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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.

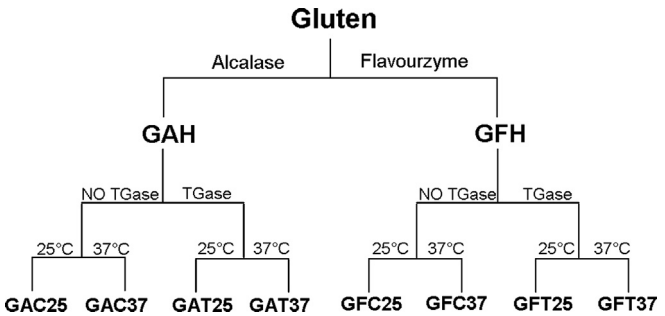


Fig. 1. Work flow for the production of hydrolysates and glycopeptides and their respective labels. Wheat gluten hydrolysates were obtained with Alcalase (GAH) and Flavourzyme (GFH). GAH and GFH represent the controls. The hydrolysates were conjugated with glucosamine (GlcN), with (GAT or GFT) or without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

2.6. Determination of peptides and glycopeptides molecular weights by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI/TOF-MS)

For the MALDI-TOF-MS analysis, the protein hydrolysate samples were diluted tenfold in 50 mL/100 mL acetonitrile/water + 0.1 mL/100 mL (v/v) trifluoroacetic acid/water. One microlitre of each sample was mixed with 1 μL matrix of α-cyano-4-hydroxycinnamic acid (4-HCCA, 10 mg/mL in 50 mL/100 mL acetonitrile/water + 0.1 mL/100 mL trifluoroacetic acid/water). One microlitre of the sample/matrix solution was then spotted onto a stainless steel target plate and allowed to air dry. All mass spectra were obtained using a Bruker Ultraflex MALDI/TOF-MS (Bruker Daltonic, GmbH). Ions were analysed in positive mode after acceleration from the ion source by 25 kV. External calibration was performed using a standard peptide mixture from Sigma–Aldrich.

2.7. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Yen and Wu (1999) as modified by Hsu (2010). The whole mixture was mixed thoroughly and incubated for 30 min in the dark at room temperature and then the absorbance at 517 nm was measured using a spectrophotometer (V-530 Jasco, Easton, Maryland). The percentage of radical scavenging activity was calculated with the following equation:

$$\text{DPPH\%} = ((\text{Abs control} - \text{Abs sample})/\text{Abs control} \times 100)$$

where Abs control is the absorbance of reference solution containing only DPPH and water, and Abs sample is the absorption of the DPPH solution with sample after 30 min. Methanol was used as a blank. The EC50 value (i.e the concentration required for a 50% reduction of the activity) was calculated using Prism 5 software.

2.8. Antimicrobial activity

The antimicrobial activity of the hydrolysates and the glycated/glycosylated hydrolysed samples was determined by the microdilution technique against *Escherichia coli* AW1.7 and *Bacillus subtilis* FAD 110 (both from University of Alberta). The bacterial strains were grown at 37 °C in Luria–Bertani Broth (LB) medium until used. The microbial cultures were prepared at 10^4 CFU/mL in the 96-well microplates. Diluted samples of protein hydrolysates were dispensed into the wells providing final concentrations in the range of 40–0.097 mg/mL. Growth controls and sterility controls were also tested. Plates were incubated at 37 °C for 24 or 48 h. The Minimal Inhibitory Concentration (MIC) value was defined as the lowest concentration preventing any discernible bacterial growth. The Minimal Bactericidal Concentration (MBC) was defined as the lowest concentration after 10 μL from a MIC well were plated in LB agar plate and incubated at 37 °C for 24 h with no discernible bacterial growth and thus representing the capacity to completely kill all the bacteria. All the MIC and MBC values were evaluated in triplicate.

