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Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS

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ABSTRACT

Enzymatic hydrolysis of proteins is influenced, either positively or negatively, by the hydrolysis conditions, temperature, enzyme concentration and pH, as well as substrate pre-treatments, e.g. heat-denaturing, glyco-conjugation and/or cross-linking. Purified bovine β-lactoglobulin (96.0% nitrogen) was hydrolysed using trypsin (EC 3.4.21.4, bovine pancrease) at between 30 and 50 °C to degrees of hydrolysis (DHs) between 1 and ~9.0%. The time taken to reach the desired DH varied greatly, being shortest at 45 and 50 °C and longest at 30 °C. The hydrolysates were analysed by tandem liquid chromatography–electrospray ionisation time-of-flight mass spectra (LC–ESI-TOF/MS) and results showed that the detectable peptides, at both 30 °C and 35 °C, were similar at DH 1%. However, not only were the detectable peptides produced at 40–50 °C different from those produced at lower temperatures, but the trypsin released peptides due to non-specific hydrolysis of β-Lg. The pattern resembled a shift of trypsinolysis towards chymotrypsinolysis, probably due to steric 'stretching' and increase of the catalytic pocket, thus allowing bulky amino acids to be processed. Hydrolysis at 30 °C to DH 5% and 10% also led to the release of peptides due to non-specific cleavage by trypsin. These results indicate that trypsin could only release peptides in a predictable manner at temperatures near, but lower than, the declared optimum of 37 °C. Above this temperature and above DH 5–10% at 30 °C, hydrolysis followed a mixed trypsin- and chymotrypsin-like activity. Lys–Pro, Lys–Ile(–Pro) and Lys–Phe bonds remained stable to trypsin at all temperatures. Some peptides with a high content of hydrophobic amino acids were undetected by ESI-TOF/MS, probably due to their poor ionisation.

1. Introduction

The hydrolysis of proteins produces peptides with improved functional, immunological and bioactive properties superior to those of the native protein. Many health effects have been found and reported in bioactive peptides. For example, blood pressure-lowering ability, cholesterol-lowering effects, antioxidant activities and enhancement of mineral absorption (Hartmann & Meisel, 2007). The bioactive peptides are used in products such as sport-drinks, infant- and hypoallergenic-foods to provide value-addition and enhance consumer health.

Many peptides, of animal protein origin, with bioactive potential have been discovered. Most of them were isolated from milk-based products or produced from milk proteins, mainly caseins and whey proteins (Hartmann & Meisel, 2007). Milk proteins consist of caseins (78.3%), whey proteins (19%) and miscellaneous proteins (2.7%). Whey proteins, which include serum albumin, immunoglobulins, α-lactalbumin and β-lactoglobulin (β-Lg), are more reactive because they dissolve in the serum. β-Lg, the major protein in whey, is responsible for most of the bioactive properties of whey proteins (Hernández-Ledesma, Recio, & Amigo, 2008). Due to their emulsifying, interfacial and gelation properties, tryptic peptides from whey proteins are of great interest to the food industry (Mota et al., 2006).

β-Lg is a small, soluble protein with a globular structure and a monomer molecular weight of 18.3 kDa. It belongs to the lipocalin family and has the ability to bind and transport small molecules, e.g. fatty acids and vitamin A, within a hydrophobic core (Ham-

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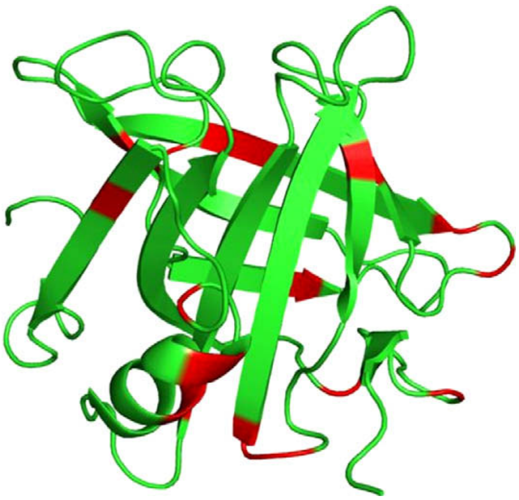

Fig. 1. Molecular structure of β-lactoglobulin with preferential cleavage sites of trypsin highlighted in red. (Compiled on http://polyview.cchmc.org on December 4th 2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bling, McAlpine, & Sawyer, 1992). β-Lg is the major whey protein in the milk of ruminants and represents approximately 10% of the total milk protein and 53% of the whey protein (Walstra, Wouters, & Geurts, 2006). It is not found in the milk of humans (Hambling et al., 1992). The primary structure consists of 162 amino acids, of which 15 are lysine and three are arginine residues. These amino acids form peptide bonds that are preferential cleavage sites for the enzyme trypsin, and are highlighted in Fig. 1.

