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ORIGINAL ARTICLE

Investigations on the activation of recombinant microbial pro-transglutaminase: in contrast to proteinase K, dispase removes the histidine-tag

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Abstract In order to produce recombinant microbial transglutaminase (rMTG) which is free of the activating protease, dispase was used to activate the pro-rMTG followed by immobilized metal affinity chromatography (IMAC). As shown by MALDI-MS, the dispase does not only cleave the pro-sequence, but unfortunately also cleaves within the *C*-terminal histidine-tag. Hence, the active rMTG cannot properly bind to the IMAC material. As an alternative, proteinase K was investigated. This protease was successfully applied for the activation of purified pro-rMTG either as free or immobilized enzyme and the free enzyme was also applicable directly in the crude cell extract of *E. coli.* Thus, it enables a simple two-step activation/purification procedure resulting in protease-free and almost pure transglutaminase preparations. The protocol has been successfully applied to both, wild-type transglutaminase of *Streptomyces mobaraensis* as well as to the highly active variant S2P. Proteinase K activates the pro-rMTG without unwanted degradation of the histidine-tag. It turned out to be very important to inhibit proteinase K activity, e.g., by PMSF, prior to protein separation by SDS–PAGE.

Keywords Dispase · *E. coli* · Proteinase K · Proteolytic activation · Recombinant microbial transglutaminase · Transglutaminase variants · IMAC purification

Abbreviations

BDM	Bio dry mass
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
HESylation	Modification of proteins by hydroxy ethyl starch (HES)
MALDI-MS	Matrix assisted laser desorption ionization mass spectrometry
His-tag	Histidine-tag
IMAC	Immobilized metal affinity chromatography
MTG	Microbial transglutaminase of *Streptomyces mobaraensis*
PMSF	Phenylmethylsulfonyl fluoride
pro-rMTG	Inactive pro-enzyme of the recombinant microbial transglutaminase
rMTG	Recombinant microbial his-tagged transglutaminase (variant of *Streptomyces mobaraensis* TG)
rMTG(S2P)	Recombinant microbial his-tagged transglutaminase (thermostable variant of *Streptomyces mobaraensis* TG)
SDS–PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TG	Transglutaminase

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Introduction

Transglutaminases (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) are a family of enzymes which have evolved for cross-linking of proteins (Mariniello and Porta 2005).

A number of prokaryotic and eukaryotic transglutaminases from various sources have been purified, characterized, and partially cloned and (over)expressed (Beninati and Piacentini 2004).

To date, the major application fields of microbial transglutaminase (MTG) are food processing, the field 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. For example, MTG treatment can be used to increase the tensile strength of wool (Cardamone 2007), for enzyme immobilization (Kamata et al. 1992; Synowiecki and Wolosowska 2006), or to coat leather (Mariniello and Porta 2005). Recently, novel applications of the MTG were described; e.g., for the production of vaccines (Chou 2009), biodegradable foils (Patzsch et al. 2010), or for the PEGylation (Sato 2002) or HESylation of (therapeutic) proteins (Besheer et al. 2009).

MTG is commercially available (Activa™) and is currently produced by fermentation of the wild-type strain *Streptomyces mobaraensis* as an extracellular protein. Activa™ contains 100 Units MTG per gram powder but only 1% protein. 99% Maltodextrin is added to stabilize the MTG and to facilitate handling. One drawback of the commercial preparation is the presence of co-secreted proteases which can potentially hydrolyze the substrate proteins that are intended to be cross-linked. Another disadvantage is the low thermostability of maltodextrin-free Activa™ and the relative low space time yield during the production of only 30 U L^{-1} h^{-1} described by Ando et al. (1989). Recently, recombinant DNA technology has been used successfully to construct heterologous expression systems. Extracellular and intracellular expression has been achieved e.g., using recombinant *Corynebacterium glutamicum* (Portilla-Rivera et al. 2009) or *Streptomyces ladakanum* (Itaya and Kikuchi 2008), and *E. coli* (Sommer et al. 2011), respectively. For application as a cross-linking enzyme, in either case, the proteolytic activation of the inactive pro-enzyme is absolutely required.