2.9. Angiotensin I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory assay was performed according to Shalaby, Zakora, and Otte (2006) and Vermeirssen, Van Camp, and Verstraete (2002). Buffers were made according to Hou, Chen, and Yaw-Huei (2003) and volumes were adjusted to fit into 96-well plates. The enzyme activity at 37 °C was based on the initial linear rate of change in absorbance at 340 nm and recorded every 3 min for 30 min. The control contained all reaction components with water instead of the protein hydrolysate sample. Blanks with no enzyme (substituted by water) or with no substrate (substituted by 50 mmol/L Tris–HCl buffer, pH 7.5, containing 0.3 mol/L NaCl) were also used. The ACE activity was expressed as the slope (m) of the decrease in absorbance at 340 nm and ACE inhibition (%) calculated as follows:

$$\%\text{ACE inhibition} = \left(1 - \left(m_{\text{sample}}/m_{\text{control}}\right)\right) \times 100$$

The IC50 values were determined by Prism 5 software.

2.10. Statistical analysis

The experiment was replicated three times, from the intact protein through to the final glycated hydrolysates. Each analysis or assay was done in triplicate. One way analysis of variance (ANOVA) was used and means were separated by using Duncan test. Means were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Characterization of wheat gluten protein and hydrolysates by size exclusion chromatography

Alc and Flv were used to obtain the peptides from wheat gluten to then produce glycopeptides enzymatically or spontaneously. Size exclusion chromatograms in Fig. 2 documented the creation of peptides with lower molecular weights (MWs). While size exclusion analysis does not give an exact value of the peptides MWs, the difference in the peak-width of Alc and Flv hydrolysates demonstrated that different pools of peptides were generated. The estimated DH by Alc was 4.70 ± 0.2%, lower than what was previously reported (Kong et al., 2007). Although the DH may depend on the selected method for its assessment, the low solubility of gluten at neutral pH could have also played an important role in the low DH obtained (Takeda, Matsumura, & Shimizu, 2001). On the contrary, Flv generated a more common DH value (11.85 ± 1.2%) as previously described (Yang, Zhang, Zou, & Chen, 2006).

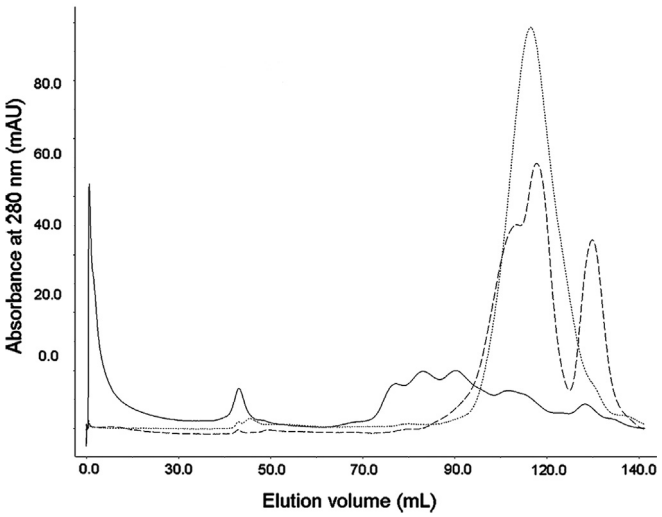
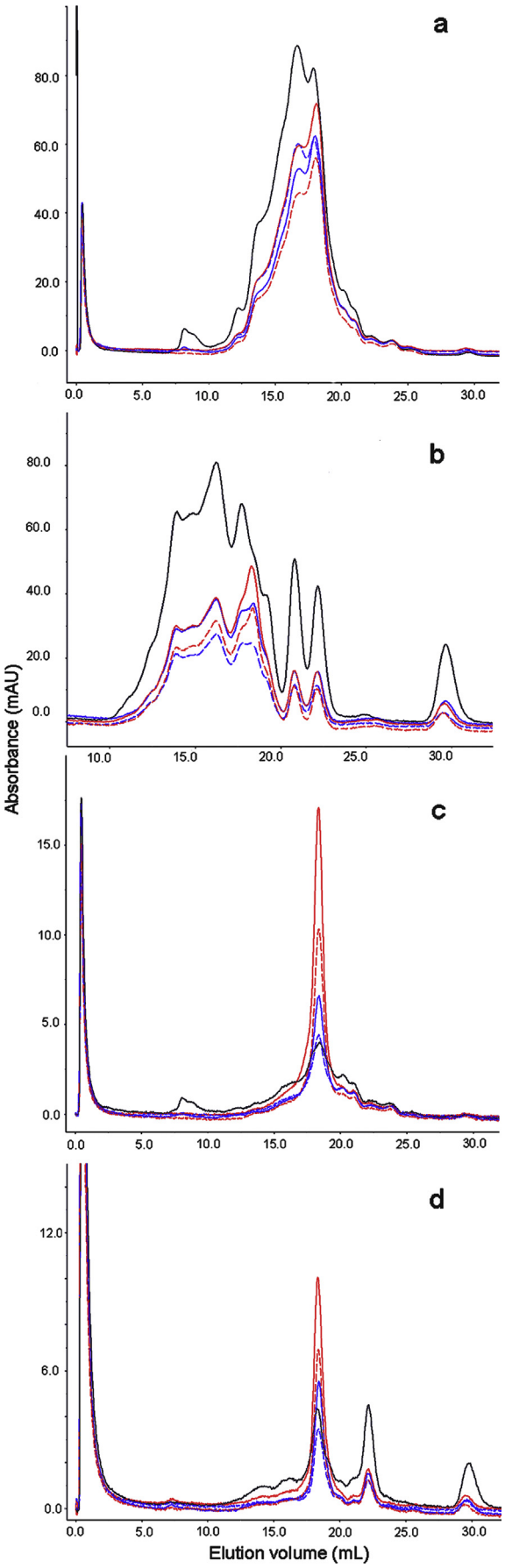


