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Research paper

Residual transglutaminase in collagen – Effects, detection, quantification, and removal

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ABSTRACT

In the present study, we developed an enzyme-linked immunosorbent assay (ELISA) for microbial transglutaminase (mTG) from *Streptomyces mobaraensis* to overcome the lack of a quantification method for mTG. We further performed a detailed follow-on-analysis of insoluble porcine collagen type I enzymatically modified with mTG primarily focusing on residuals of mTG. Repeated washing (4×) reduced mTG-levels in the washing fluids but did not quantitatively remove mTG from the material ($p < 0.000001$). Substantial amounts of up to 40% of the enzyme utilized in the crosslinking mixture remained associated with the modified collagen. Binding was non-covalent as could be demonstrated by Western blot analysis. Acidic and alkaline dialysis of mTG treated collagen material enabled complete removal the enzyme. Treatment with guanidinium chloride, urea, or sodium chloride was less effective in reducing the mTG content.

1. Introduction

Collagen is a widely used biomaterial for biomedical applications like wound healing sutures, spongy implants, injectable dispersions, or controlled release systems. However, a major drawback of sheets or porous systems made from collagen is the lack of mechanical stability. This deficiency may be overcome by applying external crosslinking to regain stability or to even provide mechanical strength beyond the natural capacity lost during the isolation processes [1,2]. Widely used chemical crosslinking methods comprise residue problems with potential or well-known toxicity [3]. Enzymatic crosslinking might help to circumvent this situation. Microbial transglutaminase (mTG) derived from *Streptomyces mobaraensis* is a commonly used enzyme in pharmaceutical research and food industry as well as in tissue engineering and biomaterials research [4–8]. It catalyzes the formation of a covalent amide bond between an ε-amino-group of a lysine residue and the γ-carboxamid group of a glutamine residue [9]. It is a 38 kDa non-glycosylated 331 amino-acid protein with one single cysteine residue, which is essential for its catalytic activity [10,11]. Crystal structure and the catalytic scheme have been discovered [12], and an activity-assay for tissue type II transglutaminase has been developed [13]. The effects of transglutaminases in tissue engineering have been intensively studied [14]. Cells seeded on mTG-treated collagen show better adhesion, proliferation, and osteogenic differentiation [15]. It has been reported that treatment with mTG increases the storage modulus of chicken sternal collagen type II [6], enhances neovascularisation of wound healing constructs made of bovine calf skin type I collagen [7], and increases tensile strength and melting temperature of porcine skin collagen type I [16]. However, in these studies, the obtained materials were analyzed without any purification and, thus, not considering potential residuals of mTG. Ciardelli et al. attempted to remove mTG by washing the specimens twice with water but did not elucidate, whether residuals were left [17]. The question arises whether the obtained results were due to mTG-mediated crosslinking of the collagen or due to other effects that can be ascribed to mTG-residuals. This has become even more important since Stachel et al. reported that mTG does not establish ε-(γ-glutamyl)-lysine bonds in native collagen type I derived from both calf skin and bovine hide [18]. Even though mTG seems to exhibit no cytotoxicity [6,16], it might provoke severe immune responses as

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any foreign protein can. From the analytical point of view, the determination of the enzymatic activity of mTG has been established in the 1960s [19] but this procedure, however, cannot be utilized to detect residuals in mTG-treated materials due to the potential activity loss resulting from material processing. The present publication closes the described analytical gap in mTG-quantification developing a specific ELISA, which can detect mTG in presence of collagen. Furthermore, it helps to give more insight into the fate of mTG after its addition to the substrate, its interactions, removal, and potential residuals.

2. Materials and methods

All water used was highly purified and deionised with the help of a USF ELGA PURELAB Plus UV/UF water purifier (Ransbach-Baumbach, Germany). All chemicals were purchased from Sigma–Aldrich, unless otherwise stated. For Elisa, Maxisorp® 96-well flat-bottom plates were purchased from Nunc Inc. (Roskilde, Denmark), rabbit anti-bacterial-transglutaminase antibody (A019) was from ZEDIRA GmbH (Darmstadt, Germany), mouse anti-bacterial-transglutaminase antibody (Cov3C7) and biotinylated rabbit anti-mouse IgG were from Covalab (Villeurbanne, France), and streptavidin poly-HRP Calbiochem® was obtained from Merck KGaA (Darmstadt, Germany).

