Food Chemistry 253 (2018) 194–202

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Comparison of the influence of pH on the selectivity of free and immobilized trypsin for β-lactoglobulin hydrolysis

Yuhong Mao[a,*], Maria Krischke[a], Claudia Hengst[a], Ulrich Kulozik[a,b]

[a] Chair of Food and Bioprocess Engineering, Technical University of Munich, Weihenstephaner Berg 1, D-85354 Freising-Weihenstephan, Germany
[b] Institute of Food & Health (ZIEL) – Technology Unit, Technical University of Munich, Weihenstephaner Berg 1, D-85354 Freising-Weihenstephan, Germany

ARTICLE INFO

Keywords:
β-lactoglobulin
Tryptic hydrolysis
Immobilization
pH
Selectivity

ABSTRACT

Although immobilized trypsin is a viable alternative to the free one in solution for producing protein hydrolysates, the change of selectivity introduced by immobilization is unclear. In this study, we compared the selectivity of free and immobilized trypsin towards different cleavage sites of β-lactoglobulin (β-Lg) with a focus on the impact of environmental pH. Both free and immobilized trypsin exhibited greater accessibility to native β-Lg at elevated pH (from pH 7.2 to 8.7). Additionally, free trypsin preferred to attack cleavage sites located at the C-terminus at pH 7.8, whereas an opposite preference for the N-terminus was observed at pH 8.7. Regarding the immobilized trypsin, the pH did not significantly influence its preference for the C- or N-terminus. Generally, immobilization of trypsin resulted in more focused cleavage within its specificity during the initial stage of hydrolysis, and some peptides were formed more rapidly by the immobilized trypsin.

1. Introduction

Enzymatic hydrolysis of β-lactoglobulin (β-Lg) produces peptides with reduced allergenicity (Selo et al., 1999) and improved functionality (Leeb, Gotz, Letzel, Cheison, & Kulozik, 2015). However, the cost of the large-scale use of enzymes in solution is very high, which severely limits their implementation at industrial scale. Correspondingly, immobilized enzymes represent an alternative approach due to the possibility of reusing enzymes and producing enzyme-free hydrolysates.

Enzyme immobilization has been exploited over the last four decades to enhance enzymatic activity and stability, which strongly depend on support properties, binding orientation, the number of formed bonds, the microenvironment of the enzyme, and other variables. Aside from measuring stability and activity, some researchers (Atacan, Cakiroglu, & Ozacar, 2016; Naldi, Černigoj, Štrancar, & Bartolini, 2017) analyzed the peptide profiles of the resulting hydrolysates to assess the enzyme specificity after immobilization. The specificity of a proteolytic enzyme describes the type of amino acid, after which it can hydrolyze a peptide bond (e.g., Lys and Arg for trypsin). Regardless of the specificity of the enzyme for individual cleavage sites, not all cleavable sites are hydrolyzed at the same time. Cheison, Brand, Leeb, and Kulozik (2011) followed the release of peptides as a function of hydrolysis time from β-Lg hydrolyzed by free trypsin during the first 10 min. They found that the N- and C-termini (Lys_8-Gly_9, Lys_{141}-Ala_{142}, and Arg_{148}-Leu_{149}) of β-Lg was cleaved early (15 s), implying the ease of trypsinolysis at the exposed termini. The results of Fernández and Riera (2013) also show the existence of areas within the intact β-Lg with different susceptibility to tryptic attack. To describe this preference in protein hydrolysis, Butre, Sforza, Gruppen, and Wierenga (2014) introduced the criteria "selectivity", referring to the rate at which individual cleavage sites in a protein substrate are hydrolyzed relative to other cleavage sites. The ability to discriminate the selectivity of an enzyme is considered essential for understanding enzymatic protein hydrolysis, especially in terms of obtaining hydrolysates consisting of preferred properties.

Upon immobilization of an enzyme, its conformation may change, thus affecting its intrinsic properties (V_{max}, k_{cat}, or K_m), especially when the enzyme is firmly fixed by multipoint covalent immobilization (Duggal & Bucholz, 1982). In addition, the properties of the supports used for immobilization, such as the charge of the stationary phase, hydrophobicity/hydrophilicity, may influence the intrinsic properties of the immobilized enzyme, particularly when charged substrates or products molecules are involved in the enzymatic process. Duggal and Bucholz (1982) presented clear evidence for significant shifts in the association constants for substrates and inhibitors due to the covalent binding of trypsin to a rigid support. Changes of the intrinsic properties of immobilized enzymes may lead to changes in selectivity.

