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Affinity of Microbial Transglutaminase to α_{s1}-, β-, and Acid Casein under Atmospheric and High Pressure Conditions

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Kinetics for the reaction of microbial transglutaminase (MTG) with individual caseins in a TRIS-acetate buffer at pH 6.0 was evaluated under atmospheric pressure (0.1 MPa) and high pressure (400 MPa) at 40 °C. The reaction was monitored under the following limitations: The kinetics from the initial velocities was obtained from nonprogressive enzymatic reactions assuming that the individual catalytic constants of reactive glutamine residues are represented by the reaction between MTG and casein monomers. Enzyme reaction kinetics carried out at 0.1 MPa at 40 °C showed Henri−Michaelis−Menten behavior with maximal velocities of $2.7 \pm 0.02 \times 10^{-3}$, $0.8 \pm 0.01 \times 10^{-3}$, and $1.3 \pm 0.30 \times 10^{-3}$ mmol/L·min and K_m values of $59 \pm 2 \times 10^{-3}$, $64 \pm 3 \times 10^{-3}$, and $50 \pm 2 \times 10^{-3}$ mmol/L for β-, α_{s1}-, and acid casein, respectively. Enzyme reaction kinetics of β-casein carried out at 400 MPa and 40 °C also showed a Henri−Michaelis−Menten behavior with a similar maximal velocity of $2.5 \pm 0.33 \times 10^{-3}$ mmol/L·min, but, comparable to a competitive inhibition, the K_m value increased to $144 \pm 34 \times 10^{-3}$ mmol/L. The reaction of MTG with α_{s1}-casein under high pressure did not fit in to Henri−Michaelis−Menten kinetics, indicating the complex influence of pressure on protein−enzyme interactions.

KEYWORDS: High hydrostatic pressure; microbial transglutaminase; α_{s1}-casein; β-casein; enzyme reaction

INTRODUCTION

Transglutaminases are enzymes that catalyze acyl-transfer reactions introducing covalent cross-links between glutamine and lysine amino acid residues of proteins (*1*). Since microbial transglutaminase is commercially available, its capacity to form ε-(γ-glutamyl) lysine isopeptide cross-links has been applied as a tool for improving functional properties of food proteins. For example, in milk products, it is used to improve physical properties such as texture in yoghurt and ice cream by increasing the breaking strength in low and full fat yoghurt with the benefit of decreasing the necessary concentration of stabilizers and solid nonfat materials (*2*).

Regarding activity and selectivity, it must be noted that there are, of course, differences between transglutaminases from different sources. For example, referring to the incorporation of lysine into food proteins, MTG shows a higher rate of product formation than guinea pig liver transglutaminase (*3*). Above that, different reaction sites could be elucidated at purified α-lactalbumin as substrate. Lee et al. (*4*) showed from cross-linked proteins that guinea pig liver transglutaminase is able to derivatize Lys16, Lys93, and Lys 122, while MTG mainly reacts on Lys5. On casein, only data for guinea pig liver transglutaminase are available. Christensen et al. (*5*) reported successful protein labeling with [^{14}C]-putrescine at Gln13, Gln108, Gln130, and Gln140 for α_{s1}-casein; at Gln179, Gln169, Gln185, and Gln 187 for α_{s2}-casein; at Gln54, Gln56, Gln72, Gln79, and Gln182 for β-casein; and at Gln29, Gln45, Gln114, and Gln163 for κ-casein.

As MTG can be produced from fermentation and hence is of industrial availability, its behavior under conditions that are relevant for food processing was a subject of several studies. Its structural changes under the influence of heat and pressure have been investigated (*6*), and it can be found that the salt content of the substrate solution is influencing heat stability (*7*). In milk systems, several investigations have demonstrated that caseins are good substrates of MTG (*8*−*10*) because they contain numerous glutamine and lysine residues. Especially for casein, the firmness of acid gels in connection with isopeptide formation via MTG has been examined, and optimal parameters for this purpose could be proposed (*11*).

Interestingly, MTG features a certain pressure stability, and its denaturation has been evaluated kinetically (*12*, *13*) using the hydroxamate method as a standard (*14*). Depending on the time scale, even at 600 MPa pressure a residual MTG activity of about 60% compared to atmospheric pressure could be found. Through this property also substrates such as β-lactoglobulin become accessible, and copolymers with casein, which were regarded to be the cause of higher gel firmness and cannot be found under atmospheric pressure, were formed (*15*). Using triglycine as labeling reagent with MTG, Partschefeld et al. (*16*) revealed seven reactive glutamine residues on β-lactoglobulin, when the reaction is carried out with 20 U MTG/g protein: Gln (72, 79,

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or 89), Gln (117, 123, 141, 146, 160, or 167), Gln182, Gln (188, 194, or 195), Gln54, Gln56, and Gln184.

The selectivity and activity of transglutaminases toward caseins as substrates have been studied qualitatively and quantitatively. With cow's and ewe's milk, it could be demonstrated that κ-casein and β-casein are more susceptible to MTG than α-casein (*10*, *17*). Kinetic data have been evaluated with human placental factor XIIIa (*8*). This enzyme was tested after purification on its cross-linking potential with different distinguished casein fractions and with whole casein, and a comparison of the measured k_{cat} and k_{cat}/K_m values is discussed along with the structural properties of the individual proteins. Similar information that characterizes the selectivity and activity of MTG is currently not available. As caseins are the main target of MTG during the processing of milk products, our objective was to investigate the affinity of MTG toward individual purified caseins and provide basic kinetic information. In addition, at β-casein we wanted to elucidate the possible reactive glutamine residues that are affected by MTG. To simplify the interpretation, all measurements of the cross-linking extent were carried out under denaturating conditions; therefore, monomeric (i.e., not covalently linked) casein represents the basis, and kinetics was calculated solely on the Henri−Michaelis−Menten law. As could be shown recently that MTG cross-linking on milk protein micelles does have a protective effect on micelle stability (*18*, *19*), we also hope to deliver reference data for the investigation of MTG activity on micellized casein. By including high pressure, our investigation shall provide basic information for the evaluation of this technique with respect to milk protein processing.

