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Cross-linking of Hen Egg White Lysozyme by Microbial Transglutaminase under High Hydrostatic Pressure: Localization of Reactive Amino Acid Side Chains

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After incubation of hen egg white lysozyme (HEWL) with microbial transglutaminase (mTG) under high pressure (400−600 MPa for 30 min at 40 °C), the formation of HEWL oligomers was observed via SDS electrophoresis. At atmospheric pressure, HEWL represents no substrate for mTG. Likewise, enzymatic treatment following a pretreatment with high pressure did not lead to oligomerization. Reactive amino acid side chains were identified by peptide mapping after tryptic digestion using RP-HPLC with ESI-TOF-MS. Isopeptide-containing peptide fragments were found only in HEWL samples simultaneously treated with enzyme and pressure. It was found that mTG exclusively cross-links HEWL under high pressure by formation of an isopeptide between lysine at position 1 and glutamine at position 121 in the peptide chain. Therefore, a pressure-induced partial and reversible unfolding of the protein with exposure of lysine and glutamine side chains has to occur, resulting in a site-directed oligomerization of HEWL by mTG. The enzymatic modification of HEWL by mTG under high pressure offers interesting perspectives for further functionalization reactions.

KEYWORDS: Microbial transglutaminase; lysozyme; glutamine; lysine; isopeptide; protein modification; high pressure treatment

INTRODUCTION

Microbial transglutaminase (mTG; γ-glutamyltransferase, EC 2.3.2.13) catalyzes an acyl-transfer reaction between glutamine and lysine side chains of proteins, resulting in the intra- and/or intermolecular formation of the isopeptide *N*-ε-(γ-glutamyl)lysine (*1*, *2*). As a consequence, the functional properties of proteins are modified. The field of technological applications covers pasta, bakery products, and other plant protein containing foodstuffs and dairy technology (*3*, *4*). Treatment of milk proteins with mTG leads to products with increased viscosity, improved water-binding capacity, higher gel strength, and reduced syneresis (*5*).

Several proteins, such as the globular whey proteins or collagen, are not substrates for mTG in their native state. Here, cross-linking by mTG can be achieved only following heat denaturation or after the addition of detergents and/or reducing agents such as mercaptoethanol. Thus, the use of mTG in the food industry is limited to certain proteins (*5*).

Recently, we have shown that mTG is remarkably stable under high hydrostatic pressure (*6*). This observation was traced back to the fact that the active site of the enzyme, which is surrounded by β strands, is nearly incompressible (*7*). The whey protein β-lactoglobulin can be converted to oligomers by mTG under high hydrostatic pressure in the range from 300 to 500 MPa, indicating that the simultaneous pressure/enzyme treatment may open new fields for the functionalization of food proteins (*8*, *9*).

Here we report the enzymatic cross-linking of lysozyme from hen egg white (HEWL; EC 3.2.1.17). On the basis of its antibacterial effect against Gram-positive microorganisms, lysozyme is used as a preserving agent in dairy products as well as in wine technology (*10*, *11*). With 129 amino acids, a molecular weight of 14.3 kDa, and 4 disulfide bonds, HEWL is very temperature and pressure stable (*12*, *13*). HEWL is not affected by transglutaminase under ambient pressure (*14*). Our study, therefore, followed the hypothesis that lysozyme can be cross-linked by mTG after a partial and reversible unfolding of the protein under high hydrostatic pressure via a selective modification at certain amino acid side chains. We aimed to identify possible reaction sites using peptide mapping and LC-MS.