Fig. 2. Size exclusion chromatogram of wheat gluten and its hydrolysates. Lines represent wheat gluten (——), the hydrolysates with Alcalase (GAH, ……) and Flavourzyme (GFH, – – –), respectively. The shift to the right of GAH and GFH peaks means formation of smaller peptides obtained after hydrolysis. Analyses were conducted in duplicate.



3.2. Characterization of glycoconjugated wheat gluten hydrolysates by size exclusion chromatography

A specific profile for both peptides and conjugated hydrolysates was determined by size exclusion chromatography using a Superdex Peptide 10/300 GL. The chromatograms were recorded at 215 nm (Fig. 3a and b). In Fig. 3a gluten hydrolysed with Alc (GAH), and its glycated (GAC25 and GAC37, at 25 and 37 °C, respectively) and glycosylated forms (GAT25 and GAT37) are shown. Gluten hydrolysed with Flv (GFH) and its glycated (GFC25 and GFC37) and glycosylated form (GFT25 and GFT37) are reported in Fig. 3b. An overall decrease of absorbance at 215 nm in both cases was observed. This decrease was more relevant for peptides obtained with Flv (Fig. 3b) than those obtained with Alc (Fig. 3a). These results likely represent the extent of peptide conjugation, since the attachment of a sugar into a peptide molecule may have an effect on the final absorbance at 215 nm. Specifically, this wavelength allows the monitoring of peptide bonds inside the molecule, hence, the extent of a sugar nearby such bonds could interfere with them, causing an overall decrease in the final absorbance at 215 nm. On the other hand, an inversely proportional behaviour in peak intensity was detected in the chromatograms obtained at 280 nm (Fig. 3c and d). Aromatic amino acids normally, such as phenylalanine and tyrosine which are extensively present in wheat gluten, absorb at this wavelength. Generally, the formation of glycated peptides through Schiff base conjugation and Amadori rearrangement cannot be seen as a stable process, if it is compared with the glycosylated process where an isopeptide bond is formed. During the Maillard reaction, the sugar/peptide rearrangement can lead to a reduction in the overall final amino acid availability (Yoong, Walters, Tester, Gomes, & Ledward, 1994), particularly in those proteins containing a high proportion of basic amino acids (Bunn et al., 1979). On the contrary, since the aromatic amino acids are less involved in this process, their relative proportion can increase to result in a higher absorbance at 280 nm.