Several proteases have been successfully used for the activation of microbial (pro-) transglutaminase. 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 albogriseolus* have been used (Kikuchi et al. 2003; Date et al. 2003). It was found that dispase does not completely remove the pro-sequence, but leaves an *N*-terminal FRAP-sequence which, however, 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). This problem could be only partially solved by on-column activation of immobilized pro-transglutaminase (Yang et al. 2009). TAMEP is not commercially available and therefore not suited for mass production of the recombinant enzyme.

In our ongoing experiments on the large scale preparation of active TG and TG-variants which were obtained by random and site-directed mutagenesis (Marx et al. 2008b) and DNA shuffling (Buettner et al. 2011), 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 IMAC. Proteinase K was reinvestigated for pro-MTG activation because of the stimulating effect that was observed at low SDS concentrations (Gross-Bellard et al. 1973). Therefore, proteinase K was inhibited by phenylmethylsulfonyl fluoride PMSF before the samples were analyzed by SDS–PAGE (Ebeling et al. 1974). Immobilized proteinase K was compared to the free enzyme in order to simplify the separation of protease and activated transglutaminase.

Materials and methods

General

Unless otherwise stated, all chemicals were of analytical grade and were purchased from Sigma-Aldrich (Taufkirchen, Germany). The protein marker (PageRuler prestained protein ladder) used for SDS-PAGE was purchased from Fermentas (St. Leon-Rot, Germany). Deionized water was used throughout the experiments. The biomass containing pro-rMTG-His_6 and variants was produced by fed-batch fermentation as described previously (Sommer et al. 2011). Dispase was purchased from BD Biosciences (Heidelberg, Germany, Product No. 354235), proteinase K was purchased from Fermentas (St. Leon-Rot, Germany, Product No. EO0491), and proteinase K immobilized on Eupergit C was purchased from Sigma (Taufkirchen, Germany, Product No. 82452).

Cell lysis by high pressure homogenization

Four grams 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 $MgCl_2$, and pH 8 to adjust the biomass concentration to 6.4 g L^{-1}. The temperature of the cell suspension was adjusted to 4°C and disintegrated by four passages at 1,000 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 centrifuged for 20 min at 10,000 g in order to separate insoluble matter. The supernatant (crude protein extract) was used either immediately or after storage at −20°C.

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 2–3 h 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 min at 16,000 g and at 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.

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-His_6, 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^{-1}; flow rate for washing and elution: 3 mL min^{-1}; binding buffer: 50 mM Tris/HCl buffer with 300 mM NaCl, 20 mM imidazole, pH 8.0; elution buffer: 50 mM Tris/HCl buffer with 300 mM NaCl, 500 mM 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 to 500 mM imidazole using the elution buffer. Transglutaminase containing fractions was analyzed by SDS–PAGE and the hydroxamate activity assay. Fractions were stored at 4°C.

Dialysis and storage of IMAC fractions

Pro-rMTG-His_6 containing fractions of IMAC was pooled and dialyzed twice against dialysis buffer (50 mM Tris/HCl buffer, 300 mM NaCl, 2 mM $CaCl_2$, and 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 $CaCl_2$, and 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 $CaCl_2$, and 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.

Pro-rMTG activation with dispase or proteinase K

An IMAC purified stock solution of 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 h at 37°C with 0.21 U mg^{-1} dispase (activation experiments), and 1.31 U mL^{-1} dispase (MALDI-TOF MS measurements) or various proteinase K concentrations according to the experimental setup.

Analytical methods

Standard activity assay

TG activity was assayed by the calorimetric hydroxamate procedure (hydroxamate assay) (Folk 1969).

SDS–PAGE

Analytical separation of proteins was performed by SDS–PAGE according to the method of Laemmli (1970). Due to the stimulating effect of low SDS concentrations on proteinase K and its high thermostability (Gross-Bellard et al. 1973), the proteinase K was inhibited in all samples prior to the SDS–PAGE (e.g. preliminary incubation at 99°C), to prevent unwanted reactions. Ebeling et al. (1974) investigated the inhibition of proteinase K and reported a complete inhibition of proteinase K by PMSF. Therefore, PMSF was added to all samples to a final concentration of 1 mM. The samples were incubated at room temperature for 30 min and then analyzed by SDS–PAGE.