MATERIALS AND METHODS

Materials. Microbial transglutaminase active MP isolated from *Streptoverticillium mobaraense* was supplied by Ajinomoto Co. Inc. (Hamburg, Germany). *N*ε-(γ-glutamyl)-L-lysine, Nα-CBZ-glutaminyl-glycine, L-glutamic acid γ-monohydroxamate, and the glutathione reduced form were purchased from Sigma (Steinheim, Germany). Ferric chloride was from Grussing (Filsum, Germany), and tris(hydroxymethyl)-aminomethane was from Merck, (Darmstadt, Germany). All other chemicals were of the highest purity available.

Determination of Enzyme Activity. Measurement of microbial transglutaminase activity was performed as described in ref *14*. Briefly, enzyme sample solution (0.5 mL) was added to 1 mL substrate solution that was previously incubated at 37 °C for 10 min. Immediately after enzyme sample solution addition, the sample was vortex mixed and further incubated at 37 °C for 10 min. Ferric chloridetrichloroacetic acid reagent (1.5 mL) was added and vigorously mixed to stop the reaction. To separate any insoluble material, the sample was centrifuged at 1500*g* for 10 min. The supernatant was measured after 20 min at 525 nm using an Ultrospec 1000 spectrophotometer (Pharmacia Biotech, Freiburg, Germany). One enzyme unit was defined as the amount of enzyme that catalyzes the formation of 0.5 μmol of hydroxamate per min in the test.

The substrate solution for microbial transglutaminase from *Streptoverticillium mobaraense* consisted of 0.1 mol/L hydroxylamine, 10 mmol/L glutathione, 30 mmol/L Nα-CBZ-Gln-Lys, and 0.2 mol/L TRIS-acetate buffer at pH 6.0.

To prepare the ferric chloride-trichloroacetic acid reagent, equal volumes of 5% (w/v) ferric chloride solution in 0.1 mol/L HCl, 12% (w/v) trichloroacetic acid, and 12% (v/v) HCl were mixed using a magnetic stirrer until a clear and transparent yellow solution was obtained.

Isolation of Individual Caseins. Acid casein was extracted by isoelectric precipitation from raw milk obtained from Milchviehanlage Grosserkmannsdorf, Saxonia, Germany using the method of Aschaffenburg (*20*). The composition of the acid casein was: 52% α_{s1}- and α_{s2}-casein, 11% κ-casein, 32% β-casein, and 5% of low molecular weight proteins (e.g., γ-casein). Isolation of α_{s1}- and β-casein was carried out by ion exchange chromatography. The procedure was performed at 20 °C using a Biological LP system with Source 30 Q ion exchange media (Pharmacia-LKB, Freiburg, Germany) packed in a 16 mm/10 cm Pharmacia column to form a bed volume of 20 mL at a flow rate of 10 mL/min using the elution buffer 0.02 mol/L imidazole (pH 7.0) containing 3.3 mol/L urea and 0.05% thioglycerine with a concentration gradient of 0.5 mol/L NaCl. Extinction of the column eluate was measured at 280 nm and collected in 10 mL volumes using a fraction collector. The different fractions were collected and dialyzed against water at 6 °C during 3 days using a dialysis membrane type 27 (Biomol Feinchemikalien, Hamburg, Germany). After desalting, the isolated fractions were freeze-dried and stored at −18 °C. Purity and characteristics of the isolated casein was analyzed by polyacrylamide gel electrophoresis following the procedure of Lane (*21*). According to this, in the β-casein fractions no other proteins could be detected and the α_{s1}-casein showed no proteins in the κ-casein region, and the residual α_{s2}-casein was less than 2% as calculated with densitometry.

Modification of β-Casein at Ambient and High Pressure. MTG-catalyzed modification of β-casein was carried out in 5 mM Tris-acetate buffer (pH 6.0), containing β-casein (1 mg/ml), MTG (4 U/g protein,) and triglycine (50 mM). The enzymatic reaction was carried out at 40 °C for 60 min at atmospheric or high hydrostatic pressure (400 MPa). For pressure treatment, the sample was poured in polypropylene tubes and placed in the preheated pressure vessels of the hydrostatic pressure unit. High-pressure levels were generated on a high-pressure plant (Bernd Dieckers GmbH, Willich, Germany) using a water/ethyleneglycol mixture for pressure transduction. The pressure was built up at a rate of 300 MPa/min, and the decompression time was less than 15 s. The nonpressure treated sample was poured into polypropylene tubes and placed into a temperated sand bath. Immediately after enzymatic treatment at atmospheric or high hydrostatic pressure, MTG was inactivated by heating up the sample to 85 °C for 2 min. Afterward, the protein solution was dialyzed against water and freeze-dried. To access the reactive glutamine residues, peptide mapping was performed according to ref *16*.

Association of Casein. Association of casein was analyzed as described by Sood and Slaterry (*22*). Particle size distribution of α_{s1}-casein solutions in a concentration of 21 to 212 μmol/L (0.5 to 5 mg/mL) in a 0.2 mol/L TRIS-acetate buffer, pH 6.0, at 40 °C and 0.1 and 400 MPa was measured with laser light scattering (ALV/DLS/SLS-5000 compact Ganiometer model with ALV/CGS-5000 system using an ALV-5000/EPPP Multiple Tau Digital Correlator and an ALV-Correlator software; ALV-Laser Vertriebsgesellschaft mbH Langen, Germany).