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MATERIALS AND METHODS

Materials. Microbial transglutaminase (mTG) Activa MP (activity = 81.8 U/g; 1 U is defined as the amount of enzyme that catalyzes the formation of 1 μmol of hydroxamate from hydroxylamine and CBZ-L-glutaminylglycine within 1 min at pH 6.0 at 37 °C) was obtained from Ajinomoto (Hamburg, Germany). Activa MP is a food grade preparation, containing enzyme [protein content of 1%; no other proteins detectable (*7*)], lactose (90%), and maltodextrin (9%) according to the manufacturer. Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid, and acetic acid were purchased from VWR International (Darmstadt, Germany). 1,4-Dithiothreitol (DTT) and iodacetamide were from Biomol (Hamburg, Germany). TPCK-treated trypsin (from bovine pancreas, essentially salt-free, lyophilized powder, 10000 BAEE units/mg of protein) and HEWL (70000 U/mg) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). All chemicals were of the highest purity available.

High-Pressure Treatment. HEWL was incubated at a concentration of 20 mg/mL in 50 mM Tris-HCl buffer (pH 7.5) in the absence or presence of mTG (40 U/g HEWL) for 30 min at 40 °C at atmospheric or high hydrostatic pressure (600 MPa) as described in ref *9*. Pressure treatment

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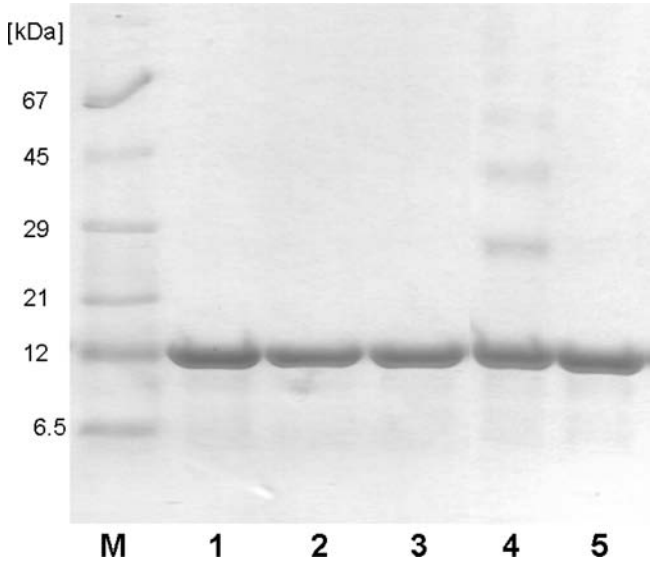

Figure 1. SDS-PAGE of HEWL samples after reduction with DTT. Lanes: 1, control (untreated); 2, treated with mTG for 30 min at 40 °C at ambient pressure; 3, treated for 30 min at 600 MPa and 40 °C; 4, treated with mTG for 30 min at 40 °C and 600 MPa; 5, treated for 30 min at 600 MPa and 40 °C, followed by incubation with mTG for 30 min at 40 °C and ambient pressure; M, standard mixture of proteins.

was performed with a high-pressure plant (Bernd Dieckers, Willich, Germany) using a mixture of water/ethylene glycol (50:50 v/v) for pressure transduction. The pressure limit of this equipment is 700 MPa. The pressure was built up at the rate of 300 MPa/min, and the decompression time was <20 s. To avoid heating of the samples by adiabatic heat generation, the temperature was kept constant using an external heating coil wrapped around the autoclaves. Immediately after enzymatic treatment, mTG was inactivated by heating the sample to 85 °C for 5 min. Afterward, samples were dialyzed at 4 °C against water, lyophilized, and stored at −20 °C until further investigations.

Polyacrylamide Gel Electrophoresis. Oligomerization of HEWL was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) after reduction of the samples using DTT according to ref *15*. The gel gradient was from $T = 4\%$ to 20%. HEWL bands were stained with Coomassie Brilliant Blue R. Semiquantitative evaluation of the electropherograms was performed by scanning band volumes using Total Lab TL120 version 2006c (Nonlinear Dynamics Ldt., Newcastle, U.K.).