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.

The *protein concentration* was determined according to the method of Bradford (Bradford 1976). BSA was used for calibration.

MALDI-MS experiments were carried out using a delayed extraction TOF mass spectrometer Voyager-DE PRO (AB Sciex, Darmstadt, Germany) equipped with a pulsed nitrogen laser ($\lambda = 337$ 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) and BSA.

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 $MgCl_2$, and pH 8) at 37°C for 2 h under constant mixing. After 2 h, the immobilized proteinase K was removed by centrifugation and the supernatant was measured for caseinolytic activity according to Marx et al. (2008a).

Results and discussion

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

In order to produce recombinant microbial transglutaminase (rMTG) which is free of activating protease, the previously screened dispase was used for the activation of the pro-rMTG (Marx et al. 2008a). The separation of the activating protease from the target enzyme was carried out by immobilized metal affinity chromatography (IMAC) as described in "Materials and methods".

As can be seen from the SDS–PAGE shown in Fig. 1a (lanes 3 and 4), the pro-sequence of pro-rMTG was completely removed within 2 h of activation time. The protein solution was then applied to the IMAC column. As can be seen from the elution profile Fig. 1b and the SDS–PAGE of the elution fractions, rMTG activated by dispase was hardly binding to the column.

In order to explain this unexpected result, it was investigated whether the histidine-tag 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. 1a, lane 4) was investigated by MALDI-TOF mass spectrometry. The obtained mass spectrum is shown in Fig. 2.

The mass spectrum in Fig. 2 shows two major peaks at *m/z* 19,428 and *m/z* 38,860 that correspond to the doubly and singly protonated species 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 cleaves the pro-sequence between serine and phenylalanine and thus 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}. The concentration of the dispase, however, 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 represent a cluster of rMTG and matrix associated molecules and can be neglected. The molecular weight was calculated from the *m/z*-value of the singly charged ion. The mass determined for the dispase-activated rMTG was found to be 38,859 g mol^{-1}. In conclusion, the molecular weight of the dispase-activated rMTG was smaller than the expected theoretical value.

The calculated molecular weights of several potential proteolysis products are listed in Table 1.

Within the mass accuracy limits of about ±500 ppm of the MALDI-TOF MS analyses there is only one calculated mass of a *C*-terminal truncation that fits to the determined mass. Furthermore, combined *N*- and *C*-terminal truncations do not match the measured values.

Altogether, there is a strong evidence that four of the six histidine residues have been cleaved, which explains the elution of the dispase-activated rMTG from the IMAC column in the flow through of non-binding proteins.

As a consequence, dispase is not suitable as TG activating protease since it cannot be removed easily using IMAC from the target enzyme.

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. (1974) described the complete inhibition of proteinase K by PMSF. Therefore, proteinase K was reinvestigated and the sample was treated with PMSF as an inhibitor prior to separation by SDS–PAGE. Without PMSF, the SDS–PAGE of proteinase K treated pro-rMTG showed a considerable proteolysis (Marx et al. 2008a).

As shown by the SDS–PAGE (Fig. 3a), 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 h, the rMTG was fully activated as also shown by the standard activity assay (Fig. 3b). Even high concentrations of proteinase K (0.62 U mL^{-1}) did not result in a reduced activity.