Abbreviations: β-Lg, β-lactoglobulin; α-La, α-lactalbumin; MITR, monolith-based immobilized trypsin reactor; HCCA, α-cyano-4-hydroxycinnamic acid; DHAP, 2,5-dihydroxyacetophenone; BAEE, Nα-benzoyl-L-arginine ethyl ester; DH, degree of hydrolysis

* Corresponding author.
E-mail address: yuhong.mao@tum.de (Y. Mao).

https://doi.org/10.1016/j.foodchem.2018.01.151
Received 29 September 2017; Received in revised form 29 November 2017; Accepted 23 January 2018
Available online 31 January 2018
0308-8146/

Furthermore, the mass transfer properties of an immobilized biocatalyst may affect the competition for the active site of the enzyme between the original protein substrate and released polypeptides, which influences the selectivity to some extent, especially in a diffusion-limited step. Thus, more attention should be devoted to comparing selectivity between free and immobilized enzymes. Rocha, Gonçalves, and Teixeira (2011) compared peptide profiles of whey protein hydrolysates from free and immobilized trypsin by reversed-phase high-performance liquid chromatography (RP-HPLC) and concluded that no significant difference was observable. However, the sensitivity of HPLC, in our eyes, is not sufficient to make this statement. Mass spectroscopy would be required to identify and quantify the resulting peptides not only in the final hydrolysates, but also during the hydrolysis. Hence, many important aspects remain uncharacterized or not well understood, especially the evolutions of peptides, which should be affected by alterations in the selectivity of an enzyme after immobilization.

Additionally, the process of hydrolysis should be performed with a focus on the holistic influence of the hydrolytic environment, including pH, ionic strength, and temperature, et al., as these parameters play important roles regarding effectiveness and selectivity of enzymes. To exert more control over the process, it is important to elucidate the influence of the hydrolytic environment on peptide composition, i.e., the selectivity of the enzyme, allowing hydrolysis to be driven by both the “speed” and the desired peptide profiles obtained at various stages along the hydrolysis process.

Among these parameters, pH is easy to adjust while dramatically affecting the process. The effect of pH on enzymes varies, but it is prominently represented by the fact that each enzyme has an optimal pH range (e.g., pH 7.8–8.1 for free trypsin) in which the highest activity is observed. Meanwhile, changes in pH disrupt hydrogen bonding and affect salt bridges, leading to changes of the secondary and tertiary structures of the substrate as well as the enzyme. In fact, evidence of the influence of pH on enzyme selectivity was revealed in a study of the hydrolysis of whey protein isolate (WPI) by *Bacillus licheniformis* (Butré, Sforza, Wierenga, & Gruppen, 2015), in which large differences in enzyme selectivity for different cleavage sites of β-Lg were observed at pH 7.0, 8.0, and 9.0 (optimal pH for *Bacillus licheniformis* is 8.0).

Our previous work (Mao, Černigoj, Zalokar, Štrancar, & Kulozik, 2017) developed a monolith-based immobilized trypsin reactor (MITR), which has been confirmed as an effective alternative tool for producing β-Lg hydrolysates. This previous work also evaluated the effects of pH on the activity of both immobilized and free trypsin using a model substrate Nα-benzoyl-L-arginine ethyl ester (BAEE). Specifically, the free trypsin showed the highest activity at pH 7.8–8.1, and remained around 80% and 60% activity at pH 7.2 and pH 8.7, respectively. The MITR exhibited 70% activity at pH 7.2 compared with that at its optimal pH range (pH 8.5–8.7). In the present study, we aimed at clarifying the selectivity of the immobilized trypsin for β-Lg hyrolysis in comparison with free trypsin. The influence of pH ranging from pH 7.2 to pH 8.7, was investigated, i.e. somewhat broader than the optimum for free trypsin, in order to also include the pH range optimal for the immobilized one. The hydrolysis was characterized using three descriptors: (i) degree of hydrolysis (DH); (ii) the amount of depleted or remaining intact β-Lg as a function of DH (two genetic variants, β-Lg A and B, were compared); and (iii) the peptide profiles and molecular mass distribution depending on DH. Peptides with a mass of less than 4000 Da were assigned to specific sequences, and the selected peptides were further quantified to follow their dynamic evolutions.

