Investigations of the production, purification, activation and characterization of a novel, recombinant, highly active Transglutaminases

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von

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Declaration of Academic Honesty

Hereby, I declare that I have carried out this PhD research project on my own, having used only the listed resources and tools.

Christian Sommer Halle (Saale), 28.02. 2012

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1. Index and abbreviations

1.1 Index

1.2 Abbreviations

ACN	Acetonitrile
BDM / CDW	Bio / Cell dry mass
BWM	Bio wet mass
BSA	Bovine Serum Albumin
CV	column volume
Conc.	Concentration
CBZ-Q-G	Benzyloxy carbonyl-L-Gln-Gly
DoE	Design of experiment
equ.	equation
e.g.	For example
Fig.	Figure
GSH	Gluthation
HPLC	High pressure liquid chromatography
his-tag	Histidine-tag
IB	Inclusion body
IUPAC	International Union of Pure and Applied Chemistry
IPTG	Isopropyl-β-D-thiogalactopyranosid
IMAC	Immobilized metal affinity chromatography
MALDI	Matrix assisted laser desorption mass spectrometry
MTG	Microbial Transglutaminase
MDC	Monodansylcadaverine
MW	Molecular weight [kDa oder $g \cdot mol^{-1}$]
OFAT	One factor at a time approach to optimize a multivariable system
OD	Optical density
PAGE	Polyacrylamide gelelectrophoresis
PMSF	Phenylmethylsulfonylfluoride
rMTG	Recombinant microbial transglutaminase (variant of *Streptomyces mobaraensis* enzyme with his-tag)
rMTG(S2P)	Recombinant microbial transglutaminase (S2P variant of *Streptomyces mobaraensis* enzyme with his-tag)
RMSE	root mean square error
SDS	Natriumdodecylsulfat
SM-TAP	Tripeptidyl-Aminopeptidase
TAMEP	Transglutaminase activating Metalloprotease
TG	Transglutaminase
Tab.	Table
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)-Aminomethan
U	Unit ($\mu mol\ min^{-1}$)
UV	Ultraviolet

The amino acid abbreviations (one letter amino acid code) correlate to the IUPAC nomenclature.

Amino acids

Ala	A	Alanin
Arg	R	Arginin
Asn	N	Asparagin
Asp	D	Aspartat
Cys	C	Cystein
Gln	Q	Glutamin
Glu	E	Glutamat
Gly	G	Glycin
His	H	Histidin
Ile	I	Isoleucin
Leu	L	Leucin
Lys	K	Lysin
Met	M	Methionin
Phe	F	Phenylalanin
Pro	P	Prolin
Ser	S	Serin
Thr	T	Threonin
Trp	W	Tryptophan
Tyr	Y	Tyrosin
Val	V	Valin

2. Introduction

2.1 State of the art

2.1.1 Production of Transglutaminase

Transglutaminases (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) are a family of enzymes that have been evolved for the cross-linking of proteins [Mariniello and Porta, 2005]. A number of prokaryotic and eukaryotic transglutaminases from various sources have been purified, characterized, and some genes have also been cloned and (over)expressed.

Until today, the major application of microbial transglutaminase (MTG) is found in food processing, the field of application for which the enzyme was originally developed. Enzymatic protein cross-linking results in the restructuring of protein-rich food such as meat or fish and alters the texture of the food [Mariniello and Porta, 2005]

Besides the food industries, there are various other applications for transglutaminases. MTG treatment, for example, can be used to manufacture biodegradable protein films [Patzsch et al., 2010], for the PEGylation or HESylation of (therapeutic) proteins [Sato, 2002] [Besheer et al., 2009], for vaccine production [Chou, 2009], to increase the tensile strength of wool [Cardamone, 2007], for enzyme immobilization [Kamata et al., 1992; Synowiecki and Wolosowska, 2006], to coat leather [Mariniello and Porta, 2005] and various other applications.

MTG is commercially available (trade mark Activa^{TM}) and is produced by fermentation of *Streptomyces mobaraensis* as extracellular protein. Activa^{WM} contains 100 Units MTG per gram powder but only 1 % protein. 99% Maltodextrin is added to stabilize the MTG and to improve handling properties. One drawback of the commercial preparation besides the high Maltodextrin content is the presence of prote-

ases, which can hydrolyze proteins that are intended to be cross-linked. Another drawback is the low thermostability of maltodextrin-free Activa^{TM}.

Several attempts have been made to overproduce MTG in recombinant form. First attempts to express MTG of *Streptomyces mobaraensis* in *E. coli* failed due to the formation of inclusion bodies and low recovery yields. In other investigations, coryneform bacteria (e.g. *C. glutamicum, C. ammoniagenes*) were developed as expression systems. MTG expression levels of 2,500 mg L^{-1} after 71 hours have been obtained with *C. ammoniagenes* [Itaya and Kikuchi, 2008]. This number corresponds to a space time yield of 810 mg L^{-1} h^{-1}.

Recently, the problem of inclusion body (IB) formation and the overexpression of a his-tagged MTG of *Streptomyces mobaraensis* (rMTG) recombinant in *E. coli* in a soluble form was possible by decreasing the temperature during the protein expresion [Marx et al., 2007a]. The soluble expression allowed for optimization of the enzyme by directed evolution. Compared to the wild-type enzyme, a transglutaminase variant possessing a single amino acid exchange (serin to prolin; S2P) was remarkably more thermostable. Additionally, this variant showed a doubled specific activity [Marx et al., 2008b]. In Table 1 an overview over the most important studies concerning the MTG expression is given.

Table 1: Comparison of yields of transglutaminase production in different host strains. To calculate missing data, the specific activity of 22.6 U mg^{-1} was used for the native transglutaminase from *S. mobaraensis* [Ando et al., 1989], 23 U mg^{-1} for the recombinant transglutaminase expressed in *C. ammoniageneses* [Itaya and Kikuchi, 2008] and 26 U mg^{-1} for the recombinant transglutaminase expressed in *C. glutamicum* [Date et al., 2004]. The specific activity of the rMTG(S2P) variant produced in the present paper was 46.1 U mg^{-1} [Marx et al., 2007b]. Table sorted according to increasing space time yield.

Reference	Host strain	MTG yield [mg L^{-1}]	MTG yield [U L^{-1}]	Bio dry mass related yield [U g^{-1}]	Bio dry mass yield [g L^{-1}]	Cultivation time [h]	Space-time yield [U L^{-1} h^{-1}]	Remarks
[Takehana et al., 1994]	*E. coli*	5	113	--	--	--	--	No pro sequence, ompA sequence added, activity estimated from band intensity of western blot
[Yurimoto et al., 2004]	*P. pastoris*	1	23	--	--	68	0.3	Methanol fed-batch, extracellular expression, pro sequence
[Portilla-Rivera et al., 2009]	*S. ladakanum*	20	452	38	11.9	72	6	Complex medium (sugar cane molasses), batch cultivation
[Yurimoto et al., 2004]	*C. boidinii*	87	1966	72	27	125	16	Glycerol fed-batch, methanol feeding for induction, extracellular expression, pro sequence
[Ando et al., 1989]	*S. mobaraensis*	93	2102	125	16.8	70	30	Original strain, secreted, values calculated from figure in the reference
[Yokoyama et al., 2000]	*E. coli*	45	1017	--	--	20	51	Codon usage adjusted to *E. coli*; inclusion bodies 15 % activity after refolding
[Kikuchi et al., 2003]	*C. glutamicum*	142	3692	--	--	70	53	Secreted and activated, co-expressed protease
[Marx et al., 2007a]	*E. coli*	65	1469	638	2.3	24	61	IPTG, temperature profile, > 90 % soluble, batch cultivation
[Marx et al., 2008a]	*E. coli*	68	1537	--	--	19	81	Intracellular, 90 % soluble, lactose induction, batch cultivation
[Kawai et al., 1997]	*E. coli*	60	1356	--	--	10	136	Inclusion bodies, 20 % activity after refolding
[Date et al., 2004]	*C. glutamicum*	881	22906	--	--	40	573	Secreted, activated; chimeric pro sequence, batch cultivation
[Itaya and Kikuchi, 2008]	*C. ammoniagenes*	2500	57500	1150	50	71	810	Secreted, activated, batch cultivation

The aim of this thesis is to provide large amounts of the thermostable, recombinant S2P variant (rMTG(S2P)) of the wild-type enzyme, which is free of maltodextrin and contaminating proteases. The results were discussed later in terms of advantages of intra- or extracellular expression, space time yield as well as final product concentrations and yields.

Therefore in the present thesis rMTG(S2P) production was investigated by developing an optimized *E. coli* fed-batch fermentation protocol. Fed-batch processes have been developed to overcome nutrient limitations and are currently the standard for most fermentation processes. The addition of concentrated medium and carbon sources prevents nutrient depletion and prolongs the growth phase leading to higher biomass concentrations. Higher biomass concentrations finally result in higher product concentrations [Sauer et al., 2000].

Static and dynamic parameters have to be determined in order to optimize the product yield of *E. coli* fed-batch fermentations. Static parameters, such as temperature and inductor concentration, do not only influence the protein expression but also the formation of inclusion bodies and the plasmid stability. Optimal parameters for the product formation can be determined by unstructured [Lye et al., 2003], one-factor-at-a-time (OFAT) [Islam et al., 2007], and statistical design of experiments (DoE) approaches [Swalley et al., 2006]. Unstructured and OFAT approaches are usually time and resource intensive and therefore DOE is today the preferred method. In addition, the resulting model is capable of predicting the overall optimal parameter values for a maximum soluble protein yield while it is sensitive to small changes in the underlying variables unlike OFAT approaches.

Dynamic parameters are for example feed rate, cultivation and induction time, growth rate and biomass concentration profile [Patkar et al., 1993]. In order to describe the complex interaction and dependence of such dynamic parameters and to

minimize experimental efforts, usually mathematical models of the process are developed. Therefore, the following steps, as suggested by d'Anjou and Daugulis, were made [d'Anjou and Daugulis, 1997]: (i) determination of raw data by basic experiment / cultivation (e. g. 20 g L^{-1} biomass concentration; 5,000 U L^{-1}), (ii) development of a model describing the basic cultivation as good as possible and model parameter identification by comparison with the raw data, and (iii) simulation and testing of an optimized cultivation.

Mathematical models for high cell density cultivation of *E. coli* have been developed for example by Galvanauskas et al. [Galvanauskas et al., 2004]. They are usually based on simple Monod kinetics describing the biomass production and are usually specific for a certain product. The aim of this thesis is the development of the mathematical model for the MTG overexpression providing a means to further investigate and optimized the MTG production.

The product formation in Monod based models are described by a special π(μ)-relationship. The effect of growth suppression by the specific product concentration can be modeled by a fixed parametric form, which is essentially a bell shaped function [Gnoth et al., 2008]. In addition, plasmid stability and formation of inclusion bodies have to be considered to find an optimal working point after induction. A combination of these models was used in the present work to increase the MTG expression in E. coli.

2.1.2 Purification and activation of Transglutaminase

For the activation of microbial (pro-) transglutaminases, several proteases have been used. Amongst others, dispase, bovine trypsin [Pasternack et al., 1998], proteinase K [Marx et al., 2008a], the endogenous proteases TAMEP and SM-TAP [Zotzel et al., 2003] and SAM-P45 a recombinant protease from *Streptomyces*

albogriseolous have been used [Kikuchi et al., 2003] [Date et al., 2003]. It was found that dispase is not completely removing the pro-sequence but leaves an N-terminal FRAP-sequence which does not affect the activity. Trypsin is not specific for the removal of the pro-sequence but also hydrolyzes the active transglutaminase [Marx et al., 2008a], a problem which could only partially be solved by on-column activation of column-bound pro-transglutaminase [Yang et al., 2009]. TAMEP is not commercially available and therefore not suited for mass production of the recombinant enzyme. In ongoing experiments on the large scale preparation of active rMTG and rMTG-variants which were obtained by random and site-directed mutagenesis [Marx et al., 2008b], dispase from *Bacillus polymyxa* was used for activation. Previously, dispase was shown to activate pro-transglutaminase without degradation and activity loss [Marx et al., 2008a].

2.1.3 Investigation of the substrate specificity of Transglutaminase

Though, many substrates of the transglutaminase are known like Casein, α-Lactalbumine, soy protein 11S and 7S and some wheat proteins e.g. ß-Lactoglobulin [Yokoyama et al., 2004]; [Nieuwenhuizen et al., 2004], not every protein respectively every glutamin and lysine residue is accessible by the rMTG for the cross linking or modification. For most of the proteins the actual location in the protein, where the cross linking takes places, is not known. In a first approach to determine the substrate and region specificity Ando et al. investigated 8 different peptides [Ando et al., 1989] based on the standard substrate CBZ-Q-G for the hydroxamate test as described by Folk et al. [Folk and Cole, 1966]. All investigated peptides showed a considerable lower activity than the CBZ-Q-G. Even the G-Q-G peptide was not accepted as a substrate (0% activity) which proves that it is not

enough to provide an available glutamine residue and emphasized the importance of the peripheral groups of the glutamine for the transglutaminase activity.

Ohtsuka et al. used the transglutaminase successfully to crosslink simple amides with functional groups and sugar residues [Ohtsuka et al., 2000b]. In addition Ohtsuka et al. investigated various peptides composed of 7 amino acids with a central glutamine, surrounded by five glycines and one variable amino acid position shifting through every position around the glutamine (10 proteinogenic amino acids were investigated). These peptides were crosslinked by MTG with the fluorescent dye MDC and the reactivity measured by HPLC was compared [Ohtsuka et al., 2000a].

Similar to the experimental result found by Ando et al. [Ando et al., 1989], all investigated peptides have a significant lower reactivity than CBZ-Q-G. The best combinations were peptide combinations in the form of GGXQGG whereas the X represents leucine or glutamic acid which both reached a reactivity of around 30 %. The reactivity of the other combinations reactivity was lower, most of them below 10 %.

To determine the overall influence of the flanking amino acids every combination and not only one substitution should be investigated. Sugimura et al. used a phage-display method with a phage-peptide library of 1.5×10^{11} combinations to investigate the influence of flanking amino acids in peptides consisting of 12 amino acids on the MTG crosslinking [Sugimura et al., 2008]. This phage-peptide library was incubated with biotinylated cadaverine and MTG. The cross linked phage-peptide / biotinylated cadaverine complexes were isolated using a mono-avidin affinity chromatography. The selected phages were propagated and these steps were repeated 5 times. Then from the selected peptides 27 randomly chosen peptides were sequenced to identify the flanking amino acids.

The results of Sugimura et al. indicated a preference for hydrophobic amino acids and arginine as flanking amino acids [Sugimura et al., 2008]. However, one sequenced peptide, which did not contained any glutamine, was selected by this procedure but was not further discussed by Sugimura et al. It remains unclear how this peptide could be crosslinked with biotinylated cadaverine and therefore selected via the mono-avidin affinity chromatography. One hypothesis might be the display of lysine or glutamine on other phage areas accessible to the MTG which would put the whole study into question. However, due to the peptide size of 12 amino acid a total of 20^{12}= $2.048x10^{14}$ combinations are possible and therefore even this widespread assay is not sufficient to investigate the whole influence of all flanking amino acids in such big peptides.

Fontana et al. investigated the MTG substrate specificity by MTG mediated PEGylation of different proteins and consecutive identification of the modified glutamines [Fontana et al., 2008]. The results indicated a preference for glutamines which are not located in a α-helix or ß-sheet and are exposed on the surface. Only in one example the glutamine on position 40 located in a α-helix in the human growth hormone was modified by MTG. Fontana et al. utilized the B-factor to explain these results. The B-factor describes the flexibility of single amino acids in a polypeptide chain determined by crystallography. The B-factor diagram for the human growth hormone shows a maximum around the positions 40 to 50 and between 135 and 150. So not only flanking amino acids but also the flexibility of the nearby region influences the substrate specificity of TG.

2.2 Aim of the work

The first objective of this work was the bulk quantity production of pro-rMTG(S2P) / pro-rMTG containing biomass. Therefore, *E. coli BL21 Gold*, containing plasmids capable of synthesizing pro-rMTG or pro-rMTG(S2P) by IPTG-induction, was first adapted from complex to minimal medium. Then, the ideal static cultivation parameters for the protein expression (temperature and inductor concentration) were determined by DoE investigations and non-linear modeling. The dynamic fed batch parameters (e.g. feed rate, growth phase length, final biomass etc.) were determined, optimized using a mathematical model and finally verified in a high-cell density fed-batch cultivation.

Following the bulk quantity production of pro-rMTG(S2P) / pro-rMTG containing biomass a large scale purification and activation method to produce a protease-free transglutaminase preparation had to be developed. Therefore, the present work investigated the activation of bulk quantities of the pro-rMTG(S2P) / pro-rMTG and the separation of the dispase from the active histidine-tagged transglutaminase by immobilized metal affinity chromatography (IMAC). Due to some negative side effects of the dispase activation proteinase K was also investigated. Immobilized proteinase K was compared to the free enzyme in order to simplify the separation of protease and activated transglutaminase.

In the last part of the present work the substrate specificity of the purified rMTG / rMTG(S2P) was investigated by a peptide library screening utilizing immobilized peptides consisting of 3 amino acids with a central glutamine and variable flanking amino acids. These results could be applied in combination with the B-factor to pre-

dict substrate preferences of the MTG followed by the prediction of new MTG substrates by e.g. a Database search which could lead to novel substrates and applications.

3. Materials and methods

3.1 General

Unless otherwise stated, all chemicals were of analytical grade and were purchased from Sigma Aldrich (Taufkirchen, Germany). The protein marker (PageRuler Prestained Proteinladder) used for SDS-PAGE was purchased from Fermentas (St. Leon-Rot, Germany). Deionized water was used throughout the experiments. Dispase was purchased from BD Biosciences (Heidelberg, Germany, order N° 354235), proteinase K was purchased from Fermentas (St. Leon-Rot, Germany, order N° EO0491), and proteinase K immobilized on Eupergit C was purchased from Sigma (Taufkirchen, Germany, order N° 82452). The protein concentration was determined according to the method of Bradford [Bradford, 1976]. BSA was used for calibration. The plasmid stability was determined according to the literature [Chen et al., 2008].

3.2 Bacterial strain

E. coli BL21Gold(DE3) was provided from Stratagene (Amsterdam, Netherlands). It was previously transformed by Marx et al. with the plasmid pCM203 containing an ampicillin resistance and the gene of the recombinant his-tagged pro-rMTG(S2P) [Marx et al., 2008b]; Genbank accession no: HM047086. The pro-sequence inactivates the pro-rMTG(S2P) to protect the host organism and a his-tag allows for easy purification. In order to produce the active rMTG(S2P) enzyme, the pro-sequence was cleaved off as described in section 3.14.2.

E. coli BL21Gold(DE3) pDJ13 biomass containing pro-MTG was obtained as described in Marx et al. [Marx et al., 2008a].

E. coli BL21Gold(DE3) pCM203 and E*. coli* BL21Gold(DE3) pDJ13 were stored at -80°C in 1 mL aliquots with 50% glycerol and an optical density of 2 at 600 nm (OD).