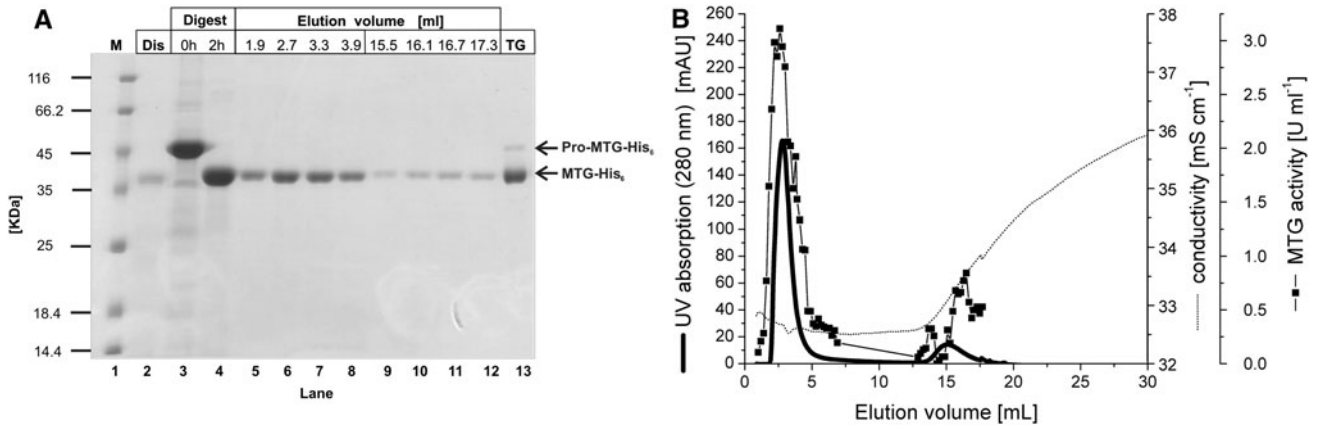

Fig. 1 IMAC purification of dispase-activated rMTG. IMAC purified pro-rMTG solution (2.5 mg mL^{-1} pro-MTG in storage buffer with 1 mM GSH and 23% glycerol) was incubated for 2 h with 0.21 U mL^{-1} dispase and then transferred to a streamline chelating Ni–NTA IMAC column: XK 5/5, CV: 1.2 mL, flow rate 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 1

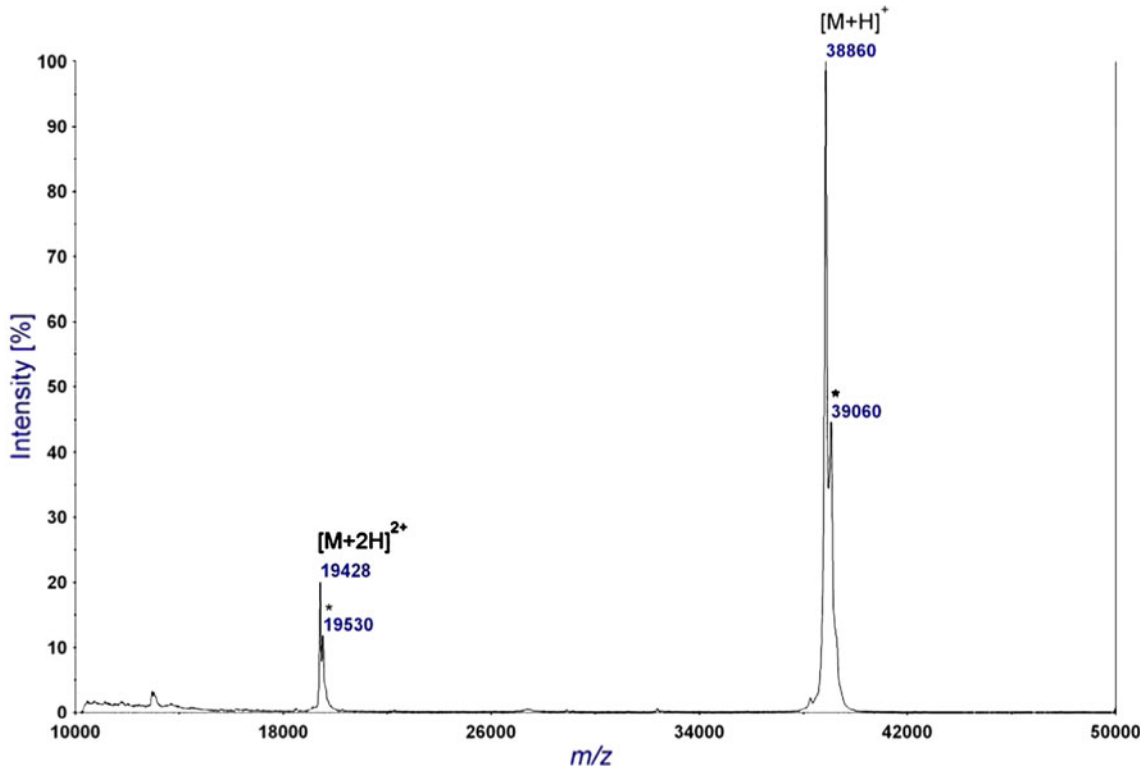

Fig. 2 Positive ion MALDI-TOF mass spectrum of dispase-activated rMTG (1 mg mL^{-1} pro-rMTG in storage buffer and 1.31 U mL^{-1} dispase). The sample was prepared using the dried-droplet method by mixing with sinapinic acid as matrix. *Asterisk* denotes matrix adducts of the protein