2.1. mTG modification of collagen

Porcine-skin derived collagen-suspension was prepared according to Meyer et al. [20] and freeze-dried (Christ Epsilon 2-6D®, Osterode am Harz, Germany) to obtain insoluble porcine collagen type I raw material. The dried collagen (nonxl) was dispersed in 800 mL Soerensen-buffer (pH 6.0) at a concentration of 0.5% (w/w) as transglutaminase is more active around neutral pH. AktivaWM® transglutaminase (activity: 51.8 U/g) from Ajinomoto (Hamburg, Germany) was added at 2 g/L for "tgxl" samples and 5 g/L for "tgxl high" samples. The enzymatic activity therefore was 20.7 U/g collagen for tgxl and 51.8 U/g collagen for tgxl high, respectively. The mTG-preparation was used without further purification and therefore contained 99% maltodextrin. The initial content of pure mTG in tgxl and tgxl high collagen was then calculated to be 0.4% and 1%, respectively. The dispersion was incubated at 30 °C for 2 h and centrifuged at 2739*g* (Sigma 4K15®, Osterode am Harz, Germany) after incubation. The supernatants (770 mL of the initial 800 mL) were removed, and pH values were measured before freezing the material at −40 °C for further ELISA-analysis. The pellets were washed four times by resuspending each with 150 mL water (pH adjusted to 3.7 with concentrated acetic acid). This pH condition (pH 3.7) was chosen to ensure that both, collagen with an isoelectric point of 7.8 and mTG, for which an isoelectric point of 9 has been reported by Ando et al. [10], show a highly positive net charge resulting in substantial repulsive interaction. After centrifugation, the washing solutions were collected, pH-values were measured, and the solutions were subsequently deep-frozen at −40 °C for further analysis. The mTG-modified collagen pellets were freeze dried. Collagen treated with either Kristallpur RC 0811 maltodextrin "malto" (Hafen-Muehlen-Werke GmbH, Bremen, Germany) or heat inactivated mTG (95 °C, 45 min.) "inactivated" were processed in the same manner as tgxl-material to obtain references.

2.2. Collagen sample preparation

Samples were prepared by dispersing the different collagen materials (nonxl, tgxl, tgxl high, malto, inactivated) in water (pH adjusted to 3.7 with concentrated acetic acid) at a concentration of 2 % (w/w). After swelling for 4 h, the dispersions were homogenized with an ESGE® immersion blender (Unold AG, Germany). Dispersion-aliquots of 1.5 mL per well were distributed in Costar® polystyrene 24-well plates (Corning Inc., Corning, USA) and freeze dried.

2.3. Melting temperature

Melting temperatures were determined using a Mettler Toledo DSC821[e] differential scanning calorimeter. Therefore, 3 mg samples ($n = 3$) were incubated with 10 μL PBS-buffer in 40 μL aluminum crucibles (Mettler, ME-26763) for at least 2 h at room temperature and then heated from 20 to 90 °C with a heating rate of 10 K/min.

2.4. Enzymatic degradation

Collagen samples of 6 mg ($n = 3$) were incubated in 1 ml TES-buffer (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, adjusted to pH 7.4 with 1 M sodium hydroxide) at 37 °C for one hour to allow wetting and swelling of the collagen. 20 μg collagenase (*Clostridium histolyticum* type H, Sigma) was added to each sample and incubated for 2, 5, or 24 h. Samples were centrifuged with 186,000*g* (Optima TLX-CA, Beckman Coulter, Brea, CA, USA) for 45 min at 4 °C, and the supernatants were removed. Supernatants of the 24-h incubation were analyzed by ELISA and Western blot. To evaluate whether mTG was degraded by collagenase under these conditions, mTG alone was treated in the same way. After vacuum drying, the weight loss was determined gravimetrically.

2.5. Tensile strength measurement

For tensile strength measurements, strips of 10×50 mm displaying a height of 3–5 mm (determined with a caliper) were manufactured by dispersing the collagen materials of interest in water (pH adjusted to 3.7 with concentrated acetic acid) at a concentration of 2%, followed by swelling for 4 h, homogenization with an ESGE® immersion blender (Unold AG, Germany) and freeze drying in polyethylene molds. Cutting with a scalpel rendered the desired dimensions. The obtained strips were incubated in PBS for at least 2 h at room temperature (RT). The wet specimens ($n = 6$) were elongated with a Texture Analyser XTPlus (Stable Microsystems, UK) at a speed of 0.5 mm/s at RT until rupture and the highest force measured was divided through the cross-section areas of the corresponding collagen strips.