Reaction Kinetics. Reaction mixtures (5 mL) containing casein concentrations from 5.2 to 125 μmol/L (0.125 to 3 mg/mL) and an enzyme activity of 10 U MTG/L, which corresponds to an enzyme concentration of 0.02 μmol/L as calculated from protein contents (1 g/100 g) and activity measurements (120 U/g) of the supplied powder, in 0.2 M TRIS-acetate buffer at pH 6.0 at 6 °C were applied to 2 mL polypropylene tubes without trapping air bubbles and placed into preheated pressure vessels of the hydrostatic pressure plant. The samples were treated at 0.1 and 400 MPa at 40 °C for 0, 5, 10, 15, and 20 min. The pressure was built up at the rate of 300 MPa/min, and the decompression time was less than 30 s. To stop the reaction, MTG−casein solutions were heated up to 80 °C for 2 min. The effectiveness of inactivation was tested on enzyme solutions with and without substrate addition (data not shown). Calibration curves were recorded with monomers of the different caseins in a range from 5.2 to 125 μmol/L (0.125 to 3 mg/mL), incubated with thermally inactivated MTG (10 U/L) under the conditions mentioned above. The concentration units expressed originally in mg/mL were converted to mmol/mL considering the molecular weight of 23 980 g/mol for β-casein, 23 610 g/mol for α_{s1}-casein, and 22 000 g/mol as an average of the molecular weight of the major proteins contained in whole casein (*23*).

Reaction kinetics of MTG with individual caseins was analyzed by monitoring casein oligomerization under reducing and denaturing conditions using gel permeation chromatography. After high pressure treatment, 0.54 g of urea, 8 mg of 1,4-dithiothreitol, and 1 mg of 3-[(3-cholamidopropyl)dimethylammonium]-1-propansesulfonate were dissolved in 1.5 mL volumes of each MTG-casein sample and incubated at 4 °C for 20 h, then filtered through a 0.45 μm membrane filter before

injecting 500 μL portions to a gel permeation chromatography column. Gel permeation chromatographic analysis was performed at 25 °C with a Knauer WellChrom HPLC system (Knauer, Berlin, Germany) using a Superdex 200 HR 10/30 column (Pharmacia, Freiburg, Germany) at a flow rate of 0.5 mL/min using 0.1 mol/L sodium phosphate buffer (pH 6.8) containing 6 mol/L urea, 0.1 mmol/L sodium chloride, and 0.1% 3-[(3-cholamidopropyl)dimethylammonium]-1-propansesulfonate as eluent. Detection was performed at 280 nm, and the chromatograms were analyzed by curve-fitting software EuroChrom 2000 (Knauer, Berlin, Germany).

Curve Fitting. The mathematical adjustment of the plots of reaction velocities as a function of casein concentration, as well as the calculation of V_{max} and K_m values were performed by a linear regression using Hanes diagram and by a nonlinear regression from experimental data using Sigma plot software (Systat GmbH, Germany).

Prerequisites for the Interpretation of the Kinetic Data. Kinetic analysis of the enzyme reaction with protein substrates is more complex than the reaction with peptide substrates containing only a single functionality. The proteins have multiple sites as substrates, and furthermore, the enzyme can catalyze a progressive chemical reaction with the formed products (*24*). Christensen et al. (*5*) reported that for α_{s1}-casein only 4 of 15 and for β-casein only 5 of 21 glutamine residues were found to be catalytically competent for an acyl-transfer reaction catalyzed by TG. If it is considered that these 4 and 5 glutamine residues of α_{s1}-casein or β-casein act as substrates in a nonprogressive enzymatic reaction, this would result in several microscopic kinetic parameters. MTG would bind to each casein monomer in *n* different ways according to *n* available glutamine residues, to produce *n* catalytic complexes that result in *n* unique final products. That means that the reaction of MTG with casein reflects a complex mechanism in which the reaction of each of the glutamine residues is governed by individual constants $(k_{cat}/K_m)_n$. As measuring these individual constants goes beyond the scope of this article, the following assumption had to be made in the reaction model.

In the present work, the following conditions were established to analyze the kinetic parameters of MTG using protein substrates: (1) The initial reaction velocity was regarded to derive from a nonprogressive enzymatic reaction of the polymeric substrate. (2) The substrate concentration exceeded MTG concentration by the ratio of 2500 to 6000. (3) Because of the complex kinetics, it was attempted to interpret the results of the experiments from the reaction of MTG with the individual caseins at the stage of a single MTG−monomeric casein complex formation. The analytical data were interpreted considering that the enzyme-catalyzed reaction involves the conversion of a single substrate into a product. Where *M* is the monomer of individual caseins containing a number of *n* glutamine residues, *A* is the enzyme MTG, and *P* is the polymerization product (eq 1).

$$A + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A:M \xrightarrow{k_{cat}} p \qquad (1)$$

Thus a reaction kinetic according to Henri−Michaelis−Menten was regarded to be valid within the regarded time period (15 min) (*24*−*26*).

Evaluation of the Kinetic Parameters. The kinetic parameters V_{max}, K_m, and k_{cat}, which define the behavior of the enzyme MTG as a function of the casein concentration as substrate, were determined using mathematical adjustments based on the following considerations: The value of K_m and k_{cat} represents the sum of $\Sigma_{i=1}^{n} K_{m_i}$ and $\Sigma_{i=1}^{n} k_{cat_i}$ of the individual kinetics of the *n* numbers of glutamine residues in the monomeric casein. In the same way, the $\Sigma_{i=1}^{n} k_{cat_i}/K_{m_i}$ ratio symbolizes the turnover of the Henri−Michaelis−Menten complex between MTG and monomeric casein.