In summary, the effect of proteinase K activation by SDS and the importance of inhibition of proteinase K containing samples prior to SDS–PAGE have been confirmed.

As a consequence, proteinase K is suitable for the activation of the pro-rMTG. No degradation was detected for at least 3 h. 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 indeed binds to the affinity column and can be separated from non-binding proteins.

Table 1 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 the determined weight of 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

Bold letters indicate the most probable variant after proteolysis

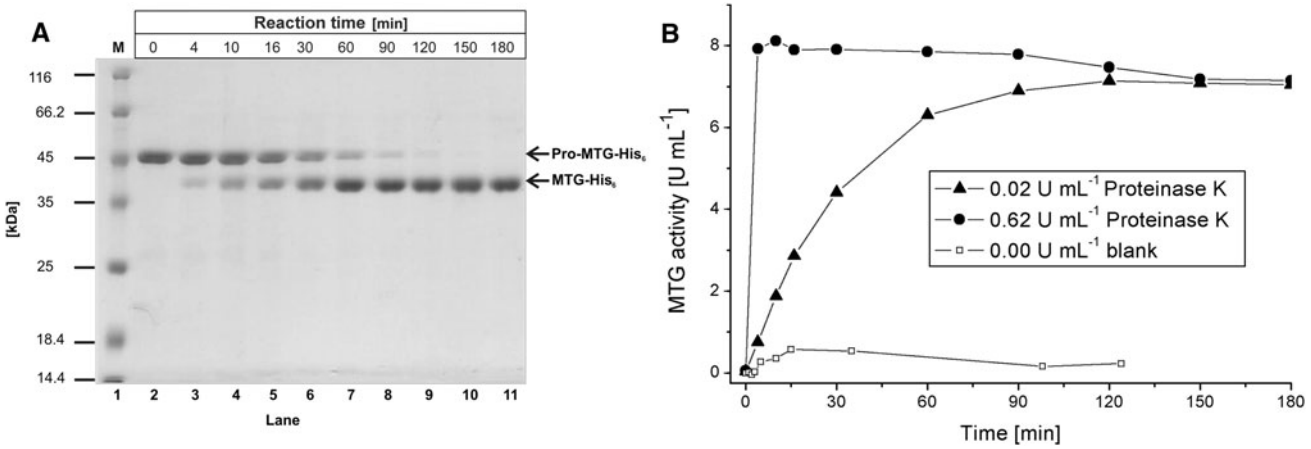

Fig. 3 Pro-rMTG activation with proteinase K and PMSF inhibition. Proteinase K (final concentration 0.02 U mL^{-1} and 0.62 U mL^{-1}) was applied to IMAC purified pro-rMTG (0.25 mg mL^{-1}) in storage buffer with 1 mM and 23% glycerol and incubated at 37°C. Samples were taken before the addition of 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 at 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, *lanes 2 to 11* activation samples, **b** time dependence of the pro-rMTG activation, rMTG activity was measured directly after sampling

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).

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

As shown before, proteinase K is suitable for the activation of IMAC purified pro-rMTG. The disadvantage of this method is 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 step. Therefore, the activation of pro-rMTG by proteinase K was carried out directly in the centrifuged and 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 protein 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. 4 reveal a fast conversion of pro-rMTG to active rMTG. The intensity of the bands corresponding to the *E. coli* host cell protein decreases over time. During the treatment with proteinase K some of the (partially) hydrolyzed *E. coli* proteins became less soluble and formed a precipitate. A similar behavior was observed for the purification of antigen II from *Streptococcus mutants* during hydrolysis with pronase (Russell et al. 1980). This precipitate can be removed by centrifugation or by expanded bed

