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Effect of Maillard induced glycation on protein hydrolysis by lysine/arginine and non-lysine/arginine specific proteases

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ABSTRACT

Enzymatic protein hydrolysis is sensitive to modifications of protein structure, e.g. Maillard reaction. In early stages of the reaction glycation takes place, modifying the protein primary structure. In later stages protein aggregation occurs. The specific effect of glycation on protein hydrolysis was studied using α-lactalbumin glycated with D-glucose at 50 °C (0–10 h). This resulted in proteins with different degrees of glycation (DG = 0–63%) without changes in secondary, tertiary and quaternary structure. These glycated proteins were hydrolyzed by lysine/arginine specific proteases (bovine and porcine trypsin) or by non-lysine/arginine specific proteases (*Bacillus licheniformis* protease (BLP), α-chymotrypsin and subtilisin A). For bovine and porcine trypsin, the maximal degree of hydrolysis decreased linearly with 65% from untreated to maximal glycated protein (DG = 63%). This means trypsin cannot hydrolyze glycated cleavage sites. BLP and subtilisin A hydrolyses were independent of glycation, while α-chymotrypsin cannot hydrolyze cleavage sites with glycated binding sites. This means for non-lysine/arginine specific proteases, the effect of glycation depends on the enzyme sensitivity towards modifications on binding sites. Since not all cleavage sites are efficiently used by the enzymes, the extent of the effects depends on the enzyme selectivity towards cleavage sites (for trypsin) or cleavage sites near glycation sites (for α-chymotrypsin). Combining the results of all proteases, an equation was derived describing the effect of modification of protein primary structure on the extent of hydrolysis based on the enzyme specificity, selectivity and binding site sensitivity.

1. Introduction

Enzymatic protein hydrolysis is sensitive to modifications of the protein structure, which can be induced by industrial processes, e.g. Maillard reaction. In food production, the Maillard reaction is sometimes applied to improve the techno-functional properties of proteins, e.g. emulsion stability (Darewicz & Dziuba, 2001) and foam stability (Wierenga, Van Norél, & Basheva, 2009). In other cases, the reaction is a non-desired side-effect of production and storage of food products. The Maillard reaction results in glycation of lysine residues, but the proteins can also undergo unfolding or aggregation. These factors may all affect protein hydrolysis during digestion, as well as during industrial production of protein hydrolysates. The effects may further depend on the enzymes used for the protein hydrolysis. This study focusses on a quantitative understanding of the effects of glycation on enzymatic protein hydrolysis.

The early stage of the Maillard reaction changes the protein primary structure. In the first step of the Maillard reaction, also called glycation, the reducing end of a carbohydrate reacts with a free amino group, which results in Amadori compounds. This amino group can be the side chain of a lysine residue, an arginine residue or at the N-terminal amino acid in a protein. For example, the glycation of α-lactalbumin with glucose at 60 °C with 65% relative humidity for 8 h resulted in a mixture of variants with 6–13 glucoses on the α-lactalbumin molecule (ter Haar, Schols, & Gruppen, 2011). In perspective of protein hydrolysis, the glycation can change the protein primary structure. In later stages of the reaction, secondary reactions take place, which lead to protein aggregation (Chevalier, Chobert, Mollé, & Haertlé, 2001), e.g. through lysine-arginine cross-links (Biemel, Reihl, Conrad, & Lederer, 2001). In addition, the heating itself may result in aggregation through lysinoalanyl cross-links (Fritsch, Hoffmann, & Klostermeyer, 1983) and/or non-covalent aggregation. These

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reactions can influence the secondary, tertiary and quaternary structure of the protein. Each of these structural changes can influence the susceptibility of the protein to enzymatic hydrolysis. The occurrence of the secondary reactions also confuse published results of the influence of glycation on protein hydrolysis. For example, one study showed that the pancreatic hydrolysis rate of whey protein was decreased by glycation with dextrans (Böttger, Etzel, & Lucey, 2013). Also, the hydrolysis of two infant formulas made of soy protein isolates by a mixture of trypsin, chymotrypsin and intestinal peptidase showed that the sample with the highest degree of glycation resulted in the lowest degree of hydrolysis (Pereyra Gonzáles, Naranjo, Malec, & Vigo, 2003). In contrast to these findings, other studies showed that glycation has been shown to increase the extent of protein hydrolysis. In the same study, when the hydrolysis of two infant formulas made of bovine milk powder were compared, the sample with the highest glycation had the highest degree of hydrolysis (Pereyra Gonzáles et al., 2003). Another indication of increased hydrolysis with increased glycation was obtained from the comparison of the hydrolysis of ultra-high-temperature (UHT) milk compared to pasteurized milk by pepsin and pancreatin. The amount of intact proteins remaining after hydrolysis was lower in the UHT milk than in the pasteurized milk, despite higher levels of Maillard reaction indicators (carboxymethyllysine and lactulosylysine) in the UHT milk (Wada & Lönnerdal, 2014). The inconsistent reports on the effects of Maillard reaction on subsequent enzymatic hydrolysis may be in part due to the fact that not only glycation, but also aggregation may have occurred in the samples. It is not clear whether the dominant factor of the influence was glycation or aggregation because aggregation was also reported to affect hydrolysis (Pinto et al., 2014). One study reported that the proportion of total glycated amino groups in β-casein samples was up to 40%, while 60% of the proteins were covalently aggregated (Bhatt et al., 2014). It was observed that the initial rate of plasmin hydrolysis linearly decreased 60% with the maximal glycated sample, which also had the highest amounts of aggregates (Bhatt et al., 2014). Another reason for the variety in reported effects of glycation on hydrolysis could be that different enzymes were used.

