induced at 37°C than at 25°C and 30°C (Fig. 32). Therefore, the co-production of DnaK/ClpB may enhance the resolubilization of the IBs at high urea concentrations when the cultures were induced at high temperature (37°C).

5. SUMMARY

In this thesis, production of soluble rPA protein in the periplasm and culture supernatant with the assistance of DsbA as a coexpressed helper protein in shake flask and bioreactor scale was investigated. For the expression of α-glucosidase as a model protein in the cytosol, a new technique DLS using the Zetasizer 3000 (Malvern, UK) was applied to determine the impact of production conditions on the size distributions of IBs of α-glucosidase and on the solubility of the protein.

- DsbA co-expression, appropriate cultivation conditions (temperature, pH medium and culture media) and addition of different additives (L-arginine, redox system, glycine and Triton X-100) enhanced yield of native rPA in the periplasm and in the culture supernatant in shake flask scale. The additives e.g. glycine and Triton X-100 had a remarkable impact on the secretion of native rPA into periplasm and culture supernatant. The presence of L-arginine and redox system with glycine and Triton X-100 was necessary for cell growth. Only with the presence of 0.4M L-Arg, an equimolar mixture with 2.5 mM each of reduced and oxidized glutathione, addition of 2% glycine and 1% Triton X-100 to the culture after induction, rPA activities increased not only in the periplasm but also in the culture medium. Compared to the experiments without the use of glycine and Triton X-100, accumulation of active rPA in the periplasm and culture supernatant reached 2.6 and 100 µg/L increasing 10 and 12fold, respectively. However, proper cultivation conditions in bioreactor scale need to be developed to control foam during fermentation.

The production of native rPA in the periplasm and culture supernatant in 8L fermentator on defined medium was optimized in the presence of different additives.
Growth rates had strong effect on the secreted protein yield in the periplasm. rPA accumulation was slow within 5-10 h after induction but steady for another 40 h. Feeding rates aiming at specific growth rates between 0.06 and 0.2 h^{-1} did not influence the specific rPA activity profiles.

Specific growth rates and the time of induction influenced the native recombinant protein in the periplasm and in the culture supernatant. In fed-batch cultivation, slow feeding at 0.06 h^{-1} retarded glucose accumulation but also decreased final OD leading

low volumetric rPA activity. As rPA production induced during a prolonged batch phase followed by slow feeding at 0.06 h^{-1}, the specific activity in the periplasm was lower than with induction in the fed-batch phase. However, rPA activities up to 1300 µg/ L (about 3 times higher than with induction in fed-batch mode) were found in the culture supernatant. But, in contrast to low feeding rate of 0.06 h^{-1}, at higher growth rate of 0.12 h^{-1}, induction of rPA during prolonged batch phase did not enhance the yield of rPA in the culture medium. Volumetric activities in the culture medium of the cultivations with the induction at the prolonged batch phase and fed-batch phase were of 500, 1600 µg/L, respectively.

- By using Zetasizer 3000, IBs size of α-glucosidase formed in cytosol of *E.coli* during shake flask and bioreactor cultivation were measured.

Cell disruption methods (sonication and homogenizer) had no effect on sizes of the IBs. Z-average sizes of the inclusion bodies from the *dnaK* mutant and Hsps co-expression strain were approximately of 600 and 300 nm, respectively.

Cultivation media (LB, HDF medium) had a little influence on size of IBs. Z-average sizes of IBs of the culture grown in LB and HDF medium were of about 600 and 700 nm, respectively.

Temperature had a strong impact on the formation of inclusion bodies: larger sizes of IBs were obtained at higher induction temperature. Z-average size of the IBs reached about 800 nm as the culture was induced at 42°C , nearly 2 times larger than that induced at 30°C.

Solubility of α-glucosidase of the control, *dnaK* mutant and Hsps co-expression strain was inversely proportional to the size of their IBs. Lower soluble protein activities were obtained, larger sizes of IBs were observed.

Co-production of the chaperones e.g. DnaK and ClpB influenced the dissolution of the IBs and size of the remnant IBs after incubation of IBs with increasing concentrations of urea.

The co-production of DnaK/ClpB probably promoted more resolubilization of the IBs at high urea concentrations when the cultures were induced at high temperature (37°C) than at lower temperatures (25°C and 30°C).

6. REFERENCES

Agerkvist I, Enfors SO. Characterization of *E.coli* cell disintegrates from a bead mill and high pressure homogenizers. Biotechnol. Bioeng. 1990, 36, 1083-1089

Akiyama Y, Kamitani S, Kusukawa N and Ito K. In vitro catalysis of oxidative folding of disulfide-bonded proteins by the *Escherichia coli* dsbA (ppfA) gene product. J Biol Chem. 1992, 22440-22445

Allen SP, Polazzi JO, Gierse JK and Easton AM. Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli*. J Bacteriol. 1992, 174, 6938-6947

Arneborg N, Salskov-Iversen AS and Mathiasen TE. The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. Appl Microbiol Biotechnol. 1993, 39, 353-357

Anspach FB, Curbelo D, Garke G and Deckwer WD. Expanded-bed chromatography in primary protein purification. J Chromatogr A. 1999, 865, 129-144

Arakawa T, Tsumoto K. The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. Biochem Biophys Res Commun. 2003, 25, 304(1), 148-52.

Åslund F, Berndt KD and Holmgren A. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. J Biol Chem.1997, 272(49), 30780-30786.