Peptide Mapping. Lyophilized HEWL samples (0.1% w/v) were digested with TPCK-treated trypsin in 50 mM Tris-HCl, pH 7.5, at an enzyme/substrate ratio of 1:100 at 37 °C for 6 h. Afterward, reduction was performed with DTT (final concentration of 1%, w/v) incubated overnight at 6 °C. Tryptic peptides were analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC) with electrospray ionization−time-of-flight mass spectrometry (ESI-TOF-MS) using equipment and general conditions according to ref *9*. A Zorbax 300 Extend-C18 column (Agilent, Waldbronn, Germany) was used. Solvent A was ammonium acetate, 10 mM, pH 5.5, and solvent B was 84% acetonitrile in 10 mM ammonium acetate. The gradient was as described in ref *9*. Absorbance was measured at 220 and 280 nm. The mass spectrometer was a Mariner workstation equipped with an electrospray ion source (PerSeptive Biosystems, Framingham, MA). ESI was performed in the positive ionization mode. Operating conditions of the mass spectrometer were as described in ref *9*. Data acquisition and handling were performed using the software Data Explorer version 4.0.0.1 (Applied Biosystems, Foster City, CA). Every sample was analyzed at least in triplicate.

RESULTS AND DISCUSSION

The aim of our study was to investigate mTG-catalyzed modification of HEWL under high hydrostatic pressure. As can be seen from the SDS electropherogram shown in **Figure 1**, no cross-linking of HEWL was detectable after incubation with mTG for 30 min under ambient pressure (lane 2). Furthermore, high-pressure treatment without the addition of mTG did not lead to protein oligomerization (lane 3). The formation of oligomers with apparent masses corresponding to dimers, trimers, tetramers, and pentamers of HEWL was observed only for samples in which HEWL had been treated simultaneously with mTG and high hydrostatic pressure (lane 4). The observation that mTG treatment following a pressure treatment (lane 5) did not lead to protein oligomerization points to the fact that reversible unfolding of HEWL must occur under high pressure. In this denatured state, lysine and glutamine side chains become available as substrates for mTG, resulting in the formation of isopeptides at specific sites of the protein.

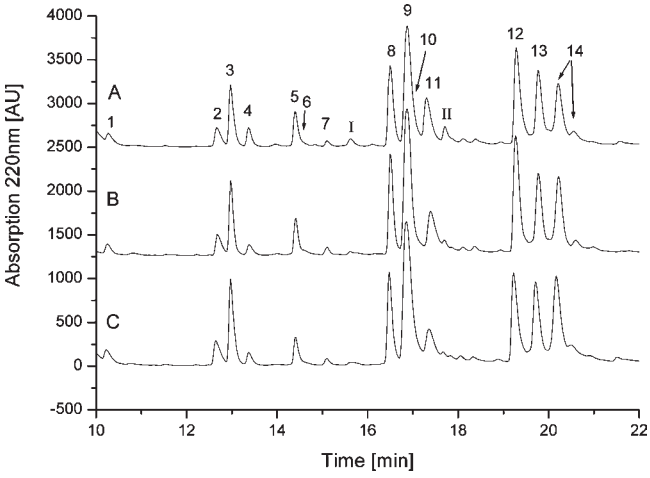

Figure 2. RP-HPLC of tryptic digests of HEWL: (**A**) untreated HEWL; (**B**) HEWL treated with mTG for 30 min at 40 °C and ambient pressure; (**C**) HEWL treated with mTG for 30 min at 40 °C and 600 MPa.