RESULTS AND DISCUSSION

Monitoring of the Enzymatic Reaction. When β-casein (31 μmol/L; 0.75 mg/mL) was incubated with MTG (0.010 U/mL) at 0.1 and 400 MPa at 40 °C for 0, 5, 10, and 15 min, it was possible to monitor the reaction by measuring the decrease of monomeric casein, as irreversible covalently cross-linked oligomers were formed with increasing time. A higher decline in the concentration of monomeric casein was observed during treatment at atmospheric pressure compared to high pressure (**Figure 1**). Comparing the oligomer patterns of the different samples revealed a more pronounced formation of higher oligomers at atmospheric pressure than at 400 MPa. A qualitatively similar monomer decrease was observed for 32 μmol/L (0.75 mg/mL) α_{s1}-casein after treatment with 0.010 U/mL MTG in the same system and conditions. However, the reaction between MTG and α_{s1}-casein as substrate leads, compared to β-casein, resulted in more of an increase of the peak in the dimer region of the chromatogram. Comparing the gel chromatograms of α_{s1}-casein, similar to β-casein, more of the monomer is converted to dimers or oligomers at atmospheric pressure than at high pressure (**Figure 2**).

Reaction progress curves of α_{s1}-, β-, and acid casein were plotted from the remaining monomeric casein [*M*] versus time. The starting concentration ranged from 5.2 to 125 μmol/L (0.125 to 3 mg/mL), and reaction times were 0, 5, 10, and 15 min. The initial velocity (v_o) for each starting concentration was calculated as the slope of these linear plots (data not shown).

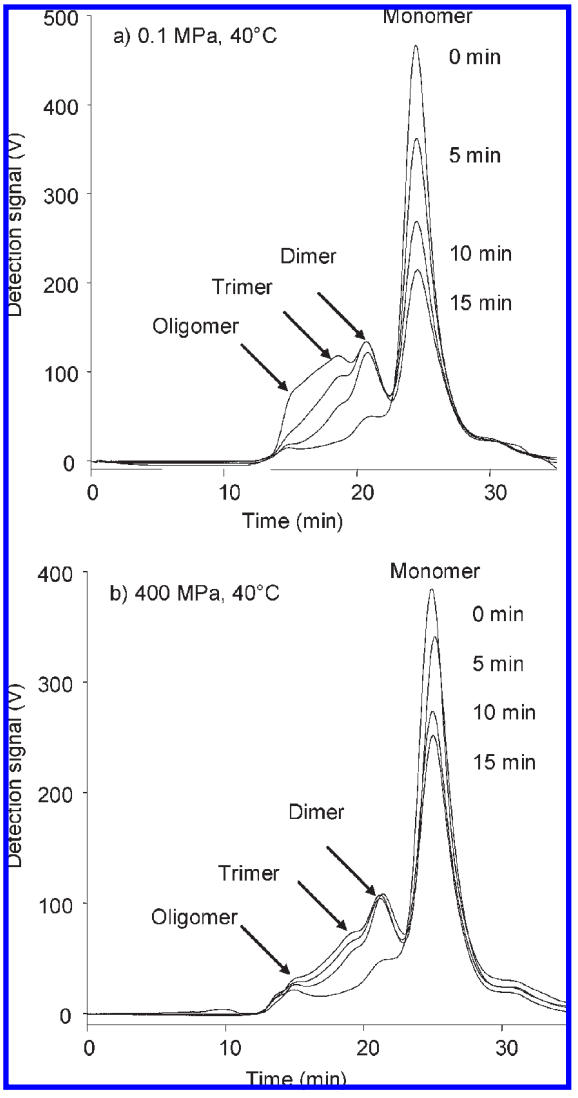

Figure 1. Gel permeation chromatograms of β-casein samples (31 μmol/L; 0.75 mg/mL) incubated with MTG (0.010 U/mL) in a 0.2 mol/L TRIS-acetate buffer at pH 6.0 at 40 °C for 0 to 15 min. (**a**) Reaction at 0.1 MPa. (**b**) Reaction at 400 MPa. UV-detection: λ = 280 nm.

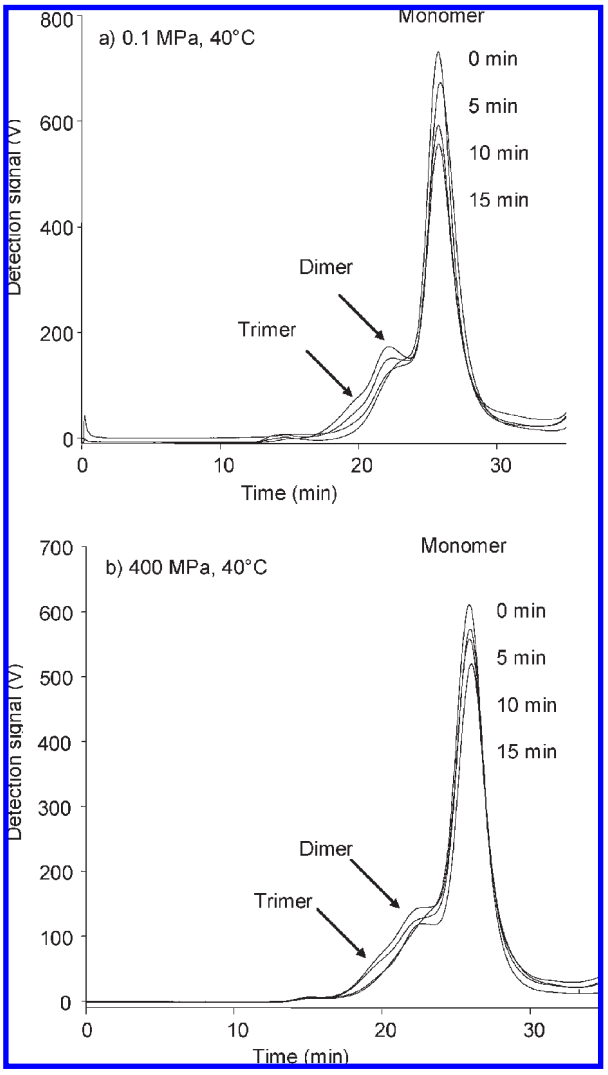

Figure 2. Gel permeation chromatograms of α_{s1}-casein samples (32 μmol/L; 0.75 mg/mL) incubated with MTG (0.010 U/mL) in a 0.2 mol/L TRIS-acetate buffer at pH 6.0 at 40 °C for 0 to 15 min. (**a**) Reaction at 0.1 MPa. (**b**) Reaction at 400 MPa. UV-detection: λ = 280 nm.