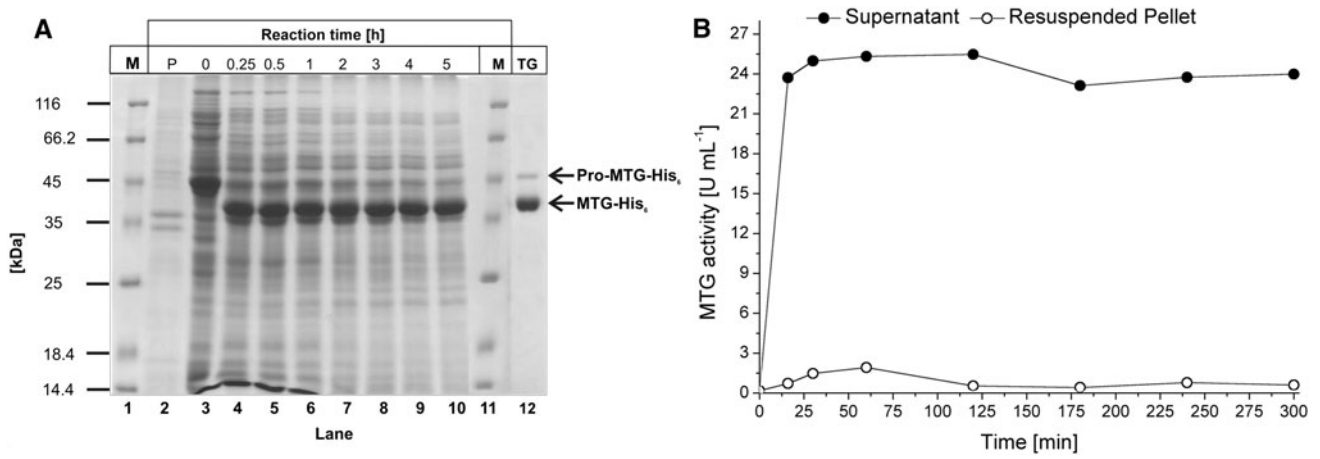

Fig. 4 Proteinase K activation of pro-rMTG in crude protein extract. Proteinase K (final concentration 0.076 U $mg^{-1}_{protein}$) was added to crude protein extract and incubated at 37°C. Sampling was done before proteinase K addition and after 15, and 30 min, 1, 2, 3, 4 and 5 h; **a** SDS–PAGE gel, all samples were diluted at 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, *lanes 1* and *11* molecular weight marker, *lane 2* pellet 10 mg mL^{-1}, which precipitated during −80°C storage, *lanes 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

chromatography. Ideally, the latter method can be combined with IMAC purification of the histidine tagged rMTG. The activated rMTG was stable for at least 300 min, indicating that *E. coli* proteases did not hydrolyze the target enzyme. This is long enough to further process bulk quantities of crude protein extract containing active rMTG.

In order to separate the activated rMTG from proteinase K and remaining *E. coli* host cell proteins, IMAC purification was carried out as described in "Materials and methods". The elution profile of rMTG and the SDS–PAGE are shown in Fig. 5, the purification table is shown in Table 2.

As can be seen in Fig. 5, proteinase K-activated rMTG did bind to the IMAC column and could be eluted by imidazole, as usual for immobilized metal affinity chromatography. 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 −21°C. Proteinase K bands (reference "PK" in Fig. 5a, lane 2) are not visible in the final rMTG preparation. However, the concentration might be too small to be detected by SDS–PAGE. However, a strong proof for the absence of proteinase K is the observation that the proteinase K inhibitor PMSF was only necessary in the sample applied to the column to inhibit rMTG degradation. In the final product, without PMSF there is no degradation of rMTG.

The purification results 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).

Production of a transglutaminase variant (rMTG(S2P)-His_6)

In previous studies, Marx et al. (2008b) selected a variant of microbial transglutaminase (rMTG(S2P)) which exhibits more than twice the specific activity and 270% higher thermostability at 60°C compared to the wild-type enzyme. The proteinase K catalyzed the 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 3), the purification protocol of this variant gives essentially the same results as for the wild-type enzyme.

