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Conjugation of gluten hydrolysates with glucosamine at mild temperatures enhances antioxidant and antimicrobial properties

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ABSTRACT

Gluten represents one of the principal by-products of the wheat starch industry. Peptides obtained by wheat hydrolysis can be used for specific functional and biological activities, albeit at relatively low yields. Although the Maillard reaction (glycation) is widely used to increase functionality of proteins, its main disadvantage is the production of undesirable compounds due to high processing temperature. In this research, functional and biologically active glycopeptides were obtained from gluten. Alcalase or Flavourzyme proteases were used to hydrolyse gluten protein, and the resulting peptides were conjugated with glucosamine by enzymatic glycosylation, using transglutaminase, or through glycation. Both reactions were performed at mild temperatures (25 or 37 °C). The formation of glycopeptides depended mostly on the glycation process, as demonstrated by MALDI-TOF-MS. The bioactivities of the conjugated hydrolysates were compared to the native hydrolysates. Although a reduction in the anti-ACE activity was detected, improved DPPH scavenging activity and enhanced antimicrobial activity against *Escherichia coli* were observed in the glycated Alcalase-derived hydrolysates and in the glycated Flavourzyme-derived hydrolysates, respectively. This study showed that mild conditions are an alternate approach to the traditional Maillard process conducted at elevated temperatures in creating conjugated gluten hydrolysates with enhanced bioactivities.

1. Introduction

Wheat gluten, a by-product of the wheat starch industry, is produced in large scale throughout the world. Due to its modest price, it competes with milk and soy proteins as a protein source (Kong, Zhou, & Qian, 2007). Wheat gluten peptides obtained by hydrolysis has attracted the interest of food industries since they possess functional properties, such as solubility, foaming and emulsifying capacities (Kong et al., 2007; Wang, Zhao, Zhao, Bao, & Jiang, 2007) and can create hypoallergenic nutritional mixtures (Daya, Augustina, Bateyb, & Wrigleyb, 2006). Additional studies reported some antioxidant properties of gluten hydrolysates, including the capacity to inhibit the linoleic acid peroxidation or to quench the DPPH radical.

The enhancement of the functional and biological properties of glycoproteins is a new frontier being explored by several teams (Bielikowicz et al., 2010; Liu, Ru, & Ding, 2012). The importance of specific glycopeptides has been emphasised in some physiological processes, such as the immune-system and inflammation, brain development, endocrine system and fertilization (Spiro, 2002). The conjugation of sugars and amino acids can be produced both through enzymes and spontaneously, under specific conditions. Enzymatic glycosylation is one of the main post-translational processes occurring in eukaryotic and prokaryotic cells (Spiro, 2002). On the other hand, glycation is the term universally used to define the chemical bonding of sugars with proteins or peptides, and occurs spontaneously both in the human body and in food systems (Liu et al., 2012). As reviewed by Oliver, Stanley, and Melton (2006), glycation via the Maillard reaction is one of the most studied processes in food science. Although the Maillard reaction improves several functional properties of food proteins (Liu et al., 2012), the main disadvantages are: i) the use of high temperatures or prolonged heat treatment, and ii) the formation of undesirable and unhealthy compounds during the late stages of the reaction (Brands, Alink, Boekel, & Jongen, 2000; Guerra-Hernandez, Gomez, Garcia-Villanova, Sanchez, & Gomez, 2002). Heat is necessary in

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order to overcome the low reactivity of amino acids and, in particular, sugars (Wang & Ismail, 2012). On the contrary, Kraehenbuehl, Davidek, Devaud, and Mauroux (2008) reported that amino-sugars had higher reactivity compared to normal sugars. In fact, Hrynets, Ndagijimana, and Betti (2013) demonstrated that the amino-sugar glucosamine (GlcN) is a potential candidate for protein glycation at mild temperatures (37–40 °C). GlcN can be obtained through the hydrolysis of chitosan, the main by-product from shrimp and other crustacean shell processing. The acetylated form of GlcN is also fundamental for the formation of bacterial cell wall and human cartilage (Wang, Laverty, Dumitriu, Plaas, & Grynpas, 2007). Binding amino-sugars with peptides through transglutaminase (TGase) has already been proposed by Jiang and Zhao (2010), however their results were inconclusive since they did not give any direct evidence (no chemical proof) of glycosylation. TGase derived from eukaryotic and prokaryotic sources is extensively used in food processing, and is responsible for different reactions depending on the situation. On one hand, the reaction can be driven towards the formation of inter- and intra-molecular cross-linkages if the primary amino groups derive only from another amino acid, such as lysine, or towards deamidation in the absence of primary amino groups. On the other hand, the acyl-transfer between the γ-carboxyamide group of a molecule and the primary amino group in another can occur, creating a stable bond that can resist proteolysis (Greenberg, Birckbichler, & Rice, 1991).

The objective of this study was to produce glycopeptides at mild temperature (25 and 37 °C) and to evaluate their bioactivities. Two different methodologies were used: i) glycosylation, utilizing the amino group of GlcN and the high glutamine (Gln) content of gluten peptides as substrates for the TGase and ii) glycation, using the high reactivity of GlcN to conjugate it with wheat gluten hydrolysates. The antioxidant, antimicrobial and anti-hypertensive bioactivities of the conjugated hydrolysates were compared to the native hydrolysates.