2.6. ELISA-procedure

For the development of an ELISA for mTG, all incubation steps were performed at RT on an orbital shaker (Titramax 101, Heidolph Instruments GmbH, Schwabach, Germany) at 150 RPM unless otherwise stated. Microplate wells were coated for 4 h with 100 μL of 2 μg/mL rabbit anti-mTG antibody (ZEDIRA) in 50 mM carbonate buffer (pH 9.6). Plates were rinsed five times with wash/dilution buffer (10 mM phosphate buffer (pH 7.4) containing 0.1% Polysorbate-20 and 0.5 M sodium chloride) and blocked with 200 μL of 0.5% BSA in wash/dilution buffer for one hour followed by five washing steps with wash/dilution buffer. For preparation of the calibration curves, mTG-preparation was diluted to concentrations between 100 ng/mL and 5 μg/mL (i.e., mTG concentration: 1–50 ng/mL) in TES-buffer (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) adjusted to pH 7.4 with 1 M sodium hydroxide and incubated for 24 h at 37 °C in the presence or absence of collagenase (19.6 μg/mL). Calibration standards and protein samples were added to the wells (100 μL) and incubated

overnight at 4 °C without agitation. After rinsing five times with wash/dilution buffer, 100 μL of mouse anti-mTG antibody (Covalab; 1 μg/mL in wash/dilution buffer) were added to each well and incubated for 1 h and subsequently washed five times. 100 μL of biotinylated IgG H + L (Covalab; 1 μg/mL in wash/dilution buffer) was added to each well and incubated for 1 h, followed by five washing steps and the subsequent addition of 100 μL streptavidin poly-HRP (Merck; 0.1 μg/mL in wash/dilution buffer). After one hour of incubation, the plate was rinsed five times with wash/dilution buffer before the addition of 100 μL citric acid buffer solution (pH 5) containing 0.005% H_2O_2 and 0.5 mg/mL o-phenylenediamin. The reaction was stopped by adding 100 μL of 1 N H2SO4 after 6 min, and the absorbance was measured at 490 nm with a FLUOstar Omega® microplate reader (BMG LABTECH GmbH, Offenburg, Germany).

2.7. Western blot analysis

Samples were prepared according to 2.4 or without the addition of collagenase. Standard Laemmli sample buffer was added, and samples were boiled for 5 min at 95 °C. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE, Bio-Rad, Munich, Germany) was performed, and separated proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) by tank electroblotting. Membranes were blocked with bovine serum albumin (Sigma Aldrich). A rabbit polyclonal anti-mTG antibody (1:1000, overnight incubation) from ZEDIRA (Darmstadt, Germany) was used as primary antibody. For detection, horseradish peroxidase-labeled goat anti-rabbit antibody (1:10,000, 2 h incubation) from DIANOVA (Hamburg, Germany) and ECL Plus Western Blotting Detection Reagent from GE Healthcare (Munich, Germany) were used. The appearing luminescence was detected by exposure (1 min.) of the membrane to an X-ray film (Super RX, Fuji, Düsseldorf, Germany), which was subsequently developed with a Curix 60 developing system (AGFA-Gaevert, Cologne, Germany).

2.8. Dialysis

Dialysis was performed to reduce mTG concentration in tgxl samples. Collagen material (tgxl) and mTG as reference were dispersed at concentrations of 10% in HCl (pH 2) or NaOH (pH 12). After swelling for 2 h, the materials were placed in Spectra/Por® PVDF membranes with a 500 kDa cut-off (Spectrum® Laboratories Inc., Rancho Dominguez, CA, USA) and dialyzed in three consecutive steps (3 h, 18 h, and 3 h) against 1.5 L HCl (10 mM, pH 2) or NaOH (10 mM, pH 12) per g collagen or mTG, respectively. The dialyzed samples were freeze dried to obtain material for Western blot and ELISA determination after enzymatic degradation. As reference, the materials (tgxl and mTG) were treated accordingly at pH 2 or pH 12 without dialysis.

2.9. Washing with additives

Nonxl and tgxl collagens were dispersed at a concentration of 1% (m/m) in 100 mL water containing either 1 M sodium chloride, guanidinium chloride, or urea with an ESGE® immersion blender (Unold AG, Germany) and incubated for 18 h at RT. The dispersions were sonified for 5 min after 1, 16, and 17 h using a USC1200TH ultrasonic device (VWR, Darmstadt, Germany). After centrifugation at 2739*g* (Sigma 4K15®, Osterode am Harz, Germany), the supernatants were removed and the collagens were washed twice with 100 mL water. Finally, the collagen materials were lyophilized and analyzed by Western blot (see Section 2.7).