The final specific activity was 43.9 U mg^{-1} which is nearly as high as the published specific activity of 46.1 U mg^{-1} (Sommer et al. 2011).

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 also enables the reuse of the biocatalyst.

First it was investigated, whether any proteinase K is bleeding from the carrier material (see "Materials and methods"). No soluble proteinase K activity could be detected in the washing buffer used.

Then, the carrier-fixed proteinase K was investigated for its ability to activate pro-rMTG(S2P). Two different

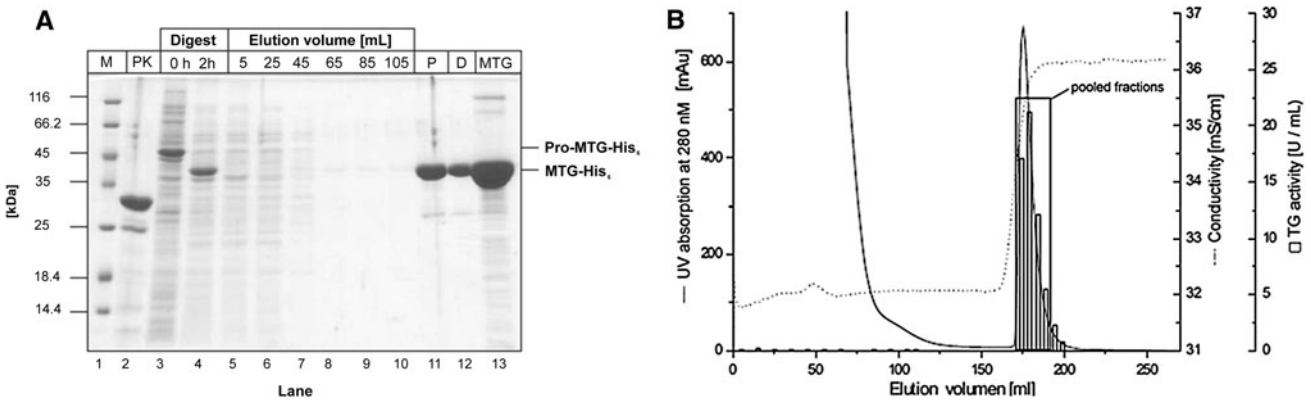

Fig. 5 Purification of active rMTG from cell lysate by IMAC. **a** SDS–PAGE, all samples were diluted at 1:2 with sampling buffer; *lane 2* molecular weight marker (*M*), *lane 3* proteinase K (30 U mL^{-1}), *lane 4* pro-rMTG containing cell lysate prior to the digest, *lane 5* cell lysate after 2 h digestion (PMSF was added to a final concentration of 1 mM and incubated for 30 min at room temperature), *lanes 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; elution 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; fraction 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* indicates the pooled fractions

Table 2 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)	Specific activity (U mg^{-1})	Yield (%)	Purification factor (–)
Cell lysate	200	12.6	–	–	–	–	–
Proteinase K activation	120	7.2	12.5	1,500	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

Table 3 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)	Specific activity (U mg^{-1})	Yield (%)	Purification factor (–)
Crude protein extract	200	2.5					
Proteinase K activation	150	2.2	23.6	3,540	10.7	100	1
IMAC	12	4.9	201.8	2,422	41.2	68.4	2.8
Dialysis	12.6	4.3	185.2	2,334	43.1	65.9	2.9

concentrations were used to hydrolyze a centrifuged particle-free extract of *E. coli*. The results are shown in Fig. 6.

As can be seen from Fig. 6, 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 min. Compared to the activation by free proteinase K (Fig. 4), there is a reduced activity which can be explained by the diffusion limitation of rMTG into the particle. The diffusion rate of rMTG is obviously slower than that 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 3-dimensional structure of rMTG which is lacking for casein.