Bader MW, Xie T, Yu CA and Bardwell JC. Disulfide bonds are generated by quinone reduction. J Biol Chem. 2000, 275, 26082-26088

Bailey SM, Blum PH and Meagher MM. Improved homogenization of recombinant *Escherichia coli* following pretreatment with guanidine hydrochroride. Biotechnol. Prog. 1995, 11, 533-539

Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol. 1999, 10, 411-421

Baneyx F, Mujacic M. Recombinant protein folding and misfolding in *Escherichia coli*, Nat Biotechnol. 2004. 22, 1399-1408

Bardwell JC, Lee JO, Jander G, Martin N, Belin D and Beckwith J. A pathway for disulfide bond formation *in vivo*. Proc Natl Acad Sci USA. 1993, 90, 1038-1042

Bardwell JC, McGovern K and Beckwith J. Identification of a protein required for disulfide bond formation in vivo. Cell. 1991, 67, 581-589

Barrett CML, Ray N, Thomas JD, Robinson C and Bolhuis A. Quantitative export of a reporter protein, GFP, by the twin-arginine translocation pathway in *Escherichia coli*. Biochem Biophys Res Comm. 2003, 304, 279-284

Bessette PH, Åslund F, Beckwith J and Georgiou G. Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasma. Proc Natl Acad Sci USA. 1999, 96, 13703-13708

Bessette PH, Cotto JJ and Gilbert HF, Georgiou G. In vivo and in vitro function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG. J Biol Chem. 1999, 274, 7784-7792

Boström M, Markland K, Sandén AM, Hedhammar M, Hober S and Larsson G. Effect of substrate feeding rate on recombinant protein secretion, degradation and inclusion body formation in *Escherichia coli*. Appl Microbiol Biotechnol. 2005, 68, 82-90

Bothmann H, Plückthun A. Selection for a periplasmic factor improving phage display and functional periplasmic expression. Nat Biotechnol. 1998, 16, 376-380

Bothmann H, Plückthun A. The periplasmic *Escherichia coli* peptidylprolyl *cis, trans*-isomerase FkpA.I. Increased functional expression of antibody fragments with and without *cis*-prolines. J Biol Chem. 2000, 275, 17100-17105

Brehmer D, Gässler C, Rist W, Mayer MP and Bukau B. Influence of GrpE on DnaK substrate interactions. J Bio Chem. 2004, 279(27), 27957-27964

Brinkmann U, Mattes RE and Buckel P. High-level expression of recombinant genes in *Escherichia coli* is dependent on the availability of the *dnaY* gene product. Gene 1989, 85, 109-114

Buchner J, Walter S. Molecular chaperones-cellular machines for protein folding. Angew Chem Int. 2002, 41, 1098-1113

Bukau B, Horwich AL. The Hsp70 and Hsp60 chaperone machines. Cell.1998, 9, 351-366

Carrel, Lomas DA. Lancet.1997, 350,134-138

Carrio MM, Villaverde A. Protein aggregation as bacterial inclusion bodies is reversible. FEBS. 2001, 489, 29-33

Carrio MM, Villaverde A. Role of molecular chaperones in inclusion body formation. FEBS Letters. 2003, 537, 215-221

Chen C, Snedecor B, Nishihara JC, Joly JC, McFarland N, Andersen DC, Battersby JE and Champion KM. High-level accumulation of a recombinant antibody fragment in the periplasm of *Escherichia coli* requires a triple-mutant (*degP prc spr*) host strain. Biotechnol Bioeng. 2004. 85, 463-474

Choi JH, Jeong KJ, Kim SC and Lee SY. Efficient secretory production of alkaline phosphatase by high cell density culture of recombinant *Escherichia coli* using the *Bacillus* sp. endoxylanase signal sequence. Appl Microbiol Biotechnol. 2000. 53, 640-645

Choi JH, Lee SY. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol. 2004, 64, 625-635

Chou CY, Lin YL, Huang YC, Sheu SY, Lin TH, Tsay HJ, Chang, GG and Shiao MS. Structural variation in human apolipoprotein E3 and E4: secondary structure, tertiary structure, and size distribution. J Biophys. 2005. 88(1), 455- 466

Clark ED. Protein refolding for industrial processes. Curr.Opin. Biotechnol. 2001, 12, 201-207

Connolly L, De Las Penas A, Alba BM and Gross CA. The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. Genes Dev. 1997, 11, 2012-2021

Curless CE, Forrer PD, Mann MB, Fenton DM and Tsai LB. Chemostat study of kinetics of human lymphokine synthesis in recombinant *Escherichia coli*. Biotechnol Bioeng. 1989, 34, 415-421

Curless CE, Pope J, Loredo L and Tsai LB. Effect of preinduction specific growth rate on secretion of granulocyte macrophage colony stimulating factor by *Escherichia coli*. Biotechnol Prog. 1994, 10, 467-471

De Marco A, de Marco V. Bacteria co-transformed with recombinant proteins and chaperones cloned in independent plasmids are suitable for expression tuning. J Biotechnol. 2004, 8,109(1-2), 45-52

De Leeuw E, Granjon T, Porcelli I, Alami M, Carr SB, Müller BM, Sargen F, Palmer T and Berks BC. Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes. J Mol Bio. 2002, 4, 322(5), 1135-1146

DeLisa MP, Tullman D and Georgiou G. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. Proc Natl Acad Sci USA. 2003, 100, 6115-6120

Diez-Gonzalez F, Karaibrahimoglu Y. Comparision of the glutamate-, arginine- and lysine-dependent acid resistance systems in *Escherichia coli* O157:H7. Appl Microbiol. 2004, 96, 1237-1244

Dresler K, van den Heuvel J, Müller RJ and Deckwer WD. Production of a recombinant polyester-cleaving hydrolase from *Thermobifida fusca* in *Escherichia coli*. Bioprocess Biosys Eng. 2006, 29, 169-183

Ehrnsperger M, Gräber S, Gaestel M and Buchner J. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. EMBO J. 1997, 16, 221-229