3. Results and discussion

3.1. Melting temperature

The melting temperature (T_m) or denaturation temperature is a parameter obtained during DSC-analysis of collagen samples. T_m strongly depends on the hydration of the collagen molecules. This hydration obviously depends on the water content of the samples [21,22] and T_m increases with decreasing water content. But T_m also increases with increasing crosslinking degree [23]. To compare different crosslinking degrees of the collagen samples, the measurements were performed in the presence of an excess of water. Thus, a fully hydrated state is achieved, and differences caused by a different water content on T_m can be ruled out [22]. The stating material used in our studies displayed a T_m of 60.1 ± 0.6 °C. This T_m is in agreement with native fibrillar collagen. The T_m for mTG-treated collagen (tgxl) was 58.6 ± 0.5 °C. The melting temperature for "tgxl high" material was 58.4 ± 0.9 °C. This finding is in accordance with Collighan et al. who previously reported no effect on T_m for mTG and guinea pig liver transglutaminase (gTG) treatment of bovine hide or soluble rat collagen [24]. Furthermore, the results correspond to the findings of Stachel et al. who reported that acid soluble collagen type I does not act as a substrate for mTG in the native state [18]. However, Ciardelli et al. presented contradictory results revealing an increase of T_m by ~18 K for acid soluble calf skin collagen type I incubated with mTG (0.025 U/mg of protein) for 30 min at 37 °C [17]. Since these latter authors used an incubation temperature of 37 °C, their results are again in good agreement with the findings of Stachel et al. who reported that mTG-derived crosslinks are formed at 37 °C, whereas they are not established at 30 °C incubation temperature [18]. Taking these findings into account, the effect of mTG-treatment of collagen on T_m depends on the raw material being soluble or insoluble, on the incubation temperature as well as on alkaline or acidic pretreatments. Furthermore, in the case of the insoluble, acid derived porcine material presented here, the crosslinking degree of the raw material is already at a high level and incubation temperature was low to maintain its native state. Therefore, mTG-treatment did not lead to a further increase in T_m.

3.2. Enzymatic degradation

For collagen material characterization, the resistance to enzymatic degradation by collagenase is a commonly used in vitro

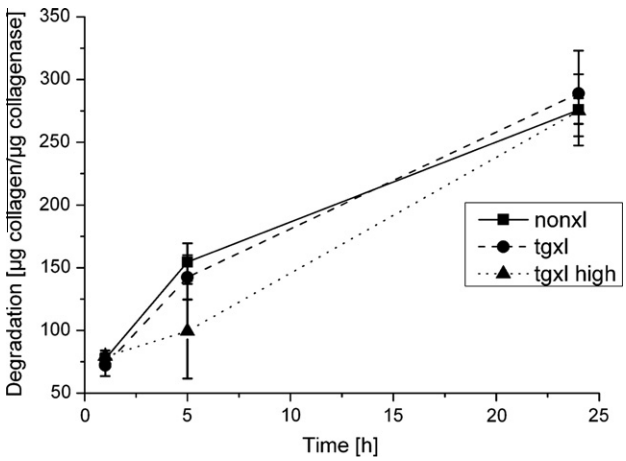

Fig. 1. Degradation rates as a function of time of collagen raw material (nonxl) and mTG-treated collagen material (tgxl (0.004 g mTG/g collagen) & tgxl high (0.01 g mTG/g collagen)) subjected to 20 μg *Clostridium histolyticum* collagenase per 6 mg collagen.

model. The gravimetrical determination is an established method since the work of Olde Damink et al. [25,26]. Rault et al. showed that crosslinking of calf skin collagen with hexamethylene-diisocyanate (HMDC) and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) reduces enzymatic degradation [27]. In Fig. 1, the degradation rate of collagen is presented as a function of time. After 2 and 24 h, the degraded amount is not influenced by mTG-treatment, contradicting Ciardelli et al. who reported reduced enzymatic degradation by ~50% for calf skin collagen type I incubated with mTG (0.025 U/mg of protein) for 30 min at 37 °C [17]. However, after 5 h of incubation, the values of the samples that had been modified with the higher mTG-concentration indicate a slightly, but not significantly, higher resistance to collagenase. It is important to note that ultracentrifugation was mandatory to obtain meaningful data, whereas centrifugation at only 2739g did render irreproducible and varying results.