The reaction velocity was measured during the early phase of the reaction; for this reason, $v = v_0$ was considered to be valid. The effect of individual casein concentrations on the reaction velocities was analyzed by plotting reaction velocities $-\Delta[M]/\Delta t$ from β- and α_{s1}- and acid casein, as a function of substrate concentrations (**Figure 3**). Reaction velocities at 0.1 MPa and 40 °C for β-, α_{s1}-, and acid caseins at low substrate concentrations displayed first-order behavior, and at high substrate concentration, the velocity switches to zero-order behavior, implying no dependence on substrate concentration. The obtained models resemble a hyperbolic behavior, which could be interpreted according to the Henri−Michaelis−Menten law (*24*, *26*). In contrast, a hyperbolic model was not obtained for the reaction of α_{s1}-casein under 400 MPa and 40 °C. The experimental data were adjusted by nonlinear regression (**Table 1**) of our experimental data with the Henri−Michaelis−Menten equation. In addition, to confirm the kinetic parameters, linear regressions using Hanes diagrams were also plotted (**Figures 4** and **5**). The adjusted values from both procedures were not significantly different ($P > 0.05$).

Evaluation of Reaction Kinetics at Atmospheric Pressure. The resulting kinetic parameters from adjusted hyperbolic curves of MTG reactions with individual caseins under atmospheric

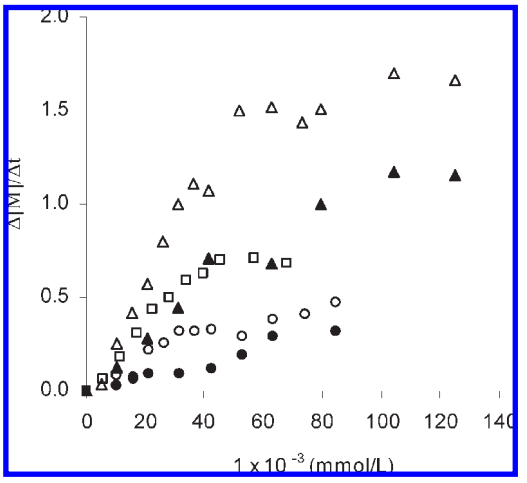

Figure 3. Reaction of β-casein with MTG at 40 °C at 0.1 MPa (Δ) and 400 MPa (▲). Reaction of α_{s1}-casein with MTG at 40 °C at 0.1 MPa (○) and 400 MPa (●). Reaction of acid casein with MTG at 40 °C at 0.1 MPa (□).

conditions are exposed in **Table 1**. The statistical evaluation of the values reported in **Table 1** show that the mathematical model fits were significant ($P < 0.05$) for experiments with α_{s1}-, β-, and acid casein carried out at 0.1 MPa and 40 °C. This indicates that the adjusted values of V_{max} and K_m are acceptable. Maximal velocities of $2.66 \pm 0.02 \times 10^{-3}$, $0.79 \pm 0.01 \times 10^{-3}$, and $1.32 \pm 0.3 \times 10^{-3}$ mmol/L·min were obtained from β-, α_{s1}-, and acid casein, respectively.

The first step in the Henri−Michaelis−Menten reaction model describes the binding of the substrate to the enzyme and the constant K_m corresponds to the dissociation constant of the enzyme−substrate complex, as long as the product formation is very slow compared to the dissociation process of this complex (*24*, *26*). The K_m values of $59 \pm 2 \times 10^{-3}$ for β-casein, $64 \pm 3 \times 10^{-3}$ for α_{s1}-casein, and $50 \pm 2 \times 10^{-3}$ mmol/L for acid casein suggest that although MTG achieved maximal velocity with β-casein, it has the highest affinity to acid casein followed by β-casein and finally α_{s1}-casein. The latter exhibited only 45% affinity compared to acid casein. The high MTG affinity to acid casein could be explained by the contribution of the potential reactive glutamine residues of κ-casein (*5*). The Gln114 and Gln113 are located in the high soluble regions of κ-caseins, which are exposed to solvent. These regions surround the casein micelles, and with regard to this, the affinity of MTG toward individual caseins at 0.1 MPa at 40 °C followed the order acid casein > β-casein > α_{s1}-casein. These findings are well in line with the affinity orders that can be found in the literature, although without supplied K_m values (*10*, *11*). Comparable kinetic parameters can be found on human placental factor $XIII_a$ (*8*). The published K_m values of 100×10^{-3}, 34×10^{-3}, 50×10^{-3}, and 130×10^{-3} mmol/L, for α_s-, β-, κ-, and whole casein, respectively, show the same order of magnitude and make clear that MTG has an equivalent affinity to the named substrates, with the benefit of not requiring activators. The differences in acid and whole casein cannot be retraced but may be attributable to the different compositions of these substrates.