Fernandez LA, Sola I, Enjuanes L and De Lorenzo V. Specific secretion of active single-chain Fv antibodies into the supernatants of *Escherichia coli* cultures by use of the hemolysin system. Appl Environ Microbiol. 2000, 66, 5024-5029

Fu J, Wilson DB and Shuler ML. Continous , high level production and excretion of a plasmid-encoded protein by *Escherichia coli* in a two-stage chemostat. Biotechnol Bioeng. 1993, 41, 937-946

Fu Z, Hamid SB, Razak CA, Basri M, Salleh AB and Rahman RN. Secretory expression in *Escherichia coli* and single-step purification of a heat-stable alkaline protease. Protein Expr Purif. 2003, 28, 63-68

Fujiyama K, Maki H, Kinoshita S and Yoshida T. Purification and characterization of the recombinant alginate lyase from *Pseudomonas* sp. leaked by *E.coli* upon addition of glycine. FEMS Microbiol Lett. 1995, 125, 19-24

Georgiou G, Segatori L. Preparative expression of secreted proteins in bacteria: status report and future prospects. Curr.Opin.Biotechnol. 2005, 16, 538-545

Georgiou G, Valax P. Expression of correctly folded proteins in *Escherichia coli*. Curr.Opin.Biotechnol. 1996, 7, 190-197

Glover JR., Lindquist S. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell. 1998, 94: 73-82

Goloubinoff P, Mogk A, Zvi AP, Tomayasu T and Bukau B. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc* Natl Acad Sci USA. 1999, 96: 13732-13737

Gorman PM, Yip CM, Fraser PE and Chakrabartty A. Alternate aggregation pathways of the Alzheimer beta-amyloid peptide: Abeta association kinetics at endosomal pH. J Mol Biol. 2003, 325(4), 743-757

Grauschopf, Fritz A and Glockshuber R. Mechanism of the electron transfer catalyst DsbB from *Escherichia coli*. Embo J. 2003, 22 , 3503-3513

Hammes W, Scheifer K and Kandler O. Mode of action of glycine on the biosynthesis of peptidoglycan. J Bacteriol. 1973, 116,1029-1053

Han L, Doverskog M, Enfors SO and Häggström L. Effect of glycine on the cell yield and growth rate of *Escherichia coli*: evidence for cell-density-dependent glycine degradation as determined by ^{13}C NMR spectroscopy. J Biotechnol. 2002, 92, 237-249

Han MJ, Park SJ, Park TJ and Lee SY. Roles and applications of small heat shock proteins in the production of recombinant proteins in Escherichia Coli. Biotechnol Bioeng. 2004, 88(4), 426-436

Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in *Escherichia coli*. Trends Biotechnol. 1998.16, 54-60

Hayhurst A, Harris WJ. *Escherichia coli* Skp chaperone coexpression improves solubility and phage display of single-chain antibody fragments. Protein Expr Purif. 1999, 15, 336-343

Hellmuth K, Korz DJ, Sanders EA and Deckwer WD. Effect of growth rate on stability and gene expression of recombinant plasmids during contious and high cell density cultivation of *Escherichia coli* TG1. Biotechnol. 1994, 32, 289-298

Hoffmann F, Rinas U. On-line estimation of the metabolic burden resulting from synthesis of plasmid-encoded and heat-shock proteins by monitoring respiratory energy generation. Biotechnol Bioeng. 2001, 333-340

Hoffmann F, Rinas U. Roles of Heat shock chaperones in the production of recombinant proteins in *Escherichia coli.* Adv Biochem Engin Biotechnol.2004, 89, 143-161

Hoffmann F, van den Heuvel JJ, Zidek N and Rinas U. Minimizing inclusion body formation during recombinant protein production in *Escherichia coli* at bench and pilot plant scale. Enzyme Microbial Technol. 2004, 34, 235-241

Hsiung HM, Cantrell A, Luirink J, Oudega B, Veros AJ and Becker GW. Use of bacteriocin release protein in *E. coli* for excretion of human growth hormone into the culture medium. Biotechnol. 1989, 7, 267-271

Hu X, O'Hara L, White S, Magner E, Kane M and Wall JG. Optimisation of production of a domoic acid-binding scFv antibody fragment in *Escherichia coli* using

molecular chaperones and functional immobilisation on a mesoporous silicate support. Protein Expr Purif. 2007, 52 (1), 194-201

Huber- Wunderlich M, Glockshuber R. A single dipeptide sequence modulates the redox properties of a whole enzyme family. Fold Des. 1993, 3, 161-171

Hunke S, Betton JM, Temperature effect on inclusion body formation and stress response in the periplasm of *Escherichia coli*. Mol Microbiol. 2003, 50, 1579-1589

Ikura Y. Effect of glycine and ist derivatives on production and release of β-galactosidase by E.coli. Argic.Biol.Chem. 1986, 50, 2747-2753

Jaenicke R. Protein self-organization *in vitro* and *in vivo*: partitioning between physical biochemistry and cell biology. Biol Chem. 1998, 379(3), 237-243

Jander, Martin NL and Beckwith J. Two cysteines in each periplasmic domain of the membrane protein DsbB are required for its function in protein disulfide bond formation. Embo J. 1994, 13, 5121-5127

Jang KH, Seo KB, Song KB, Kim CH and Rhee SK. Extracellular secretion of levansucrase from *Zymomonas mobilis* in *Escherichia coli*. Bioproc Eng. 1999, 21, 453-458

Jeong KJ, Lee SY. Secretory production of human leptin in *Escherichia coli.* Biotechnol Bioeng. 2000, 67:398-407

Jeong KJ, Lee SY. Excretion of human beta-endorphin into culture medium by using outer membrane protein F as a fusion partner in recombinant *Escherichia coli*. Appl Environ Microbiol. 2002, 68, 4979-4985