3.3. Tensile strength

Fig. 2 shows the displacement data of the nonxl and tgxl collagen strips during their elongation until rupture. The highest force obtained for each sample was taken to calculate the force upon rupture of the wet strips, which is presented in Fig. 3. The maximum force increased from 3.7 mN/mm^2 for the unmodified insoluble collagen to 16.3 mN/mm^2 for the mTG-treated tgxl material. The difference was proven to be statistically significant ($p < 0.01$) using a t-test. The general finding of increasing tensile strength by mTG-treatment is in accordance with literature, even though some of the reported data in literature lack control values for untreated raw material [16]. The increase in tensile strength being 440 % for the mTG-treated material is remarkable considering that it cannot be ascribed to the formation of ε-(γ-glutamyl)-lysine bonds, which is the typical reaction product of mTG-catalysis [9], since Stachel et al. showed that these bonds are not established in native acid soluble bovine calf skin collagen type I under the tested conditions [18] and insoluble collagen material should be a less suitable substrate. Freeze-drying might induce crosslinking by dehydrating conditions, but the nonxl material as well as the controls (maltodextrin only and heat inactivated transglutaminase) was freeze dried according to the same protocol. The tensile strength values obtained for collagen treated with inactivated mTG or maltodextrin were similar to the nonxl material. Therefore, we can exclude a contribution of the freeze-drying steps to the increased tensile strength. What in fact increases tensile strength of the mTG treated collagen samples can only be hypothesized. We assume that mTG itself interacts with the collagen leading to better coherence. The tgxl high collagen exhibited a tensile strength comparable to the untreated material. After incubation with mTG, the dispersion appeared grainy and homogeneity could not be achieved during treatment with the immersion blender. Therefore, finding the ideal incubation

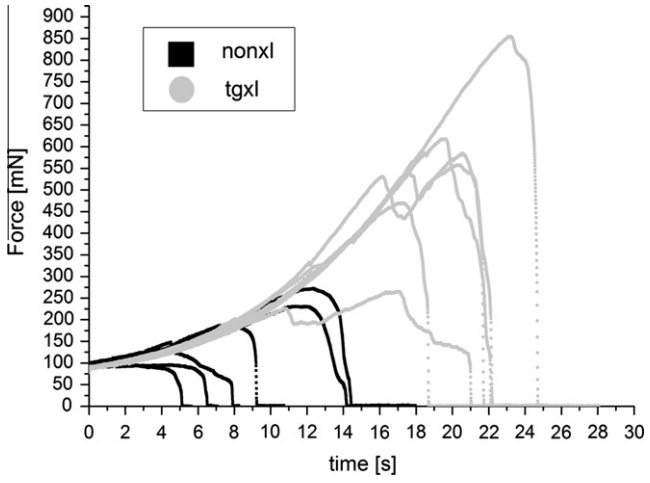

Fig. 2. Displacement-data (0.5 mm/s) of elongated wet collagen strips (2% collagen content), raw material (nonxl), and mTG-treated collagen (tgxl (0.004 g mTG/g collagen)) recorded with a Texture Analyser XTPlus.

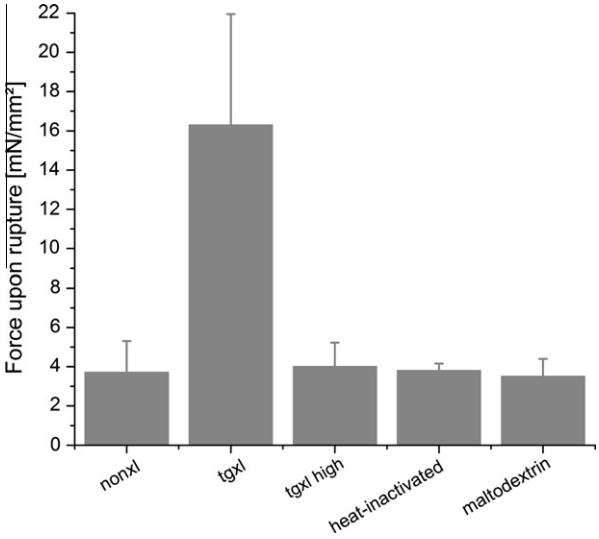

Fig. 3. Force upon rupture of wet collagen samples: untreated material (nonxl), mTG-treated collagen (tgxl (0.004 g mTG/g collagen), tgxl high (0.01 g mTG/g collagen), heat inactivated), and maltodextrin-treated collagen.