3.3 Cultivation media

LB_{amp} medium containing 10.0 g L^{-1} tryptone / peptone, 10.0 g L^{-1} NaCl, 5.0 g L^{-1} yeast extract, pH 7.5, 100 mg L^{-1} ampicillin and a glucose based minimal medium containing 2.0 g L^{-1} Na_2SO_4; 2.68 g L^{-1} $(NH_4)_2SO_4$; 0.5 g L^{-1} NH_4Cl; 14.6 g L^{-1} K_2HPO_4; 4.0 g L^{-1} $Na_2HPO_4 \times 2\ H_20$; 1.0 g L^{-1} $(NH_4)_2$-citrate; 0.5 g L^{-1} $MgSO_4 \times 7\ H_2O$; 0.01 g L^{-1} thiamine; 3 mL L^{-1} trace element solution (TES) [Wilms et al., 2001] were used for cultivation. The trace element solution consists of 0.5 g L^{-1} $CaCl_2$; 0.18 g L^{-1} $ZnSO_4 \times 7\ H_2O$; 0.1 g L^{-1} $MnSO_4 \times H_2O$; 10.05 g L^{-1} Na_2–EDTA; 8.35 g L^{-1} $FeCl_3$; 0.16 g L^{-1} $CuSO_4 \times 5\ H_2O$ and 0.18 g L^{-1} $CoCl_2 \times 6H_2O$. Ampicillin was added to the medium in a final concentration of 100 mg L^{-1}.

3.4 Adaptation of *E. coli* from LB_{amp} to minimal medium

In order to substitute the complex medium by the minimal medium according to Wilms et al. [Wilms et al., 2001], the host organism was first grown in 100 mL LB_{amp} medium. After reaching a bio dry mass (BDM) concentration of 0.264 g L^{-1}, 10 mL of the biomass suspension were transferred to 90 mL fresh minimal medium. If the next culture was reaching a biomass concentration of 0.264 g L^{-1}, again 10 mL of the biomass suspension was transferred to 90 mL fresh minimal medium. These steps were repeated until two successive batch cultivations were showing the same growth behavior. The adaptation was then considered as successful. To verify that the adapted strain still contains the plasmid, another cultivation with 500 mL minimal medium was inoculated from the last successful adapted batch. The protein expres-

sion was induced by addition of 0.4 mM IPTG after reaching a biomass concentration of 0.15 g L^{-1}. Prior to the induction, the incubation temperature was decreased to 24°C to prevent the formation of inclusion bodies. The MTG expression was monitored using the hydroxamate assay as described in section 3.14.2.

To produce cryo stock cultures, 0.5 mL samples were taken from the last not induced cultivation in minimal medium after the biomass concentration reached 1.32 g_{BDM} L^{-1}. The samples were diluted 1:1 with glycerol and stored at -80°C.

3.5 Satellite batch cultivations

To investigate the influence of the static cultivation parameters (temperature and inductor concentration) on the transglutaminase expression in *E. coli* BL21, satellite batch cultivations were carried out according to the literature [Dodge and Gerstner, 2002]. The different temperatures and IPTG concentrations were mapped out by a design of experiment software (MODDE 8.0; Umetrics AB, Umea, Sweden).

The batch cultivations of *E. coli* BL21 Gold (DE3) were performed in 2 L shaking flasks with an initial culture volume of 0.5 L minimal medium and an initial biomass concentration of 0.81 g_{BDM} L^{-1}. The biomass to inoculate the shaking flasks was provided from the batch phase of a fed-batch cultivation as described in section 3.6. To evaluate the optimal protein expression parameters, the inoculated shaking flasks were incubated at various temperature/inductor combinations (details see Table 2) at 80rpm (Multitron II, Inforce HT, Bottmingen, Switzerland). After 2 hours of incubation, the specific activity of rMTG(S2P), plasmid stability and inclusion body formation were measured as described in section 3.14. The relatively low biomass concentration and the short induction time were chosen to minimize the risk for a pH shift or oxygen limitation. Therefore, the influence of the environmental parameters

(e.g. substrate concentration, dissolved oxygen concentration etc.) was minimized to investigate the influence of the chosen parameters (temperature and inducer concentration) on the protein expression.

3.6 Fed-batch cultivation

The fed-batch cultivations with *E. coli* BL21 Gold (DE3) were performed in a 20 L Biostat C20-3 Bioreactor (Braun Biotech AG, Melsungen, Germany) with an initial culture volume of 11.5 L minimal medium.

The basic scheme for the fed-batch cultivation consisted of 3 phases: 1. batch phase, 2. feeding phase and 3. protein expression with feeding. The temperature for the batch and feeding phase was 37 °C and was decreased to 29 °C prior to the protein expression phase. The dissolved oxygen concentration was maintained above 20 % saturation by operating the stirrer speed and the airflow in a predetermined profile. In the optimized fed-batch cultivation, the stirrer speed was adjusted by a linear ramp from 500 rpm to 1000 rpm for the first 9 hours, then keeping it at 1000 rpm for the next 3 hours and then decreasing the speed linear from 1000 rpm to 500 rpm over the next 4 hours. The airflow was adjusted in the first 2 hours to 5 L min^{-1}, from 2 hours to 12 hours it was linear increased to 16 L min^{-1} and from 12 hours to 16 hours it was linear decreased to 8 L min^{-1}. According to the bioreactor configuration (maximum stirrer speed, stirrer geometry, maximum air flow etc.), a k_La value (up to 60 h^{-1} at 1200 rpm and 20 L min^{-1} airflow) sufficient to support biomass concentrations up to 20 g_{BDM} L^{-1} using air could be realized. Biomass concentrations higher than 25 g_{BDM} L^{-1} were critical. Therefore, the maximal biomass concentration was adjusted to 20 g_{BDM} L^{-1}. The pH was kept to 7.0 by automated dosage of base (NH_4OH, 25%) and acid (phosphoric acid, 20 %). The formation of foam

was monitored online and suppressed by automated addition of silicone antifoaming emulsion purchased from Carl Roth GmbH (order No 0865.2, Karlsruhe, Germany).

To prepare the inoculum for the cultivations, six 2 L shaking flasks with 500 mL minimal medium were inoculated with 2 mL cryo stock culture and grown for 8 h at 37 °C and 90 rpm (preculture I). The biomass was then harvested (4000 g, 30 min, Avanti^{TM} J-30I, Beckmann Coulter GmbH, Krefeld, Germany) and resuspended in a lower volume of minimal medium (approximately 400 mL). The OD was measured and the necessary volume for the inoculation of the bioreactor with an initial concentration of 0.15 g L^{-1} was calculated. The batch phase was started with an initial volume of 11.5 L minimal medium containing 100 mg L^{-1} ampicillin and 20 g L^{-1} glucose and the addition of the inoculum. The second phase (feeding of glucose) was started after 3.5 h cultivation time before the initial amount of glucose was consumed. Hereby, it was possible to equalize initial biomass concentration deviations and to avoid the starvation stress otherwise observed during the shift from batch to fed-batch conditions [Jenzsch et al., 2006]. An exponentially feeding rate was realized to adjust the biomass growth rate (μ) to a certain value which was calculated by the mathematical model. The applied growth rate was ranging from 0.505 h^{-1} for full batch growth down to 0.1 h^{-1}. The feeding profile was formed according to equ. 1 where *Fi*1 was the control value for the feeding rate. The feeding function followed an exponential profile to control the growth rate μ in the fed-batch phase and in the post induction phase by the means of glucose limitation.

$$Fi1 = \left(\frac{\mu_{set}}{Y_{\frac{X}{S}}} + m \right) V_{ts} X_{ts} e^{\mu_{set}(t-ts)} \quad \text{equ. 1}$$

To optimize the cultivation profile, the parameters of the feed function are summarized in three functional parameters. These functional parameters were the main optimization variables of the cultivation as shown in equ. 2.

$$Fi1 = a e^{b_{(t-c)}} \quad \text{equ. 2}$$

The feeding solution consisted of minimal medium as described in section 3.3 and 500 g glucose per kg solution. The biomass was then grown until a defined concentration and then induced with 0.7 mM IPTG to start the protein expression phase while the temperature was set to 29 °C. Additional Ampicillin to a final concentration of 100 mg L^{-1} was added directly after adding the inductor to suppress all cells without the plasmid responsible for the rMTG(S2P) expression and ampicillin resistance. The induction period usually lasted between 3 and 6 hours depending on the predicted time course of the protein expression. The cultivation was then finished and the biomass harvested by centrifugation (4000 g, 30 min, Avanti™ J-30I, Beckmann Coulter GmbH, Krefeld, Germany).

3.7 Cell lysis by high pressure homogenization

Four gram of frozen *E. coli* cells were resuspended in 150 mL of 50 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$, pH 8 to adjust the biomass concentration to 6.4 g L^{-1}. The cell suspension was tempered to 4 °C and disintegrated by four passages at 1000 bar using a high pressure homogenizer (EmulsiFlex-C5, Avestin Europe, Mannheim, Germany). After each passage, the suspension was cooled to 4°C before starting the next disintegration cycle. The suspension was then centri-

fuged for 20 min at 10.000 g in order to separate insoluble matter. The supernatant (crude protein extract) was used immediately or sometimes after short storage at -20 °C.

3.8 Pro-rMTG and pro-rMTG(S2P) activation with proteinase K in crude protein extract

The crude protein extract was incubated at 37 °C in a stirred bioreactor for 2 to 3 hours with free proteinase K (0.02 and 0.62 U mL^{-1}), and immobilized proteinase K (0.862 and 2.03 U mL^{-1}). After regular time intervals, samples were centrifuged for 1 minute at 16000 g and 4°C. The supernatant was assayed for activity using the standard activity assay. After complete activation, the supernatant was purified by Immobilized Metal Ion Affinity Chromatography (IMAC).

3.9 Immobilized Metal Ion Affinity Chromatography (IMAC)

The IMAC based purification of different protein samples (crude protein extract containing pro-rMTG-His_6 or activated rMTG(S2P)-His_6 / rMTG(S2P)-His_6 and IMAC purified pro-rMTG-His_6) was carried out as published previously [Marx et al., 2008a]. Support: Streamline Chelating, Ni-NTA; column: XK 16 / 20; CV: 30 mL; flow rate for sample application: 3 mL / min; flow rate for washing and elution: 3 mL / min; binding buffer: 50mM Tris/HCl buffer with 300 mM NaCl, 20 mM imidazole, pH 8.0; elution buffer: 50mM Tris/HCl buffer with 300 mM NaCl, 500mM imidazole, pH 8.0; fraction volume: 1 mL. After loading and washing the column with 3 CV of binding buffer, step elution was carried out from 20 mM to 500 mM imidazole using the elu-

tion buffer. Transglutaminase containing fractions were analyzed by SDS–PAGE and the hydroxamate activity assay. Fractions were stored at 4 °C.

3.10 Dialysis and storage of IMAC fractions

Pro-rMTG-His_6 containing fractions of IMAC were pooled and dialyzed twice against dialysis buffer (50 mM Tris/HCl buffer, 300 mM NaCl, 2 mM $CaCl_2$ pH 8.0) at 4 °C. The imidazole free transglutaminase preparations were stored at -80°C in storage buffer as stock solution (4.58 mg ml^{-1} pro-rMTG in storage buffer (50 mM Tris/HCl buffer, 300 mM NaCl, 1 mM GSH, 2 mM $CaCl_2$ pH 8.0 in 50 % glycerol).

Proteinase K activated rMTG-His_6 or rMTG(S2P)-His_6 IMAC purified from cell lysate was dialyzed against dialysis buffer without NaCl or $CaCl_2$ (50 mM Tris/HCl buffer, 300 mM NaCl, 2 mM $CaCl_2$ pH 8.0) at 4 °C. During the dialysis, protein precipitation was observed. The supernatant was removed and the pellet containing the transglutaminase was stored at -80°C.

3.11 Pro-rMTG activation with dispase or proteinase K

An IMAC purified stock solution with 4.58 mg mL^{-1} pro-rMTG-His_6 in storage buffer was diluted to different concentrations (0.25 mg mL^{-1} for the activation experiments using proteases, 1 mg mL^{-1} for the MALDI-TOF MS measurement and 2.5 mg mL^{-1} for the proteolytic activation and consecutive IMAC purification experiments). These solutions were incubated for 2 hours at 37 °C with 0.21 U mg^{-1} dispase (activation experiments), 1.31 U mL^{-1} dispase (MALDI-TOF MS measurements) or various proteinase K concentrations according to the experimental set up.

3.12 Peptide synthesis and immobilisation providing immobilized tripeptide library papers

All amino acids derivatives, resins and coupling reagents were obtained from Calbiochem-Novabiochem. Other solvents and chemicals were purchased from Sigma-Aldrich. Peptides were synthesized and immobilized by Dr. Malesevic, Max Planck Institut Halle Saale.

3.13 Investigation of the rMTG / rMTG(S2P) substrate specificity using immobilized tripeptide libraries

The peptide libraries immobilized on the paper matrix contained 400 tripeptide-spots of the kind X-Q-Y-linker, where X and Y represent the variable flanking amino acids around the central glutamine or lysine. The flanking amino acids consisted of all 20 proteinogenic amino acids and were varied to provide every possible combination. All steps were done in hybridization flask bought from Ochs GmbH (Bovenden, Lenglern, Germany). The loaded paper matrix was washed 2 times with Tris buffer (50 mM Tris-HCl pH 8) at 37 °C and then incubated for 30 min at 37 °C in 25.6 mL MDC / rMTG or rMTG(S2P) solution. For the preparation of the fluorescence marker MDC with a final concentration of 140 µM and TG (rMTG or rMTG(S2P)) with a final concentration of 1 U / mL were prepared in 25.6 mL of a 50 mM Tris-HCl pH 8 buffer. During the incubation the rMTG / rMTG(S2P) crosslinked the tripeptides on the paper matrix with MDC. After incubation the MDC / TG solution was removed and the paper matrix was washed five times with Tris buffer (50 mM Tris-HCl pH 8). To remove eventual fluorescing TG / MDC precipitates the paper matrix was washed with 50 mL methanol followed by five times washing with dist. water and 3 times repetition of this step.

To determine the substrate specificity of the rMTG / rMTG(S2P) toward the different tripeptides on the paper matrix the fluorescence of every spot on the paper matrix was measure with the Bio-Vision+ Detector (Vilver-Lourmat Deutschland GmbH, Eberhardzell, Germany) at 312 nm excitation and the measurement was done at 520 nm. The evaluation was done using the Software „Bio-1D“.

3.14 Analytical methods

3.14.1 Off-line measurements of the fed-batch cultivation

Off-line measurements of the fed-batch cultivation such as biomass and glucose concentrations were performed every 30 minutes. The biomass concentration was calculated from OD_{600} measurements (OD_{600} of 1 equates to 0.3 g_{BDM} L^{-1}). Glucose concentration was measured quantitatively by using a glucose analyzer (YSI 2700 SELECT, YSI Incorporated, Yellow Springs, Ohio, USA]. During the protein expression phase, 1/OD samples were taken, centrifuged (16100 g, 5 min), the supernatant was removed and the pellet was stored at -20°C.

3.14.2 Determination of enzymatic activity in biomass or purified samples

Cells from 1/OD samples were resuspended in 130 µL lysis buffer (50 mM Tris-HCL, 20 mM $MgCl_2$, 10 U mL^{-1} Benzonase) and enzymatically lysed with 3 mg mL^{-1} lysozyme for 60 min at 37°C. The insoluble pellet was centrifuged off (16100 g, 5 min, Eppendorf 5415R, Eppendorf AG, Hamburg, Germany) and the MTG containing supernatant, called crude protein extract, was activated. Dispase (1 U mL^{-1}, 30 min, 37°C, BD Biosciences, Heidelberg, Germany) was used as activating protease. The high enzyme concentration and long incubation times ensured sufficient and comparable cell lysis and activation equal for every 1/OD sample in the experiments.

The activity of the transglutaminases in crude protein extract or purified samples was determined by the standard assay [Folk and Cole, 1966].

In order to relate published and obtained rMTG activity [U] to rMTG protein amount [mg], the published values of the specific activity of the wild-type MTG from *Streptomyces mobaraensis* (22.6 U mg^{-1} protein) was used [Ando et al., 1989]. For the rMTG(S2P) a specific activity of 46.1 U mg^{-1} was published [Marx et al., 2007b].

3.14.3 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [Laemmli, 1970] using a 4.5 % collecting gel and a 12.5 % separation gel. Cells from 1/OD samples were enzymatically lysed with lysozyme (3 mg mL^{-1}) and the insoluble pellet was centrifuged off (16100 g, 5 min, Eppendorf 5415R, Eppendorf AG, Hamburg, Germany). The soluble fraction was then analyzed by SDS-Page directly. The insoluble fraction was resuspended, washed two times with a 0.05 M Tris/HCl buffer, pH 8, dissolved in SDS-buffer and then analyzed by SDS-PAGE.

Due to the stimulating effect of low SDS-concentrations on proteinase K and its high thermostability [Gross-Bellard et al., 1973] prior to the SDS-PAGE (e.g. preliminary incubation at 99°C) the proteinase K was inhibited in all samples to prevent unwanted reactions. Ebeling et al. investigated the inhibition of proteinase K and described a complete inhibition of proteinase K by Phenylmethylsulfonyl fluoride (PMSF) [Ebeling et al., 1974]. Therefore, PMSF was added to all samples to a final concentration of 1 mM. The samples were incubated at room temperature for 30 minutes and then analyzed by SDS-PAGE.

3.14.4 N-terminal sequence determination

The N-terminal Edman-sequencing of IMAC purified and proteinase K activated rMTG was done by Proteome Factory AG (Berlin, Germany). Prior to the analysis, the protein sample was separated by SDS-PAGE and the proteins were transferred from the resulting gel to a transfer membrane in a transfer chamber with 15 V / 0.44 A for 20 min in transfer buffer (10 mM CAPS and 10 % methanol in water at pH 11, the pH was adjusted with 2 M NaOH). The dried transfer membrane was then sent for sequencing.

3.14.5 Measurement of the bleeding of immobilized proteinase K

The potential bleeding of immobilized proteinase K was investigated by incubation of 10 U mL^{-1} of immobilized proteinase K in buffer (50 mM Tris/HCl, 300 mM NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$, pH 8) at 37°C for 2 hours under constant mixing. After 2 hours, the immobilized proteinase K was completely removed by centrifugation and the supernatant was measured for caseinolytic activity according to Marx et al. [Marx et al., 2008a].

3.14.6 Mass spectrometric investigation of the dispase activated rMTG

MALDI-MS experiments were carried out by Dr. Schmelzer, Institut of Pharmacy, Martin Luther University using a delayed extraction TOF mass spectrometer Voyager-DE PRO (AB Sciex, Darmstadt, Germany) equipped with a pulsed nitrogen laser (λ = 337 nm). Sinapinic acid was used as matrix solution and mixed with the sample solution 10:1 (V/V) and then dried in a stream of air. Measurements were performed operating in the positive ion linear mode at a total acceleration voltage of 25 kV, grid voltage set to 92 %, 0.15 % guide wire voltage and an extraction delay of 700 ns. A low mass gate was set to *m/z* 10,000 to prevent detector saturation from low mass compounds. The instrument was externally calibrated using calibration mixture 3 of the Sequazyme Peptide Standards Kit (AB Sciex, Darmstadt, Germany) and BSA.

3.14.7 Mass spectrometric investigation of immobilized tripeptides from a paper matrix

The mass spectrometric analysis was done with a MALDI-ToF REFLEX mass spectrometer (Bruker Daltronik). The sample was prepared from a previously rMTG cross linked spot (K-Q-R) of the paper matrix and analyzed by Dr. Malesevic, Max Planck Research Unit for Enzymology of Protein Folding, Halle Saale.

