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Influence of denaturation and aggregation of β-lactoglobulin on its tryptic hydrolysis and the release of functional peptides

Elena Leeb [a,*], Alexander Götz [a,b], Thomas Letzel [c], Seronei Chelulei Cheison [a,d], Ulrich Kulozik [a]

[a] Technische Universität München, Chair for Food Process Engineering and Dairy Technology, ZIEL Research Centre for Nutrition and Food Sciences, Weihenstephaner Berg 1, 85354 Freising-Weihenstephan, Germany
[b] Technische Universität München, Fakultät für Physik E14, Maximus-von-Imhof-Forum 4, 85354 Freising, Germany
[c] Technische Universität München, Analytical Research Group, Chair of Urban Water Systems Engineering, Am Coulombwall 8, 85748 Garching, Germany
[d] Global Applied Science & Technology (GAST), Mars GmbH, Eitzerstr. 215, D-27283 Verden, Germany

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ABSTRACT

Whereas previous studies showed that thermal pre-treatment of whey proteins promote their enzymatic hydrolysis, to date no correlation between the conformation of denatured protein and the release of individual peptides has been considered. Hence, in this study total denaturation of β-lactoglobulin was performed at defined pH-values to enable the generation of different denatured particles. The denatured proteins were used as substrate for tryptic hydrolysis and the hydrolysis progress was characterised by the degree of hydrolysis (DH) and the release of functional peptides, detected using LC–ESI-TOF/MS. Denaturation and subsequent aggregation of β-lactoglobulin, induced by thermal treatment at pH 5.1, altered the DH slightly, whereas the release of investigated peptides was significantly decreased. Contrary, denaturation at pH 6.8 and 8.0 led to formation of non-native monomers and reduced the DH to 75%, but showed promoting as well as reducing effects on the release of peptides, depending on their location within the protein.

1. Introduction

By enzymatic hydrolysis of food proteins, peptides with enhanced techno-functional properties as well as peptides acting as regulatory compounds with hormone-like effects are released (Korhonen & Pihlanto, 2006). One well studied protein in this regard is the major whey protein, β-lactoglobulin (β-LG), acting as precursor for several functional peptides with antihypertensive, antiviral, antibacterial and hypoglycaemic properties (Chatterton, Smithers, Roupas, & Brodkorb, 2006; Hernández-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007; Pihlanto-Leppälä, Rokka, & Korhonen, 1998). Due to careful enzyme selection, based on specific cleavage affinities for certain peptide bonds, the composition of the hydrolysate can be steered (Adjonu, Doran, Torley, & Agboola, 2013). The use of the serine protease Trypsin (EC 3.4.21.4), which preferably cleaves the C-terminal peptide bonds of Arginine (Arg/R) and Lysine (Lys/K), leads to the release of five biofunctional peptides when hydrolysis proceeds to its maximum possible degree of hydrolysis (DH_{max}). The position of these peptides within the structure of β-LG, their amino acid sequence and reported functional properties are listed in Table 1.

Native β-LG is a globular protein, consisting of 162 amino acids with a total molecular mass of 18.4 kDa. The secondary structure of bovine β-LG, schematically illustrated in Fig. 1A, consists of an eight-stranded, flattened β-barrel (strand A – H), which folds into a calyx and a flanking three-turn α-helix. In its native state two intramolecular disulphide bridges (represented with dotted lines) are formed between four of the five Cysteine (Cys/C) residues, whereby Cys at the position 121 (Cys_{121}) represents a free sulfhydryl group (Hoffmann & van Mil, 1997). The tertiary structure of the β-LG monomer, shown in Fig. 1B, is sensitive to unfolding at temperatures above 60 °C, leading to the exposure of interior hydrophobic residues and the free sulfhydryl group of Cys_{121} (Manderson, Hardman, & Creamer, 1998). These reactive intermediates, characterised as molten globule state, react with each other upon further heat treatment. It is well known that the physicochemical properties of formed β-LG aggregates are influenced by the environmental conditions during denaturation like pH, ionic strength, protein and lactose concentration as well as temperature–time combinations (Chen et al., 2005; Hoffmann & van Mil, 1997; Tolkach & Kulozik, 2005, 2007; Toro-Sierra, Schumann, & Kulozik, 2013; Toro-Sierra, Tolkach, & Kulozik, 2013). The aggregates can be stabilized by non-covalent interactions and/or covalently due to thiol-disulphide exchange reactions (Mounsey & O'Kennedy, 2007). The formation of non-covalently