3.3. Evaluation of glycoconjugation of gluten peptides by MALDI-TOF/TOF-MS

MALDI-TOF/TOF-MS analysis was conducted to obtain a qualitative description of the glycopeptides produced and the MW profiles of the conjugated gluten hydrolysates were determined. Analysis of the controls (GAH and GFH) confirmed the production of different profiles when Alc or Flv were used. The Alc hydrolysates ranged from 500 to 3000 Da, whereas those obtained with Flv ranged from 300 to 4100 Da. Alc is an endopeptidase that generates a homogeneous distribution of MWs, whereas Flv produces a more scattered profile including low and higher MW, due to its mixture of endo- and exo-peptidases.

To estimate the extent of peptide glycation, all the signals present in the spectra were checked for their mass before and after the

Fig. 3. Overlay of size exclusion chromatograms corresponding to the hydrolysed samples before and after glycation/glycosylation. a and c, samples derived from Alcalase hydrolysis; b and d, samples derived from Flavourzyme hydrolysis. a and b represent the chromatograms obtained at 215 nm, while c and d represent chromatograms obtained at 280 nm. a and b showed a transition from a higher absorbance intensity at 215 nm in the hydrolysates (GAH and GFH, ——) to a lower absorbance intensity after glycation (25 °C, and 37 °C; —— and ——, respectively) and glycosylation (25 °C, and 37 °C, – – – and – – –, respectively). c and d showed a transition from a lower absorbance intensity at 280 nm in the hydrolysates (GAH and GFH, black line) to a higher absorbance intensity after glycation (25 °C, and 37 °C; blue and red solid lines, respectively) and glycosylation (25 °C, and 37 °C, blue and red dotted lines, respectively). Analyses were conducted in duplicate. Abbreviations are the same as in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reaction. In particular, the MW of glycopeptides was calculated by adding a mass shift of 161 Da, or any multiple of it, in case of additional sugars attached, to the original peptide MW, due to the production of 1 mol of water. Hrynets et al. (2013) proposed that a potential glycation reaction, with an initial release of water and a subsequent ammonia molecule, may occur with an additional mass shift of 144 Da to be added to the peptide MW. Also, a mass shift of 162 Da in the MW of the peptides was considered to identify the glycosylated peptide in the presence of TGase, due to the release of 1 mol of ammonia. According to these calculations, at least two different glycopeptides were produced through glycation of Flv hydrolysates, independently of the temperature used (Table 1). The same number of glycopeptides was also determined for the Alc hydrolysates (Table 2). The largest number of glycopeptides (four) was observed in GFC25 (Table 1). The MW profiles of the glycopeptides depended on their origin, with gluten Alc glycopeptides ranging mostly from 1095.5 to 3000.7 Da, and gluten Flv glycopeptides ranging from 1436.6 to 3177.7 Da. Since gluten is mainly composed of Gln/Glutamic acid (Glu) (~40%) (Wieser, 2007), the relatively low amount of glycosylated peptide obtained was unexpected. Indeed, only one glycosylated peptide was found in GFT37 (Table 1). In a previous preliminary experiment we considered an excess of sugar (peptide/GlcN, 1:3 w/w) to promote both glycation and glycosylation. However, five times less glycopeptides were produced comparing to the total amount obtained in the current experiment (data not shown). This may be due to the high reactivity of GlcN that undergoes to auto-condensation, especially if it is present in excess.