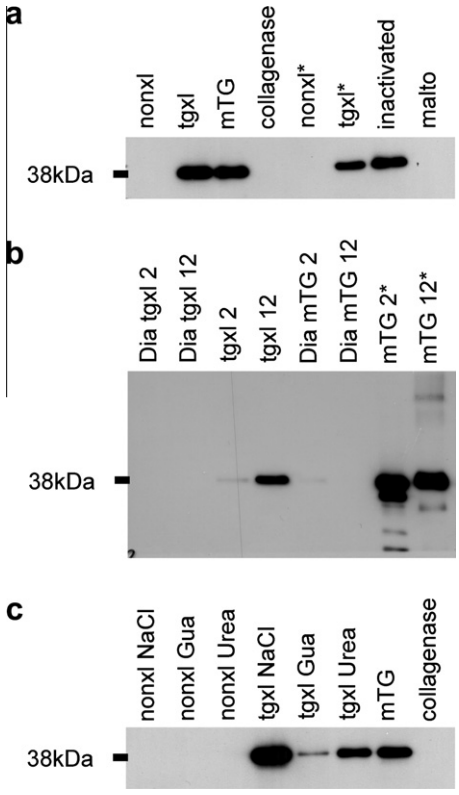

Fig. 4. Western blot, samples marked with (∗) were not subjected to collagenase; (a) collagen raw material (nonxl), mTG-treated collagen (tgxl (0.004 g mTG/g collagen)), mTG-only, collagenase-only, collagen treated with heat inactivated mTG (inactivated) and maltodextrin-only control; (b) dialyzed mTG (Dia mTG) and dialyzed mTG-treated collagen (Dia tgxl) at pH 2 & 12 and undialyzed controls; and (c) mTG-treated collagen (tgxl (0.004 g mTG/g collagen)) after washing with NaCl, urea, or guanidiniumchloride and controls.

conditions for modification with mTG is of crucial interest for material modification. After dialysis at pH 2 or pH 12, a tensile strength-determination is not feasible due to amide-bond hydrolysis occurring under these conditions that led to a strong decrease in tensile strength (data not shown).

3.4. Western blot analysis

Western blot analysis was performed to evaluate, whether the washing steps enable the removal of mTG from the treated collagen material, and to gain insight into the type of interactions between collagen and mTG. Fig. 4a shows a band for mTG at around 38 kDa, which is in accordance with previously reported results [10]. The mTG band is equally present in the enzymatically degraded tgxl collagen (lane 2 from the left) as well as in the tgxl sample not treated with collagenase (lane 6 from the left). As expected, mTG was not detected in the nonxl materials or controls. Obviously, mTG was not degraded by collagenase under the tested conditions and was therefore still accessible for analytical methods. Furthermore, the results show that the enzyme could not be quantitatively removed during washing steps. The distinct mTG band in the non-degraded tgxl material revealed that mTG was not completely removed from mTG-treated collagen materials by four washing steps with water (pH adjusted to 3.7 with concentrated acetic acid). A clear quantification was not feasible, but the band intensity indicated the presence of substantial amounts of mTG. Microbial transglutaminase was not covalently bound to collagen as it could still be separated during electrophoresis.

3.5. ELISA of washing solutions

Despite numerous washing steps with water (pH adjusted to 3.7 with concentrated acetic acid), Western blot analysis rendered considerable amounts of mTG that remained in the tgxl-material. Quantification of mTG in the incubation medium after mTG-modification as well as in the washing fluids (see Section 2.1) should allow the calculation of the total amount of mTG that had been removed from the reaction mixture and consequently the mTG-amount remaining in the tgxl material. Fig. 5 demonstrates the decrease of mTG-concentration in the washing fluids with each washing step. The concentration determined in the supernatant after incubation and centrifugation was 8.54 μg/mL ± 0.68 μg/mL for tgxl and 16.80 μg/mL ± 2.68 μg/mL for tgxl high, respectively, whereas the initial concentration of mTG added for modification was either 20 μg/mL or 50 μg/mL. The third and the fourth washing step could only remove marginal amounts of the enzyme. The total amount of mTG that had been removed by all of the performed centrifugation and washing steps was 42.5 ± 7.8% for the treatment leading to tgxl and 33.2 ± 16.1% for the treatment leading to tgxl high. From these values, the residual amount of mTG in the collagen materials, based on the initial mTG amount during incubation, 0.4% for tgxl and 1% for tgxl high, can be calculated to be 0.23 ± 0.04 % mTG in the tgxl-collagen material and 0.66 ± 0.16 % mTG in the tgxl high collagen material, assuming that collagen itself was not removed during washing. This finding confirms the Western blot observation that mTG cannot be quantitatively removed by washing steps. The pH-value decreased with each washing step from 5.9 to 4.1 to 3.7 and finally to 3.7. This ensures that both, collagen with an isoelectric point of 7.8 and mTG, for which an isoelectric point of 9 has been reported by Ando et al. [10], show a highly positive net charge resulting in substantial repulsive interaction. Additionally, Western blot analysis proved that the interaction is non-covalent (see Section 3.4). Furthermore, hydrophobic interactions are expected to be less pronounced between the two rather hydrophilic molecules. Consequently, we conclude that mTG is mechanically entrapped in a network created by its own catalytic activity in the mTG-treated collagen materials.