Table 1. Identification of Tryptic Peptides from Native Lysozyme by RP-HPLC with ESI-TOF-MS

peak	time (min)	*m/z* $(M+H)^+$	*m/z* $(M+2H)^{2+}$	tryptic peptide	theoretical peptide mass
1	10.3	448.3		126−129	447.23
2	12.8	606.4		1−5	605.36
3	13.0	874.5	437.7	15−21	873.41
4	13.5	776.4		117−123	775.35
5	14.5	1428.9	714.9	34−45	1427.64
6	14.7	836.5		6−13	835.39
7	15.1	992.7	496.8	6−14	991.49
8	16.5	1045.7	523.31	117−125	1044.53
9	16.9	936.5	468.7	62−68	935.37
10	17.0	1276.7	638.9	115−125	1275.64
11	17.4	1754.6	877.5	46−61	1752.83
12	19.3	1268.7	634.9	22−33	1267.60
13	19.8	1805.1	902.6	97−112	1802.89
14	20.3	1676.4	838.5	98−112	1674.79
14	20.6	1676.4	838.5	98−112	1674.79
I	15.7	497.3		unknown artifact	
II	17.75	1288.9		unknown artifact	

The reaction sites for mTG were identified using peptide mapping. Tryptic peptides of HEWL samples were analyzed via RP-HPLC with UV detection and ESI-TOF-MS. A chromatogram for a peptide map of an untreated sample of HEWL is shown in **Figure 2A**. For 14 peaks, tryptic peptides resulting from the cleavage of the peptidase at the C-terminal side of lysine and arginine residues were identified by comparing their mass spectrometric data with theoretically achievable tryptic peptides of HEWL (**Table 1**). Thus, around 80% of the peptide sequence

of HEWL (104 of 124 amino acids) was recombined, including 5 of 6 lysine residues (Lys1, Lys13, Lys33, Lys97, and Lys 116) and all 3 glutamine residues (Gln41, Gln57, and Gln121) as potential substrates for mTG.

No differences were observed between the peptide maps obtained at a detection wavelength of 220 nm for native lysozyme (**Figure 2A**) and HEWL treated with mTG for 30 min at 40 °C under ambient or high hydrostatic pressure (**Figure 2B**,**C**), indicating that the conditions used for treatment of HEWL had no significant influence on the digestibility by trypsin. For identifying the reaction sites, we followed the assumption that mTG may use six possible lysine and three possible glutamine side chains of HEWL under high pressure. Thus, theoretically a limited number of 18 tryptic peptides linked by an ε-(γ-glutamyl)lysine isopeptides are possible, taking into account that C-terminal peptide bonds of lysine residues incorporated into an isopeptide are not substrates for trypsin cleavage. The corresponding monoisotopic masses of all theoretically possible tryptic peptides containing isopeptides were calculated (data not shown) and were searched for in the tryptic hydrolysates via mass spectrometry. Using this approach, no relevant mass peaks were found in the tryptic hydrolysates of either native HEWL or from HEWL treated exclusively with mTG of high pressure, again confirming that lysozyme is not accessible to mTG under ambient pressure (*14*). However, one specific peak with an m/z of 817.49 ($z = 2$), originating from a peptide eluting with a retention time at 18.1 min, was exclusively detectable in the tryptic hydrolysates of simultaneously mTG- and high-pressure-treated HEWL samples (**Figure 3**). This peak corresponds to a peptide with a putative monoisotopic mass of 1633.0 Da and was identified as originating from combining the HEWL peptide segments 1−5 (monoisotopic molar mass of 605.4 Da) and 117−125 (1044.5 Da) minus the molar mass of ammonia (17.0 Da) due to the enzymatic formation of the isopeptide, resulting in a calculated monoisotopic mass of 1632.9 Da (**Figure 4**). The found and calculated masses are in perfect agreement within the accuracy of the mass determinations, which is around ±0.02% or ±0.3 Da. On this basis, we can conclude that mTG under pressure selectively cross-links HEWL via the N-terminal lysine residue (Lys1) and glutamine at position 121 (Gln121). The resulting isopeptide must have been formed predominantly intermolecularly between individual lysozyme molecules, leading to the observed oligomerization. At present, however, an intramolecular reaction between Lys1 and Gln121 within one lysozyme molecule cannot be ruled out.