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

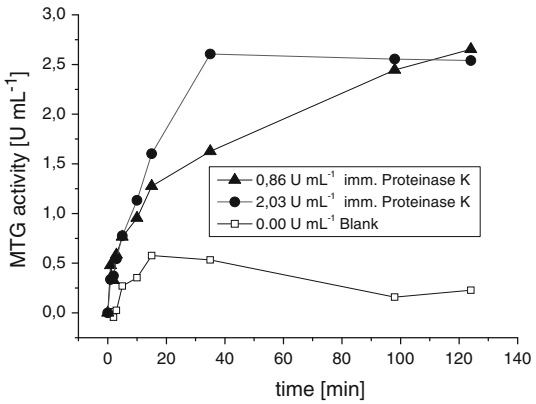

Fig. 6 Pro-rMTG(S2P) activation with immobilized proteinase K. Two different proteinase K concentrations were applied to the crude protein extract and incubated at 37°C for 2 h. Activity was measured directly after sampling with the hydroxamate assay

Conclusions

Microbial transglutaminase is commercially available (Activa™) and is currently produced by fermentation of the wild-type strain *Streptomyces mobaraensis* as an extracellular protein. Activa™ contains 100 Units MTG per gram powder but only 1% protein. 99% Maltodextrin is added to stabilize the MTG and to facilitate handling. One drawback of the commercial preparation is the presence of co-secreted proteases, which can potentially hydrolyze the substrate proteins that are intended to be cross-linked. Another disadvantage is the relatively low thermostability of maltodextrin-free Activa™ and the relative low space time yield during the production of only 30 U L^{-1} h^{-1} (Ando et al. 1989). The thermostability of recombinant TG and TG-variants was significantly improved by random and site-directed mutagenesis and DNA shuffling (Marx et al. 2008b; Buettner et al. 2011). The volumetric yield and the space time yield of inactive pro-transglutaminase have been increased by fermentation technology and the construction of heterologous expression systems (Portilla-Rivera et al. 2009; Itaya and Kikuchi 2008; Sommer et al. 2011). For application as a cross-linking enzyme, in either case, the proteolytic activation of the inactive pro-enzyme is absolutely required. Several proteases have been successfully used for the activation of microbial (pro-) transglutaminase. 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 albogriseolus* have been used (Kikuchi et al. 2003; Date et al. 2003). It was found that most proteases like dispase do not completely remove the pro-sequence, but leave an *N*-terminal FRAP-sequence which, however, does not affect the activity. Trypsin is not specific for the removal of the pro-sequence, also hydrolyzes the active transglutaminase (Marx et al. 2008a). This problem could be only partially solved by on-column activation of immobilized pro-transglutaminase (Yang et al. 2009). TAMEP is not commercially available and therefore not suited for mass production of the recombinant enzyme.

In the present paper, we have investigated the applicability of dispase for the activation of pro-rMTG. Surprisingly, it was found that the dispase not only cleaves the pro-sequence but, unfortunately, also degrades the *C*-terminal His-tag from 6 to 2 histidine residues. The cleavage of the His-tag results in a serious problem for the subsequent IMAC purification as the shortened his-tag is not sufficient to bind the active rMTG properly to the IMAC material. Therefore, dispase is not applicable for a fast, easy and efficient pro-rMTG activation and simple removal of the activating protease using the affinity tag. The removal of the activating protease is absolutely required, since an unspecific enzyme like dispase or proteinase K in contrast to the highly specific endogenous enzymes TAMEP and SM-TAP is also destroying the protein substrate.

As an alternative, proteinase K was investigated and found to be an applicable alternative. Proteinase K can be applied as free or immobilized enzyme. It activates the pro-rMTG without degradation. The *N*-terminal cleavage pattern is identical to the endogenous protease TAMEP and dispase and the *C*-terminal His-tag remains intact. Host cell proteins of *E. coli* can be partially removed by direct application of the protease to the cell extract. The activated rMTG is sufficiently stable against proteolysis by *E. coli* proteases. The procedures developed here can be applied to wild-type rMTG and variants as shown for the S2P mutant enzyme. Especially the use of the immobilized proteinase K provides easy access to large amounts of active transglutaminase. The immobilized protease can easily be separated from the cross-linking enzyme by filtration. Thereby, expensive chromatographic separation steps like IMAC can be omitted, preferentially if the *E. coli* host cell proteins are accepted in the application of the transglutaminase. This is the case, e. g., if the transglutaminase is used to produce protein-based materials like films which are not intended to be used in food technology (Patzsch et al. 2010).

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