3.6. ELISA of collagen samples

To confirm the amount of mTG calculated to be still present in the collagen after reduction of the enzyme by washing steps, a direct quantification method for mTG in the collagen was evaluated. Therefore, the collagen material needed to be hydrolyzed by collagenase. Additionally, it had to be clarified whether or not collagenase interfered with the ELISA. Two calibration curves of mTG were prepared, one in the presence and one in the absence of collagenase. These curves are presented in Fig. 6. As can be seen, no significant difference was observed, confirming that mTG is not degraded by collagenase under the tested conditions (mTG degraded according to Section 2.4) and that the enzyme is still accessible for Western blot and ELISA analysis. Therefore, the desired samples were enzymatically degraded (see Section 2.4), and mTG concentrations in the supernatants of the 24 h samples were determined with the newly developed ELISA after 1:1000 dilution. Nonxl collagen samples treated with the same conditions served as blanks. Table 1 shows the residual mTG amounts

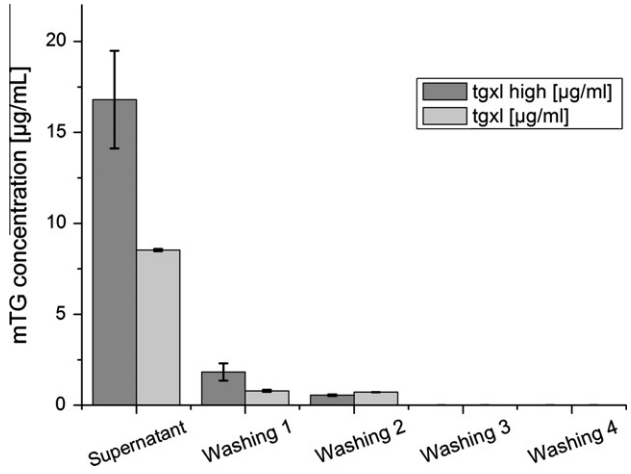

Fig. 5. Concentration of mTG in incubation-solution and in the washing fluids (washing 1–4) of mTG-treated collagen (tgxl (0.004 g mTG/g collagen) & tgxl high (0.01 g mTG/g collagen)) determined by ELISA.

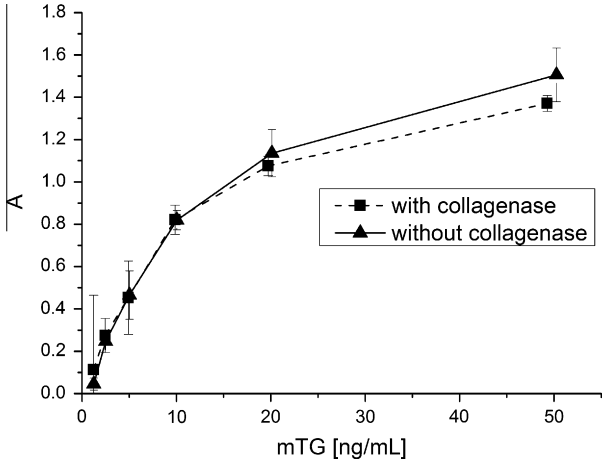

Fig. 6. ELISA calibration curves (mTG-quantification) in the absence and in the presence of *Clostridium histolyticum* collagenase type H (20 μg collagenase, 24 h incubation, 37 °C).

Table 1
mTG content in mTG-treated collagen before and after washing (tgxl (0.004 g mTG/g collagen) & tgxl high (0.01 g mTG/g collagen)).

	tgxl	tgxl High
Before washing	0.398%	0.990%
After washing	0.157 ± 0.008%	0.406 ± 0.004%

determined being 0.157 ± 0.008 % in the tgxl collagen and 0.406 ± 0.004 % in the tgxl high samples. These amounts are lower than the calculated values based on washing fluid concentrations (see Section 3.5). This indicates a removal of collagen during washing procedure creating a systematic error of the calculation. The direct quantification of mTG in the collagen samples is therefore regarded as superior and much more reproducible considering the standard deviations.