* Corresponding author. Tel.: +49 8161 71 3719; fax: +49 8161 71 4384.
E-mail address: elena.leeb@tum.de (E. Leeb).

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Table 1
Bioactive peptides released by tryptic hydrolysis of β-lactoglobulin.

Protein fragment	Amino acid sequence	Functional property	
f(9–14)	GLDIQK	[1]ACE-inhibitor	Pihlanto-Leppälä et al. (1998)
f(71–75)	IIAEK	Hypocholesterolemic	Nagaoka et al. (2001)
f(78–83)	IPAVFK	Bactericidal	Pellegrini, Dettling, Thomas, and Hunziker (2001)
f(92–100)	VLVLDTDYK	Bactericidal	Pellegrini et al. (2001)
f(142–148)	ALPMHIR	[1]ACE-inhibitor	Mullally, Meisel, and FitzGerald (1997)

[1] ACE – angiotensin-I-converting enzyme.

linked aggregates was shown to mainly go along with changes in the tertiary structure and only small differences in the secondary structure (Carrotta, Bauer, Waninge, & Rischel, 2001). Investigations of Hoffmann and van Mil (1997) showed that the formation of covalently linked aggregates via intermolecular disulfide bonds induced by denaturation of β-LG at neutral and slightly alkaline pH-values prevent the reversibility of modifications in the tertiary structure of native β-LG and further association of the modified proteins via non-covalent interactions. A special case related to thiol-exchange reactions during β-LG denaturation is the formation of stable molten globule states, called non-native monomers via intramolecular thiol-exchange reactions due to heat denaturation at neutral pH-values (Croguennec, Mollé, Mehra, & Bouhallab, 2004). These intermediates, are stabilized by the formation of a new disulphide-bond between Cys_{106} and Cys_{121} whereby Cysteine at position 119 (Cys_{119}) is present with a free thiol group. These stable intermediates have only a low affinity to aggregate and therefore remain in this molten globule state by appropriate denaturation conditions.

To which extent the formation of different denatured particles alters the enzymatic hydrolysis is until today not satisfactory determined. Whereas the hydrolysis kinetics and the time dependent release of peptides during tryptic hydrolysis of native β-LG are well described by Cheison, Schmitt, Leeb, Letzel, and Kulozik (2010) and Fernández and Riera (2013), detailed information on the hydrolysis of denatured β-LG and the impact of denaturation on the release of peptides is missing. To date, thermally induced denaturation of the substrate prior to its hydrolysis was investigated for whey protein isolate (WPI) and whey protein concentrate (WPC) by Adjonu et al. (2013), O'Loughlin, Murray, Kelly, FitzGerald, and Brodkorb (2012), O'Loughlin et al. (2013), Stanciuc, Hintoiu, Stanciu, and Rapeanu (2010), Zhang and Vardhanabhuti (2014). In addition, reports on heat denaturation of pure β-LG as substrate for subsequent hydrolysis have been published (Creamer et al., 2004; Peram, Loveday, Ye, & Singh, 2013; Stanciuc, van der Plancken, Rotaru, & Hendrickx, 2008).