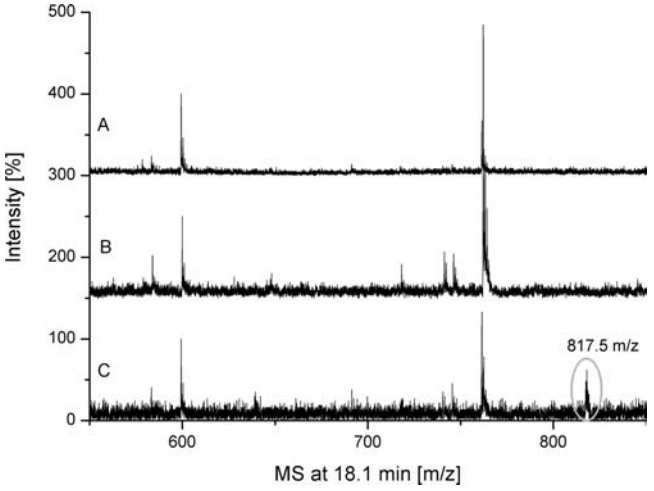

Figure 3. Mass spectrum at a retention time of 18.1 min (corresponding with **Figure 2**).

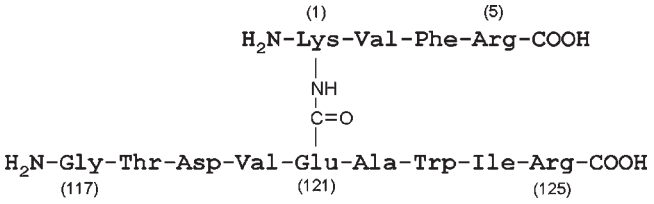

Figure 4. Sequence of isopeptide-containing fragment, identified in tryptic hydrolysates of HEWL treated with mTG for 30 min at 40 °C and 600 MPa. Numbers refer to position of amino acid in HEWL sequence (see text for details).

Table 2. Partial Amino Acid Sequence of Four Amino Acids around Lysine and Glutamine Residues of Lysozyme

residue	amino acid sequence adjacent
Lys1	**K**VFGR
Lys13	AAAM**K**RHGL
Lys33	VCAA**K**FESN
Lys96	VNCA**K**KIVS
Lys97	NCAK**K**IVSD
Lys116	RNRC**K**GTDV
residue	amino acid sequence adjacent
Gln41	NFNT**Q**ATNR
Gln57	YGIL**Q**INSR
Gln121	GTDV**Q**AWIR

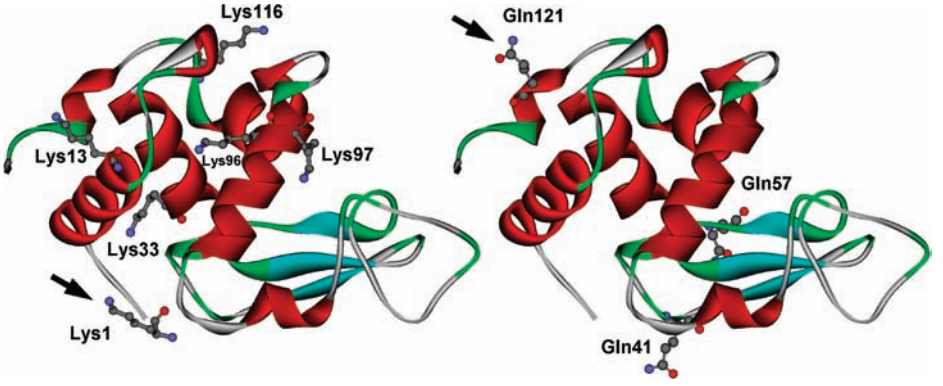

Figure 5. Visualization of lysine and glutamine residues within the tertiary structure of HEWL (pdb-file: 1gxv). Lys1 and Gln121 were identified as accessible for mTG under high hydrostatic pressure. α-Helices are colored in red, β-sheets in blue, turns in green, and random coils in light gray (created using Discovery Studio Visualizer, v2.5, Accelrys Software Inc., San Diego, CA).