Jevsevar S, Gaberc-Porekar V, Fonda I, Podobnik B, Grdadolnik J and Menart V. Production of nonclassical inclusion bodies from which correctly folded protein can be extracted. Biotechnol Prog. 2005, 21, 632-639

Joly JC, Leung WS and Swartz JR. Overexpression of *Escherichia coli* oxidoreductases increases recombinant insulin-like growth factor-I accumulation. Proc Natl Acad Sci USA. 1998, 95, 2773-2777

Joly JC, Swartz JR. Protein folding activities of *Escherichia coli* protein disulfide isomerase. Biochemistry. 1994, 33, 4231-4236

Jonda S, Huber-Wunderlich M, Glockshuber R and Mössner E. Complementation of DsbA deficiency with secreted thioredoxin variants reveals the crucial role of an efficient dithiol oxidant for catalyzed protein folding in the bacterial periplasm. EMBO J. 1999, 3271-3281

Kaderbhai MA, Ugochukwu CC, Lamb DC and Kelly S. Targeting of active human cytochrome P4501A1 (CYP1A1) to the periplasmic space of *Escherichia coli*. Biochem Biophys Res Comm. 2000, 279, 803–807

Kaderbhai N, Karim A, Hankey W, Jenkins G, Venning A and Kaderbhai MA. Glycine-induced extracellular secretion of a recombinant cytochrome expressed in *Escherichia coli*. Biotechnol Appl Biochem. 1997, 25, 53-61

Kadokura H, Katzen F and Beckwith J. Protein disulfide bond formation in prokaryotes. Annu Rev Biochem. 2003, 72, 111-135

Kaszuba M, Connah M and Mattison K. High concentration particle size measurements using dynamic light scattering. LabPlus international. 2004

Kim J.Y, Fogarty E.A, Lu F.J, Zhu H, Wheelock G.D, Henderson L.A and DeLisa M.P. Twin-Arginine Translocation of Active Human Tissue Plasminogen Activator in *Escherichia coli* . Appl Environ Microbiol. 2005, 71(12), 8451–8459

Kim JR, Muresan A, Lee KY and Murphy RM. Urea modulation of β-amyloid fibril growth: experimental studies and kinetic models. Protein science. 2004, 13, 2888-2898

Kim SS, Kim EK and Rhee JS. Effects of growth rate on the production *of Pseudomonas fluorescens* lipase during fed-batch cultivation of *Escherichia coli*. Biotechnol Prog. 1996, 12, 718-722

Kishigami S, Akiyama Y and Ito K. Redox states of DsbA in the periplasm of *Escherichia coli.* FEBS Lett. 1995, 364, 55-58

Kitagawa M, Miyakawa M, Matsumura Y and Tsuchido T. *Escherichia coli* small heat shock proteins IbpA and IbpB, protect enzymes from inactivation by heat shock and oxidants. Eur J Biochem. 2002, 269, 2907-2917

Kleist S, Miksch G, Hitzmann B, Arndt M, Friehs K and Flaschel E. Optimization of the extracellular production of a bacterial phytase with *Escherichia coli* by using different fed-batch fermentation strategies. Appl Microbiol Biotechnol. 2003, 61, 456–462

Knivett VA, Cullen J. Some factors affecting cyclopropane acid formation in *Escherichia coli.* Biochem J. 1965, 96, 771-776

Kohnert U, Rudolph R, Verheijen JH, Weening-Verhoeff EJ, Stern A, Opitz U, Martin U, Lill H, Prinz H, Lechner M, Kresse GB, Buckel P and Fischer S. Biochemical properties of the kringle-2 and protease domains are maintained in the refolded t-PA deletion variant BM06.022. Protein Eng. 1992, 5, 93-100

Kopetzki E, Buckel P and Schumacher G. Cloning and characterization of baker´s yeast alpha-glucosidase: over-expression in a yeast strain devoid of vacuolar proteinases. Yeast. 1989a, 5, 11-24

Korz DJ, Rinas U, Hellmuth K, Sanders EA and Deckwer W-D. Simple fed-batch technique for high-cell density cultivation of *Escherichia coli*. Biotechnol. 1995, 39, 59-65

Krzewska J, LangerT and Liberek K. Mitochondrial Hsp78, a member of the Clp/Hsp100 family in *Saccharomyces cerevisiae*, cooperates with Hsp70 in protein refolding. FEBS Lett. 2001, 489:92–96

Kucharczyk K, Laskowska E and Taylor A. Response of *Escherichia coli* cell membranes to induction of λcI857 prophage by heat shock. Mol Microbiol. 1991, *5*, 2935-2945

Kucharzyk K, Laskowska E and Taylor A. Response of E.coli cell membranes to induction of Lamda cl1857 prophage by heat shock. Mol Microbiol. 1991, 5, 2935-2945

Kuczynska-Winsnik D, Kedzierska S, Matuszewska E, Lun P, Taylor A, Lipinska B and Laskowska E. The *Escherichia coli* small heat heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured in vivo during extreme heat shock. Microbiology.2002, 148, 1757-1765

Kujau MJ, Hoischen C, Riesenberg D and Gumpert J. Expression and secretion of functional miniantibodies McPC603scFvDhlx in cell-wall-less L-form strains of *Proteus mirabilis* and *Escherichia coli*: A comparison of the synthesis capacities of L-form strains with an *E. coli* producer strain. Appl Microbiol Biotechnol. 1998, 49, 51–58

Kula M-R, Schütte H, Vogels G and Frank A. Cell disintegration for the purification of intracellular proteins. Food Biotechnol. 1990, 4, 169-183

Kurokawa Y, Yanagi H and Yura T. Overexpression of protein disulfide isomerase DsbC stabilizes multiple-disulfide-bonded recombinant protein produced and transported to the periplasm in *Escherichia coli*. Appl Environ Microbiol. 2000, 66, 3960-3965