Even though the glycated process gave the best results in the present work, the glycosylation represents a novel approach and the use of TGase requires further investigation. In fact, in another work, Hong, Gottardi, Ndagijimana, and Betti (2014) showed that it was possible to obtain more glycosylated peptides using a different substrate (gelatin peptides). At the same time, an enhanced glycosylation of gluten peptides can be useful for other applications. Elli et al. (2012) reported the use of TGase as a tool to incorporate lysine into gluten to promote a higher lysine availability in wheat products, which is significant since lysine is a major limiting essential amino acid. Also, the incorporation of molecules to "protect" Gln to reduce the toxicity of gluten has not been widely studied.

Table 1
Mass shift of Flavourzyme-hydrolysed wheat gluten peptides subjected to glycoconjugation. To estimate the extent of glycation or glycosylation, the masses of the peptides present in the spectra were determined before (native peptide, GFH) and after the incubation with glucosamine at either 25 or 37 °C, both with (GFT25 and GFT37) or without transglutaminase (TGase) (GFC25 and GFC37). Only peptides showing a mass shift are reported; MALDI-TOF/TOF-MS analyses were conducted in duplicate. For the glycation, the MW of glycopeptides was calculated by adding a mass shift of 161 or 144 Da (or multiple) to the MW of the original peptide, depending on the release of 1 mol of H_2O or 1 mol of H_2O plus 1 mol of NH_3, respectively. For the glycosylation, a mass shift of 162 Da (or multiple) from the MW of the native peptide was considered, depending on the release of 1 mol of NH_3 after TGase reaction.

Treatment	*m/z* native peptide	Mass shift (Da)	*m/z* detected peptide	Type of modification	Intensity
GFC25	1291.5	483.4	1774.9	Glycation	1289.7
	1418.4	432.3	1850.7	Glycation	1741.5
	1546.3	432.3	1978.6	Glycation	1477.1
	2536.1	576.4	3112.5	Glycation	1006.9
GFT25	1418.4	432.3	1850.7	Glycation	1120.0
	1546.3	432.3	1978.6	Glycation	865.0
GFC37	1828.9	322.3	2151.2	Glycation	2970.2
	2150.0	576.4	2726.4	Glycation	2546.6
	2855.4	322.3	3177.7	Glycation	1460.0
GFT37	1292.5	144.1	1436.6	Glycation	3342.7
	1812.7	162.1	1974.8	Glycosylation	4799.2
	2855.4	322.3	3177.7	Glycation	1741.1

Table 2
Mass shift of Alcalase-hydrolysed wheat gluten peptides subjected to glycoconjugation. To estimate the extent of glycation or glycosylation, the masses of the peptides present in the spectra were determined before (native peptide, GAH) and after the incubation with glucosamine at either 25 or 37 °C, both with (GAT25 and GAT37) or without transglutaminase (TGase) (GAC25 and GAC37). Only peptides showing a mass shift are reported; MALDI-TOF/TOF-MS analyses were conducted in duplicate. For the glycation, the MW of glycopeptides was calculated by adding a mass shift of 161 or 144 Da (or multiple) to the MW of the original peptide, depending on the release of 1 mol of H_2O or 1 mol of H_2O plus 1 mol of NH_3, respectively. For the glycosylation, a mass shift of 162 Da (or multiple) from the MW of the native peptide was considered, depending on the release of 1 mol of NH_3 after TGase reaction.

Treatment	*m/z* native peptide	Mass shift (Da)	*m/z* detected peptide	Type of modification	Intensity
GAC25	838.9	483.4	1322.3	Glycation	10154.9
	951.4	144.1	1095.5	Glycation	9469.5
	1193.5	483.4	1676.9	Glycation	8141.5
GAT25	838.9	483.4	1322.3	Glycation	9620.9
	1193.5	483.4	1676.9	Glycation	8593.1
	2856.6	144.1	3000.7	Glycation	2175.3
GAC37	838.9	483.4	1322.3	Glycation	7078.8
	1193.5	483.4	1676.9	Glycation	7608.8
GAT37	838.9	483.4	1322.3	Glycation	1485.0
	1193.5	483.4	1676.9	Glycation	1494.0
	1882.2	288.2	2170.4	Glycation	1185.0