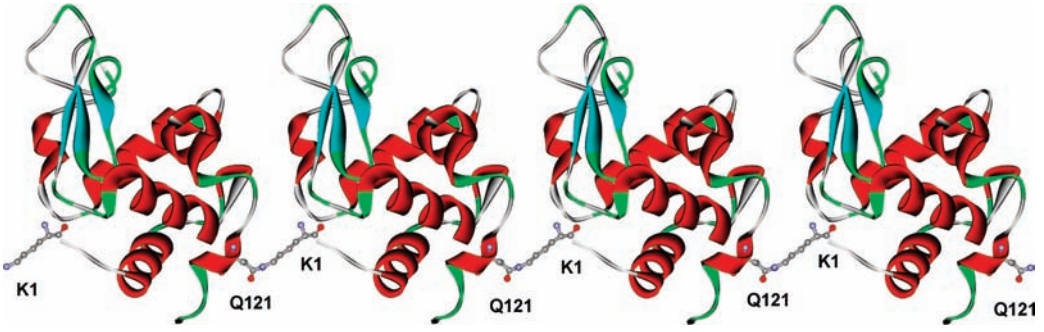

Figure 6. Proposed site-directed oligomerization of HEWL via isopeptide formation between Lys1 and Gln121 (created using Discovery Studio Visualizer, v2.5, Accelrys Software Inc., San Diego, CA).

The results reported here show that, with the given parameters, one of three glutamine residues (Gln121) and one of six lysine residues (Lys1) become accessible for mTG at 600 MPa (**Figure 5**). Within the native structure of HEWL, four lysine residues (Lys13, Lys33, Lys96, and Lys97) are located in extended helical regions of the protein (**Figure 5**). Murthy et al. (*14*) recently have shown that transglutaminase 2, an enzyme found in many cells and also in the extracellular matrix, does not modify lysine residues in HEWL under ambient pressure, although most of the side chains of HEWL are exposed to the solvent. The authors speculate that for lysine residues, location in a highly flexible portion of the protein is important to become accessible for transglutaminases. Lys1 and Lys116 are positioned in random coil regions, from which Lys1 may become more exposed due to partial unfolding of the protein under pressure when compared to the sequence surrounding Lys116, which is near the "stiff" disulfide bridge between Cys115 and Cys30 and therefore may be less flexible. When the three glutamine residues are compared, it is obvious that Gln57 is buried within the tertiary structure of the protein, whereas Gln41 and Gln121 are found on the surface of the protein (**Figure 5**). Steric hindrance, therefore, may block mTG cross-linking at Gln57. With regard to the sequence around Gln41 and Gln121 (**Table 2**), it is interesting to note that Gln41 has threonine (Thr40) at the N-terminal and alanine (Ala42) at the C-terminal side, whereas Gln121 is neighbored by valine (Val120) and alanine (Ala122). To date, there is only limited information concerning the influence of the amino acid sequence on the reactivity of glutamine residues. Studies published by Ohtsuka et al. (*16*), however, point to the fact that hydrophobic amino acids such as valine and leucine on the N-terminal side seem to enhance the accessibility of glutamine side chains for mTG. This may be one aspect favoring the site-directed modification exclusively at Gln121 compared to Gln41. Furthermore, studies by Refaee et al. (*17*) show that the helical region of HEWL around Gln121 turns into a flexible random coil at 200 MPa. Although our experiments were performed at much higher pressure, this observation may underscore the theory that reversible pressure-induced unfolding leads to increased accessibility for mTG in dedicated regions of HEWL.

In conclusion, mTG under high hydrostatic pressure can induce a site-directed cross-linking of lysozyme, resulting in the formation of well-defined lysozyme oligomers as visualized in **Figure 6**. The formation of fibrillar oligomers with substantially altered functional properties is highly likely. Corresponding studies are underway in our laboratory.

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