The second reaction step describes the catalytic rate or the rate of product formation and is referred to as the turnover number k_{cat}, which is defined as the maximal number of product per active site per time unit ($k_{cat} = V_{max}/[E_T]$). The obtained k_{cat} values were 133 ± 2 min^{-1} for β-casein, 40 ± 1 min^{-1} for α_{s1}-casein, and 65 ± 3 min^{-1} for acid casein at 0.1 MPa. The value of the ratio k_{cat}/K_m is generally considered to be a

Table 1. Kinetic Values of the Reaction between MTG and Individual Caseins from Adjusted Data Using Sigma Plot Software[a]

	β-Casein		α_{s1}-Casein		Acid casein	
nonlinear regression	coefficient	$P > F$	coefficient	$P > F$	coefficient	$P > F$
V_{max} (1×10^{-3} mmol/L·min)						
0.1 MPa, 40 °C	2.66	0.0001	0.79	0.0011	1.30	0.0004
400 MPa, 40 °C	2.56	0.0048	no fit	0.9900	n.d	
K_m (1×10^{-3} mmol/L)						
0.1 MPa, 40 °C	58.90	0.0006	64.38	0.04	49.90	0.0117
400 MPa, 40 °C	144.10	0.0367	no fit		n.d	

[a] Values given are the mean of two measurements. Coefficients significant at 95% confidence level. Values of $P > F$ less than 0.05 indicate that model terms are significant. Values greater than 0.10 indicate that the model terms are not significant. n.d not determined.

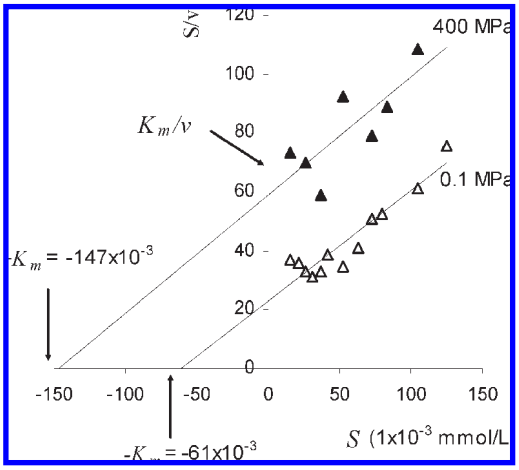

Figure 4. Hanes diagram. Reaction of β-casein with MTG at 40 °C at 0.1 MPa; linear regression $y = 0.37x + 22.86$; V_{max} = 2.65 μmol/min; K_m = 61 μmol (Δ). Reaction of β-casein with MTG at 40 °C at 400 MPa; linear regression $y = 0.40x + 59.05$; V_{max} = 2.49 μmol/min; K_m = 147 μmol (▲).

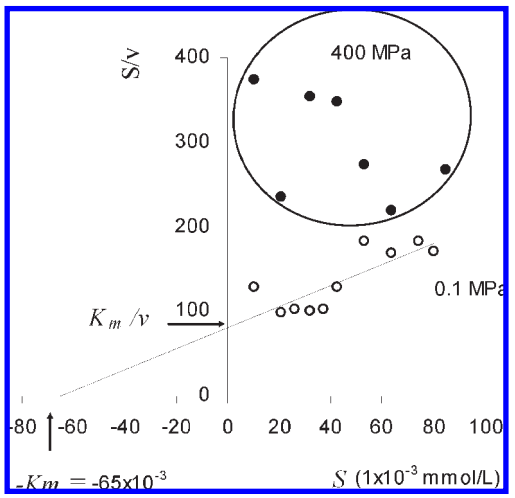

Figure 5. Hanes diagram. Reaction of α_{s1}-casein with MTG at 40 °C at 0.1 MPa; linear regression $y = 1.25x + 81.19$; V_{max} = 0.83 μmol/min; K_m = 65 μmol (○). Reaction of α_{s1}-casein with MTG at 40 °C and 400 MPa (●).

parameter of the catalytic efficiency of an enzyme (*24*). Thereby, the obtained k_{cat}/K_m values of 2258 ± 47 L/mmol·min for β-casein, 625 ± 29 L/mmol·min for α_{s1}-casein, and 1302 ± 59 L/mmol·min for acid casein indicate that at 0.1 MPa and 40 °C, MTG in a 0.2 mol/L TRIS-acetate buffer at pH 6 most efficiently modifies β-casein followed by acid- and α_{s1}-casein. The tendencies of K_m and k_{cat} values as well as the k_{cat}/K_m ratio are in agreement with Traoré and Meunier (*8*), who analyzed the transfer reaction of Ca^{2+} activated TG from human placental factor $XIII_a$ with individual caseins at 37 °C by the formation of ammonia. The kinetic reactions of individual caseins followed the Michaelis−Menten's law. k_{cat} values of 97, 364, and 300 min^{-1} for α_s-, β-, and whole casein were reported, respectively. This demonstrates that β- and whole casein are more susceptible to cross-linking by factor $XIII_a$ than α_{s1}-casein. The tendency of substrate specificity is also similar, and k_{cat}/K_m ratios of 966 L/mmol·min for α_s-casein, 10694 L/mmol·min for β-casein, and 2308 L/mmol·min for whole casein were reported.

The different affinities of MTG to individual caseins can be discussed under several considerations. For example, the affinity of the enzyme to casein depends also on the type of transglutaminase. Studies by Gorman and Folk (*27*) reported that distinct transglutaminases may recognize the same protein as substrate, but often with different affinity or with specificity for different glutamine residues. A further subject of consideration is the amino acid sequence of caseins. Several authors (*28*, *29*) explain that the amino acid sequence of proteins determines the reactivity of the glutamine residue, but there are yet no rules given to decide when a glutamine is potentially reactive. Traoré and Meunier (*8*) reported that the relationship of glutamine/lysine residues of individual caseins also correlates with casein reactivity. Another very important consideration is the localization of potential glutamine residues on the casein molecule. The primary structure of the casein as well as its conformational structure determines whether a glutamine residue can be reactive. Currently, there is no really clear information about this subject in literature. Most of the potential glutamine residues of β-casein proposed by Christensen et al. (*5*) are located in hydrophobic regions, which contradicts the theory of Aeschlimann et al. (*29*), who stated that the reactive glutamines are located in surface regions exposed to the solvent or flexible extension of the proteins, as patches of hydrophilic residues are often found in their vicinity. As the substrates or enzymes were different from ours, we labeled β-casein at ambient and high pressure with triglycine. With liquid chromatography/mass spectrometry, we elucidated Gln72 (peak 5), Gln79 (peak 4), one residue between Gln117 and Gln167 (peak 6), Gln175 (peak 2), Gln182 (peak 1), and Gln188 (peak 3) to be potent for a cross-linking reaction (**Figure 6**) and could not observe differences with the application of pressure. The residues located between Gln117 and Gln167, most probably Gln 117 or 123, as well as Gln 175 make the difference between MTG and guinea pig liver transglutaminase and find themselves in hydrophilic regions of the protein.

