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Thermal pre-treatment of β-Lactoglobulin as a tool to steer enzymatic hydrolysis and control the release of peptides

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Abstract

Tryptic hydrolysis of β-Lactoglobulin (β-Lg) has been shown as an excellent way to produce several functional peptides. However enzymatic hydrolysis results in a mixture of numerous different peptides, which is problematic regarding the enrichment of one functional peptide in foods. Therefore, a new approach was investigated to control enzymatic hydrolysis by thermal pre-treatment of the substrate to produce specifically desired peptides. For this a 5% β-Lg solution (w/w) was denatured at 80°C and the influence of the surrounding pH (pH 4.6, 6.8 and 8) on the formed denatured particles was characterised by degree of denaturation (DD) and particle size. The native, as well as the denatured protein was then hydrolysed by trypsin (EC 3.4.21.4) at enzyme optimal conditions (pH 8 and 37°C). Hydrolysis was monitored by degree of hydrolysis (DH) using the pH-stat method and the hydrolysates were analysed by MALDI-TOF-MS/MS after several DHs. It could be demonstrated that the attacked cleavage sites during tryptic hydrolysis of native β-Lg followed no specific order. Denaturation independent of the environmental conditions led to a decrease of the DH during the total hydrolysis. Of special interest was the formation of non-native monomers by thermal denaturation at pH 8 and the subsequent decrease of attacked peptide bonds to only five different cleavage sites after a DH of 1%. This conditions were favorable, due to the release of a slightly heterogeneous hydrolysate, containing the functional peptide f(142-148), with reported ACE-inhibitory activity.

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Keywords: β-Lactoglobulin; thermal denaturation; tryptic hydrolysis;bioactive peptides;

1. Introduction

Functional compounds, such as bioactive peptides and their application in different food systems are of growing interest in the food industry. To enhance the customer acceptance of these functional foods, the

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production of bioactive peptides out of natural sources is favoured. However, to date, processes to obtain and enrich functional peptides in several foods are not feasible, though different proteins are known to be potential sources for different functional peptides. Milk proteins are especially known to be an excellent source for several bioactive and techno-functional peptides [1,2]. β-Lactoglobulin (β-Lg), the major whey protein is a source of a wide range of peptides with different biofunctionalities like antibacterial, hypocholesterolemic activity and in particular angiotensin-I converting enzyme (ACE)-inhibitory properties [3]. To release these desired peptides, enzymatic hydrolysis is the method of choice.

Currently, the release of peptides by means of enzymatic hydrolysis results in a mixture of numerous different peptides. Nevertheless even with the application of specific enzymes, the variety of possible cleavage sites is very broad. A new approach to reduce undesired cleavages and increase the hydrolysis of desired peptide bonds from the outset of the hydrolysis, is the pre-treatment of the substrate. Thereby conformational changes within the protein structure prevent the hydrolysis of undesired, and increase the accessibility of the enzyme to desired peptide, bonds.

Some different possibilities have already been investigated to affect the enzymatic hydrolysis of whey proteins. Thereby the influence of high hydrostatic pressure, as well as thermal treatment of the substrate has been examined. Knudsen et al. [4] hydrolysed native and pressure-treated β-Lg with different proteases, to determine the influence of pressure treatment on the protein conformation. The effect of thermal pre-treatment of whey proteins on subsequent enzymatic hydrolysis was also investigated [5] including thermal denaturation on tryptic hydrolysis of β-Lg [6]. All these works determined an increase in the accessibility of the enzyme to possible peptide bonds within the denatured protein, with limited denaturation. As a result hydrolysis could be increased by specified time-temperature combinations. Diminished hydrolysis rates were explained by the polymerisation of the denatured β-Lg particles, resulting in lower accessibility of the enzyme to specific cleavage sites. However, to date there is still no specific information about a thermal pre-treatment on the released peptides during the hydrolysis process. Furthermore, no information is available on how environmental conditions during thermal treatment affect the denatured particle formation and the relationship to subsequent hydrolysis patterns. At the same time this information is of major interest, when the control of enzymatic hydrolysis and the released peptides is based on the conformational changes of the protein and subsequent steric hindrance to enzyme access. It is well known, that denaturation of the globular protein β-Lg is dependent on several environmental factors. Besides the temperature, environmental conditions like protein concentration and pH can affect the denaturation kinetics and the polymerisation behaviour of denatured particles [7,8].

Therefore the aim of this study was to determine the influence of pH on the denaturation behaviour of β-Lg and the formed aggregates after thermal treatment. In addition, the release of functional peptides by subsequent controlled enzymatic hydrolysis was investigated using the ACE-inhibitory peptide f(142-148) as an index. Based on these results a platform for the production of functional peptides shall be created, which includes also the possibility of substrate pre-treatment.