Laskowska E, Bohdanowicz J, Kuczynska-Wisnik D, Matuszewska E, Kedzierska S and Taylor A. Aggregation of heat-shock-denatured, endogenous proteins and distribution of the ibpA/B and Fda marker-proteins in *Escherichia coli* WT and grpE280 cells. Microbiology. 2004, 150, 247-259

Laskowska E, Wawrzynow A and Taylor A. IbpA and IbpB, the new heat-shock proteins, bind to endogenous *Escherichia coli* proteins aggregated intracellularly by heat shock. Biochemie. 1996, 78, 117-122

Le TH. Optimization of active recombinant protein production, exploring the impact of small heat shock proteins of *Escherichia coli*, IbpA and IbpB, on *in vivo* reactivation of inclusion bodies. PhD thesis. The Martin Luther Universiy Halle-Wittenberg. 2005

Le Thanh H, Hoffmann F. Processes for production of active α-glucosidase by in-vivo-reactivation from inclusion bodies. Evaluation of processes using in vivo reactivation from inclusion bodies. Biotechnol Prog. 2005, 21, 1053-1061

Lee S, Sowa ME, Watanabe Y, Sigler PB, Chiu W, Yoshida M and Tsai FT. The structure of ClpB. A molecular chaperone that rescues proteins from an aggregated state. Cell. 2003, 115, 229-240

Lee SY, Choi JH and Lee SJ. Secretory production of therapeutic proteins in *Escherichia coli*. Methods Mol Biol. 2005, 308, 31-42

Lee SY. High cell density culture of *Escherichia coli*. Trends Biotechnol. 1996, 14, 98-105

Lethanh H, Neubauer P and Hoffmann F. The small heat-shock proteins IbpA and IbpB reduce the stress load of recombinant *Escherichia coli* and delay degradation of inclusion bodies. J Microbial Cell Factories. 2005, 4:6

Li Y, Chen CX, von Specht B and Hahn HP. Cloning and hemolysin-mediated secretory expression of a codon-optimized synthetic human interleukin-6 gene in *Escherichia coli*. Gene. 2002, 25, 437-447

Lin WJ, Huang SW and Chou CP. DegP-coexpression minimizes inclusion-body formation upon overproduction of recombinant penicillin acylase in *Escherichia coli*. Biotechnol Bioeng. 2001a, 484-492

Lin WJ, Huang SW and Chou CP. High-level extracellular production of penicillin acylase by genetic engineering of *Escherichia coli*. J Chem Technol Biotechnol. 2001b, 76, 1030-1037

Lin, HY. Cellular responses to the induction of recombinant genes in *Escherichia coli* fed-batch cultures. PhD thesis. The Martin Luther University of Halle-Wittenberg. 2000

Luirink J, Van der Sande C, Tommassen J, Veltkamp E, De Graaf FK and Oudega B. Mode of action of protein H encoded by plasmid CloDF13: effects of culture conditions and of mutations affecting phospholipase A activity on excretion of Cloacin DF13 and growth and lysis of host cells. J Gen Microbiol. 1986, 132, 825-834

Mak KW, Loh KC and Yap MG. Production & purification of recombinant tumour necrosis factor-beta. Autralas Biotechnol. 1993, 3(4), 206-12.

Malik A, Rudolph R and Söhling B. A novel fusion protein system for the production of native human pepsinogen in the bacterial periplasm. Protein Expr Purif. 2006, 47(2), 662-671

Malvern. The importance of sample viscosity in dynamic light scattering measurements. Application note. Recognising the quality of data from photon correlation spectroscopy measurements. Technical note. Malvern Instruments. 2005

Manosroi J, Tayapiwatana C, Götz F, Werner RG and Manosroi A. Secretion of active recombinant human tissue plasminogen activator derivatives in *Escherichia coli*. Appl.Environ.Microbiol. 2001, 67, 2657-2664

Martin JL, Bardwell JC and Kuriyan J. Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*. Nature.1993, 365**,** 464- 468

Messens J, Collet JF. Pathways of disulfide bond formation in *Escherichia coli.* Int J Biochem Cell Biol. 2006, 38(7), 1050-1062

Meyer SC, Huerta C and Ghosh I. Single-Site Mutations in a Hyperthermophilic Variant of the B1 Domain of Protein G Result in Self-Assembled Oligomers.Biochemistry, 2005**,** 44(7), 2360 – 2368

Middelberg A PJ. Preparative protein refolding. Trends in Biotechnol. 2002, 20, 437-443

Middelberg APJ, Bogle IDL and Snoswell MA. Sizing biological samples by photosedimentation techniques. Biotechnol. Prog. 1990, 6, 255-261

Miksch G, Neitzel R, Fiedler E, Friehs K and Flaschel E. Extracellular production of a hybrid β-glucanase from *Bacillus* by *Escherichia coli* under different cultivation conditions in shaking cultures and bioreactors. Appl Microbiol Biotechnol. 1997, 47, 120-126

Miksch G, Kleist S, Friehs K and Flaschel E. Overexpression of the phytase from *Escherichia coli* and its extracellular production in bioreactors. Appl Microbiol Biotechnol. 2002, 56, 685–694

Missiakas D, Raina S. Protein folding in the bacterial periplasm. J Bacteriol. 1997, 179(8), 2465-2471

Mitraki A, King J. Biotechnol.1989, 7, 690-697

Mogk A, Deuerling E, Vorderwülbecke S, Vierling E and Buckau B. Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. Molecular Microbiology. 2003, 50(2), 585-595

Mogk A, Mayer MP and Deuerling E. Mechanisms of protein folding: molecular chaperones and their application in Biotechnology. Chem Biochem. 2002, 3, 807-814