3.4. DPPH radical scavenging activity

The quenching activity of the samples against DPPH was assessed and the estimated EC50 values are reported in Table 3. As shown in Table 3, all the glyconjugated samples had improved antioxidant activity, and neither GAH nor GFH possessed a concentration-dependent activity. Among GA samples, GAC37 was the most active with an EC50 of 2.0 ± 0.1 mg/mL, followed by GAT25, GAT37 and GAC25. Nevertheless, their final EC50 values were not significantly different amongst them ($P < 0.05$). The same extent of glycation represents a possible explanation for these results. GFT37, having a glycopeptide obtained through glycosylation, was the most active among GF samples (EC50 = 1.0 ± 0.2 mg/mL). DPPH measures the free radical scavenging capacity of a sample, based on a combination of hydrogen atom and electron transfer reactions (Huang, Ou, & Prior, 2005). These activities may depend mainly on the sugar moiety of a glycopeptide, which can be both

Table 3
Bioactivities of the wheat gluten hydrolysates before and after conjugation. DPPH scavenging activity reported as EC50 (mg/mL) with the respective R^2; antimicrobial activity against *E. coli* AW1.7, the Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal concentration (MBC) are expressed in mg/mL; antihypertensive activity (anti-ACE) reported as IC50 (mg/mL) with the respective R^2. Results are presented as mean ± standard deviation ($n = 3$). All samples were analysed in triplicate ($n = 3$). Abbreviations as in Fig. 1.

Sample	DPPH scavenging activity		Antimicrobial activity against *E. coli*		Anti-ACE	
	EC50 (mg/mL)	R^2	MIC (mg/mL)	MBC (mg/mL)	IC50 (mg/mL)	R^2
GAH	n.d.		–	–	0.17 ± 0.05	0.72
GAC25	2.7 ± 0.2	0.98	–	–	0.23 ± 0.03	0.60
GAC37	2.0 ± 0.1	0.99	–	–	0.44 ± 0.10	0.67
GAT25	2.2 ± 0.1	0.99	–	–	0.31 ± 0.09	0.79
GAT37	2.5 ± 0.3	0.99	40	40	0.44 ± 0.02	0.70
GFH	n.d.		–	–	0.41 ± 0.03	0.84
GFC25	3.2 ± 0.3	0.99	40	40	0.86 ± 0.02	0.92
GFC37	3.5 ± 0.1	0.97	40	–	0.43 ± 0.06	0.89
GFT25	2.2 ± 0.2	0.99	40	–	0.78 ± 0.10	0.80
GFT37	1.0 ± 0.2	0.64	40	40	1.1 ± 0.1	0.91

n.d.: not detectable.
–: no activity was observed in the range of concentrations used.

electron donor and acceptor (van Boekel, 2001). In fact, Wu and Wang (2009) showed that the antioxidant activity of a glycopeptide isolated from the fruiting bodies of *Ganoderma lucidum* strictly depended on the carbohydrate moiety, whereas the peptide moiety appeared to be nonessential. On the other hand, Rajapakse, Mendis, Jung, Je, and Kim (2005) reported that aromatic amino acids can also have electron scavenging activity. Even if the antioxidant activities reported here were not temperature-dependent, the possible formation of Maillard reaction products (MRPs) or GlcN autocondensation cannot be excluded. Regardless, the use of glucosamine for conjugation may generate a product with enhanced antioxidant capacity.