An important task in the production of functional foods containing bioactive peptides is the production of those peptides during food processing or by deliberate enzyme hydrolysis of proteins. To make that possible it is important to know how hydrolysis is influenced by different parameters, e.g. hydrolysis pH and temperature. Hydrolysis releases free carboxyl and free amino groups which are more or less ionised, depending on the pH of the reaction. At a pH value of 7.5, at which the experiments of this work took place, the amino group is half protonated while the carboxyl group is fully dissociated. If left uncontrolled during hydrolysis at alkaline pH, the pH would decrease, leading to enzyme denaturing. Keeping the pH constant (or static, hence pH-stat) is possible by the addition of a known amount of base, e.g. NaOH, of a known concentration. The relationship between the volume of base consumed and the peptide bonds cleaved during hydrolysis is proportional. This proportionality is utilised to calculate the degree of hydrolysis (DH) by the pH-stat method. The DH is defined as the percentage of peptide bonds cleaved (h) compared to the total available peptide bonds (h_{tot}) in a protein substrate (Eq. (1)). It is this relationship which most closely reflects the catalytic action of the enzyme (Adler-Nissen, 1986).

$$\mathrm{DH} = \frac{h \cdot 100\%}{h_{\text{tot}}} \tag{1}$$

Using Eq. (1), it is possible to follow the DH during the enzyme reaction. The DH is calculated directly from the consumption of base during the hydrolytic degradation, which is the principle of the pH-stat technique, according to Eq. (2).

$$\mathrm{DH} = \frac{V_{\mathrm{B}} \cdot N_{\mathrm{B}} \cdot 100\%}{\alpha \cdot \mathrm{MP} \cdot h_{\text{tot}}} \tag{2}$$

where, V_{B}, base consumption in ml; N_{B}, normality of the base; α average degree of dissociation of the -NH groups; MP, mass of protein in g; h, the hydrolysis equivalents in meqv/g protein; h_{tot}, total number of peptide bonds in the protein substrate (7.2 meqv/g of protein in this work)

For our work, we calculated the value of h_{tot} to be 7.20 for β-Lg, which is probably the first time it has ever been reported in the literature.

β-Lg exists as a dimer at physiological pH and temperature values (Hambling et al., 1992). A disulfide bond near the C-terminus (Cys_{66}–Cys_{160}) and one in the interior of the molecule (Cys_{106}–Cys_{119}) stabilize the compact globular conformation. When exposed to higher temperatures or pH values (above 8), the dimer dissociates. Below pH 5.5, β-Lg associates to particles of up to eight units (Fig. 2). At a pH below 3.5 no association occurs (Walstra et al., 2006). These properties are useful for both the manufacture and during the hydrolysis of β-Lg because they influence its solubility, and hence enzyme penetration and hydrolysis.

Bovine trypsin (EC 3.4.21.4) is a serine protease with a molecular weight of 23.3 kDa and consisting of 226 amino acids. Due to its nucleophilic active site, with an aspartic acid 189 sitting in a specificity pocket, the enzyme cleaves predominantly at the positively charged side chains of arginine-X and lysine-X, with X being any amino acid except a proline (Olsen, Ong, & Mann, 2004). As the lysine residue in β-Lg at position 47 is followed by a proline residue, this peptide bond is unlikely to be cleaved during hydrolysis by trypsin, with proline's imino acid "kink-like" bond structure providing a hindrance to trypsinolysis. Trypsin's optimal performance conditions are at pH 7.8 and 37 °C (Olsen et al., 2004) although it retains varied levels of activity at other pH and temperature values as well.

For the hydrolysis of β-Lg, in addition to substrate concentration and enzyme to substrate ratio (E/S), external parameters, such as the hydrolysis pH and temperature, have to be considered. Every enzyme has a specific pH optimum at which it works best. Lowering or increasing that value in the hydrolysis environment, leads to an influence on the enzyme activity. In the pH range 7–9 trypsin seems not to be affected by a change of pH (Mota et al., 2006). Conversely, the pH has a strong influence on the solubility and viscosity of protein solutions (Pelegrine & Gasparetto, 2005). This is because the side groups of proteins are deprotonated at a high pH and protonated at a low pH to a large extent. Additionally, β-Lg undergoes autolysis under high alkaline conditions (Christen-

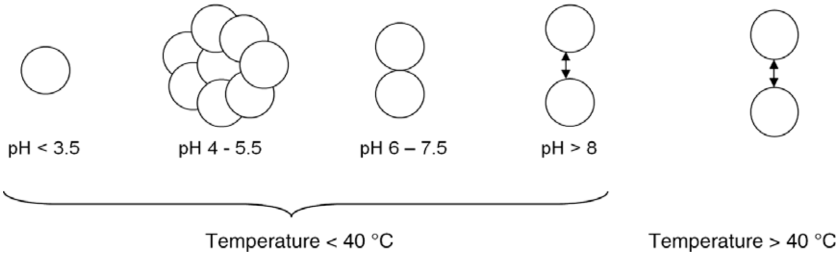

Fig. 2. Conformation of β-lactoglobulin in different environments.