However, existing studies showed promoting as well as reducing effects on the hydrolysis rate and degradation of the initial proteins due to thermal substrate pre-treatment. Zhang and Vardhanabhuti (2014) investigated the impact of the heating pH on the aggregation of whey proteins in WPI and their subsequent digestibility. Thermal treatment at 85 °C for 30 min at acidic pH values did not lead to denaturation and subsequent aggregation of whey proteins, so that the proteins remained relatively resistant against peptic hydrolysis. In contrast to that, denaturation at pH values above the pI of β-LG (5.1) induces the formation of large aggregates with higher accessibility for the enzyme pepsin (Zhang & Vardhanabhuti, 2014). The authors concluded that by aggregation of the whey proteins due to unfolding of the molecule, more hydrophobic residues were exposed leading to an enhanced accessibility of peptide bonds. Furthermore, an influence of the stabilizing mechanisms of the aggregates on the digestibility of the proteins was implied, namely that non-covalently linked aggregates are easier to be digested than disulphide-bonded aggregates. On the other hand the influence of denaturation-time at varying temperatures on the digestibility of whey proteins in WPI and WPC was investigated by Stanciuc et al. (2010), O'Loughlin, Murray, Kelly, FitzGerald, and Brodkorb (2012), respectively. Because both working groups used short heating times and moderate protein concentrations, high amounts of residual native protein were present. However, O'Loughlin et al. (2012) determined the amount of residual native protein and took into account that the initial substrate was a mixture of both, native and denatured proteins. A more detailed investigation by O'Loughlin et al. (2013) showed that although the thermal pre-treatment of WPI solutions

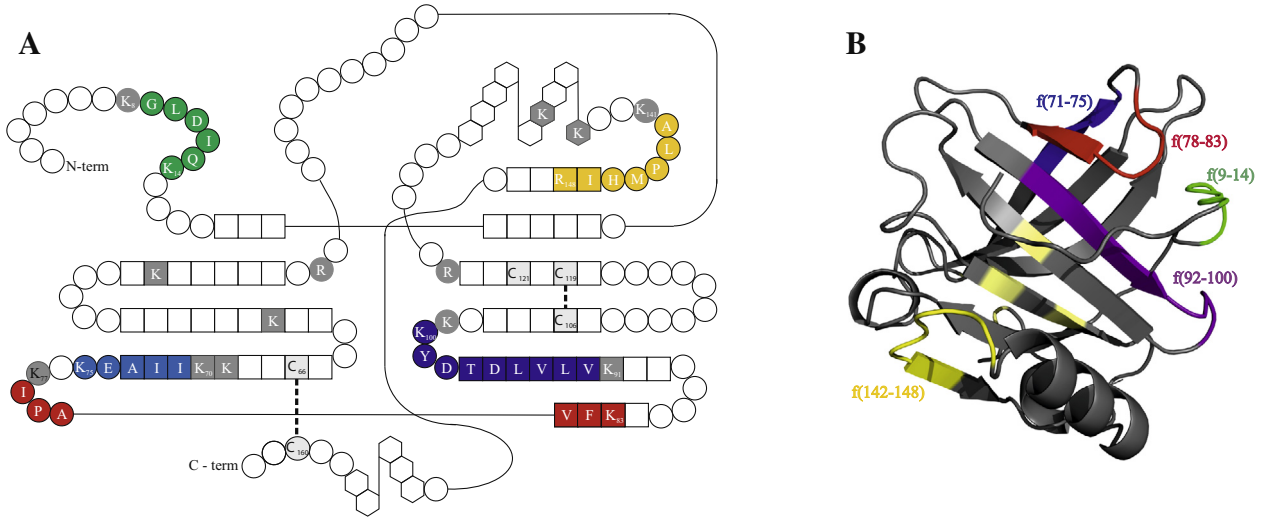

Fig. 1. (A) Schematic diagram of the secondary structure of native β-LG; amino acids presented as hexagons represent α-helixes, squares represent β-sheets and circles represent loops. Preferred cleavage sites for trypsin are highlighted in grey (Arg/R & Lys/K) and the bioactive peptide f(9–14) in green, f(71–75) in blue, f(78–83) in red, f(92–100) in violet and f(142–148) in yellow. Modified according to Sakurai, Konuma, Yagi, & Goto, 2009. (B) 3-dimensional figure of native β-LG (pdb code 1BSQ). The positions of biofunctional peptides within the globular monomer are highlighted. The five Cysteine residues are highlighted in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)