The lower affinity of MTG to the monomer of α_{s1}-casein compared to β-casein may be due to the fact that the former contains fewer reactive glutamine residues and furthermore

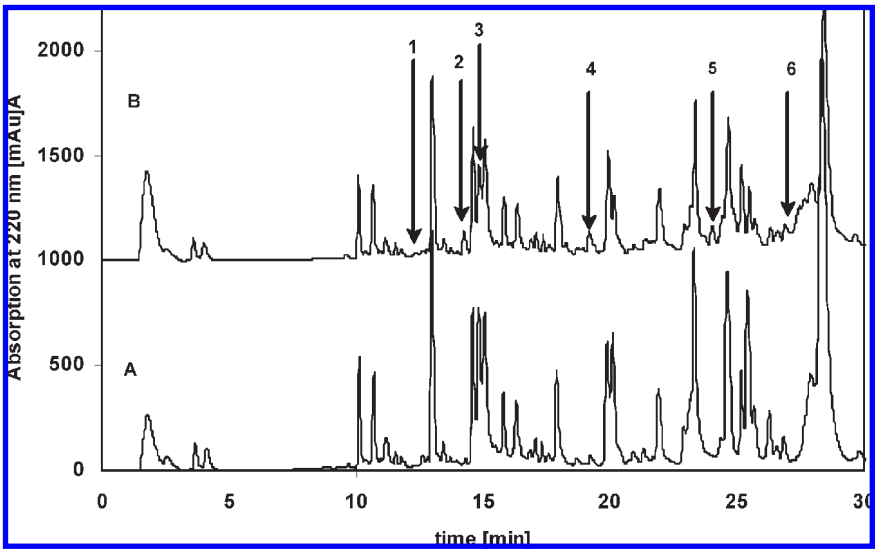

Figure 6. Chromatograms of tryptic digestions of β-casein (**A**) and β-casein labeled with triglycine and MTG (**B**) at atmospheric pressure, 40 °C, and pH 6.0.

that the hydrophobic and charged residues are not uniformly distributed; therefore, glutamine residues are masked when the protein tends to self-associate, e.g., in 0.2 mol/L TRIS-acetate buffer at pH 6. Among the potential reactive glutamines of α_{s1}-casein proposed by Christensen et al. (*5*), Gln13 is located in the hydrophilic region, but Gln108, Gln130, and Gln140 are located among the residues 100 to 199, which have a high degree of hydrophobicity (*29*) and are probably responsible, in part, for the pronounced self-association of the α_{s1}-monomer association in aqueous solutions.

Evaluation of Reaction Kinetics at High Pressure. The resulting parameters V_{max} and K_m from the adjustment of the kinetic data of MTG reactions with individual caseins under high pressure conditions (400 MPa and 40 °C) are summarized in **Table 1**. The kinetically adjusted values pointed out different reaction orders compared to those at 0.1 MPa at 40 °C. The coefficient of the estimated hyperbola was significant ($P < 0.05$) for β-casein but not significant ($P > 0.05$) for α_{s1}-casein, which demonstrated that the reaction of MTG with α_{s1}-casein under high pressure had non-Henri−Michaelis−Menten kinetics. For β-casein, a k_{cat} value of 128 ± 16 min^{-1} and a k_{cat}/K_m ratio of 888 ± 41 L/mmol·min at 400 MPa could be found.

The non-Michaelian kinetic reaction behavior of α_{s1}-casein under high pressure could be explained by the low solubility of α_{s1}-casein. Limitations of α_{s1}-casein solubility in 0.2 mol/L Tris-acetate buffer at pH 6 could cause errors in calculating kinetic parameters. The experiments were limited to 80 μmol/L α_{s1}-casein (1.9 mg /mL) with possible effects on the estimation of V_{max} and K_m. In addition, self-association of α_{s1}-casein occurs with increasing concentration. In our study, analyzed by dynamic light scattering, the self-association behavior of α_{s1}-casein in a 0.2 mol/L Tris-acetate buffer, pH 6.0, at 0.1 MPa and 40 °C showed that under these conditions, the molecules occur as monomers with a size of 10 nm radius and also as associates with radii from 36 to 119 nm. Increasing the α_{s1}-casein concentration to 169 μmol/L (4 mg/mL) and 212 μmol/L (5 mg/mL) caused increasing agglomerate sizes from 22 to 168 nm and from 23 to 215 nm, respectively. A solution of α_{s1}-casein in a concentration of 127 μmol/L (3 mg/mL) in the same buffer treated at 400 MPa and 40 °C for 15 min also shows higher agglomerate size (about 22 to 650 nm radius) compared to that of 0.1 MPa and 40 °C samples. Although, López Faldino et al. (*30*) assumed that the pressure-induced dissociation of the casein micelle occurs by weakening of hydrophobic and electrostatic interactions between the micelle subunits and the solubilization of colloidal calcium phosphate, at pressure levels around 250−300 MPa, the higher compressibility of the free water compared with that of bound water could effectively enhance association of casein molecules through hydrophobic bonds, leading to hydrophobic interactions of high protein density (*31*). Thus, it cannot be excluded that even in the regarded range up to 80 μmol/L increasing amounts of associated α_{s1}-casein were obtained by increasing the protein concentration and that high pressure treatment also favored the formation of associates. Under these conditions, Gln108, Gln130, and Gln140 of α_{s1}-casein could be hidden in the associated hydrophobic region and are therefore not accessible to MTG.