3.5. Antimicrobial activity

The antimicrobial activity of samples, before and after conjugation, was tested against two bacterial strains: *E. coli* and *B. subtilis* (Gram negative and positive, respectively). The peptide mixtures before the treatment (GAH and GFH) did not show any antimicrobial activity. After conjugation, the majority of the samples were active against *E. coli* (Table 3). Specifically, all the conjugated GF samples inhibited cell growth at 40 mg/mL. Moreover GFC25 and GFT37 also showed a bactericidal activity. Among the GA samples, GAT37 was both inhibitor and bactericidal (40 mg/mL) against *E. coli*. The activity against *B. subtilis* was not reported in Table 3 since none of the samples was active. This demonstrated that glycation at moderate temperatures contributed for the improvement of the antimicrobial activity. In particular, this effect was more relevant in GF than GA. Glycopeptides are generally more active against Gram-negative. For example, Drosocin, a small proline-rich glycopeptide isolated from *Drosophila melanogaster*, resulted active against different bacteria such as *E. coli* (Otvos, Bulet, Varga, & Hoffmann, 2002). The sugar moiety is able to enhance the antimicrobial effect of the native peptide by approximately 100 fold (Otvos et al., 2002). Although this activity of glycopeptides has been reported, (Bulet & Stöcklin, 2005; Kahne, Leimkuhler, Lu, & Walsh, 2005), the mechanisms were not well described. Kragol et al. (2001) suggested that the Drosocin related glycopeptides may inhibit protein folding by interacting with the chaperonine/heat shock proteins GroEL and DnaK. This interaction occurs only when the sugar moiety is present, since it should determine a spatial rearrangement of the peptide (McManus, Otvos, Hoffmann, & Craik, 1999). The high proline content of wheat gluten (Wieser, 2007) and the GlcN moiety of the glycopeptides may be responsible for the antimicrobial activity inside the cell.

3.6. Anti-ACE activity

Samples were tested for their anti-hypertensive activity by measuring the capacity to inhibit the ACE enzyme, and the IC50 values were estimated (Table 3). Glycation reduced the ACE inhibitory activity in almost all samples, in particular in GF samples. In fact, their IC50 values, except GFC37, were at least double than the control. GAC37, GAT25 and the GAT37 also showed higher IC50 values compared to GAH ($P < 0.05$). ACE inhibitory activity is strongly linked to the peptide amino acid composition and the primary amino acid sequence. Anti-ACE gluten peptides have already reported, where the main activity of the hydrolysates is attributed to the low MW peptides since the inhibitory activity increased by four-fold, after sample fractionation (Kim, Whang, & Suh, 2004). On the other hand, Kinoshita, Yamakoshi, and Ikuchi (1993) reported that the high MW peptide fraction reduced the blood pressure in hypertensive rats. Je, Park, Kwon, and Kim (2004) reported that the presence of hydrophobic amino acids enhances the ACE-inhibitory ability of the peptides. Since the glycation process can change the final hydrophobicity of the peptides, it may be responsible for the decreased anti-hypertensive activity. This aspect must be taken into consideration when antihypertensive peptides are formed during the production or added into baked food, in particular baked goods, because the formation of glycopeptides, also originated from the Maillard reaction at high temperature, may reduce their activity.

4. Conclusion

The potential to glycate and glycosylate gluten peptides at mild temperatures to increase their bioactivities was investigated. The production of gluten glycopeptides occurred at mild temperatures (25 and 37 °C), representing a promising step for industrial production. The advantage of this mild-temperature process is the production of conjugated mixtures of peptides possessing increased antioxidant and antimicrobial potential, while minimizing the formation of Maillard by-products. The two main factors influencing the bioactive properties were: 1) the type of proteases used for the hydrolysis of the protein. For instance, glycated Flv hydrolysates showed higher antimicrobial activity, while glycated Alc hydrolysates had increased antioxidant activity; 2) the occurrence of the sugar–peptide conjugation that was able to influence positively the antioxidant and the antimicrobial activities but not the anti-ACE property. Since the conjugated hydrolysates included glycated and non-glycated peptides, fractionation and purification of the active compounds could enhance these activities. Additional studies could improve the final yield of the glycation and glycosylation processes, and test the stability of these new compounds. The current study demonstrates that the use of GlcN, either alone or in combination with TGase, can be employed to create new functional glycopeptides or mixtures of peptides at mild temperature from different plant sources.

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