Mogk A, Schlieker C, L.Friedrich K, Schönfeld H-J, Vierling E and Bukau B. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most effiently by ClpB/DnaK. Biological Chemistry. 2003, 278(33), 31033-31042

Mogk A, Tomoyasu T, Goloubinoff P, Rüdiger S, Röder D, Langen H and Bukau B. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. EMBO J. 1999, 18(24), 6934-6949

Moriya H, Kaneko J, Nakamura S and Osumi M. Electron microscopic observation of recombinant *Escherichia coli* cells overproducing human tumor necrosis factor-alpha mutant as inclusion bodies. J Electron Microsc. 1991, 40, 364-367

Nagahari K, Kanaya S, Munakata D, Aoyagi Y and Mizushima S. Secretion into the culture medium of a foreign gene product from *Escherichia coli*: use of the *ompF* gene for secretion of human ß-endorphin. EMBO J. 1985, 16, 3589–3592

Nakamoto H, Bardwell J CA. Catalysis of disulfide bond formation and isomerization in the *Escherichia coli* periplasm. Biochemica et Biophysica Acta. 2004, 1694,111-119

Neubauer P, Lin HY and Mathiszik B. Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli*. Biotechnol Bioeng. 2003, 83, 53-64

Nguyen VS, Patil G and Hoffmann F. Optimum glucose supply strategy for protein secretion by recombinant *Escherichia coli* in presence of an inhibitory medium additive. Unpublished paper. 2006

Nichols MR, Moss MA, Reed DK, Cratic-McDaniel S, Hoh JH and Rosenberry TL. Amyloid- Protofibrils Differ from Amyloid- Aggregates Induced in Dilute Hexafluoroisopropanol in Stability and Morphology. J Bio Chem. 2005, 280(4), 2471-2480

Nishihara K, Kanemori M, Yanagi H and Yura T. Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. Appl Environ Microbiol. 2000.66, 884-889

Oelschlaeger P, Lange S, Schmitt J, Siemann M, Reuss M and Schmid RD. Identification of factors impeding the production of a single-chain antibody fragment in *Escherichia coli* by comparing in vivo and in vitro expression. Appl Microbiol Biotechnol. 2003, 61, 123-132

Oliver DB. Periplasm. In *Escherichia coli* and Salmonell-Cellular and molecular biology, 2nd ed., Neidhardt FC, Ed, ASM Press, Washington DC. 1996, 88-103

O'Neal D, Harrip P, Dragicevic G, Rae D and Best JD. A comparison of LDL size determination using gradient gel electrophoresis and light-scattering methods. J. Lipid Research. 1998, 39, 2086-2090

Pan K-L, Hsiao H-C, Weng C-L, Wu M-S and Chou CP. Roles of DegP in prevention of protein misfolding in the periplasm upon overexpression of penicillin acylase in *Escherichia coli*. J Bacteriol. 2003, 185, 3020-3030

Pavlou AK, Reichert JM. Recombinant protein therapeutics- success rates, market trends and values to 2010. Nat Biotechnol. 2004, 22 , 1513–1519

Popplewell AG, Sehdev M, Spitali M and Weir AN. Expression of antibody fragments by periplasmic secretion in *Escherichia coli*. Methods Mol Biol. 2005, 308, 17-30

Pugsley AP. The complete general secretory pathway in gram-negative bacteria. Microbiol Rev.1993, 57, 50-108

Qiu J, Swartz JR and Georgiou G. Expression of active human tissue-type plasminogen activator in *Escherichia coli*. Appl.Environ Microbiol. 1998, 64, 4891-4896

Rawle, A. PCS in 30 minutes. Malvern Instruments Inc. Malvern, UK, 1995

Reddy K RC, Lilie H, Rudolph R and Lange C. L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. Protein Science. 2005, 14, 929-935

Rinas U, Hoffmann F. Selective leakage of host-cell proteins during high-cell-density cultivation of recombinant and non-recombinant *Escherichia coli*. Biotechnol Prog. 2004, 20, 679-687

Rippmann JF, Klein M, Hoischen C, Brocks B, Rettig WJ, Gumpert J, Pfizenmaier K, Mattes R and Moosmayer D. Procaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of *Proteus mirabilis* leads to active product and overcomes the limitations of periplasmic expression in *Escherichia coli*. Appl Environ Microbiol. 1998, 64(12), 4862-4869.

Robbens J, Raeymaekers A, Steidler L, Fiers W and Remaut E. Production of soluble and active recombinant murine interleukin-2 in *Escherichia coli*: high level expression, *Kil*-induced release, and purification. Protein Expr Purif. 1995, 6(4), 481-486

Rozhkova A, Stirnimann CU, Frei P, Grauschopf U, Brunisholz R and Grutter MG. Structural basis and kinetics of inter- and intramolecular disulfide exchange in the redox catalyst DsbD. EMBO J. 2004, 23, 1709–1719

Ruddock LW, Hirst TR and Freedman RB. pH-dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol:disulphide oxidoreductase) and protein disulphide-isomerase: studies with a novel simple peptide substrate. Biochem J. 1996, 315, 1001-1005

Rudolph R, Böhm G, Lilie H and Jaenicke R. A practical approach. Creighton, T.E., ed. 1997, 57-99

Rudolph R, Lilie H. *In vitro* folding of inclusion body proteins. FASEB J. 1996, 10, 49-56

Rudolph R. Successful protein folding on an industrial scale. Protein Engineering: Principles and Practice (Cleland JL & Craik SC., ed.) John Wiley and Sons, NY. 1996, 283-298

Sambrock J, Fritsch EF and Maniatis T. Molecular cloning. A laboratory manual. Cold Spring Habor Laboratory Press, Cold Spring Habor, NY, 1989