4. Results

The first aim of this thesis was the mass production of a soluble recombinant pro-transglutaminase variant which is (after proteolytic removal of the pro-sequence) 100 % more active and 270 % more stable against thermal denaturation than the wild-type transglutaminase from *Streptomyces mobaraensis*. Therefore, the S2P variant of MTG which was isolated during the random mutagenesis experiments [Marx et al., 2008b] was selected for overproduction. In contrast to the wild-type enzyme, this variant carries a proline instead of a serine residue at position two of the primary sequence and a C-terminal histidine-tag which allows for the purification by affinity chromatography. In order to be able to produce large amounts of rMTG(S2P), an *E. coli* high-cell density fed-batch process was developed.

The second aim of this thesis was the development of a purification and activation method for bulk quantities of the intracellular expressed pro-rMTG(S2P) from bio wt mass.

In the third part of the thesis the substrate specificity of the rMTG(S2P) was investigated to better understand its cross-linking behavior to optimized its application and to enhance the prediction of possible new rMTG(S2P) substrates.

4.1 Adaptation of the host organism to a minimal medium

In order to allow reproducible growth and product yield, minimal medium was used throughout the fed-batch cultivations. The production organism was adapted to the minimal medium by sequential passaging as described in section 3.4. After 6 passages a portion of only 1 ppm LB_{amp} medium is left. As can be seen from Fig. 1, the specific growth rates on minimal medium after the 4^{th} – 6^{th} passage are identical to the growth rates observed in the complex medium which indicated a successful ad-

aptation. Using the adapted cells, a reproducible time management for the entire production can be achieved. A comparable adaptation behavior was observed for *Saccharomyces cerevisiae* cells, which were adapted from a medium with glucose as sole carbon source to a medium with xylose as the sole carbon source [Madhavan et al., 2009].

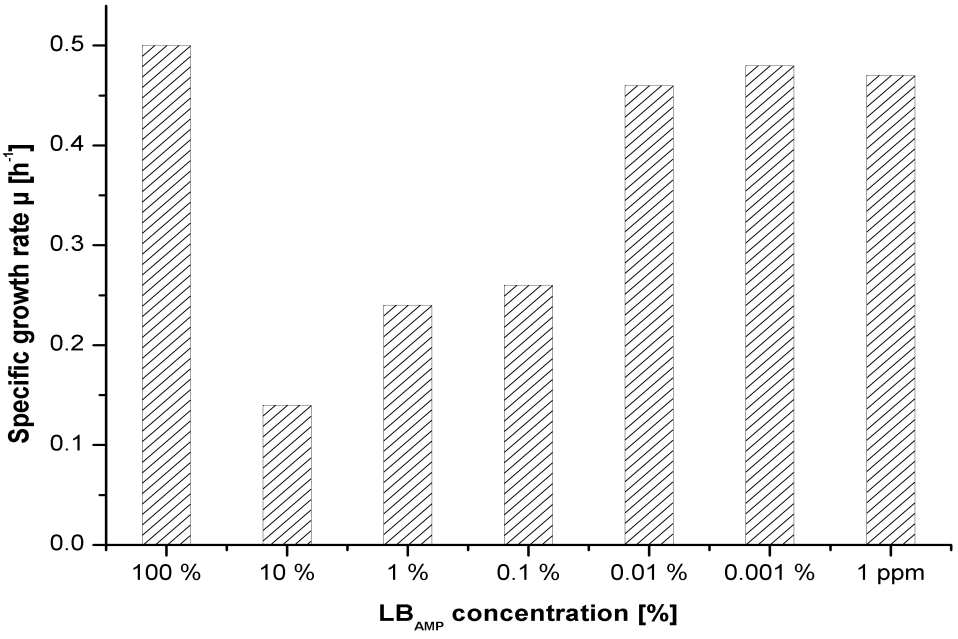

Fig. 1: Adaptation of *E. coli* pCM203(S2P) from LB_{amp} to minimal medium by sequential passaging. The specific growth rates are plotted over the LBamp concentrations remaining in the minimal medium. The first cultivation was carried out using 100 % LBamp medium. After reaching a bio dry mass (BDM) concentration of 0.264 g L-1, 10 mL of the biomass suspension were transferred to 90 mL fresh minimal medium. If the next culture was reaching a biomass concentration of 0.264 g L-1, again 10 mL of the biomass suspension was transferred to 90 mL fresh minimal medium. These steps were repeated for all cultivations.

To check if the strain is still able to express pro-rMTG(S2P), the adapted strain was cultivated in a control batch cultivation as described in section 3.4. The control cultivation (Fig. 2) shows clearly the protein expression after induction with IPTG. Thereby, the host organism was successfully adapted to the minimal medium ac-

cording to Wilms et al. [Wilms et al., 2001] and still does contain the plasmid to produce the pro-rMTG(S2P).

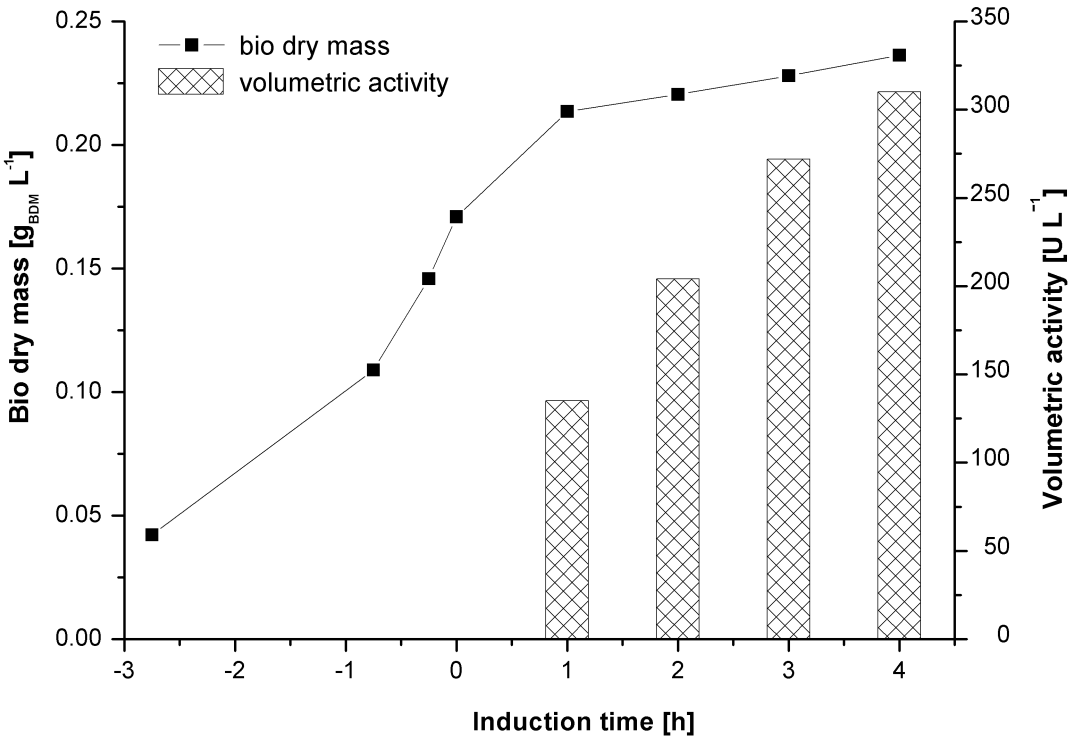

Fig. 2: Cultivation of adapted *E. coli* pCM203(S2P) cells using minimal medium according to Wilms et al. [Wilms et al., 2001] and time course of the formation of pro-rMTG(S2P). At a biomass concentration of 0.165 g L^{-1} the induction of the adapted cells was done by adding IPTG to a final concentration of 0.4 mM. The incubation temperature of the adapted cells was decreased after induction to 24°C to prevent the formation of inclusion bodies. 1/OD samples were taken in the first 4 h after induction, cells were enzymatically lysed and the pro-rMTG(S2P) was activated by cleaving the pro-sequence with dispase, followed by the standard hydroxamate assay.

4.2 Optimization of the static product expression parameters using satellite cultivations mapped out by a design of experiments method

To investigate the ideal static product expression parameters (temperature and IPTG concentration), satellite batch cultivations were carried out. The parameter pattern shown in Table 2 (row 1 and 2) was mapped out by a Design of Experiment method based on the literature [Marx et al., 2007a]. The experimental conditions are described in section 3.5. Besides biomass concentration and specific activity, the plasmid stability was measured.

Table 2: Experimentation pattern and results for the satellite cultivation to investigate the influence of the protein expression parameters temperature and inductor concentration on the pro-rMTG(S2P) expression in *E. coli* pCM203(S2P) mapped out by a Design of Experiment method.

Temperature [°C]	IPTG conc. [mM]	Biodrymass [$g_{BDM}\ L^{-1}$]	Spec. activity [$U\ g^{-1}_{BDM}$]	Plasmid stability [%]
24	0.4	0.969	780.1	100
24	0.7	0.873	785.3	97
24	1.0	0.945	719.0	97
29	0.4	1.047	1318.2	96
29	0.7	1.038	1364.4	82
29	0.7	1.005	1411.3	93
29	0.7	1.014	1383.3	88
29	1.0	1.017	1342.1	93
34	0.4	1.107	1095.7	83
34	0.7	1.071	945.6	87
34	1.0	1.089	977.3	95

The results shown in Table 2 were used to develop the statistical model to describe the influence of temperature and inductor concentration. This model was used to calculate the resulting specific activity and plasmid stability for various temperature / IPTG concentration combinations as shown in Fig. 3. According to the model, the

highest specific activity can be obtained at 29 °C and an IPTG concentration of 0.7 mM.

The probability value P is defined as the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true. It describes the statistical reliability and should be usually lower than 0.05 for statistical significance.

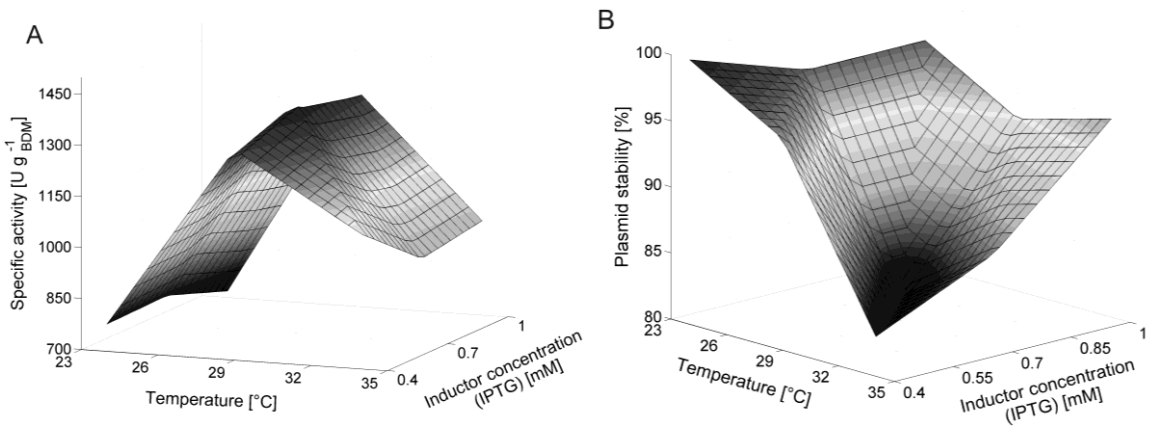

Fig. 3: 3D-plot for the influence of temperature and inductor concentration on the pro-rMTG(S2P) expression in *E. coli* pCM203. Batch cultivations were carried out using 0.5 L minimal medium in 2 L shaking flasks. The flasks were inoculated with 0.81 g_{BDM} L^{-1} initial biomass obtained from the batch phase of a fed-batch cultivation. The flasks were incubated at various temperature / IPTG concentration combinations and the resulting specific activities after 2 hours were used to develop a statistical model describing the influence of temperature and IPTG concentration on the biomass related specific activity (A) and the plasmid stability (B).

In the experiment, P indicated for the temperature a statistically significant influence (P = 0.003) on the bio dry mass related specific activity. For the influence of the IPTG concentration P is equal to 0.3 and therefore these results are statistically uncertain. The plasmid stability did also show a statistically significant temperature dependence (P=0.043) and is decreasing with higher temperatures. Again, the statistical uncertainty was to high to verify a significant influence for the IPTG concentration (P = 0.6) on the plasmid stability.

Though, the statistical indicators do not provide proof for an IPTG concentration influence on the protein expression of the plasmid stability the raw results still indicate an influence of the IPTG concentration. For the aim of this thesis, the optimization of the pro-rMTG(S2P) expression, the obtained results are sufficient. However, it would be interesting to investigate the IPTG influence in another more detailed study further.

These results lead to the conclusion that the induction of the fed-batch cultivation should be done at 29 °C in order to obtain the highest specific activity. At this temperature high plasmid stability is ensured and possible formation of inclusion bodies avoided. IPTG could be applied in concentrations from 0.4 mM to 1 mM. Finally, a standard IPTG concentration of 0.7 mM was chosen for the consecutive cultivations.

4.3 Production of pro-rMTG(S2P) by fed-batch cultivation of *E. coli* BL21Gold(DE3), modeling and simulation

4.3.1 Determination of raw / experimental data

The state of the art for producing recombinant proteins in *E. coli* (like pro-rMTG(S2P)) is a fed-batch regime. This method allows reproducible production of high cell densities, provides high product yields within relatively short time intervals and thus a high space-time yield. The principle fed-batch regime for *E. coli* is widely known and for example described by Kavanagh et. al [Kavanagh and Barton, 2008]. However, for the production of a novel protein the process parameters can not be taken from fermentation of a different protein. They have to be investigated for exactly this process to obtain a maximum product yield and to avoid problems like acetate formation or substrate / oxygen limitation. Therefore, a first fed-batch cultivation using the optimized static product expression parameters (see chapter 4.2) was car-

ried out in order to gather raw data for the parameter estimation. The feed profile developed by Korz et al. was used to make sure the biomass concentration doesn´t reach the critical area, to provide results useful for the development of a mathematical model for this process and was characterized by the growth rates as listed below [Korz et al., 1995]. The results are shown in Fig. 4.

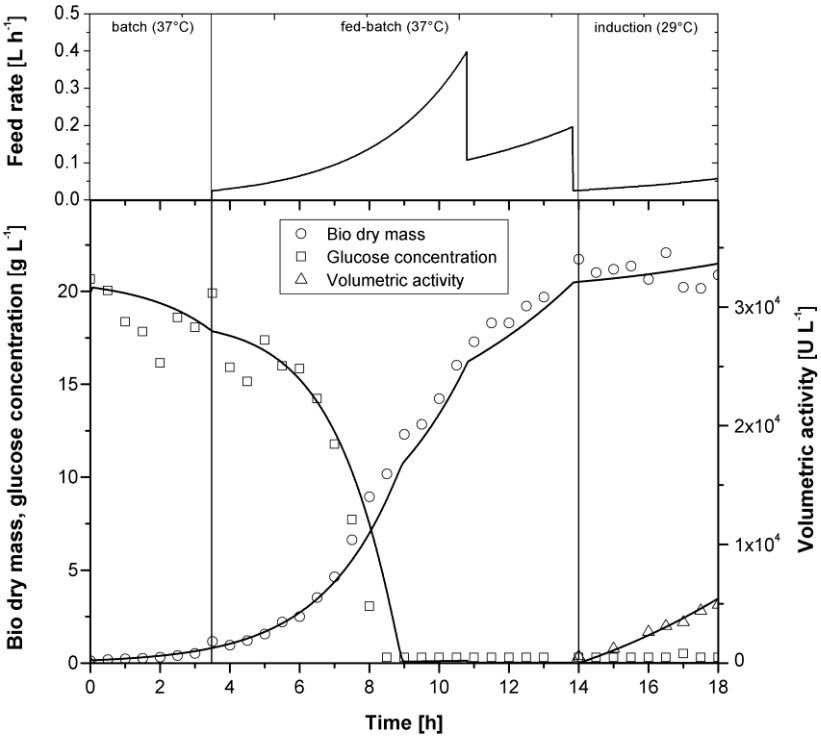

Fig. 4: Basic fed-batch cultivation: pro-rMTG(S2P) production in *E. coli* pCM203(S2P). Cells were grown in a bioreactor with an initial volume of 11.5 L of minimal medium, containing 0.1 g L^{-1} ampicillin at 37 °C. After 14 hours of cell growth pro-rMTG(S2P) production was induced with 0.7 mM IPTG after lowering the temperature to 29 °C. Ampicillin was added to a final concentration of 0.1 g L^{-1}. 1/OD samples were taken every 0.5 hours after induction, cells were enzymatically lysed and the pro-rMTG(S2P) was activated by cleaving the pro-sequence with dispase, followed by the hydroxamate assay. The upper graph shows the feeding profile for the substrate solution (500g glucose per kg minimal medium) according to the profile calculated by the mathematical model. The lower graph shows bio dry mass and glucose concentration and volumetric rMTG(S2P) activity over time during the cultivation. Raw data are shown as symbols and the drawn lines represent the model simulation.

In the batch phase, *E. coli* was growing with a maximum growth rate of 0.49 h^{-1} for 9 hours until the glucose in the medium was consumed. The exponential feeding was started at 3.5 hours before glucose was completely exhausted. Thereby, the stress caused by the shift from batch to the fed-batch phase can be reduced [Jenzsch et al., 2006]. After 9 hours, the excess glucose was consumed and the growth rate was adjusted by the exponential feeding from the maximum growth rate to 0.22 h^{-1} for 2 hours. After 11 hours of cultivation the growth rate was decreased to 0.08 h^{-1} by means of decreasing the feed rate for the next 3 hours to keep the biomass below the critical area and to provide condition for the initial product formation (see diagram A of Fig. 4).

The induction was done by a biomass concentration of around 20 g_{BDM} L^{-1}. After decreasing the temperature to 29°C, IPTG was added to the medium to a final concentration of 0.7 mM. Not only the temperature but also the growth rate was decreased to 0.01 h^{-1} by decreasing the feed. Thereby, a relatively slow pro-rMTG(S2P) formation rate was realized ensuring the soluble expression of the protein by avoiding high local protein concentrations in the cytoplasma which could lead to inclusion body formation. The relatively high deviation in the beginning for the glucose and at the end for the bio mass concentration were caused by the increasing but necessary number of sample dilution step prior to the analysis. Additionally the increasing foam formation with higher bio mass concentrations due to higher protein concentrations in the cell free medium also increased the deviation.

The product concentration increased within 4 hours to approximately 5000 Units pro-rMTG(S2P) per L medium. Then, the biomass (1.4 kg wet and approx. 300 g dry mass, respectively) was harvested. In total, approximately 85,000 U (1,850 mg) of rMTG(S2P) were obtained from 13 L cultivation volume within 18 hours, which corresponds to a space time yield of 363 U_{TG} L^{-1} h^{-1}).

4.3.2 Model design

The raw data obtained from the first cultivation (see Fig. 4) were used to develop a mathematical model according to Galvanauskas et al. to simulate the whole process [Galvanauskas et al., 2004]. All used parameters are listed in Table 3.

Table 3: Variables used for the modeling of the fed-batch cultivation of *E. coli* pCM203(S2P).