2. Materials and Methods

2.1. Material

Bovine β-Lg was prepared out of whey protein isolate from Fonterra Co-operative Group Ltd (Auckland, New Zealand) as described by Gezan-Guizou et al. [9] using an optimised method. It was possible to gain β-Lg with a purity of 95.7 %. Trypsin (T9201 from bovine pancreas, activity of ≥ 7500 units/mg solid) was purchased from Sigma-Aldrich (St Lois, MO, USA). Other chemicals and equipment are mentioned under respective methods. All used solvents and chemical reagents were of analytical grade.

2.2. Heat treatment of β-Lactoglobulin solutions

Aqueous β-Lg solutions (50 g/L) were pH-adjusted (4.6, 6.8, 8) and thermally denatured at 80 °C. The thermal treatment was performed in a thermostatic water bath with constant temperature and aliquots were taken after different time intervals (0 – 120 min). The obtained samples were characterised by degree of denaturation (DD) and particle size. The DD was calculated by the disappearance of native β-Lg using equation (1), where C_0 and C_t are the concentrations of native β-Lg before and after heat-treatment.

$$DD = 1 - \frac{C_t}{C_0} \cdot 100\% \quad (1)$$

The particle size of the protein molecules was analysed by determination of the hydrodynamic diameter using a Zetasizer Nano System (Malvern Instruments Inc, Worecester, UK).

2.3. Tryptic hydrolysis of β-Lactoglobulin solutions

The native as well as the denatured β-Lg was hydrolysed with trypsin at the enzyme optimal conditions (38 °C, pH 8) with an Enzyme-to-Substrate-Ratio = 0,1%. An auto-titrator (TitroLine alpha plus, Schott AG, Mainz, Germany) was used to kept the pH constant. In the case of thermal pre-treatment of β-Lg a 5% solution (w/w) was heated for 2 h at pH 8 and 3 h at pH 4.6 and 6.8 to reach the maximum possible DD. These solutions were cooled and subsequently used for enzymatic hydrolysis.

During the hydrolysis experiments the pH was kept constant by addition of 0.5 M NaOH. Based on the consumption of base the number of cleaved peptides bonds was estimated. The percentage of cleaved peptide bonds is an appropriate measurement to specify the progress of hydrolysis. Therefore the degree of hydrolysis (DH) was calculated based on following equation [10].

$$\mathrm{DH}\,(\%) = \frac{B \cdot N_b}{\alpha \cdot MP \cdot h_{tot}} \cdot 100 \quad (2)$$

Whereby B is the amount of base consumption [mL], N_b the molarity of the base, α is the average degree of dissociation of the α-NH groups, MP is the mass of the protein, and h_{tot} corresponds to the total number of peptide bonds in the substrate protein [meqv/g protein].

2.4. Analysis of the β-Lactoglobulin and the hydrolysates

The initial native and residual non-hydrolysed protein content was determined by means of reversed-phase high performance liquid chromatography (RP-HPLC) [11]. The peptide composition of the sampled aliquots at DH 1%, 5% and maximum possible DH (DH_{max}) were analysed by means of matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) as already described in our earlier work [12].

3. Results and Discussion

3.1 Denaturation behaviour of β-Lactoglobulin depending on the environmental pH

The denaturation behaviour of β-Lg was investigated at three different pH-values (4.6, 6.8 and 8.0). These pH-values were deliberately selected since the native protein is present in different conformational stages at those pH values. At pH 8, native β-Lg exists mainly as monomers, whereas at pH 6.8 the monomers tend to associate to dimers. At pH 4.6, near the isoelectric point (pI) of β-Lg, the association of β-Lg monomers is at its height and octamers are formed, particularly for β-Lg genetic variant A (β-Lg A).

Similar to the effect on the native conformation of β-Lg, the environmental pH also had affects on its denaturation behaviour and the thereby formed denatured particles. Depending on the pH of the β-Lg solution, different DDs were detected after thermal treatment at 80 °C (Figure 1).

At alkaline conditions (pH 8) the denaturation proceeded faster and the maximal possible DD of approximately 96 % was reached after 45 min. At pH 6.8 the maximum possible DD was comparable to that reached at pH 8, but the denaturation proceeded a little bit slowly. Hence, the maximum possible DD of 97 % was reached after 90 min. In contrast to the other two pH values, denaturation of β-Lg was very slow at pH 4.6. Even after thermal treatment of 150 min, a DD of only 55 % was reached. The different denaturation behaviour could also be detected by characterisation of the denatured β-Lg particles by means of dynamic light scattering (Figure 2).