2. Materials and methods

2.1. Materials

Alcalase (Alc, EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g) and Flavourzyme (Flv, EC 3.4.11.1, from *Aspergillus oryzae*, 500 U/g), GlcN hydrochloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Angiotensin I-converting enzyme (ACE), N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FA-PGG), TGase from guinea pig liver were purchased from Sigma–Aldrich (St. Louise, MO). All chemicals used in Size Exclusion Chromatography (SEC) and MALDI-TOF were of HPLC grade supplied by Sigma–Aldrich (St. Louise, MO), whereas other chemicals were of analytical grade.

2.2. Production of hydrolysates

Wheat gluten, 5 g/100 mL, was resuspended in a 0.05 mol/L $(NH_4)HCO_3/NH_4OH$ buffer at a final pH of 8. It was mixed with a homogeniser (Fisher Scientific, Power Gen 1000 S1, Schwerte, Germany) for 2 min and then heated at 80 °C for 10 min. The gluten mixture was cooled to 50 °C before adding Alc or Flv at the enzyme-to-substrate ratio 1:10 (mL:g). Then the mixture was incubated in a shaker (200 rpm) at 50 °C for 3.5 h. In order to stop the proteolysis, a subsequent incubation at 80 °C for 10 min was performed. The hydrolysates were centrifuged at 10,000 × g (10 °C) for 15 min, and then filtered by Whatman No. 1 filter paper. The amount of filtrate collected was 98.8 mL and 97.8 mL starting from 100 mL of Alacalse and Flavourzyme hydrolysates, respectively. The filtrate was lyophilized and stored at −18 °C.

2.3. Preparation of glycated/glycosylated peptides

Exactly 1.5 g of the lyophilized hydrolysate powder (from Alc or Flv, respectively) and an equal amount of GlcN was dissolved in 30 mL of 0.05 mol/L $(NH_4)HCO_3/NH_4OH$ buffer (pH 7.0 ± 0.5) and each incubated at 25 °C and 37 °C, respectively, for 3.5 h in the presence of 5 mmol/L calcium chloride. Samples with GlcN were subjected to incubation with or without TGase (2 Unit/g of lyophilized hydrolysate) at pH 7.5. Controls containing only lyophilized hydrolysate were incubated at the same temperature without GlcN. The work plan and sample labels are summarized in Fig. 1. At the end of the incubation, all the mixtures were passed through a 0.2 μm syringe filter (13 mm, Mandel, Ontario) followed by ultrafiltration with a molecular weight cut-off membrane of 10 kDa (3900 × g, 20 min, 10 °C, Amicon Ultra Centrifugal filters (Millipore, Cork, Ireland)) in order to remove the TGase. Whereas, the excess of GlcN was removed by dialysis membrane with a molecular weight cut-off of 100–500 Da (Spectrum Laboratories, TX). The retentates were collected, lyophilized, and stored at −18 °C until thawed for chemical and bioactive characterization.

2.4. Degree of hydrolysis (DH)

The measurement of DH was carried out according to Nielsen, Petersen, and Dambmann (2001). Serine has been used as standard for hydrolysis determination. Protein content of each gluten sample was assessed. The percent DH was calculated according to Alder-Nissen (1986).

2.5. Size exclusion chromatography

Samples were subjected to size exclusion chromatography using a 120 mL HiLoad 16/60 Superdex 200 pg column (GE Healthcare Amersham Biosciences) connected to fast protein liquid chromatography (GE Healthcare Amersham Biosciences) immediately after hydrolysis. A sample volume of 500 μL (1 mg/mL) was injected and eluted isocratically at 1.2 mL/min with 50 mmol/L phosphate buffer containing 0.15 mol/L NaCl. The gluten peptides and the glycated/glycosylated peptides were subjected to size exclusion chromatography using a Superdex Peptide 10/300 GL (GE Healthcare Amersham Biosciences). A sample volume of 100 μL (1 mg/mL) was injected and eluted isocratically at 0.5 mL/min with 50 mmol/L phosphate buffer containing 0.15 mol/L NaCl. Eluted molecules were detected at 215 nm and 280 nm. The mass calibration was performed using a protein mixture (200–12.4 kDa) or a peptide mixture (12340–76 Da), both obtained from Sigma–Aldrich.

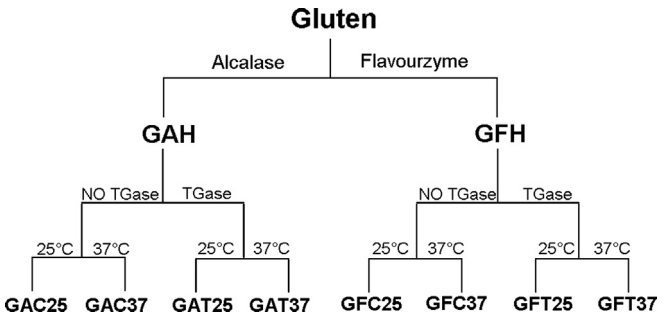

Fig. 1. Work flow for the production of hydrolysates and glycopeptides and their respective labels. Wheat gluten hydrolysates were obtained with Alcalase (GAH) and Flavourzyme (GFH). GAH and GFH represent the controls. The hydrolysates were conjugated with glucosamine (GlcN), with (GAT or GFT) or without (GAC or GFC) the action of Transglutaminase (TGase), at 25 or 37 °C.