Value	Description	Unit
μ_{max}	maximal specific growth rate	h^{-1}
μ_{maxind}	maximal specific growth rate after induction	h^{-1}
$Y_{X/S}$	yield bio dry mass obtained from substrate before induction	$g\ g^{-1}$
μ	growth rate	h^{-1}
X	bio dry mass concentration	$g\ L^{-1}$
S	substrate concentration	$g\ L^{-1}$
S_f	substrate concentration of the feed solution	$g\ L^{-1}$
σ	substrate consumption rate	$g\ L^{-1}$
π	product formation rate	$g\ h^{-1}$
$Y_{X/Sind}$	yield bio dry mass obtained from substrate after induction	$g\ g^{-1}$
$Y_{P/X}$	yield product from bio dry mass after induction	$g\ g^{-1}$
$Y_{X/S}$	yield bio dry mass from substrate	$g\ g^{-1}$
K_S	monod constant	$g\ L^{-1}$
K1	metabolic burden constant growth	$g\ U^{-1}$
K2	metabolic burden constant production	$g\ U^{-1}$
Fi1	feed rate for the substrate solution	$L\ h^{-1}$
Fi2	flow rate for control solutions like acid, base or antifoam agent which enter the system	$L\ h^{-1}$
Fa1	flow rate of cell and substrate containing volume which leaves the system by sampling	$L\ h^{-1}$
Fa2	flow rate of cell and substrate free volume which leaves the system by evaporation	$L\ h^{-1}$
V	Volume	L
T	Time	h
t_0	induction time	h
P	product concentration	$g\ L^{-1}$

The developed model consists of an equation system of four terms. The first term describs the biomass development over time ($dX\ dt^{-1}$) during the cultivation and is shown in equ. 3:

$$\frac{dX}{dt} = \mu X + \frac{(Fi1 + Fi2 - Fa2)}{V} * X \qquad \text{equ. 3}$$

The first term represents the biomass growth by multiplication the biomass concentration with the growth rate. The second term adjusts the calculated biomass concentration to the volume alteration by feeding, sampling or evaporation. The growth rate is determined by equ. 4:

$$\mu = \begin{cases} \dfrac{\mu_{max} * S}{K_S + S} & t < t_{ind} \\ \dfrac{\mu_{maxind} * S}{K_S + S} * e^{-K1\frac{P}{X}} & t > t_{ind} \end{cases} \qquad \text{equ. 4}$$

The first part simulats the growth rate from inoculation until induction by means of a normal Monod kinetic. To adjust the model to the real process, the host specific parameters μ_{max} and K_s were identified as shown later in section 4.3.3.

The second part describs the growth rate during the product formation (induction) phase. Here, the normal Monod kinetic was used but modified by multiplication with a second term which describs the influence of the product formation on the growth rate by an exponential function.

This exponential function accounts for the product concentration, the biomass concentration [g L^{-1}] and K_1, which was specific to *E. coli* BL21Gold(DE3) pCM203. K_1 was identified by fitting the model to the raw data in a least square approach discussed in section 4.3.3. It was found that an increase in the product concentration was decreasing the growth of *E. coli* BL21Gold(DE3) pCM203 until finally the biomass growth stopped.

The second part of the equation system describs the time dependence of the substrate concentration during the cultivation. It consists of 2 terms like the equation system for the biomass discussed above. The main equation (equ. 5) simulates the substrate consumption by biomass in the first term. The second term represents the substrate concentration increase by the addition of the feeding solution. The third term describes the influence on the substrate concentration of solutions which do

not contain substrate like acid, base and anti foam agent (represented by the Fi2 value). The loss of volume by evaporation was represents by Fa2, which was subtracted from Fi2.

$$\frac{dS}{dt} = -\sigma X + \frac{Fi1}{V} * \left(S_f - S\right) - \frac{(Fi2 - Fa2)}{V} * S \qquad \text{equ. 5}$$

The substrate consumption rate σ is calculated by equ. 6 similar to the calculation of the growth rate µ.

$$\sigma = \begin{cases} \frac{1}{Y_{\frac{X}{S}}} * \frac{\mu_{max} * S}{K_S + S} & t < t_{ind} \\ \frac{1}{Y_{\frac{X}{S}ind}} * \frac{\mu_{maxind} * S}{K_S + S} * e^{-K1\frac{P}{X}} & t > t_{ind} \end{cases} \qquad \text{equ. 6}$$

Before the induction, the substrate consumption was calculated by a conventional Monod equation. The biomass yield from the substrate ($Y_{X/S}$) was identified according to section 4.3.3. and is specific for *E. coli* BL21Gold(DE3) pCM203. After induction, the Monod kinetic was modified by multiplication with an exponential function whereas the exponent $-K_1^{\frac{P}{X}}$ described the growth suppression caused by product expression as mentioned earlier.

The third part of the equation system calculates the product concentration over time. The main equation is given in equ. 7:

$$\frac{dP}{dt} = \pi X - \frac{(Fi1 + Fi2 - Fa2)}{V} * P \qquad \text{equ. 7}$$

The first term describes the pro-rMTG(S2P) production by multiplying the product formation rate and the biomass.

The second term considers the dilution of the product. The flow rates of the product free fluids like acid, base, anti foam agent and volume loss by evaporation are used to correct the product concentration. The product formation rate was calculated according to equ. 8.

$$\pi = \begin{cases} = 0 & t < t_{ind} \\ Y_{P/X} * \frac{\mu_{maxind} * S}{K_S + S} * e^{-K2\frac{P}{X}} & t > t_{ind} \end{cases} \quad \text{equ. 8}$$

Product formation due to an eventual leakage of the T7 expression system could not be detected in the experiments. Therefore, in equ. 8 the product formation rate was defined as zero for the time from inoculation until induction.

After induction, the Monod kinetic was utilized once more and multiplied with the product yield to calculate the product formation rate. Again, for a good fitting to the raw data, a suppression factor as a second correction term was necessary. The best solution was again to multiply with an exponential function with $-K_2^{\frac{P}{X}}$ as exponent. Thereby, the effect of growth suppression by the product rMTG(S2P) was introduced to the calculation of the product formation rate. K_2 is another host specific parameter and had to be identified by fitting the model to the raw data in a least square approach as done in section 4.3.3.

The last term of the equation system simulates the variation of the cultivation volume (equ. 9) and was required for balancing the system and to enable the exact identification of the parameters mentioned above.

$$\frac{dV}{dt} = Fi1 + Fi2 - Fa1 - Fa2 \quad \text{equ. 9}$$

In equ. 9, the different flow rates like Fi1 (feeding rate of substrate solution), Fi2 (control fluids like acid, base and anti foaming agent), Fa1 (volume loss due to sampling) and Fa2 (volume loss due to evaporation) were added up.

4.3.3 Model parameter identification

In section 4.3.2, the equation system to describe a fed-batch cultivation of *E. coli* BL21Gold(DE3) pCM203 to produce pro-rMTG(S2P) was established. The model had then to be fitted to the experimental data to identify the host specific parameters μ_{max}, K_s, K_1, K_2, $Y_{x/s}$ and $Y_{P/X}$.

The identification was done with an iterative least square approach algorithm. In Fig. 4, the raw data are shown as symbols and the fitting is presented by the drawn lines. To identify the specific parameters with an iterative least square approach, an initial guess is used to calculate the development of the fed-batch cultivation. Then, the results are compared with the experimental data and the specific parameters are adjusted to a new value that fits better to the raw data. This cycle is repeated until the sum of squared residuals has its least value, a residual being the difference between the raw data and the value given by the model. The identified values are shown in the Table 4.

Table 4: Parameters identified by the mathematical model for the fed-batch cultivation to produce pro-rMTG(S2P) in *E. coli*. Cells were grown in a 20 L bioreactor with minimal medium containing 0.1 g L^{-1} ampicillin at 37 °C. After 14 hours of cell growth, pro-rMTG(S2P) production was induced with 0.7 mM IPTG after lowering the temperature to 29 °C.

Before induction			**After induction**		
μ_{max}	0.49	h^{-1}	μ_{max}	0.20	h^{-1}
$Y_{X/S}$	0.28	g g^{-1}	$Y_{X/S}$	0.19	g g^{-1}
K_S	0.1	g L^{-1}	$Y_{P/X}$	5159	g g^{-1}
			K_1	0.00049	g U^{-1}
			K_2	0.00088	g U^{-1}

4.4 Estimation of the state space in order to determine optimal cultivation conditions for a maximal product yield

The basic fed-batch cultivation provided the experimental data to establish the model and to identify the host specific parameters. In the second step, the fed-batch strategy was optimized in order to increase the product yield. To predict the optimal conditions for such a cultivation strategy, a state space for the glucose dependency of the product formation in relation to the specific activity was simulated, based on the mathematical model (Fig. 5).

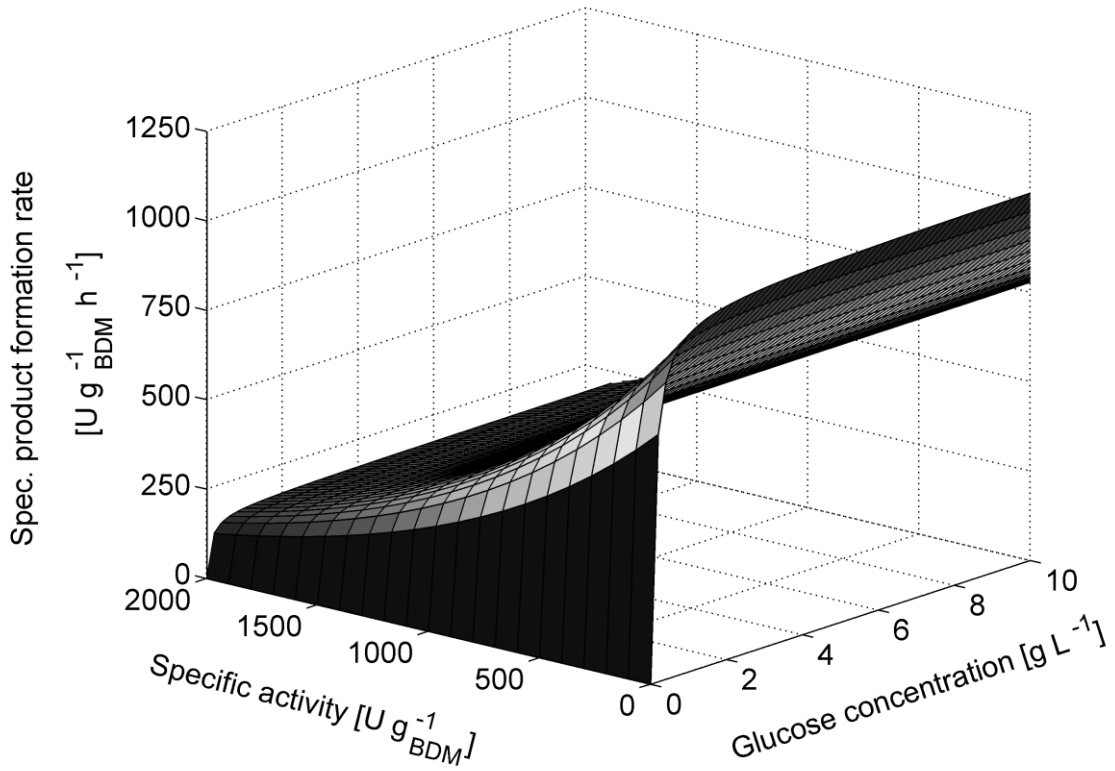

Fig. 5: Model based simulation of the cultivation space. Dependence of the product formation rate on the glucose concentration in relation to the specific activity of the rMTG(S2P) per gram bio dry mass**.**

The negative slope from 0 U g^{-1}_{BDM} to 1500 U g^{-1}_{BDM} for the product formation rate was caused by the growth suppression of the product as formulated in equ. 8. With

an increase of the specific product concentration in the host cells, the repression term $e^{-K_2\frac{P}{X}}$ becomes smaller and lowers the product formation.

The model shows the requirement of glucose concentrations above 5 g L^{-1} for maximal product formation rates (see Fig. 5) leading to improved protein expression. The substrate excess during the pro-rMTG(S2P) expression provides the energy required for the increasing maintenance and protein expression of the cells. Bhattacharya et al. reported that the metabolic maintenance requirements of induced cells was two times higher than that of uninduced cells [Bhattacharya and Dubey, 1995].

However, the higher maintenance requirements alone did not always result in a reduction in the growth rate as reported by Gill et al. [Gill and Ballesteros, 2000]. Bonomo et al. demonstrated that the primary sequence of a recombinant protein can negatively influence translation velocities and strongly decrease the growth rate. For example if a recombinant protein mainly consists of one amino acid the protein expression will consume this amino acid very fast. This leads to concentrations of this amino acid to low for undisturbed cell growth or in the extreme even for the normal metabolic maintenance [Bonomo and Gill, 2005]. Also, the expression of heat shock proteins could provoke stress and a higher metabolic burden on the cells [Parsell and Sauer, 1989].

As a result, glucose concentrations above 5 g L^{-1} were required for a maximal product formation rate that leads to an optimal space time yield. For *E. coli* BL21Gold(DE3) it was shown in a previous batch cultivation that between glucose concentrations of 8 – 20 g L^{-1}, the maximal acetate concentration reached only a level of 0.2 g L^{-1}, an uncritical concentration for overall fed batch performance.

4.5 Optimized fed-batch cultivation for the production of pro-rMTG(S2P) in *E. coli* BL21Gold(DE3)

To realize the glucose excess during the induction period, which is necessary for maximal product formation, in the next experiment the feed rate was decreased in a way that the growth rate was linearly decreasing from 0.505 h^{-1} (μ_{max}) to 0.2 h^{-1} from the point of induction.

Similar to the previous experiment, the feeding started at 3.5 h. After theoretical glucose exhaustion (8 hours) the growth rate was adjusted to 0.505 h^{-1}. 12 hours after process start, the protein expression was induced and the growth rate was set to a variable decreasing rate as described above. The results and the feed profiles are shown in Fig. 6.

After 4 hours of induction, approximately 23,000 U pro-rMTG(S2P) per liter cultivation volume were obtained. In total, in 13 L cultivation volume 300,000 U pro-rMTG(S2P) were produced, which correlated to a 400 % higher space time yield of approx. 1,450 U L^{-1} h^{-1} compared to the basic fed-batch cultivation.

Directly after the induction, the glucose concentration was increasing as intended and the resulting product formation was significantly higher than in the previous experiment. After 16 hours, the cultivation was terminated.

Altogether, it was successfully shown, that the elevated glucose concentration after induction provides enough energy for a high product formation.

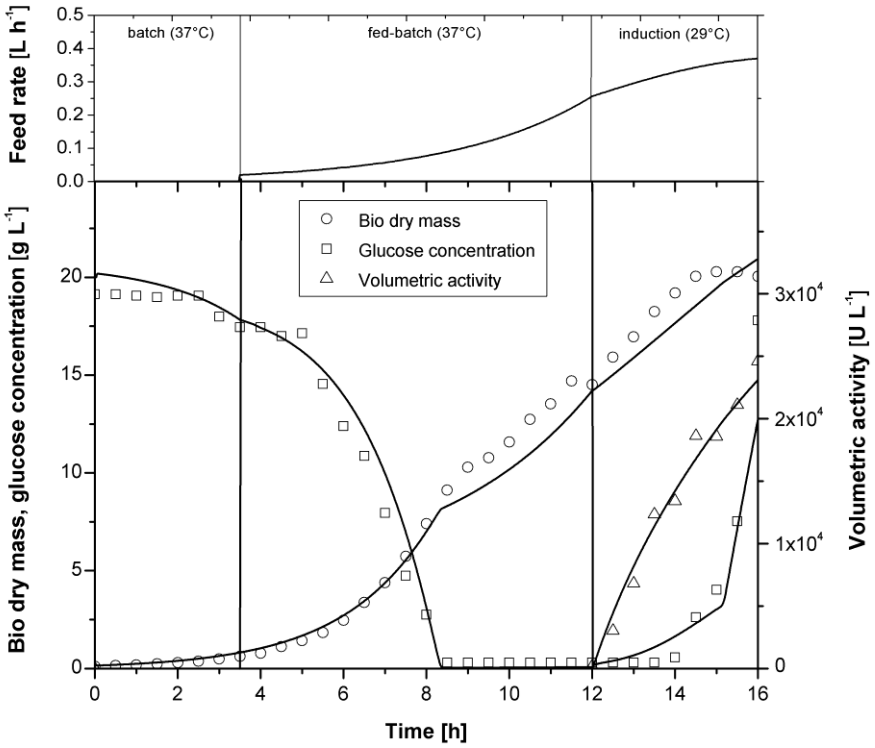

Fig. 6: Optimized fed-batch cultivation: Pro-rMTG(S2P) production in *E. coli* pCM203**.** Cells were grown in a bioreactor with 11.5 L minimal medium as the start volume, containing 0.1 g L^{-1} ampicillin at 37 °C. After 12 hours of cell growth, temperature was lowered to 29 °C and pro-rMTG(S2P) production was induced by addition of IPTG to a final concentration of 0.7 mM. 1/OD samples were taken every 0.5 hours after induction, cells were enzymatically lysed and the pro-rMTG(S2P) was activated by cleaving the pro-sequence with dispase, followed by the hydroxamate assay. The upper graph shows the feeding profile for the substrate solution (500g glucose per kg minimal medium) according to the profile calculated by the mathematical model. The lower graph shows bio dry mass and glucose concentration and volumetric rMTG(S2P) activity over time during the cultivation. Raw data are shown as symbols and the drawn lines represent the model simulation.

Simultaneously with the increased product formation rate, the risk for the formation of pro-rMTG(S2P) inclusion bodies is also increasing. As discussed above, in the batch cultivation the formation of inclusion bodies was successfully suppressed by adjusting the temperature to 29 °C during the induction phase. Samples of the optimized fed-batch cultivation were analyzed by SDS-PAGE (Fig. 7).

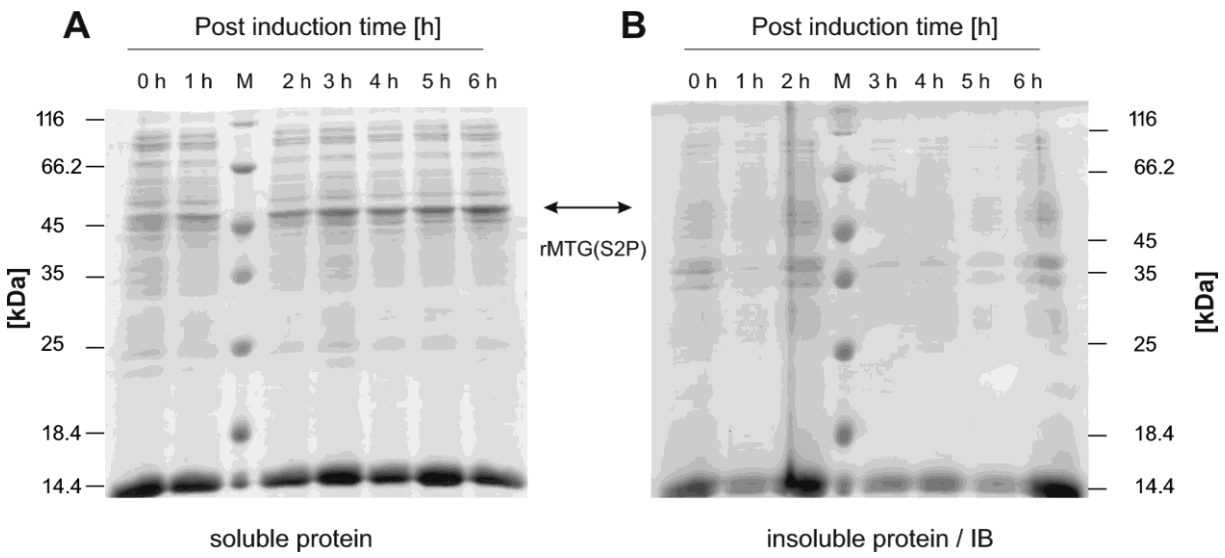

Fig. 7: SDS-PAGE showing the amount of pro-rMTG(S2P) in the soluble (A) and insoluble fractions (B) of 1/OD samples of the optimized fed-batch cultivation. Growth and induction conditions are the same as for Fig. 6. 1/OD samples were taken every 0.5 hour after induction, enzymatically lysed, centrifuged with 16,100 g for 5 min and treated further as described in the materials and methods section.