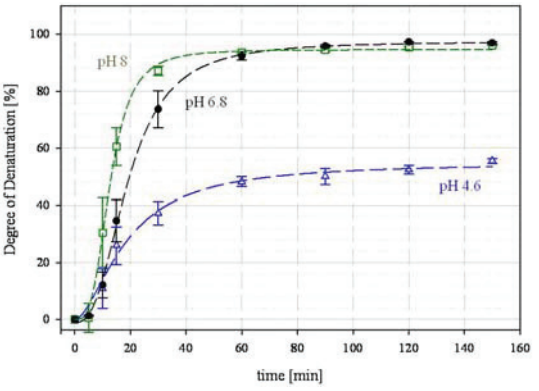

Fig. 1. Influence of pH on the degree of denaturation during thermal treatment of a 5 % β-Lg-solution at 80°C

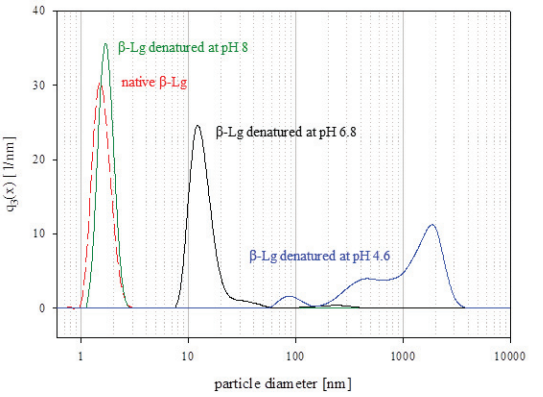

Fig. 2. Particle size distribution of native (---) as well as denatured (—) β-Lg at pH 4.6, 6.8 and 8

It was shown, that at pH 8, aggregation of denatured β-Lg particles did not occur. The particle size distribution corresponded almost to the monomodal distribution of the native monomer with a mean diameter of 1.8 nm, comparable to that of native β-Lg. At alkaline pH thermal treatment results in the formation of the so-called non-native monomers. At these environmental conditions β-Lg undergoes the unfolding mechanism, but does not aggregate. This is the result of the high reactivity of the free thiol group at this pH. Based on intramolecular disulphide exchange reactions, the reaction equilibrium is shifted to the irreversible formation of non-native monomers and aggregation of the denatured particles by intermolecular disulphide bridges is hindered. In contrast to that, with decreasing pH the maximal possible DD decreased and the heterogeneity of particle size distribution increased. Whereby at pH 8 a monomodal size distribution of denatured particles was detectable, denaturation at pH 6.8 led to the formation of bigger particles with a polymodal distribution in which the main fraction peak had a molecular size between 8 and 25 nm. With further decrease of the pH, the heterogeneity in particle size increased. Denaturation at pH 4.6 leaded to the formation of a very heterogenic particle size distribution aggregates with particle sizes up to 4 µm. These differences in particle size with monomodal distribution at pH 8 and polymodal distributions at pH 6.8 and 4.6 are attributed both to the decrease in the reactivity of the thiol group with decreasing pH and also to the different interactions between the β-Lg particles depending on the environmental pH. At pH 6.8 and even stronger at pH 4.6, aggregation of the denatured particles plays an important role. Due to decreasing repulsive forces with decreasing pH the denatured particles participate with each in hydrophbic interactions and form aggregates.

3.2. Enzymatic hydrolysis of native and denatured β-Lactoglobulin

Due to different conformational changes with varying pH, differences in the enzymatic hydrolysis were detected. Depending on the denaturation behaviour, not only different cleavage sites were exposed on the molecular surface, even the number of accessible cleavage sites was restricted by aggregation of denatured particles. The limitation in the accessibility of the enzyme to possible cleavage sites occurred independent of the denaturation condition (Figure 3).

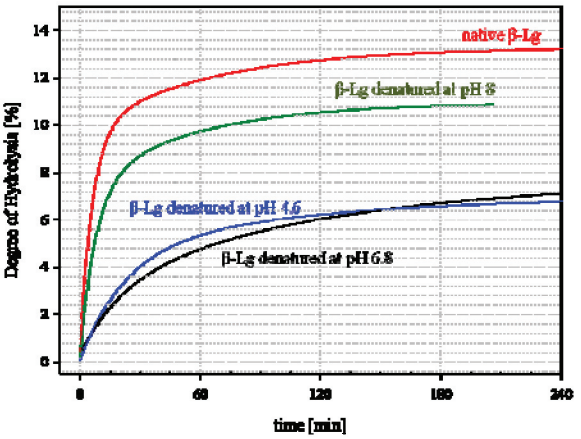

Fig. 3. Influence of pH (4.6, 6.8, 8) during thermal pre-treatment of β-Lg on the tryptic hydrolysis and the released peptides in comparison to the tryptic hydrolysis of native β-Lg

By thermal treatment of β-Lg at pH 8 to the maximal possible DD, the DH_{max} was reduced from 13.2% in the case of native protein as substrate to 10.9%. This reduction of possible cleavage sited to 82% is depended on the refolding of β-Lg through the formation of non-native monomers. The additional aggregation of β-Lg during thermal treatment at pH 4.6 and 6.8 further diminished the hydrolysis rate. For β-Lg denatured at pH 6.8, a DH_{max} of 7.1% was reached, whereas denaturation at pH 4.6 led to marked reduction of the DH_{max} to 6.7%. Thus denaturation of β-Lg with a subsequent aggregation led to a reduction of possible cleavage sites to about 50% only.