The analyzed kinetic parameters showed that the affinity of MTG to β- and α_{s1}-casein was higher at atmospheric pressure than at high pressure. The k_{cat} values of β-casein at 0.1 MPa (133 ± 2 min^{-1}) and at 400 MPa (128 ± 16 min^{-1}) were not significantly different ($P > 0.05$) and suggest that the number of formed $N\varepsilon$-(γ-glutamyl) lysine isopeptides per active site per min in the reaction of MTG with β-casein at atmospheric and high pressure is equivalent. This is in agreement with our labeling experiment, which showed no change in the numbers of reactive glutamine residues of β-casein treated under similar conditions. However, the k_{cat}/K_m ratio of the reaction of MTG with β-casein carried out at 0.1 MPa and 40 °C was 2258 L/mmol·min, whereas the k_{cat}/K_m value decreased to 888 L/mmol·min when the same reaction was performed at 400 MPa and 40 °C. This behavior showed that the reaction efficiency of MTG with β-casein falls drastically under high pressure treatment. Furthermore, the k_{cat}/K_m ratio of the reaction from α_{s1}-casein with MTG performed at 400 MPa and 40 °C could not be calculated because this reaction shows a non-Michaelian kinetic behavior.

The affinity of MTG to individual caseins under atmospheric and high pressure treatment is different from the affinity of MTG to other substrates. For example, Lee and Park (*13*) reported a kinetic study from the reaction of MTG with (CBZ)-L-glutamylglycine at 25 °C in a pressure range from 0.1 to 600 MPa. A K_m value around 25 mM and a V_{max} of 928 U of the reaction at 0.1 MPa and 25 °C were reported. Increasing pressure gave similar K_m values of 29 and 27 and 30 mM and maximal velocities of 1042, 858, and 756 units for 400, 500, and 600 MPa, respectively. The kinetic data from this system

indicate that the affinities of the MTG to (CBZ)-L-glutamylglycine remained similar and that only the V_{max} of pressurized MTG was changed. In contrast, for MTG-β-casein systems we observed that the obtained V_{max} remained between $2.7 \pm 0.03 \times 10^{-3}$ and $2.6 \pm 0.33 \times 10^{-3}$ mmol/L·min after treatment at 40 °C and 0.1 and 400 MPa, respectively, whereas the K_m increased from $59 \pm 2 \times 10^{-3}$ to $144 \pm 34 \times 10^{-3}$ mmol/L. As an explanation, it could be suggested, that the conformational structure of (CBZ)-L-glutamylglycine, a small molecule (337 g/gmol), was not drastically affected at 400 MPa and 25 °C. The single active glutamine residue of the peptide remains free to react with MTG, and for this reason, the affinity of this enzyme−substrate is similar under atmospheric and high pressure conditions. In contrast, caseins are more complex systems because they are big molecules with a high molecular mass (~22,000 to ~24,000 g/gmol). In addition, their hydrophilic and hydrophobic regions can be affected by high pressure.

The loss of affinity in the MTG−casein complex and the corresponding enzyme efficiency can be explained by the following reasons: (1) inactivation of the enzyme at high pressure. The inactivation kinetics and the influence of high hydrostatic pressure on microbial transglutaminase studies demonstrate that the enzyme activity of MTG decreased to 74% after treatment at 400 MPa and 40 °C for 15 min (*5*). (2) The constancy of potential reactive glutamine under different pressure. The analysis of peptide fragments demonstrated that the number of reactive glutamine residues of β-casein in samples treated at atmospheric and high pressure was the same. (3) Conformational changes of casein structures under high pressure treatment.

Therefore, with small molecules, such as (CBZ)-L-glutamylglycine, the influence of pressure resembles a noncompetitive inhibition with a loss in $V_{\max}$ but an almost constant K_{m}. The conformational changes of the enzyme's active site may explain this observation. With big molecules, the conformation of the substrate plays a significant role, and in the case of β-casein, it leads to a kinetic behavior that reminds one more or less of a competitive inhibition. As no new reaction sites can be found, this may indicate an enhanced susceptibility.

Pressure is also able to influence the exhibition of reactive glutamine residues to the solvent by inducing aggregation. Under pressure, different forms of aggregates are described for β-casein and α_{s1}-casein (*32*). If, as suggested, in β-casein aggregates glutamines are still well accessible to MTG, this means a local increase in substrate concentration, and with respect to $V_{\max}$, this effect possibly equals the changes at the active site and the consequences of enzyme inactivation. However, through the aggregation of α_{s1}-casein the number of reactive glutamines is virtually reduced.

In conclusion, the reaction between MTG and α_{s1}- or β-casein at atmospheric pressure (0.1 MPa) and 40 °C for 15 min induced casein polymerization, demonstrating that this enzyme accepts both caseins as substrates with a better affinity to β-casein than to α_{s1}-casein. With acid casein, the influence of the κ-casein content has to be taken into account. With respect to dairy processing, not only orders of reactivity but also kinetic data under realistic conditions are given. On the basis of these results, differences to micellized milk proteins may now be described. Finally, pressure offers an interesting feature for product innovations, and introducing kinetic data with β-casein may be helpful for further investigations on new dairy products. The results of α_{s1}-casein also show that the situation becomes more complicated with pressure and that the Henri−Michaelis−Menten law is not suitable to describe the recorded data in this case.

ABBREVIATIONS USED

MTG, microbial transglutaminase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Gln, glutamine residue.

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