No inclusion bodies could be detected, thus the produced pro-rMTG(S2P) is completely soluble.

Investigations of the plasmid stability revealed a significant loss of plasmid containing cells during the induction phase. At 1.5 h and 3.5 h after induction, just 89 % resp. 70 % of plasmid containing cells were found. The yield of the target protein could be further increased by developing an expression system with a higher plasmid stability.

4.6 Investigations on the pro-rMTG activation with dispase and consecutive IMAC purification

In order to produce enough recombinant microbial transglutaminase (rMTG / rMTG(S2P)) which is free of activating protease, the previously screened dispase was used for activation of the pro-rMTG [Marx et al., 2008a].

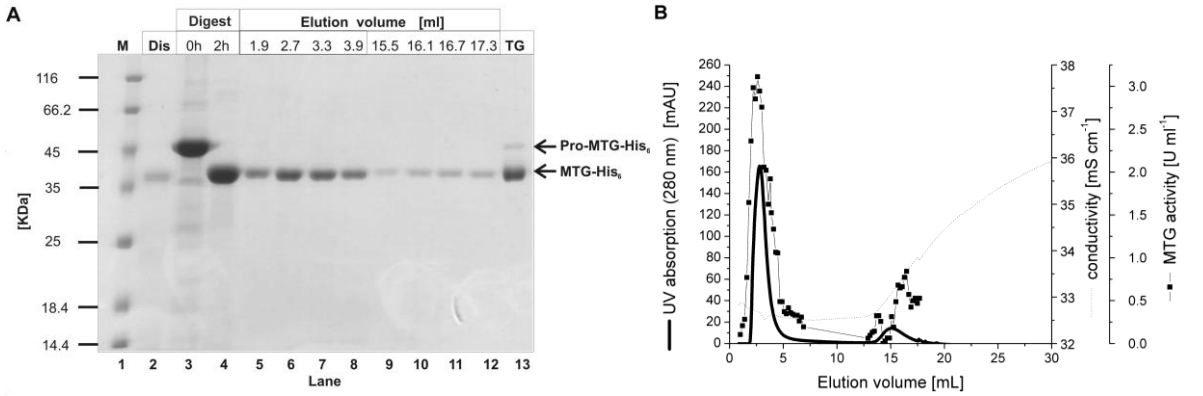

Fig. 8: IMAC purification of dispase activated rMTG. IMAC purified pro-rMTG solution (2.5 mg mL $^{-1}$ pro-MTG in storage buffer with 1 mM GSH and 23 % glycerol) was incubated for 2 hours with 0.21 U mL^{-1} dispase and then transferred to a Streamline Chelating Ni-NTA IMAC column: XK 5/5, CV: 1.2 ml, flow rate loading: 0.5 mL min^{-1}, flow rate washing / elution: 0.5 ml min^{-1}, binding and equilibrating buffer: 50 mM Tris/HCl-buffer with 300 mM NaCl pH 8 and 20 mM imidazole, elution buffer: 50 mM Tris/HCl buffer with 300 mM NaCl pH 8 and 500 mM imidazole. **A**: SDS-PAGE of the dispase activation and IMAC purification, **B**: IMAC chromatogram of the active rMTG in storage buffer I

The separation of the activating protease from the target enzyme was carried out by immobilized metal affinity chromatography (IMAC) as described in material and methods.

As can be seen from the SDS-PAGE shown in Fig. 8A (lanes 3 and 4), the pro-sequence of pro-rMTG was completely removed within 2 hours of activation time. The protein solution was then applied to the IMAC column. As can be seen from the elution profile Fig. 8B and the SDS-PAGE of the elution fractions, rMTG activated by dispase was hardly binding to the column.

After checking the binding capacity and reproducing the result several times using various controls with the same result, it was finally investigated, if the histidine-tag, which is necessary for binding to the IMAC column, was still present at the C-terminus of the rMTG after dispase treatment.

Therefore, a sample of the activated rMTG (Fig. 8A, lane 4) was investigated by MALDI-TOF mass spectrometry. The obtained mass spectrum is shown in Fig. 9.

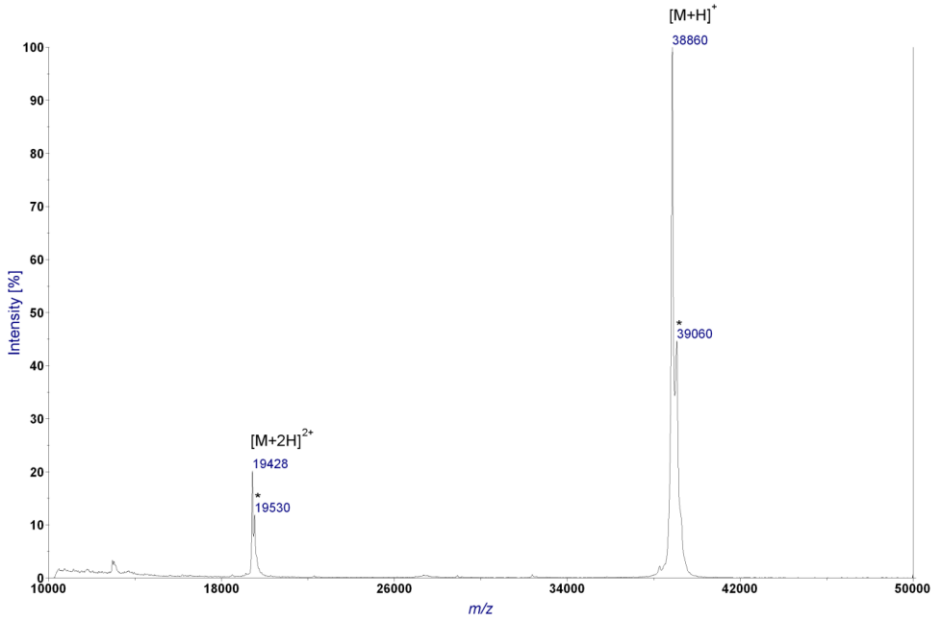

Fig. 9: Positive ion MALDI-TOF mass spectrum of dispase activated rMTG (1 mg mL^{-1} pro-rMTG in storage buffer and 1.31 U mL^{-1} dispase). The sample was prepared using the dried-droplet method by mixing with sinapinic acid as matrix. The asterisk denotes matrix adducts of the protein.

The mass spectrum in Fig. 9 showed two major peaks at *m/z* 19,428 and *m/z* 38,860 that corresponded to the doubly and singly protonated ions of rMTG, respectively. The N-terminal sequence of dispase activated pro-MTG isolated from *Streptomyces mobaraensis* (i. e. wild-type enzyme without his-tag) was previously investigated [Pasternack et al., 1998]. It was further shown by N-terminal Edman-sequencing, that the dispase was cleaving the pro-sequence between serine and phenylalanine leaving a FRAP sequence attached to the activated rMTG.

The molecular weight of FRAP-rMTG-His_6 is 39,399 g mol^{-1} and of dispase 36,000 g mol^{-1}. Obviously, the concentration of the dispase was too low to be detected.

For each peak, the mass with the highest intensity represents the rMTG and the bigger but less intense ions represents a cluster of rMTG and matrix associated molecules and could be neglected. The molecular weight was calculated from the *m/z*-value of the singly charged ion. The obtained mass for the dispase activated

rMTG was found to be 38,859 Da (singly charged ion). In conclusion, the molecular weight of the dispase-activated rMTG was smaller than the theoretical value.

The calculated molecular weights of several potential proteolysis products are listed in Table 5.

Table 5: Overview of the calculated molecular weight for FRAP-rMTG-His_6 and hypothetical variants produced by dispase treatment in correlation to the MALDI-TOF MS measured molecular weight (38,859 g mol^{-1}).

Variations	Theoretical MW [g mol^{-1}]	Difference to singly charged rMTG [g mol^{-1}]
FRAP-[rMTG]-LEHHHHHH	39,399	
C-terminal cleavage:		
FRAP-[rMTG]-LEHHH	38,988	129
FRAP-[rMTG]-LEHH	**38,851**	**- 8**
FRAP-[rMTG]-LEH	38,713	- 146
Combined N- and C-terminal cleavage:		
-[rMTG]-LEHHHHHH	38,790	- 69
P-[rMTG]-LEHHHHH	38,887	28
AP-[rMTG]-LEHH	38,821	- 38
RAP-[rMTG]-LEHHH	38,840	- 19

Within the mass accuracy limits of ± 500 ppm of the MALDI-TOF MS analyses there was only one calculated mass of a C-terminal truncation that fited to the determined mass. Furthermore, combined N- and C-terminal truncations did not fit to the measured values.

Altogether, there was strong evidence that four of the six histidine residues had been cleaved off, which explained the elution of the dispase activated rMTG from the IMAC column in the flow through of non binding proteins.

As a consequence, dispase was not suitable as MTG activating protease since it could not be removed from the active MTG easily using IMAC from the target enzyme.

4.7 Investigations of the activation of pro-rMTG using proteinase K

Proteinase K was shown previously to be stimulated by SDS and to possess a high thermostability [Gross-Bellard et al., 1973]. Due to the stimulating effect of SDS, proteinase K had to be inhibited prior to the SDS-PAGE analysis to prevent unwanted proteolysis of the samples before separation. Ebeling et al. described a complete inhibition of proteinase K by phenylmethylsulfonyl fluoride (PMSF) [Ebeling et al., 1974]. Therefore, proteinase K was reinvestigated and the sample was treated by PMSF as an inhibitor before separation by SDS-PAGE. Without PMSF, the SDS-PAGE of proteinase K treated pro-rMTG was showing considerable proteolysis [Marx et al., 2008a].

As shown by the SDS-PAGE (Fig. 10A), the activation of purified pro-rMTG by proteinase K (0.02 U mL^{-1}) resulted in only one band corresponding to the activated rMTG. After 2 hours, the rMTG was fully activated as shown also by the standard activity assay (Fig. 10B). Even high concentrations of proteinase K (0.62 U mL^{-1}) did not result in a reduced activity as seen previously by Marx et al. [Marx et al., 2008a]. Altogether, the effect of proteinase K activation by SDS and the importance of inhibition of proteinase K containing samples prior to SDS-PAGE has been confirmed.

As a consequence, proteinase K is suitable to activate the pro-rMTG. No degradation is detected over at least 3 hours. This period is long enough to activate pro-rMTG and to separate proteinase K from the active rMTG, e. g. by IMAC. As shown below, the proteinase K activated rMTG is indeed binding to the affinity column and can be separated from non binding proteins.

In order to determine the cleavage site of proteinase K, the N-terminal sequence of activated rMTG was determined. By N-terminal Edman-sequencing, the same

FRAP-sequence was determined which was found for TAMEP, the endogenous protease of *S. mobaraensis* [Zotzel et al., 2003] and dispase [Pasternack et al., 1998].

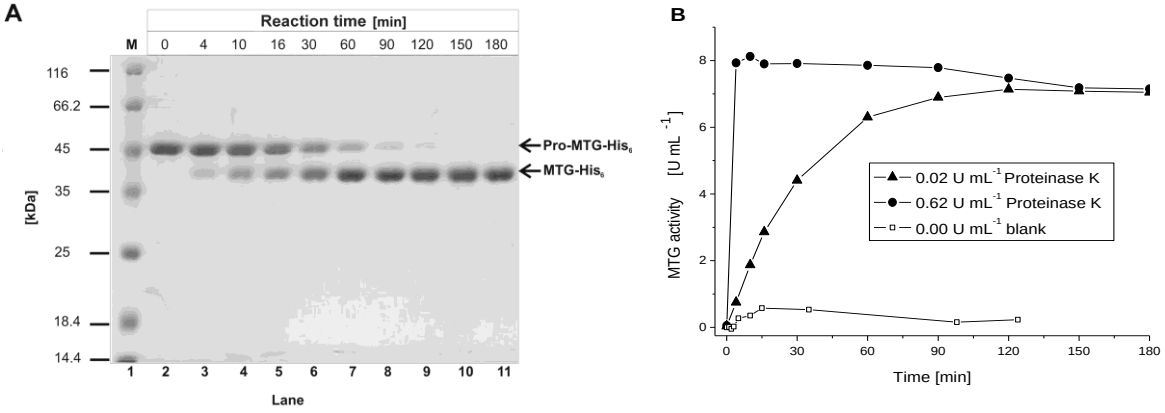

Fig. 10: Pro-rMTG activation with proteinase K and PMSF inhibition. Proteinase K (final concentration 0.02 U ml^{-1} and 0.62 U ml^{-1}) was applied to IMAC purified pro-rMTG (0.25 mg ml^{-1}) in storage buffer with 23 % glycerol and incubated at 37°C. Samples were taken before the addition of proteinase K and after 4, 10, 16, 30, 60, 90, 120, 150 and 180 min. **A:** SDS-PAGE-Gel of the pro-rMTG activation with 0.02 U mL^{-1} proteinase K, all samples were diluted 1:2 with sampling buffer and to every sample PMSF was added to a final concentration of 1 mM and incubated for 30 min at room temperature, lane 1: molecular weight marker, lane 2 to 11: activation samples, **B**: time dependence of the pro-rMTG activation, rMTG activity was measured directly after sampling

4.8 Activation of pro-rMTG in crude protein extract with proteinase K and consecutive IMAC purification

As shown before, proteinase K was suitable for the activation of IMAC purified pro-rMTG. The disadvantage of this method was the impurity presented by the proteinase K in the resulting rMTG solution. To produce a pure rMTG free of activating proteinase K, either a second chromatographic step has to be applied or the activation has to be carried out prior to the first IMAC. Therefore, the activation of pro-rMTG by proteinase K was carried out directly in the centrifuged, particle free crude protein extract of *E. coli*. Since host cell proteins of *E. coli* are also substrates for the proteinase K, the amount of the protease was calculated with respect to the total pro-

tein amount. A proteinase K activity of 0.076 U per mg soluble *E. coli* protein was applied. The SDS PAGE and the activity profile shown in Fig. 11 reveal a fast conversion of pro-rMTG to active rMTG.

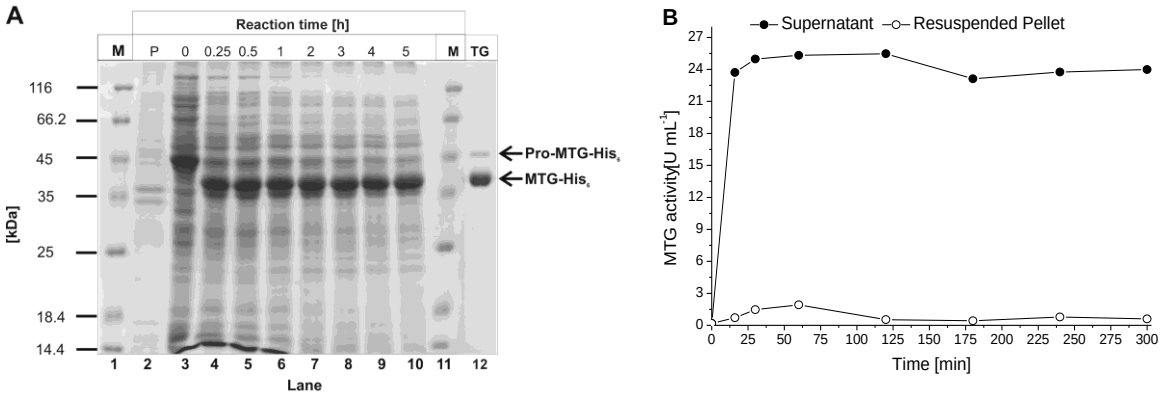

Fig. 11: Proteinase K activation of pro-rMTG in crude protein extract. Proteinase K (final concentration 0.076 U $mg^{-1}_{protein}$) were added to crude protein extract and incubated at 37°C. Sampling was done before proteinase K addition and after 15 min, 30 min, 1, 2, 3, 4 and 5h; **A**: SDS-PAGE-Gel, all samples were diluted 1:2 with sampling buffer and to every sample PMSF was added to a final concentration of 1 mM and incubated for 30 min at room temperature, lane 1 and 11: molecular weight marker, lane 2: pellet 10 mg ml^{-1}, which precipitated during -80°C storage, lane 3-10: activation samples of the reaction supernatant, lane 12: pro-rMTG / rMTG marker; **B**: time dependence of the rMTG activation in the supernatant and pellet, rMTG activity was measured directly after sampling

The bands corresponding to the *E. coli* host cell proteins are decreasing over time. During proteolysis by proteinase K a precipitate was formed. Obviously, some of the (partially) hydrolyzed *E. coli* proteins became less soluble. A similar behavior was observed for the purification of antigen II from *Streptococcus mutants* during hydrolysis with pronase [Russell et al., 1980]. The precipitate formed can be removed by centrifugation or by expanded bed chromatography. The latter method could be ideally combined with IMAC purification of the histidine tagged rMTG. The activated rMTG is stable for at least 300 min, indicating that *E. coli* proteases are not hydrolyzing the target enzyme. This is enough time to further process bulk quantities of crude protein extract containing active rMTG.

In order to remove activated rMTG from proteinase K and remaining *E. coli* host cell proteins, IMAC purification was carried out after proteinase K activation as described in material and methods. The elution profile of the rMTG can be seen in Fig. 12.

Proteinase K activated rMTG did bind to the IMAC column and could be eluted by 500 mM imidazole. The pooled fractions were dialyzed at 4°C to remove the imidazole. Under these conditions, the rMTG precipitated and was centrifuged off and stored at -20°C. Proteinase K bands (reference “PK” in Fig. 12A, lane 2) are not visible in the final rMTG preparation.