3.7. Dialysis

Our findings obtained so far clearly show that mTG could not be quantitatively removed at pH 3.7 although numerous washing steps were performed but instead remained in the mTG-treated collagen material to a high extent. As an alternative approach, a dialysis procedure was carried out to remove unbound mTG by means of a concentration gradient, enforced by application of either more acidic (pH 2) or strong alkaline conditions (pH 12). ELISA quantification of mTG in dialyzed collagen samples after degradation by collagenase revealed that mTG could not be detected in mTG-modified collagen material at both pH conditions during dialysis. This finding indicates that mTG is removed by these dialysis procedures. Furthermore, Western blot analysis after enzymatic degradation by collagenase (see Section 2.4) showed that mTG was no longer present in the mTG treated materials and therefore had been quantitatively removed by the dialysis procedures (Fig. 4b, lanes 1&2 from the left). Although treatment at pH 2 led to partial fragmentation of mTG (Fig. 4b, lane 7 from the left) and treatment at pH 12 caused partial aggregation (Fig. 4b, lane 8 from the left), mTG remained detectable for Western blot analysis. Treatment of tgxl collagen at pH 2 or 12 without dialysis was not able to quantitatively remove mTG (Fig. 4b, lanes 3&4 from the left). However, these harsh pH conditions led to a decrease in tensile strength due to amide bond hydrolysis (data not shown) and thereby interfered with the beneficial use of mTG.

3.8. Washing with additives

The understanding of the effects of specific ions on proteins is currently changing. There is increasing evidence that salting-in and salting-out effects of ions on proteins need to be ascribed to ion-protein and ion-shell-water-molecule interaction rather than on ions changing the water structure and thereby affecting protein behavior [28]. To test whether the reduction of ionic interactions may enable mTG removal from the collagen samples, the collagen materials were subjected to washing with sodium chloride, guanidinium chloride, or urea. Western blot analysis showed that the mTG content in tgxl collagen increased in the following order: guanidinium chloride < urea < sodium chloride (Fig. 4c). Although the used additives are known to have a lyotropic effect on collagen [29,30], only treatment with guanidinium chloride seems to be an effective tool to decrease mTG content but is less effective than dialysis (see Section 3.7).

4. Conclusion

In the present study, we investigated the effects of *Streptomyces mobaraensis* derived transglutaminase (mTG) treatment on insoluble porcine skin collagen type I. The results showed neither increased melting temperatures nor reduced enzymatic degradation assuming that the tested material is an improper substrate for mTG. The use of soluble collagen would have been beneficial for that purpose. Nevertheless, an increase in tensile strength was detected demonstrating a significant effect on material characteristics, even though native collagen type I is not a good substrate for mTG regarding the installation of ε-(γ-glutamyl)-lysine bonds under the tested conditions [18]. To address the question whether mTG could be removed by numerous washing steps, a Western blot analysis of the collagen materials was performed, revealing that mTG was still present in mTG-treated collagen material. The Western blot analysis further showed that mTG was not covalently bound to the collagen material and that it was not degraded by *Clostridium histolyticum* collagenase type H being still accessible for further analysis. To determine the amount of mTG that can be removed via centrifugation and washing, an enzyme-linked immunosorbent assay (ELISA) was developed to quantify mTG in the washing solutions. ELISA-analysis showed that mTG could only be partially removed by centrifugation and washing. It was shown by the direct quantification after degradation by collagenase that around 40% of the initially applied enzyme remained in the mTG-treated collagen materials. To further reduce mTG-residuals, dialysis procedures at acidic or alkaline pH and incubation with different additives were performed. Western blot analysis showed that especially guanidinium chloride treatment reduced the mTG content. The dialysis procedure was superior to all other treatments and enabled to quantitatively remove mTG. However, this method comprises the lack of amide bond hydrolysis leading to decreased tensile strength. These findings strongly suggest that future studies using transglutaminase for the "crosslinking" treatment of collagen should address potential residuals of mTG and investigate, whether observed effects are due to mTG-induced changes of the collagen structure or due to remaining transglutaminase itself. If mTG cannot be quantitatively removed, the question of potential immunogenicity of mTG needs to be addressed in future studies.

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