Santini CL, Ize B, Chanal A, Muller M, Giordano G and Wu LF. A novel sec-independent periplasmic protein translocation pathway in *Escherichia coli*. EMBO J. 1998, 2, 17(1), 101-112

Schäffner J, Winter J, Rudolph R and Schwarz E. Cosecretion of chaperones and low-molecular-size medium additives increases the yield of recombinant disulfide-bridged proteins. Appl Environ Microbiol. 2001, 67, 3994-4000

Schäffner J. Einfluss niedermolekularer Medienzusätze und sezernierter, ATP-unabhängiger Chaperone auf die Ausbeute therapeutischer Proteine im Periplasma von *Escherichia coli*. PhD Thesis. Martin Luther Universität Halle-Wittenberg. 2000

Scheibel T, Buchner J. Protein aggregation as a cause for disease. Handb Exp Pharmacol. 2006, 172, 199-219

Schlapschy M, Grimm S and Skerra A. A system for concomitant overexpression of four periplasmic catalysts to improve secretory protein production in *Escherichia coli.* Protein Eng Des Sel. 2006, 19(8), 385-390

Schlee S, Beinker P, Akhrymuk A and Reinstein J. A chaperone network for the resolubilization of protein aggregates: direct interaction of ClpB and DnaK. J Mol Bio. 2004, 336, 275-285

Schlieker C, Bukau B and Mogk A. Prevention and reversion of protein aggregation by molecular chaperones in the *E.coli* cytosol: implications for their applicability in biotechnology. Biotechnol. 2002, 96, 13-21

Schlieker C, Tews I, Bukau B and Mogk A. Solubilization of aggregated proteins by ClpB/DnaK relies on the continuous extraction of unfolded polypeptides. FEBS Letters. 2004, 578, 351-356

Schmidt M, Viaplana E, Hoffmann F, Marten S, Villaverde A and Rinas U. Secretion-dependent proteolysis of heterologous protein by recombinant *Escherichia coli* is connected to an increased activity of the energy-generating dissimilatory pathway. Biotechnol Bioeng. 1999, 66, 61-67

Schrödel A, de Marco A. Characterization of the aggregates formed during recombinant protein expression in bacteria. BMC Biochemistry. 2005, 6:10

Seo JH, Bailey JE. Continous cultivation of recombinant *Escherichia coli*: existence of an optimum dilution rate for maximum plasmid and gene product concentration. Biotechnol Bioeng. 1986, 28, 1590-1594

Sheartone JR, Baneyx F. Biochemical characterization of the small heat shock protein IbpB from *Escherichia coli*. J Biol Chem. 1999, 274, 9937-9945

Shen CL, Scott GL, Merchant F and Murphy RM. Light scattering analysis of fibril growth from the amino-terminal fragment β (1–28) of β -amyloid peptide. Biophys J. 1993. 65**,** 2383–2395

Shively JM. Inclusion bodies of prokaryotes. Ann Rev of Microbiol. 1974, 28, 167−187

Shokri A, Sandén AM and Larsson G. Growth rate-dependent changes in *Escherichia coli* membrane structure and protein leakage. Appl Microbiol Biotechnol. 2002, 58, 386-392

Shokri A, Sanden AM and Larsson G. Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*. Appl Microbiol Biotechnol. 2003, 60, 654-664

Siddiqi SF, Titchener-Hooker NJ and Ayazi Shamlou P. Simulation of particle size distribution changes occuring during high-pressure disruption of Bakers' yeast. Biotechnol Bioeng. 1996, 50, 145-150

Simão RC, Susin MF, Alvarez-Martinez CE and Gomes SL. Cells lacking ClpB display a prolonged shutoff phase of the heat shock response in *Caulobacter crescentus***.** Mol Microbiol. 2005, 57(2), 592-603

Sipe JD. Amyloidosis. Annu Rev Biochem.1992, 61, 947-975

Stanley NR, Palmer T and Berks BC. The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. J Biol Chem. 2000 , 21, 275(16), 11591-11596.

Stewart EJ, Katzen F and Beckwith J. Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*. Embo J. 1999, 18, 5963–5971

Stryer L. Biochemistry 4^{th} edition. W.H. Freeman and Company. 1996.

Stubbs MT, Renatus M and Bode W. An active Zymogen: Unravelling the mystery of tissue-type plasminogen activator. Biol Chem. 1998, 379, 95-103

Swarts J. Advances in *Escherichia coli* production of therapeutic proteins. Curr Opin Biotechnol. 2001, 12, 195–201

Taylor G, Hoare M, Gray DR and Marson FAO. Size and density of protein inclusion bodies. Biotechnol. 1986, 4, 553-557

Thomas JC, Middelberg APJ, Hamel JF and Snoswell MA. High-resolution particles size analysis in biotechnology process control. Biotechnol Prog. 1991, 7, 377-379

Thomas JG, Ayling A and Baneyx F. Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *Escherichia coli*. To fold or to refold. Appl Biochem Biotechnol. 1997. 66, 197–238

Thomas JG, Baneyx F. ClpB and HtpG facilitate *de novo* protein folding in stressed *Escherichia coli* cells. J Mol Microbiol. 2000. 36(6), 1360-1370

Thomas JG, Baneyx F. Roles of *Escherichia coli* small heat shock proteins IbpA and IbpB in thermal stress management: comparision with ClpA, ClpB, and HtpG in vivo. J Bacteriol.1998, 180, 5165-5172

Tomoyasu T, Mogk A, Langen H, Goloubinoff P and Bukau B. Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. Mol Microbiol. . 2001, 40, 397–413

Tsuchido T, Katsui N, Takeuchi A, Takano M and Shibasaki I. Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. Appl Environ Microbiol. 1985, *50*, 298-303