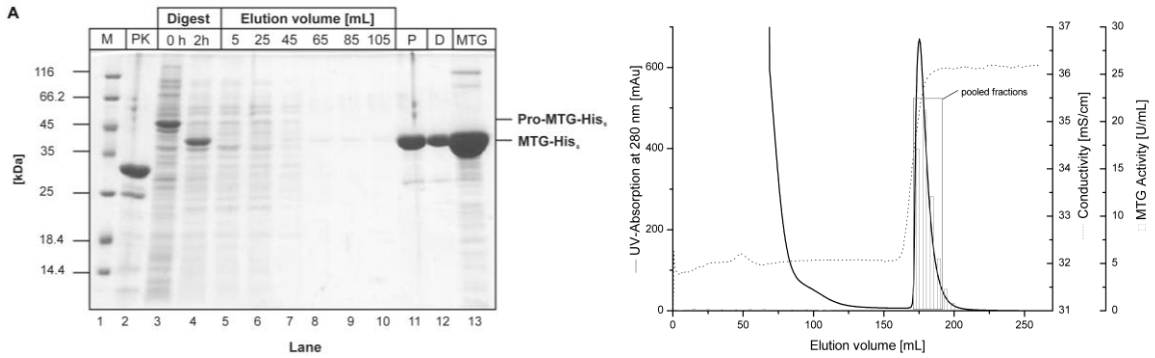

Fig. 12: Purification of active rMTG from cell lysate by IMAC. A: SDS-PAGE, all samples were 1:2 diluted with sampling buffer, lane 2: molecular weight marker (M), lane 3: proteinase K (30 U m L $^{-1}$), lane 4: cell lysate containing pro-rMTG prior to the digest, lane 5: cell lysate after 2 h digestion (PMSF was added to a final concentration of 1 mM and incubated for 30 min at room temperature), lane 6-11: washing fractions, lane 12: pooled elution fractions C 1 – C 4 (elution volume 171.59 – 191.58 mL), lane 13: dialysate, lane 14: rMTG marker; **B: IMAC chromatogram** (*column material:* streamline chelating Ni-NTA; column*:* XK 26/20; CV*:* 60 ml; loading flow rate*:* 3.0 mL min^{-1}; flow rate for washing and elution*:* 3.0 mL min^{-1}; *binding and equilibrating buffer:* 50 mM Tris/HCl buffer with 300 mM NaCl, pH 8 and 20 mM imidazole; e*lution buffer:* 50 mM Tris/HCl Buffer with 300 mM NaCl, pH 8 and 500 mM imidazole; *sample:* cell lysate was incubated for 2 h at 37°C with proteinase K (final concentration 0.076 U mg$^{-1}_{protein}$), *sample volume:* 104 mL; f*raction volume for washing:* 10 mL; fraction *volume for elution:* 5 ml). Elution was done with step gradient to 500 mM imidazole, rMTG-activity was measured with the hydroxamate assay and the box is indicating the pooled fractions

However, the concentration might be too small to be detected by SDS-PAGE. A strong proof for the absence of proteinase K is the observation that the proteinase K

inhibitor PMSF is only necessary in the samples containing proteinase K before they were applied to the column to inhibit rMTG degradation. In the final product after the IMAC even without PMSF there is no SDS-PAGE detectable degradation of rMTG.

The purification table in Table 6 gives an overview over the whole purification process. From a total volume of 200 mL crude protein extract 882 U rMTG were obtained.

Table 6: Purification table for the pro-rMTG purification from crude protein extract

step	Volume [ml]	Protein [mg ml^{-1}]	TG-activity [U ml^{-1}]	Total activity [U]	Specif. activity [U mg^{-1}]	Yield [%]	Purification-factor [-]
			-	-	-	-	-
Crude protein extract	200	12.6					
Proteinase K activation	120	7.2	12.5	1500	1.7	100	1.0
IMAC	12	3.2	77.6	931	24.3	62.1	1.6
Dialysis	12.3	2.7	71.7	882	26.8	58.8	1.8

The purification resulted in a yield of 58.8%. The loss is mainly caused by the IMAC step and shows some potential for further optimization. The final specific activity was 26.8 U mg^{-1} which is comparable to the published specific activity of 22.6 U mg^{-1} [Ando et al., 1989].

4.9 Production of an active transglutaminase variant (rMTG(S2P)-His$_6$)

In previous studies, Marx et al. selected a variant of microbial transglutaminase (rMTG(S2P)) which exhibits a more than doubled specific activity and 270 % higher thermostability at 60 °C than the wild-type enzyme [Marx et al., 2008b]. The proteinase K catalyzed activation of pro-rMTG(S2P) in the crude extract of *E. coli* and the

purification protocol, developed for the wild-type enzyme, was also applicable to the mutant. As can be seen from (Table 7) the purification protocol of this variant gives essentially the same results as for the wild-type enzyme.

Table 7: Purification table for the pro-rMTG(S2P) purification from crude protein extract

step	Volume [ml]	Protein [mg ml^{-1}]	TG-activity [U ml^{-1}]	Total activity [U]	Specif. activity [U mg^{-1}]	Yield [%]	Purification-factor [-]
Crude protein extract	200	2.5					
Proteinase K activation	150	2.2	23.6	3540	10.7	100	1
IMAC	12	4.9	201.8	2422	41.2	68.4	2.8
Dialysis	12.6	4.3	185.2	2334	43.1	65.9	2.9

The final specific activity was 43.1 U mg^{-1} which is nearly as high as the published specific activity of 46.1 U mg^{-1} and indicated the good purification efficency [Sommer et al., 2011].

4.10 Activation of pro-rMTG(S2P) by immobilized proteinase K

In order to further simplify the activation process of recombinant pro-transglutaminases and separation of the activating protease an immobilized proteinase K was investigated. The separation of the carrier-fixed proteinase K from the solution was easily achieved by centrifugation which enables also the reuse of the biocatalyst.

At first it was investigated, if any proteinase K is bleeding from the carrier material (see material and methods). No soluble proteinase K activity could be detected in the washing buffer used therefore the potential bleeding could be neglected.

Then, the carrier-fixed proteinase K was investigated for their ability to activate pro-rMTG(S2P). Two different concentrations were used to hydrolyze a centrifuged particle free crude protein extract of *E. coli*. The results are shown in Fig. 13.

As can be seen from Fig. 13, the immobilized proteinase K was indeed able to activate pro-rMTG. Using a caseinolytic activity of 2.03 U mL^{-1}, the pro-rMTG(S2P) was fully activated within 40 minutes. Compared to the activation by free proteinase K (Fig. 11), the activity was reduced which can be explained by the diffusion limitation of rMTG into the carrier particle and the limited dispersion of the immobilized proteinase K. The diffusion rate of rMTG is obviously slower than the one of casein, which was used for the determination of the specific activity of the free and immobilized proteinase K. This effect can be explained by the 3D dimensional structure of rMTG, which casein does not possess.

In summary, the application of immobilized proteinase K is feasible. For economic reasons, at least 22 reuse cycles would be necessary to compete for the reduced activity. The use of immobilized protease would be interesting for the activation of pure pro-rMTG samples, since the removal of the protease could then easily achieved just by centrifugation.

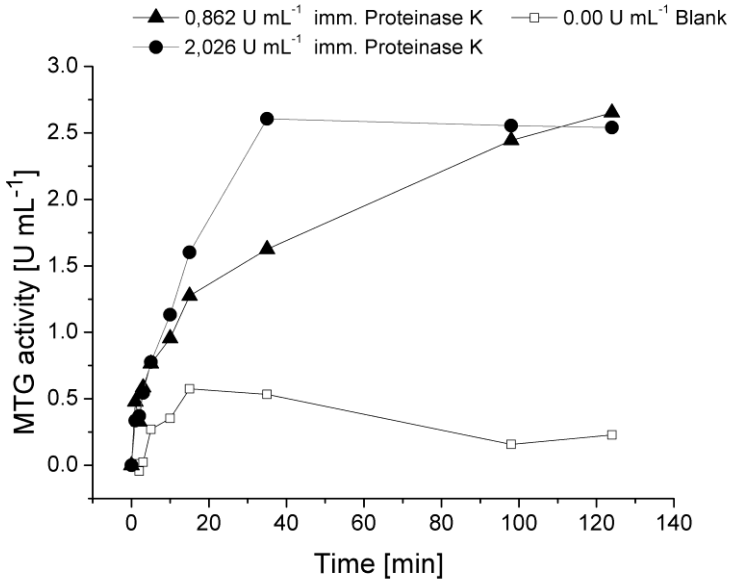

Fig. 13: Pro-rMTG(S2P) activation with immobilized proteinase K. Two different proteinase K concentrations were applied to the crude protein extract and incubated at 37°C for 2 hours. Activity was measured directly after sampling with the hydroxamate assay.

4.11 Investigation on the MTG substrate specificity using immobilized tripeptide libraries

To investigate the substrate specificity of MTG towards the flanking amino acids of a central glutamine every possible combination of tripeptides with the 20 proteinogenic amino acids were immobilized on a paper matrix as described in section 3.12. The tripeptides on the paper matrix were incubated with a fluorescence marker (MDC) which is an amine substrate suitable for cross linking by MTG.

Every spot was incubated for 30 min at 37°C by the equal distribution of the MDC and rMTG / rMTG(S2P) solution over the paper matrix. The results are shown in Fig. 14.

Differences in the substrate specificity were then the reason for different reaction velocities which led to differences in the fluorescence between the spots after washing the paper matrix. Those differences indicate then the suitability of the tripeptides for the cross linking reaction catalyzed by MTG / MTG(S2P) and therefore the influ-

ence of the flanking amino acids on the substrate specificity of the MTG / MTG(S2P).

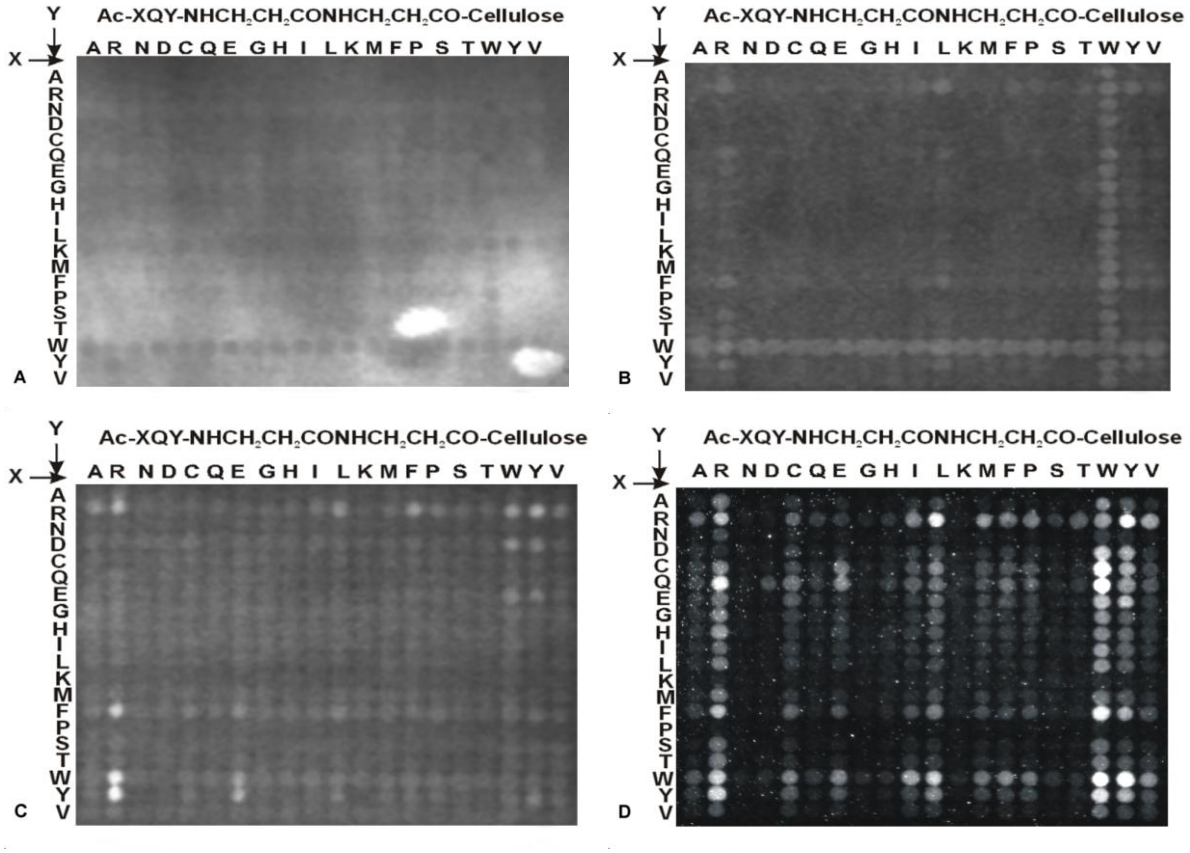

Fig. 14: Fluorescence images of the tripeptide library papers, with glutamine as central amino acid, incubated with MDC and A: Blank; B: rMTG; C: rMTG(S2P); D: rMTG (second experiment) for 30 min at 37 °C. Each spot represents one tripeptid-combination of the sequence X-Q-Y. Y and X correspond to all 20 proteinogenic amino acids in combination to each other. The experimental set up is described in chapter 3.13.

The paper A is the result of the Blank measurement without rMTG or rMTG(S2P). It showed no fluorescent spots, as expected, but two big fluorescing areas. These areas are caused by improper handling before or after incubation but fortunately do not emerge on the other tripeptide papers and can be neglected.

The second paper (paper B) was incubated with MDC and rMTG and showed a strong rMTG preference for tripeptides with flanking tryptophan and tyrosine and a

moderate preference for phenylalanine and arginine. However, the overall spot intensity was relatively low and therefore the resolution between the spots was also relatively low.

To enhance the resolution this particular result was supplemented by another run with fewer washing cycles (no washing with methanol / water at the end) resulting in paper D with a higher overall fluorescence and therefore a higher spot resolution. Due to those fewer washing steps scattered fluorescing points in the paper D can been seen. These points are a result of MDC / rMTG precipitates, which were not removed due to the decreased number of washing cycles. Fortunately they do not affect the spot analysis (shown in Fig. 15 and Table 8)

To compare the reaction pattern of rMTG and rMTG(S2P) the spot intensities in Fig. 14 were evaluated with the Bio-1D software. As an example the spot analysis for the incubation of the immobilized tripeptide library with rMTG and MDC (see Fig. 14 part D) is shown in Fig. 15 as an example for all spot analysis.

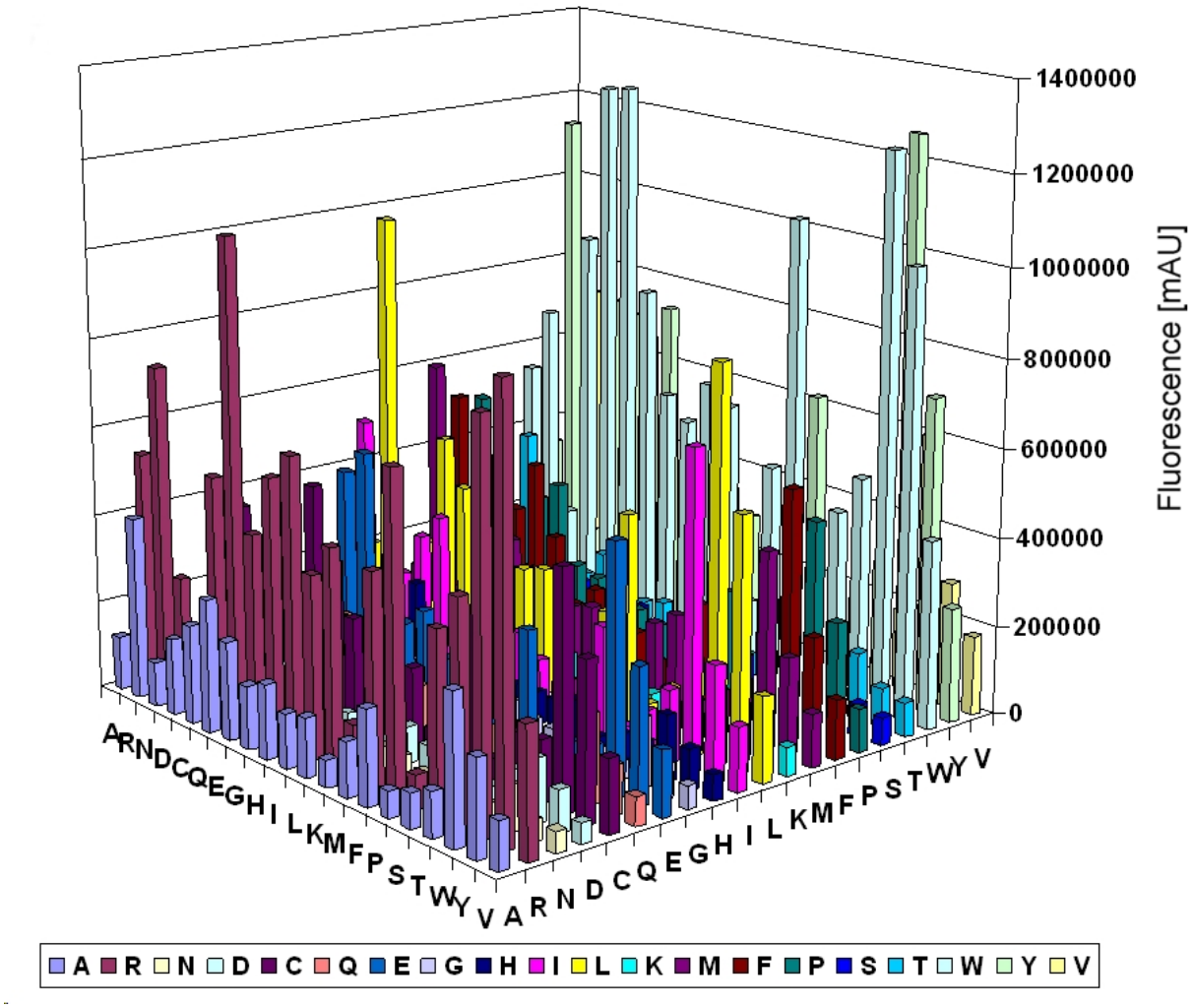

Fig. 15: Spot analysis of the image of a tripeptide library paper, with glutamine as central amino acid, incubated with MDC and rMTG for 30 min at 37 °C. Each column represents one tripeptide combination of the sequence X-Q-Y. X and Y correspond to all 20 proteinogenic amino acids in combination according to the X- and Y-Axes. The experimental set up is described in chapter 3.13

To simplify the complex spot analysis pattern given by the Bio-1D software the 15 best tripeptides were listed and compared by normalized spot intensities in Table 8.

Table 8: Comparitive ranking list of the 15 best tripeptides (X-Q-Y) of the immobilized tripeptide screened with 1 U / mL MTG respectively 1 U / mL rMTG. MDC was used as fluorescence marker, grey background indicates similarity occurrence in the top 15 of another paper, for each paper the spot with the highest fluorescence intensity was set to 100 % and all other spots were normalized against this spot to compare the tripeptides between all three papers.

rMTG [D]		rMTG [B]		rMTG(S2) [C]	
X-Q-Y	[%]	X-Q-Y	[%]	X-Q-Y	[%]
Y-Q-W	100	M-Q-W	100	R-Q-Y	100
W-Q-Q	100	I-Q-W	92	R-Q-W	96
W-Q-C	99	W-Q-R	91	R-Q-F	92
W-Q-W	98	F-Q-W	81	Y-Q-R	87
Y-Q-R	90	H-Q-W	81	R-Q-R	84
R-Q-Q	86	L-Q-W	80	R-Q-F	82
W-Q-F	83	G-Q-W	77	R-Q-W	79
W-Q-Y	79	Y-Q-R	76	F-Q-R	74
L-Q-R	77	W-Q-M	75	W-Q-D	71
R-Q-Y	76	E-Q-W	73	E-Q-Y	68
W-Q-D	70	W-Q-Q	72	E-Q-W	65
R-Q-W	69	W-Q-F	69	R-Q-A	65
L-Q-W	68	W-Q-I	67	F-Q-R	62
W-Q-E	63	W-Q-L	67	W-Q-E	54
Y-Q-E	59	W-Q-P	63	Y-Q-E	53

The better overall fluorescence intensity of paper D confirmed the MTG preference for arginine, tryptophan and additionally for tyrosine, too, as flanking amino acids at both X- and Y-position. Leucine seemed to have a positive influence on the substrate specificity more on the X- then the Y-position and the positive effect of phenylalanine was more on the Y-position.