2. Materials and methods

2.1. Materials

Bovine β-Lg was fractionated from WPI, a product developed by Fonterra Co-operative Group Ltd (Auckland, New Zealand), as described by Toro-Sierra, Tolkach, and Kulozik (2011). The obtained β-Lg powder had a protein content of 98.6% relative to the dry matter, and native β-Lg represented > 99% of the total protein content. Trypsin from bovine pancreas (Type I, approximately 10,000 BAEE units/mg protein), BAEE (B4500), Tris (hydroxymethyl)-aminomethane (TRIS), NaCl, $CaCl_2$, and NaOH were purchased from Sigma–Aldrich (St Louis, MO, USA). Deionized water was acquired using the Milli-Q System (Millipore Corporation, Bedford, USA).

The MITR was prepared as described in our earlier work (Mao et al., 2017). Aldehyde-activated Convective Interaction Media® radial column (outer diameter (D), 1.86 cm; inner diameter (d), 0.67 cm; height (h), 0.42 cm; volume, 1.0 mL) with a nominal pore size diameter of 2.1 μm was used as the immobilization support. The amount of immobilized trypsin was 5.0 ± 0.2 mg/mL monolith. After immobilization, the permeability of the MITR was 2.45×10^{-12} m^2 measured using deionized water.

2.2. Hydrolysis of β-Lg

2.2.1. Hydrolysis by free trypsin

β-Lg solution (50 mg/mL) was hydrolyzed by free trypsin with an enzyme-substrate ratio (E/S) of 0.1% (w/w) at 25 ± 1 °C. An additional experiment was conducted at E/S of 1% and pH 7.8 to reach the maximum DH. The pH of substrate solution was adjusted to 7.2, 7.8, 8.1, 8.5, and 8.7. A TitroLine alpha plus auto-titrator (Schott AG, Mainz, Germany) was used for the pH-stat hydrolysis. The detailed calculation of DH and reaction velocity was described in our published work (Mao et al., 2017).

2.2.2. Hydrolysis by immobilized trypsin

The MITR was integrated into an ÄKTA system (GE Healthcare Bio Sciences). In the single flow-through approach, a native β-Lg solution with a concentration of 3 mg/mL was pumped through the MITR at a flow rate of 0.5 or 10 mL/min. The pH of the applied solution was adjusted to 7.2, 7.8, 8.1, 8.5, and 8.7. Using the recirculation flow approach, 100 mL of a native β-Lg solution (10 mg/mL) were recirculated through the MITR at 10 mL/min, pH 7.8 and 8.7 were investigated. The pH-stat method was used to maintain a constant pH and to follow the evolution of DH.

2.3. Analysis of hydrolysates

1-mL samples were taken at different intervals (0, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min) during the pH-stat process and stored at −20 °C. When free trypsin was used, 0.5 mL of the trypsin inhibitor solution (from soybean, 10 mg/mL) was mixed with each sample immediately to stop the hydrolysis. Samples (5 mL) produced by MITR using the single flow-through approach were stored at −20 °C for further analysis.

2.3.1. Quantification of residual native β-Lg

The native β-Lg content in samples was quantitatively determined via RP-HPLC using an Agilent 1100 series HPLC system (Agilent Technologies) and a PLRP-S 300A-8 μm Latek column (150 × 4.6 mm). The detail on the gradient was previously described by Leeb, Gotz, Letzel, Cheison, and Kulozik (2015). β-Lg A (99% purity, Sigma Aldrich, L7780) and β-Lg B (99% purity, Sigma Aldrich, L8005) were used as standards to build a calibration curve.

2.3.2. Separation and quantification of select peptides

The separation of hydrolysates was performed by RP-HPLC using the system described above. All samples were diluted only with the deionized water to a concentration of 4 mg/mL, excluding the sample with the maximum DH for free trypsin that was initially incubated with dithiothreitol (DTT) for 45 min at 37 °C and then mixed with chloroacetamide in dark for 30 min. A mobile phase of solvent A containing 0.1% (v/v) trifluoroacetic acid (TFA) dissolved in Milli-Q water and