In contrast to other research works, no increase in the DH was detected by any thermal denaturation of the substrate. This confirms the hypothesis of Stanciuc et al. [5] that with polymerisation of the denatured β-Lg particles, the accessibility of the enzyme to specific cleavage sites is restricted. Therefore, with increasing particle size and, subsequently, reduced amount of attackable cleavage sites, the DH_{max} decreased. However, the formation of non-native monomers reduced the DH_{max}, which leads to the conclusion that even a refolding of the monomer diminishes the amount of possible cleavage sites irreversibly.

At different degrees of hydrolysis (DH 1%, 5% and DH_{max}) aliquots were taken and their peptide pattern analysed by MALDI-TOF-MS/MS. Based on the identified peptides, also the cleaved peptide bonds within β-Lg particles were identified. Hence the order of the enzyme attack on possible cleavage sites was investigated. The MS analysis results confirmed, that denaturation independent of the environmental pH, decreased the susceptibility of Trypsin to possible cleavage sites. Thus the variability within the peptide patterns decreased and the attacked peptide bonds within the denatured β-Lg were reduced in comparison to those in the native monomer. The cleaved peptide bonds identified within the native and the non-native β-Lg monomer at different DHs are presented in Table 1. Looking at the order

of cleaved peptide bonds within the native protein during tryptic hydrolysis, no exceptional specificity of the enzyme towards individual peptide bonds could be estimated. At the beginning of enzymatic hydrolysis (DH 1%) already 13 of the 18 possible peptide bonds were cleaved and 11 different peptides were released. At DH 5% 17 peptide bonds were cleaved and at DHmax every trypsin scissile peptide bond was cleaved so that 19 different peptides were detected.

Table 1. Identified cleavage sites within the native and non-native monomer of β-Lg, depending on the degree of hydrolysis during tryptic hydrolysis at enzyme optimal conditions

	Identified cleaved peptide bonds within the native β-Lg monomer			**Identified cleaved peptide bonds within the non-native β-Lg monomer**		
Trypsin specific cleavage sites	**DH 1 %**	**DH 5 %**	**DH_{max}**	**DH 1 %**	**DH 5 %**	**DH_{max}**
Lys^{8}	●	●	●			
Lys^{14}	●	●	●		○	○
Arg^{40}	●	●	●	○	○	○
Lys^{47}		●	●			○
Lys^{60}	●	●	●		○	○
Lys^{69}	●	●	●	○	○	○
Lys^{70}	●	●	●	○	○	○
Lys^{75}		●	●			
Lys^{77}	●	●	●			○
Lys^{83}	●	●	●			○
Lys^{91}	●	●	●		○	○
Lys^{100}		●	●			○
Lys^{101}	●	●	●			
Arg^{124}		●	●		○	○
Lys^{135}	●	●	●			○
Lys^{138}			●			
Lys^{141}	●	●	●	○	○	○
Arg^{148}	●	●	●	○	○	○

In contrast, denaturation at alkaline conditions (pH 8) and subsequent formation of non-native monomers led to the restricted cleavage of peptide bonds. The reduction of accessible cleavage sites to the peptide bonds Arg_{40}, Lys_{69}, Lys_{70}, Lys_{141} and Arg_{148} can be explained by the unfolding of the monomer and the increased exposure of these regions due to denaturation. The increased exposure of exactly these peptide bonds can be explained by the great heat sensitivity of these regions of the β-Lg molecule [13]. Due to these structural changes the peptide f(142-148) with known ACE inhibitory activity was released already at DH 1%.

4. Conclusion

The results of this study demonstrate that a thermal pre-treatment of β-Lg is an effective way to control and steer enzymatic hydrolysis. Thereby the denaturation process is affected by environmental conditions with respect to the unfolding and aggregation of β-Lg. By appropriate choice of the pH during thermal denaturation of β-Lg, the accessibility of the enzyme to individual peptide bonds can be controlled and thereby the release of desired peptides promoted. Based on these investigations not only hydrolysis

duration can be shortened, also the variety of gained peptides in the hydrolysates can be decreased and a subsequent enrichment of individual peptides is facilitated.

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