For Paper C in Fig. 14 the paper matrix was incubated with MDC and rMTG(S2P). The fluorescence pattern was over wide areas similar to the rMTG pattern, though few rMTG spots with high fluorescence intensities, like W-Q-C, W-Q-Q or W-Q-F, were not found or were considerably less intense for the rMTG(S2P) incubation. Similar to the rMTG the rMTG(S2P) exibited a clear preference for arginine, tyrosine and tryptophan as flanking amino acids at both X- and Y-position. Leucine seem to have a positive influence on the substrate specificity with a higher influence on the

X- then on the Y-position and phenylalanine exhibited a weak but measurable positive influence.

The flanking amino acids alanine, glycine, asparagine, histidine, serine and threonine lead to low or even absent fluorescence intensity for rMTG and rMTG(S2P) which indicated a decreased rMTG / rMTG(S2P) preference and therefore a negative influence on the substrate specificity.

Not surprisingly, for combinations with lysine nearly no fluorescence neither for rMTG or MTG(S2P) was detectible. Mass spectrometric investigations of the K-Q-R spot shown in Fig. 16 clearly indicated a cross linking between the spot peptides themselves (peak 1212.619) so little or no MDC could be incorporated.

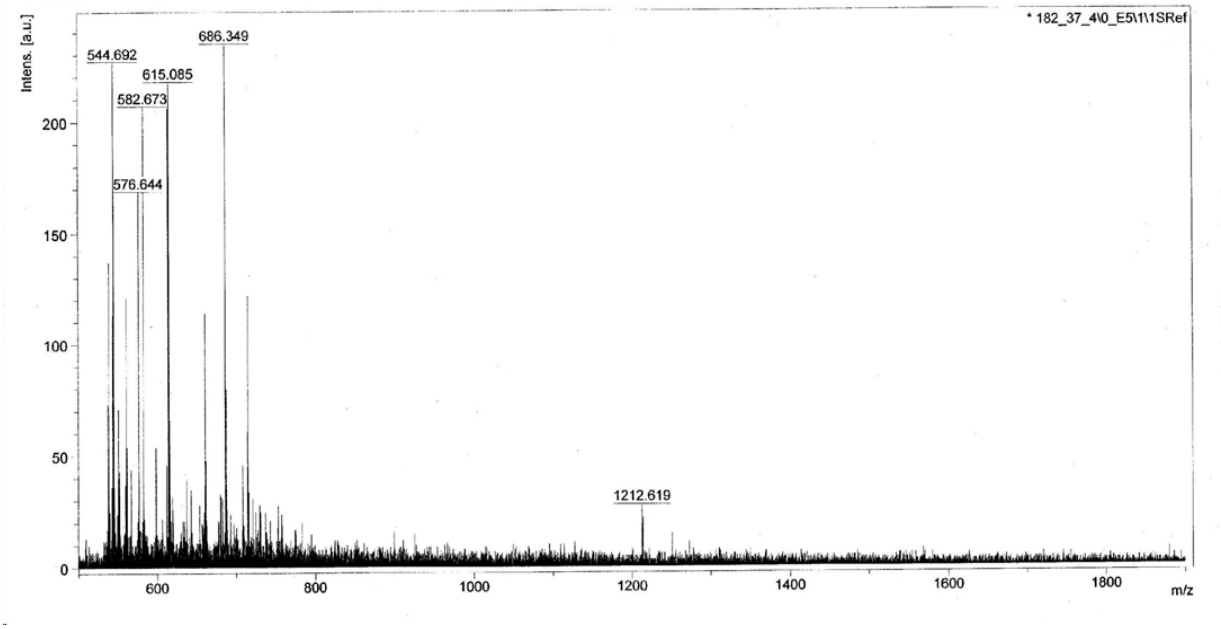

Fig. 16: Mass spectrometric analytic(ESQUIRE ion-trap mass spectrometer equipped with an ESI source [Bruker Daltronik, Bremen, Germany] and an analytic HPLC [Sykam, Fürstenfeldbrück, Germany] containing a 125/4 LiChrospher RP-8 column (Merck, Darmstadt, Germany)) of the spot K-Q-R. The peak 1212.619 m/z correlated to a dimer of K-Q-R + K-Q-R. The experimental setup was described in section 3.14.7.

5. Conclusions

A process for the production of a thermostable variant of a microbial transglutaminase was developed. The transglutaminase variant (rMTG(S2P)) produced, carried a his-tag and a single amino acid exchange (serine replaced by proline at position 2) and showed a nearly doubled specific activity of 46.1 U mg^{-1} compared to the wild-type enzyme. With the help of a model based optimization strategy, intracellular soluble production in *E. coli* was optimized. After parameter identification and two fed-batch cultivations, a space time yield of 1,438 U_{TG} L^{-1} h^{-1} was obtained which was 175 % higher than the highest values published so far (extracellular production using *Corynebacterium ammoniagenes*). High carbon source concentrations during expression were found to increase the product formation. The obtain reproducible results which are usable to develop a mathematical model of the process, the host strain was adapted from complex medium to minimal medium by serial dilution. Upon transfer to the minimal medium, initially the maximal growth rate dropped to 0.13 h^{-1}. After six consecutive cultivations the rate increased to 0.47 h^{-1} and the portion of the complex medium was reduced to 1 ppm. Using the adapted cells, temperature after induction and IPTG-concentration were investigated by satellite batch cultivation according to a Design of Experiment (DoE) plan. The product yield was strongly influenced by the temperature after induction but not by the inductor concentration. The highest specific activity of 1,386 U g^{-1} bio dry mass was obtained at 29°C and 0.7 mM IPTG.

In order to develop a process to produce active rMTG(S2P) from the pro-enzyme initial activation experiments with the recombinant his-tagged microbial transglutaminase (rMTG) were done. Aim of the investigation was the preparation of active rMTG / rMTG(S2P), which were free of the activating protease. First, dispase

was used for activation of the pro-rMTG followed by immobilized metal affinity chromatography (IMAC). As shown by MALDI-MS, the dispase is not only cleaving off the pro-sequence but is unfortunately also degrading the C-terminal his-tag from 6 to 2 histidine residues. The shortened his-tag is not sufficient to bind the active rMTG properly to the IMAC material. As an alternative, proteinase K was investigated and found to be well suited. The results showed that proteinase K can be applied as free or immobilized enzyme. The protease could also be applied directly in the crude cell extract of *E. coli* followed by an IMAC purification leading to a simple two step activation / purification procedure. The resulting preparation is then protease-free and contains almost pure transglutaminase. The protocol has been successfully applied to the rMTG and the highly active rMTG(S2P) variant. Proteinase K is activating the pro-rMTG without unwanted degradation of the his-tag. It turned out to be very important to inhibit proteinase K activity, e. g. by PMSF, prior to protein separation by SDS-PAGE to obtain realistic results due to the activation of proteinase K by SDS.

The reaction mechanism of the cross linking of lysine residues to glutamine residues by transglutaminase is well known but the high substrate and regio specificity exhibited was not or incompletely investigated in detail until today. In the present thesis the influence of peripheral amino acids flanking the glutamine residue on rMTG / rMTG(S2P) activity was investigated. Therefore tripeptides with glutamine in the centre in combination with every proteinogenic amino acid immobilized on cellulose were synthesized. A strong positive influence of hydrophobic and basic amino acids especially with Arginine, Tyrosin and Tryptophan was found.

6. Discussion

6.1 Production of pro-MTG(S2P) by fed-batch cultivation of E. coli

Today, the research on the transglutaminase (over)production is mainly focused on three groups of host organisms. Wild-type MTG, which is the only commercial product available so far, is produced by using *Streptomyces mobaraensis*. Recombinant MTGs were produced by coryneform bacteria *(Corynebacterium glutamicum, Corynebacterium ammoniagenes*) and *E. coli*, respectively. Whereas pro-MTG is secreted by *S. mobaraensis,* and coryneform bacteria, intracellular cytosolic production is obtained with *E. coli*. A comparison of the product yields of the different production systems including the results of this thesis is given in Table 9.

The wild-type strain *S. mobaraensis* is used by the company Ajinomoto Inc. for the industrial production of microbial transglutaminase. The highest MTG concentration achieved with *S. mobaraensis* was reported by Ando et al. in 1989 (93 mg L^{-1} or 2102 U L^{-1}) [Ando et al., 1989]. Other attempts with *S. mobaraensis* were made recently but only low MTG yields were achieved [Liu et al., 2006; Zheng et al., 2001]. Several attempts were made to increase the yield, e. g. by using recombinant coryneform bacteria as production organism ([Date et al., 2004; Itaya and Kikuchi, 2008; Kikuchi et al., 2003]). The highest product concentration reported so far was 2.5 g L^{-1} obtained in a 300 mL scale (bioreactor volume was 1 L). However, this corresponds to a space-time yield of only 810 mg L^{-1} h^{-1} due to the relative long cultivation time of 71 h [Itaya and Kikuchi, 2008].

Table 9: Comparison of yields of transglutaminase production in different host strains. To calculate missing data, the specific activity of 22.6 U mg^{-1} was used for the native transglutaminase from *S. mobaraensis* [Ando et al., 1989], 23 U mg^{-1} for the recombinant transglutaminase expressed in *C. ammoniageneses* [Itaya and Kikuchi, 2008] and 26 U mg^{-1} for the recombinant transglutaminase expressed in *C. glutamicum* [Date et al., 2004]. The specific activity of the rMTG(S2P) variant produced in the present paper was 46.1 U mg^{-1} [Marx et al., 2007b]. Table sorted according to increasing space time yield.

Reference	Host strain	MTG yield [mg L^{-1}]	MTG yield [U L^{-1}]	Bio dry mass related yield [U g^{-1}]	Bio dry mass yield [g L^{-1}]	Cultivation time [h]	Space-time yield [U L^{-1} h^{-1}]	Remarks
[Takehana et al., 1994]	*E. coli*	5	113	--	--	--	--	No pro sequence, ompA sequence added, activity estimated from band intensity of western blot
[Yurimoto et al., 2004]	*P. pastoris*	1	23	--	--	68	0.3	Methanol fed-batch, extracellular expression, pro sequence
[Portilla-Rivera et al., 2009]	*S. ladakanum*	20	452	38	11.9	72	6	Complex medium (sugar cane molasses), batch cultivation
[Yurimoto et al., 2004]	*C. boidinii*	87	1966	72	27	125	16	Glycerol fed-batch, methanol feeding for induction, extracellular expression, pro sequence
[Ando et al., 1989]	*S. mobaraensis*	93	2102	125	16.8	70	30	Original strain, secreted, values calculated from figure in the reference
[Yokoyama et al., 2000]	*E. coli*	45	1017	--	--	20	51	Codon usage adjusted to *E. coli*; inclusion bodies 15 % activity after refolding
[Kikuchi et al., 2003]	*C. glutamicum*	142	3692	--	--	70	53	Secreted and activated, co-expressed protease
[Marx et al., 2007a]	*E. coli*	65	1469	638	2.3	24	61	IPTG, temperature profile, > 90 % soluble, batch cultivation
[Marx et al., 2008a]	*E. coli*	68	1537	--	--	19	81	Intracellular, 90 % soluble, lactose induction, batch cultivation
[Kawai et al., 1997]	*E. coli*	60	1356	--	--	10	136	Inclusion bodies, 20 % activity after refolding
[Date et al., 2004]	*C. glutamicum*	881	22906	--	--	40	573	Secreted, activated; chimeric pro sequence, batch cultivation
[Itaya and Kikuchi, 2008]	*C. ammoniagenes*	2500	57500	1150	50	71	810	Secreted, activated, batch cultivation
This work	*E. coli*	500	23000	1150	20	16	1438	IPTG, temperature profile, fed-batch cultivation, pro-rMTG(S2P)

Early attempts to use *E. coli* as production organism resulted mainly in the formation of inclusion bodies and low refolding yields [Yokoyama et al., 2000]. Marx et al. solved this problem by reducing the translation / transcription rate during induction via decreasing the temperature. This resulted in the soluble expression of a his-tagged pro-transglutaminase in *E. coli*.

Using a complex medium, a yield of 68 mg L^{-1} rMTG was obtained [Marx et al., 2007a]. This was the highest yield obtained so far using *E. coli*. Later, this enzyme was optimized by means of random mutagenesis to obtain a more heat stable transglutaminase with a higher specific activity [Marx et al., 2008b]. This transglutaminase variant (rMTG(S2P)) was investigated in the present thesis.

The aim of the present thesis was to maximize the space time yield of the production of pro-rMTG(S2P). Therefore, a model based high-cell density fed-batch cultivation of *E. coli* harboring the expression plasmid (pCM203) was developed.

In a design of experiments (DOE) approach, the influence of various inductor / temperature combinations on the biomass related specific activity and plasmid stability were investigated. For *E. coli* it was shown previously that the temperature after induction is important for the solubility of the target protein [Marx et al., 2007a], [Baneyx, 1999], [Jana and Deb, 2005]. The optimal temperature for the soluble rMTG(S2P) expression was found to be 29°C. The influence of the IPTG concentration on the protein expression was investigated in the range from 0.4 mM to 1 mM. No statistical significant influence could be detected; therefore the IPTG concentration for further experiments was set to 0.7 mM. To optimize the product yield, two fed-batch cultivations were carried out. The fed-batch experiments were carried out several times and the model proven to be valid. The first cultivation was carried out to obtain raw data to develop a mathematical model and to indentify the model parameters. The model predicted a highly positive influence of excess glucose con-

centrations (respectively a high growth rate) on the expression of pro-rMTG(S2P). During the second fed-batch cultivation, the optimized protocol with maximal growth rate, 0.7 mM IPTG and 29 °C during the product expression was applied and a 400 % higher space time yield was obtained.

Altogether; 23,000 U rMTG(S2P) per liter cultivation volume could be obtained within 16 h. This corresponds to a space time yield of 1,438 U (L^{-1} h^{-1}) rMTG(S2P) with a nearly doubled specific activity compared to rMTG. This is almost twice as high as the highest space time yield published by Itaya et al. so far [Itaya and Kikuchi, 2008]. It was shown, that the model sufficiently described the experimental data and can be used for further optimization. However, a model is always a reduced projection of the real system and the fitting of the prediction to the measurements is influenced by various measurement errors, estimation errors and other effects, not incorporated in the model. For example, high levels of biomass increase the error of bio mass measurements due to foaming, higher necessary dilution, sedimentation etc. Such effects increase the standard deviation and therefore the fitting deviation, as can be seen during the induction phase for the fitting of the bio mass concentration. The relatively high fitting deviation of the growth curves in both fermentations was caused mainly by measurement errors. In summary however, the main purpose of developing and using the model, i. e. the maximization of the space-time yield, was successfully achieved.

Higher bio dry mass concentrations and higher specific activity would increase the space time yield further. As shown in Table 2, the highest specific activity reached 1,386 U g^{-1}_{BDM} for the soluble expression in *E. coli* (mean value of three experiments at 29°C, 0.7 mM IPTG). In the optimized cultivation, a specific activity of 1,150 U g^{-1}_{BDM} was reached, which correlates to 84 % of the maximal specific activity. This deviation is caused by the plasmid free cells which could take up to 30 % of the total

biomass. To avoid the growth of plasmid free cells, other *E. coli* strains or a different expression system (e.g. chromosomal gene incorporation, expression systems with a plasmid-chromosomal control loop to kill plasmid free cells automatically) could be used [Baneyx, 1999; Jana and Deb, 2005].

For *E. coli*, bio dry mass concentrations up to 190 g L^{-1} have been reported using a special type of reactor setup [Nakano et al., 1997]. Theoretically, such high biomass concentrations would increase the volumetric rMTG(S2P) activity nearly 10 fold to 230,000 U L^{-1}. For the production of e. g. 1 million U MTG activity, using the *C. ammoniagenes* expression system (22.4 U mg^{-1}, 2,500 mg L^{-1} [Itaya and Kikuchi, 2008]), 18 L medium for at least 71 hours (one batch). For *E. coli* (46.1 U mg^{-1}, 500 mg L^{-1}, present work,), 43 L have to be cultivated for at least 16 hours (one batch). Respectively, for the high bio mass concentration as described by Nakano et al. [Nakano et al., 1997] only 4.3 L would be necessary.

For intra- and extracellular fermentation, biomass has to be separated from the medium. In case of *C. ammoniagenes*, the 18 L product containing medium would have to be concentrated after biomass removal, e. g. by ultrafiltration. In case of *E. coli* the product is contained in approx. 4 kg wet biomass, which could easily be separated by centrifugation. The resulting biomass can be similar easily disintegrated e.g. by high pressure homogenisation prior to further purification.

Purification of the histidine-tagged recombinant enzyme can be carried out by batch adsorption using immobilized metal affinity chromatography (IMAC). In both cases, proteolytic activation of the initially produced pro-MTG is necessary. This can be achieved by co-expression of proteases as reported for *C. ammoniagenes*. [Itaya and Kikuchi, 2008]. However, the limited stability of the active wild-type MTG is not favoring this approach. In contrast to *E. coli*, C*. glutamicum, C. ammoniagenes* and the wild-type strain *S. mobaraensis* are of GRAS status (Generally Regarded As

Save). Therefore, for the conventional application of MTG in food processing, the production in *C. spec.* could be favored. For other applications of MTG, e. g. the production of biomaterials from proteins isolated from renewable resources [Patzsch et al., 2010], the *E. coli* production system is an economically feasible alternative. For this application, the histidine-tag of the variant enzyme facilitating the purification is acceptable. In contrast to commercial MTG preparations, rMTG(S2P) expressed in *E. coli* and purified by IMAC is free of proteases, which would cause protein degradation.

6.2 Pro-rMTG(S2P) purification and activation from E. coli bio mass

For the activation of MTG several proteases and methods have been previously published. Negi et al. first described in 1981 the activation of a mammalian TG with Cathepsin B [Negi et al., 1981]. Eigth years later the TG Factor XIII was activated with Thrombin by Hornyak et al. in 1989 [Hornyak et al., 1989]. TG 1 from skin and hair follicle was first activated with Dispase by Martinet et al. in 1988 [Martinet et al., 1988] and 2 years later Rice et al. investigated the activation of the same TG 1 with seven different proteases including Thrombin and found only Plasmin and Trypsin suitable for the activation [Rice et al., 1990]. The proteolytic activation of TG E from guinea pig skin with 6 different proteases was investigated by Kim et al. in 1990 [Kim et al., 1990] with good results for Dispase (100 % activation), Proteinase K (98 % activation), Trypsin (93 % activation) and Thrombin (69 % activation).

The activation of the microbial pro-transglutaminase from *Streptomyces mobaraensis* was first described by Pasternack et al. utilizing Dispase and bovine trypsin [Pasternack et al., 1998]. It was found that the Dispase is not completely removing the pro-Sequence but leaves a FRAP-sequence (one latter amino acid code). Zotzel et. al. [Zotzel et al., 2003] investigated for the same TG the activation

properties of 5 different protease and found for the activation with TAMEP, a non-commercially protease of *Streptomyces mobaraensis*, the FRAP-sequence of the pro-sequence to remain with the active MTG like the activation with Dispase. This FRAP-sequence was also found not to influence the activity of the MTG. In the same year Kikuchi et al. and Date et al. published the activation of rMTG from *Streptomyces mobaraensis* with SAM-P45, a protease from *Streptomyces albogriseolous* recombinant expressed in *Corynebacterium glutamicum* [Kikuchi et al., 2003]; [Date et al., 2003].