For enzymes that are specific for lysine and arginine residues, e.g. trypsin and plasmin, it is commonly assumed that the hydrolysis is negatively influenced by glycation because the glycated cleavage site cannot be utilized by trypsin. As a consequence, the extent of trypsin hydrolysis is expected to decrease by glycation. Most studies that annotated peptides after tryptic hydrolysis of glycated proteins did not find peptides that were cleaved after glycated lysine/arginine residues (Lapolla et al., 2004; Morgan, Léonil, Mollé, & Bouhallab, 1997). Only two previous studies reported cleavage peptide bonds after glycated lysines, based on annotation of the formed peptides. They reported that 1 out 19 (Moreno, Quintanilla-López, Lebrón-Aguilar, Olano, & Sanz, 2008) and 3 out of 16 (Carulli, Calvano, Palmisano, & Pischetsrieder, 2011) of the peptides formed during the hydrolysis were cleaved after glycated lysines, respectively. A study using model peptides showed that the replacement of the charged side chain of lysine by the neutral side chain of heptyline resulted in a decrease of the hydrolysis rate constants by a factor of 10^2-10^6 (Sanborn & Hein, 1968). The attachment of carbohydrate on the lysine side chain by glycation might have an even larger influence because not only the charge, but also the size of the side chain of lysine is modified.

For enzymes that are not specific for lysine and arginine residues, information on the influence of glycated residues proximate to the cleavage sites on protein hydrolysis has not been reported. However, it has been mentioned that the binding of the enzyme towards the cleavage site depends on the interaction between the catalytic environment of the enzyme and the binding environment of the substrate (Schechter & Berger, 1967). According to this information, the P4 to P4' positions (binding site) are the amino acids that come before and after the cleavage site (P1 position) on the primary sequence (Schechter & Berger, 1967). Hence, if the glycation sites are on the binding site positions, it is possible that the glycation influences protein hydrolysis catalyzed by non-lysine/arginine specific enzymes as well.

In addition to the above, it should be noted that during hydrolysis of native proteins, not all cleavage sites are hydrolyzed at an equal rate. This means that when hydrolyzing a protein by an enzyme, the final DH might not reach the theoretical maximum. It has been shown for instance that bovine trypsin has different hydrolysis rates towards the various lysine and arginine residues within the native β-lactoglobulin and β-casein (Cheison, Lai, Leeb, & Kulozik, 2011; Vorob'ev, Dalgalarrondo, Chobert, & Haertlé, 2000). Recently, the relative hydrolysis rate constants of the various cleavage sites on a protein were determined quantitatively and defined as selectivity (Butré, Sforza, Gruppen, & Wierenga, 2014). It was shown that 6 out of 26 of the cleavage sites in β-lactoglobulin were not cleaved by *Bacillus licheniformis* protease (BLP) and 9 cleavage sites were responsible for 95% of the total hydrolysis rate constant (Butré et al., 2014). This means that the glycation of some sites might have a larger effect on the total hydrolysis than the glycation of other sites.

In the present study, the effects of glycation on enzymatic hydrolysis were studied using α-lactalbumin and D-glucose. The hydrolysis was performed using lysine/arginine specific enzymes (i.e. bovine and porcine trypsin) and non-lysine/arginine specific enzymes (i.e. BLP, α-chymotrypsin and subtilisin A). The aim is to determine whether altering protein primary structure by glycation influences the protein hydrolysis catalyzed by enzymes with various specificities and the quantitative relationship between the degree of glycation and degree of hydrolysis.

2. Materials and methods

2.1. Materials

α-Lactalbumin (α-LA) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). The monoisotopic mass of the protein is 14,178 Da, as found in ExPASy PeptideMass (P00711) (http://web.expasy.org/peptide_mass). Mass spectrometry (MS) analysis showed that there were no naturally glycosylated variants of the protein present in the sample. Of the α-LA powder, the total protein content is ~92.8% (w/w), as determined using Dumas method (N × 6.25, based on the amino acid composition of the α-LA as described in Uniprot P00711, www.uniprot.org). Of the total protein content, ~90% is α-LA, based on the proportion of α-LA UV_{214} peak area of the total UV_{214} area using reversed phase ultra-high performance liquid chromatography (RP-UHPLC) (Butré, Sforza, Wierenga, & Gruppen, 2015). The other proteins present were β-lactoglobulin and bovine serum albumin according to MS results (data not shown). The α-LA was free of lactose and glucose, as determined by high performance anion exchange chromatography (HPAEC, data not shown). This was also confirmed by the fact that no glycation by glucose or lactose was found after heating the protein for 10 h at 50 °C with 65% relative humidity (determined using MS, data not shown). Based on the results from circular dichroism (Heijnis, Wierenga, Van Berkel, & Gruppen, 2010), ~72% of the α-LA was in the apo form (data not shown).

Bovine trypsin (EC 3.4.21.4, Sigma-Aldrich, St. Louis, MO, USA) was treated with tosyl-phenylalanyl-chloromethyl ketone (TPCK) to inactivate any chymotrypsin activity present (chymotrypsin