Turner C, Bregory ME and Turner MK. A study on the effect of specific growth rate and acetate on recombinant protein production of *Escherichia coli* JM107. Biotechnol Lett. 1994, 16, 891-896

Van der Wal FJ, Koningstein G, ten Hagen CM, Oudega B and Luirink J. Optimization of bacterocin release protein (BRP)-mediated protein release by *Escherichia coli*: random mutagenesis of the pCloDF13-Derived BRP gene to uncouple lethality and quasi-lysis from protein release. Appl Environ Microbiol. 1998, 64, 392-398

Van der Wal FJ, ten Hagen-Jongman CM, Oudega B and Luirink J. Optimization of bacteriocin-release-protein-induced protein release by *Escherichia coli*: extracellular production of the periplasmic molecular chaperone FaeE. Appl Microbiol Biotechnol. 1995, 44, 459-465

Van Hee P, Middelberg APJ, Van der Lans RG and Van der Wielen LA .Relation between cell disruption conditions, cell debris particle size, and inclusion body release. Biotechnol Bioeng. 2004, 88(1), 100-110

Van Hee P, Hoeben MA, Van der Lans RG and Van der Wielen LA. Strategy for selection of methods for separation of bioparticles from particle mixtures. Biotechnol Bioeng. 2006, 94(4), 689-709

Veinger L, Diamant S, Buchner J and Goloubinoff P. The small heat shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. J Biol Chem. 1998, 273, 11032-11037

Walter S, Buchner J. Molecular Chaperones- Cellular machines for protein folding. Angew Chem. Int. 2002, 41, 1098-1113

Wan EW, Baneyx F. TolAIII co-expression facilitates the recovery of periplasmic recombinant proteins into the growth medium of *Escherichia coli*. Protein Expr Purif. 1998, 14(1), 13-22

Weast RC. Handbook of chemistry and physics. CEC Press. 1974, 55th edition

Weibezahn J, Schlieker C, Tessarz P, Mogk A and Bukau B. Novel insights into the mechnism of chaperone-assisted protein disaggregation. Biol Chem. 2005, 386, 739-744

Winter J, Neubauer P, Glockshuber R and Rudolph R. Increased production of human proinsulin in the periplasmic space of *Escherichia coli* by fusion to DsbA. J Biotechnol. 2000, 64, 175-185

Wittrup KD, Mann MB, Fenton DM, Tsai LB and Bailey JE. Single-cell light scatter as a probe of refractile body formation in recombinant *Escherichia coli.* Biotechnol. 1988, 6, 423-426

Wong HH, O'Neill BK and Middelberg APJ. Centrifugal processing of cell debris and inclusion bodies from recombinant *Escherichia coli*. Bioseparation**.** 1997a. 6, 361-372

Wong HH, O´Neill BK and Middelberg APJ. Cumulative sedimentation analysis of *Escherichia coli* debris size. J Biotechnol and Bioengineering. 1997b, 3, 556-564

Wu MS, Pan KL and Chou CP. Effect of heat-shock proteins for relieving physiological stress and enhancing the production of penicillin acylase in *Escherichia coli*. Biotechnol Bioeng. Biotechnol Bioeng. 2007, 96(5), 956-66

Wunderlich M, Glockshuber R. In vivo control of redox potential during protein folding catalyzed by bacterial protein disulfide-isomerase (DsbA). J Biol Chem. 1993, 268, 24547-24550

Wunderlich M, Otto A, Maskos K, Mücke M, Seckler R and Glockshuber R. Efficient catalysis of disulfide formation during protein folding with a single active-site cystein. J Mol Biol. 1995, 247, 28-33

Yang J, Moyana T, Mackenzie S, Xia Q and Xiang J. One hundred Seventy-fold increase in excretion of an FV fragment tumor necrosis factor alpha fusion protein (SFV/TNF-$^{\alpha}$) from *Escherichia coli* caused by the synergistic effects of glycine and triton X-100. Appl Environ Microbiol. 1998, 64, 2869–2874

Yokoyama K, Kikuchi Y and Yasueda H. Overproduction of DnaJ in Escherichia coli improves in vivo solubility of the recombinant fish-derived transglutaminase. Biosci Biotechnol Biochem. 1998, 62, 1205-1210

Yu P, San KY. Protein release in recombinant *Escherichia coli* using bacteriocin release protein. Biotechnol Prog. 1992, *8*, 25-29

Zapun A, Bardwell JC and Creighton TE. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo*. Biochemistry. 1993, 32, 5083-5092

Zhang G, Brokx S and Weiner JH. Extracellular accumulation of recombinant proteins fused to the carrier protein YebF in *Escherichia coli*. Nat Biotechnol. 2006, 24(1), 100-104

Zhang Z, Li ZH, Wang F, Fang M, Yin CC, Zhou ZY, Lin Q and Huang HL. Overexpression of DsbC and DsbG markedly improves soluble and functional expression of single-chain Fv antibodies in *Escherichia coli*. Protein Expr Purif. 2002, 26, 218–228

Zietkiewicz S, Krzewska J and Liberek K. Succesive and synergistic action of the Hsp70 and Hsp100 chaperones in protein aggregation. J Biol Chem. 2004, 279(43), 44376-44383

Zietkiewicz S, Lewandowska A, Stocki P and Liberek K. Hsp 70 chaperone machine remodels protein aggregates at the initial step of Hsp70-Hsp100-dependent disaggregation. J Biol Chem. 2006, 281(11), 7022-7029

Zolkiewski M. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. J Biol Chem. 1999, 274(40), 28083-28086.

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DECLARATION

I certify that this Thesis is on my own work and has not been submitted to other Universities. In addition, any help and all sources of materials used in this Thesis have been acknowledged.

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