For the activation of recombinant microbial (pro-) transglutaminase expressed in *E. coli* several proteases (dispase, bovine trypsin, proteinase K, TAMEP SM-TAP and others) were investigated by Marx et al. [Marx et al., 2008a]. Trypsin is not specific for the removal of the pro-sequence but also hydrolyzes the active transglutaminase [Marx et al., 2008a], a problem which can only partially be solved by on-column activation of immobilized pro-transglutaminase [Yang et al., 2009]. TAMEP and SM-TAP are not commercially available so for bulk quantity applications not available.

In previous experiments on the large scale preparation of active TG and TG-variants produced by random and site-directed mutagenesis [Marx et al., 2008b], dispase from *Bacillus polymyxa* was used for activation. Previously, dispase was shown to activate pro-transglutaminase without degradation and activity loss [Marx et al., 2008a].

In order to produce a protease-free transglutaminase preparation, it was intended to separate the dispase from the active histidine-tagged transglutaminase by fast and efficient IMAC. Surprisingly and in contrast to the inactive pro-transglutaminase it was found that the dispase activated transglutaminase did not bind to the IMAC-column. Analysis by MALDI-TOF-MS revealed a lower molecular weight of the acti-

vated transglutaminase than expected. According to the MS analysis results the histidine-tag was partially removed by dispase. Therefore, the easy separation of the activating protease and active transglutaminase was impossible by affinity chromatography and proteinase K was investigated as an alternative.

In this thesis we were able to show that the proteinase K could be applied as free or immobilized enzyme in pure solution or in crude protein extract. It activated the pro-rMTG in an N-terminal cleavage pattern identical to the endogenous protease TAMEP or dispase without degrading the primary sequence leaving the enzyme activity intact. Also, the his-tag was not degraded by proteinase K providing an active rMTG / crude protein extract easily purified by IMAC.

Therefore, commercial available proteinase K is suitable for bulk quantities of rMTG and could be used to further develop a fast and efficient rMTG activation and purification method. An additional positive effect was the observed partial precipitation of the *E. coli* host cell proteins by direct application of the protease to the crude cell extract. During this thesis it was found that the activated rMTG / rMTG(S2P) was sufficiently stable in crude protein extract against proteolysis by *E. coli* proteases. Sufficient proteolytic stability of the active rMTG is necessary due to the increased processing times for bulk quantities and for the successfully isolation by IMAC purification.

The procedure, developed in this thesis (first activation with proteinase K in crude protein extract followed by IMAC purification), was easily applicable for wild-type rMTG and variants as shown for the S2P mutant enzyme. It provides high specific activities, high purities and high space time yields with fast, efficient and well established methods, which are easy to up-scale.

6.3 Investigations of the rMTG / rMTG(S2P) substrate specification

Until today many different application and therefore, different substrates are known for the MTG. Food proteins such as Casein, α-Lactalbumine, Soya proteine 11S and 7S and some wheat proteins e.g. ß-Lactoglobulin [Yokoyama et al., 2004]; [Nieuwenhuizen et al., 2004] and other proteins, e.g. Apomyoglobin, α-Lactalbumin [Fontana et al., 2008], Insulin A-chain [Shimba et al., 2002] and others (see Table 10) are well known as substrates for the rMTG.

Table 10: Literature overview over TG substrates and the flanking amino acids of TG recognized glutamines. Grey boxes indicate hydrophobic properties, line boxes present positive and underlining present negatively charged amino acid residues.

Protein	PDB ID	Sequence	Literature
Apomyoglobin	2VLY	-K-P-L-A-**Q**-S-H-A-T-	[Fontana et al., 2008]
α-Lactalbumin*	1F6R	-Y-G-L-F-**Q**-I-N-N-K- -Y-D-T-**Q**-A-I-V- -A-I-V-**Q**-N-N-D- -K-D-D-**Q**-N-P-H-	[Fontana et al., 2008]
α-Lactalbumin*	1F6R	-Y-G-L-F-**Q**-I-N-N-K- -Y-D-T-**Q**-A-I-V- -A-I-V-**Q**-N-N-D- -K-D-D-**Q**-N-P-H-	[Fontana et al., 2008]
hGH	3HHR	-I-P-K-E-**Q**-K-Y-S-F- -Q-I-F-K-**Q**-T-Y-S-K-	[Fontana et al., 2008]
Interleukin 2	1M47	-L-N-L-A-**Q**-S-K-N-F-	[Fontana et al., 2008]
β-Lactoglobulin	1CJ5	-L-L-D-A-**Q**-S-A-P-L- -E-I-L-L-**Q**-K-W-E-N- -D-E-C-A-**Q**-K-K-I-T- -F-N-P-T-**Q**-L-E-E-Q-	[Nieuwenhuizen et al., 2004]
Insulin A-chain	2OMH	-C-S-L-Y-**Q**-L-E-N-Y-	[Shimba et al., 2002]
Myc Epitop	--	-E-**Q**-K-L-I-S-E-E-D-	[Tanaka et al., 2005]
Ribonuclease S-Peptid	1FS3	-K-F-E-R-**Q**-H-M-D-S-	[Kamiya et al., 2003]

However, recent investigations showed that not every glutamine or lysine residue, which in theory could be accessible, is accessible by the rMTG for cross linking or modification as shown in Table 11 [Fontana et al., 2008] .

Table 11: Literature overview over glutamines and their flanking amino acids which were not accepted by MTG but in theory could be accessible. Grey boxes indicate hydrophobic, line boxes present positive and underlining present negatively charged amino acid residues.

Protein	PDB ID	Sequence	Literature
Apomyoglobin	2VLY	-E-L-G-F-**Q**-G-NH_2	[Fontana et al., 2008]
α-Lactalbumin	1F6R	H_2N-E-**Q**-L-T-K-C- -K-L-D-**Q**-W-L-C-	[Fontana et al., 2008]
hGH	3HHR	-F-L-Q-N-P-**Q**-T-S- -R-E-E-T-**Q**-**Q**-K-S- -R-T-G-**Q**-I-F-K-	[Fontana et al., 2008]
Interleukin 2	1M47	-K-H-L-**Q**-C-L-E-	[Fontana et al., 2008]

However, for most of the known MTG accessible proteins the actual location in the protein, where the cross linking takes places, is not known. In a first approach to determine the substrate and region specificity Ando et al. investigated 8 different peptides [Ando et al., 1989] based on the standard substrate CBZ-Q-G for the hydroxamate test as described by Folk et al. [Folk and Cole, 1966] All investigated peptides showed a considerable lower activity than the CBZ-Q-G. Even the G-Q-G peptide was not accepted as a substrate (0% activity) which proves that it is not enough to provide an available glutamine residue and emphasized the importance of the peripheral groups of the glutamine for the transglutaminase activity.

Ohtsuka et al. used the transglutaminase successful to crosslink simple amides, amides with functional groups and sugar residues [Ohtsuka et al., 2000b]. Similar to the experimental result found by Ando et al. [Ando et al., 1989] all investigated pep-

tides have a significant lower reactivity than CBZ-Q-G. The best combinations were peptide combinations in the form of GGXQGG whereas the X represents leucine or glutamic acid, which both reached a normalized reactivity of around 30 %. The reactivity for most of the other amino acid combinations was below 10 %.

However, to determine the overall influence of the flanking amino acids every combination and not only one substitution needs to be investigated. Sugimura et al. used a phage-display method to investigate the influence of flanking amino acids in peptides consisting of 12 amino acids on the MTG crosslinking. The published results indicated a preference for hydrophobic amino acids and arginine as flanking amino acids [Sugimura et al., 2008].

Fontana et al. investigated the TG substrate specificity by TG mediated PEGylation of different proteins and consecutive identification of the modified glutamines [Fontana et al., 2008]. The results indicate a preference for glutamines which are not located in a α-helix or ß-sheet and are exposed on the surface. Only in one example the glutamine on position 40 located in a α-helix in the human growth hormone was modified by TG. Fontana et al. utilized the B-factor to explain these results. The B-factor describes the flexibility of single amino acids in a polypeptide chain determined by crystallography. The B-factor diagram for the human growth hormone shows a maximum around the positions 40 to 50 and between 135 and 150. So not only flanking amino acids but also the flexibility of the nearby region influences the substrate specificity of TG.

This study investigated the substrate specificity of rMTG and rMTG(S2P) with immobilized tripeptide libraries containing for the first time all combinations of the proteinogenic amino acids around a central glutamine.

Another advantage of the described method is the higher sensitivity compared to previously published results, e.g. the tripeptide G-Q-G was investigated by Ando et

al. but no activity was found with the hydroxamate assay [Ando et al., 1989]. With this method even a fluorescence activity of 2.7 % compared to the tripeptide with the highest fluorescence activity could be detected. Ohtsuka et al. found for the heptapeptide G-G-G-Q-G-G-G an activity of 4 % compared to CBZ-Q-G. Ando et al. and Ohtsuka et al. investigated due to the experimental limits of their assays only a small number of combinations. To increase the combination coverage Sugimura et al. followed a different approach and investigated $1.5x10^{11}$ combinations of a peptide with 12 amino acids utilizing a phage display library and a chromatographic selection method [Sugimura et al., 2008]. Due to the long peptide of 12 amino acids even this broad approach was not able to investigate every combination. In the final analysis only 27 randomly selected peptides were sequenced to reveal the flanking amino acid combinations which lead to the highest substrate specificity.

The results of this thesis and the previously published results all indicate a MTG / rMTG preference for flanking arginine, tryptophan and tyrosin residues. Additionally tripeptides which were highly preferred by MTG / rMTG contained the amino acids phenylalanine, methionine, isoleucine, leucine, glycine, alanine and valine. All of these amino acids were hydrophobic under usual experimental conditions (e.g. pH 7, 25 °C) with the exception of arginine. Due to the hydrophobic surface around the active center of the MTG / rMTG the entropy gain of hydrophobic surfaces connecting to each other in aqueous solutions could explained the substrate preference for flanking amino acids with hydrophobic properties.

Since a 3D-structure with bound substrate / inhibitor is not available, only speculations can be made so far on the reason for the preference of hydrophobic amino acids and arginine.

The positive influence of arginine on the substrate specificity of the MTG could be explained by the negative charges of the amino acids Glu^{249}, Asp^{304} und Glu^{300}. These three amino acids were located around the active center in the 3D-structure of the rMTG / rMTG(S2P) by Kashiwagi et al. [Kashiwagi et al., 2002]. The positive charge of the flanking arginine in the tripeptide combination could lead to the higher substrate preferences of the rMTG / rMTG(S2P).

Under the chosen experimental conditions lysine is also positively charged and could lead to higher substrate preferences. Since the pH and amino acid charge in the active center depends on the local pK_a / pH, which might be different from the solution pH, only speculations can be made so far. Additionally, the results of this thesis showed intermolecular cross linking (see Fig. 16) between flanking lysine residues and the central glutamine residues which would lead to low fluorescence intensities and would explain the observed results.

Therefore, due to this second crosslink reaction even a highly positive influence of the lysine on the substrate specificity would not increase the fluorescence intensity but only the intermolecular crosslinking, which is not detectable with this assay.

The found rMTG / rMTG(S2P) preferences for flanking hydrophobic amino acids and the flanking arginine are mirrored in the flanking amino acids of the central glutamine in well known rMTG / rMTG(S2P) substrate proteins as shown before in Table 10. For the combination A-Q-S as found in Apomyoglobin, Interleukin 2 and β-Lactoglobulin only low fluorescence intensities were found with the immobilized tripeptide libraries. However, in those proteins the central glutamine was accepted by the MTG. Likewise, there are glutamines in other position which should be ac-

cessible by the rMTG / rMTG(S2P) but were not accepted as shown before in Table 11.

7. Outlook

In this thesis a fed batch fermentation method for the bulk quantity production of rMTG(S2P) was investigated. The two developed models for the protein expression and the fed batch fermentation were used to optimized parameters like fermentation temperature profile, total fermentation time, feed profile respectively substrate concentration over fermentation time, bio mass concentration at induction time and others alike.

For the influence of the inductor concentration on the protein expression or the plasmid stability no statistically significant relation could be determined in this thesis. However, it is unlikely for the inductor concentration to have no influence on the protein expression, e.g. no inductor leads to no protein and inductor concentration applied in this study lead to a high protein yield. In further experiments a broader inductor concentration range, starting at 0 mM and increasing until negative effects will be observed, should be investigated.

Another point for further experiments should be the up scaling of the developed fed batch fermentation to provide even higher space time yield by decreasing the production / maintenance ratio. The developed process was conducted in a 15 L standard bioreactor and it should be possible to up scale this process applying standard up scale procedures and knowledge. One of the possible problems, which could nevertheless occur, would be increased plasmid instability. To avoid the growth of plasmid free cells, other *E. coli* strains with a higher plasmid stability, different expression system with chromosomal gene incorporation or expression systems with a plasmid-chromosomal control loop to kill plasmid free cells automatically could be used as described by Baneyx et al. and J.K. Deb et al. [Baneyx, 1999; Jana and Deb, 2005].

In this thesis the fed batch fermentations were done with minimal medium and pure glucose as substrate for controlled and reproducible results. To develop a more cost efficient process additional experiments, investigating cheap complex media and substrates made of cheap and highly available industrial byproducts, would be interesting.

Due to the bio reactor configuration the biomass concentration limit in this thesis was around 20 to 25 g L^{-1}. An interesting topic for further investigations would be the increase of the space time yield by increasing the final biomass. Higher bio mass concentrations could be achieved with different bio reactor configurations and also different fermentation techniques such as the dialysis bioreactor as described by Nakano et al. [Nakano et al., 1997] or utilizing a chemostatic fermentation which would erase the time needed for maintenance / clean in place procedures during the fed batch cycles.

A comparison of the achievable space time yields with different media, substrates, induction methods and fermentation techniques and the analysis of the resulting costs per unit rMTG / rMTG(S2P) could actually help to evaluate further the potential of *E. coli* expressed transglutaminase.

The purification and activation of the produced rMTG(S2P) from biomass was developed in the second part of this thesis. The application of proteinase K in crude protein extract followed by IMAC purification provided an easy, fast, efficient and robust method to purify bulk quantities of rMTG(S2P) rich biomass. The next investigations to make the described method more feasible should be the investigation of the optimal proteinase K concentration for the activation. This would potentially decrease the demand of proteinase K for the activation and could therefore decrease the price per unit rMTG / rMTG(S2P). Another way to further optimize the activation

and purification would be the investigation of the optimal initial biomass concentration and incubation time. This would speed up the purification process and would allow a higher transglutaminase output with the same equipment.

The comparison of different activation techniques compared to the batch activation utilized in this thesis could also improve the process efficiency. For example could immobilized proteinase K, applied in a fixed bed or an expanded bed reactor, used to activate continuously transglutaminase in crude protein extract. The continuously obtained solution could then be applied to serial connected IMAC columns to purify the transglutaminase simultaneously.

The storage of the obtained transglutaminase was not addressed in this thesis but will be an important topic for the commercial production. Today, Ajinomoto Inc. stores transglutaminase as dry powder and adds high amounts of maltodextrin to stabilize the transglutaminase. The storage as dry powder should be the aim of future investigations, due to the usually high stability compared to storage as solution and the easy handling. It would be preferable to provide a pure and stable dry powder without stabilizing agent. Therefore, different drying techniques, such as freeze-drying or spray-drying, should be investigated. Another way to obtain pure and storable transglutaminase could be crystallization. These three techniques would be the most promising methods for further investigations and they would also be the most likely applicable methods due to the well known processes and broad application in other industrial application.

The third part of this thesis investigated the substrate specificity of rMTG / rMTG(S2P) and found a preference for flanking arginine, tryptophan and tyrosin residues. Additionally tripeptides, which were highly preferred by MTG / rMTG, con-

tained the amino acids phenylalanine, methionine, isoleucine, leucine, glycine, alanine and valine.

It was concluded from the literature that not only the properties of the flanking amino acids around the glutamine are important for the rMTG / rMTG(S2P) substrate specificity but also other factors such as the B-factor or the position relative in the secondary and tertiary structure. Due to this conclusion the influence of the linker, which connected the tripeptides to the paper matrix, should be investigated in further experiments.

Therefore, free tripetides, with a central glutamine and combinations of flanking proteinogenic amino acids, in MDC containing buffer solution should be investigated by HPLC measurement for crosslinking by transglutaminase.

These results could be compared with the results obtained in this thesis to determine the influence of the linker. The next step could be the quantization of the HPLC-results and the development of a mathematical model for the transglutaminase reaction mechanism. Such a model would provide for every tripeptide combination reaction kinetic parameter such as v_{max}, K_A and even inhibition parameters. These parameters will provide the means to compare the tripeptides very accurate and objective, removing the need for normalized comparison

The reaction kinetic parameters and additional literature information like the B-factor could then provide the means to predict new transglutaminase substrates from protein databases leading to new applications and products.

8. References

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9. Lebenslauf

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04/2006-11/2010	**Promotion** Martin-Luther-Universität Halle-Wittenberg Thema: Investigations of the production, purification, activation and characterization of a novel, recombinant, highly active Transglutaminases
10/1999-03/2006	**Abschluss Studium Bioverfahrenstechnik** Martin-Luther-Universität Halle-Wittenberg Abschluss: Diplom-Ingenieur (Dipl.-Ing.) Abschlussnote: 1,8

07/2004-09/2004 **OLSC (Ottawa, Kanada)**

Praktikum: Mitarbeit bei der Organisation zur Bionorth2004, Prüfung, Betreuung und Erweiterung der Datenbank des OLSC (Ottawa Life Science Council)

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Praktikum: Betreuung des Messestandes des Wirtschaftsraumes Halle/Saale

04/2004-06/2004 **GangaGen GmbH (Ottawa, Kanada)**

Praktikum: Anzucht *E.coli*-spezifischer Bakteriophagen und Untersuchungen auf Wirksamkeit gegen gefährliche *E.coli*-Stämme

10/2003-01/2004 **BEC GmbH (Halle/Saale)**

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10. Publikationsliste

Veröffentlichungen in Fachzeitschriften mit Gutachtersystem

Sommer C., Volk N. und Pietzsch M. (2010), „*Model based optimization of an E. coli fed batch fermentation for the soluble expression of a mutant MTG with increased specific activity and heat stability compared to MTG from Streptomyces mobaraensis",* Protein Expression and Purification, volume 7, issue 1, pages 9-19

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DFG Forschungsprojekt

DFG Bericht „*Prozesstechnische Untersuchungen zum Einsatz von Enzymen bei der Aufarbeitung biotechnologischer Produkte (Downstream Processing). Entwicklung eines Verfahrens zur Beseitigung von Endotoxinen unter Verwendung von Biokatalysatoren.*" zum DFG Projekt PI 362/7-1

Vortrag

Sommer C. und Pietzsch M. (2007), „*Application of Biocatalysts in Downstream Processing: Enzymatic Hydrolysis of Endotoxins*", DECHEMA/GVC, Aufarbeitung biotechnologischer Produkte, Osnabrück

Sommer C. und Pietzsch M. (2007), „*Application of Biocatalysts in Downstream Processing: Enzymatic Hydrolysis of Endotoxins*", 3. Mitteldeutsches Technologie-Treffen, Bayer AG, Bitterfeld