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Inhibitory activity of a Concanavalin-isolated fraction from a glucosamine-peptides reaction system against heat resistant *E. coli*

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Abstract

Alcalase-derived gelatin hydrolysates were glycosylated with glucosamine in the presence (+) or absence (–) of transglutaminase (TGase), and their antimicrobial activities toward *Escherichia coli* AW 1.7 were studied. Glycosylation treatments were subjected to concanavalin A affinity chromatography to selectively collect the glycopeptide-enriched fractions and the changes in antimicrobial activity were determined. The minimum inhibitory concentration of glycosylated hydrolysates decreased by 1.2 times compared to the native hydrolysate, with no differences between (+) or (–) TGase treatments. No difference was observed in the dicarbonyl compound concentration between the two glycosylation methods except that 3-deoxyglucosone was greater in the TGase-mediated reaction. Concanavalin A-retentate, but not the flow-through fractions, significantly improved the antimicrobial activity, however there was no difference between +TGase and –TGase glycosylated treatments. Purification of the retentate fraction from fluorescent compounds did not improve its antimicrobial activity.

Keywords: Microbiology, Food science

1. Introduction

The Maillard reaction, or non-enzymatic glycation, is one of the most important reactions occurring during industrial and domestic thermal treatment of food (Chevalier et al., 2001). The reaction is initiated with the condensation between free amino groups of protein and reducing sugars to form a Schiff base, which is stabilized through Amadori or Heyns rearrangements. These latter reactions occur through a series of steps that irreversibly create advanced glycation end products (AGEs), which are frequently chromophores and fluorophores. In the process, a variety of intermediate products, including α -dicarbonyl compounds (α -DCs) are formed. The antimicrobial effect of Maillard reaction products (MRPs), particularly those formed at the late stages (i.e. melanoidins), has been well documented (Einarsson et al., 1983; Hiramoto et al., 2004; Rurian-Henares and Morales, 2008). Compounds with reductone capacity, such as aminoreductone formed in the early stages of the Maillard reaction (Knerr et al., 1994; Pischetsrieder et al., 1998), have also attracted interest, not only for their antioxidant activity (Pischetsrieder et al., 1998) and protection against photo-degradation of riboflavin in milk (Trang et al., 2008), but also for their antimicrobial capacity. Trang et al. (2009) reported that aminoreductone inhibited 24 kinds of *Helicobacter pylori*, including antibiotic-resistant strains. The same authors found that the killing activity of aminoreductone was significantly greater than that of its derived melanoidin. The antimicrobial activity of aminoreductone against methicillin-resistant *Staphylococcus aureus* has also been demonstrated (Trang et al., 2011).

Glucosamine (GlcN, 2-amino-2-deoxyglucose, chitosamine) is an aminosaccharide that occurs in acetylated and polymerized forms of chitin, a poly-beta-1,4-N-acetylglucosamine. It is mainly used to treat osteoarthritis as a natural health product in Canada (Health Canada, 2017) or as a drug in the EU. Recently, GlcN have been used to modify different protein hydrolysates to produce taste-active, antioxidant and antimicrobial compounds (Hong et al., 2014; Hong et al., 2016; Gottardi et al., 2014). For instance, GlcN glycation of fish skin gelatin (Hong et al., 2014) or gluten (Gottardi et al., 2014) hydrolysates in the presence or absence of transglutaminase (TGase) enzyme resulted in an increased antimicrobial activity compared to the native hydrolysates. Amadori or TGase-mediated glycopeptides (Hong et al., 2014) were thought to be involved in determining this antimicrobial activity, and plausible, but not yet conclusive, evidence about glycopeptide formation was also provided using MALDI-TOF. Sugar moieties are strategically bound to a peptide by means of glycation or glycosylation reactions because certain glycopeptides possess a potent antimicrobial activity. For instance, drosocin, a proline rich glycopeptide, is very active in the low micromolar concentrations mainly against Gram-negative bacteria (Gobbo et al., 2002). Otvos et al. (2002) reported that drosocin exhibits its antimicrobial activity mainly by

interfering with the mechanism of action of the heat shocking protein DnaK. Since gelatin is a protein rich in proline and hydroxyproline, it was hypothesized that through a controlled GlcN glycation at a moderate temperature, antimicrobial glycopeptides could be produced. For instance, [Hong et al. \(2014\)](#) found these glycated hydrolysates showed more specificity towards inhibition of *Escherichia coli* (*E. coli*) compared to *Bacillus subtilis*. [Gottardi et al. \(2014\)](#) came to the same conclusion and reported that GlcN-glycated gluten hydrolysates could inhibit the growth of *E. coli* at 40 mg/mL, but not *B. subtilis*. It is expected that these antimicrobial activities are not only due to the glycopeptides alone, but also due to other chemical compounds produced, including GlcN-derived α -DCs and the GlcN self-condensation products fructosazine and deoxyfructosazine ([Hrynets et al., 2016](#); [Bhattacharjee et al., 2016](#)). Indeed, recent studies have shown that fructosazine was able to inhibit 50% of the growth of extremely heat-resistant *E. coli* AW 1.7 at 3.6 g/L at pH 5 ([Hrynets et al., 2016](#); [Bhattacharjee et al., 2016](#)). Here fructosazine exerted its antimicrobial action by permeabilizing the cell membrane, damaging membrane integrity and fragmenting DNA. Hence GlcN-peptide reaction systems may be exploited to produce several antimicrobial compounds at moderate temperatures. As reported in several studies, the advantage of using GlcN as a glycation agent is its ability to undergo Maillard reaction and caramelization reactions at a very moderate temperature (e.g. 25–50 °C) thus limiting the formation of undesired AGEs. Furthermore, a reaction system using moderate temperatures is more manageable.

The main objective of this study was to isolate a glycopeptide fraction from a GlcN-peptide reaction system in order to study its inhibitory activity against heat resistant *E. coli* AW 1.7. Here we try to demonstrate that Maillard reaction produced glycopeptides found in heated food are natural antimicrobial compounds. In this respect, fish gelatin was hydrolyzed with Alcalase and glycated with GlcN in the presence or absence of TGase at 25 °C in order to generate glycopeptides. After the glycation treatments, a glycated fraction was recovered through the affinity chromatography based on Concanavalin A, a glycoprotein with the ability to interact with glycopeptides. Recovered fractions were evaluated for their antimicrobial activity using a microdilution assay. Extremely heat-resistant *E. coli* AW1.7, a gram-negative bacteria commonly found in beef carcass ([Dluskaya et al., 2011](#)), was used in this study.

2. Materials and methods

2.1. Chemicals

D-Glucosamine hydrochloride (GlcN, $\geq 99\%$), cold water fish skin gelatin (Type A), Alcalase enzyme from *Bacillus licheniformis* (2.4 U/g), guinea pig liver transglutaminase (TGase), HPLC-grade solvents (acetonitrile (ACN), water,

methanol, formic acid, *o*-phthalaldehyde (OPA), glucosone (G, 2-keto-D-glucose; $\geq 98.0\%$), glyoxal (GO, ethanedial; 40% in H₂O), methylglyoxal (MGO, 2-oxopropanal; 40% in H₂O), diacetyl (DA, butane-2,3-dione; $\geq 95.0\%$), 1,2-diaminobenzene and Concanavalin A (Con A) were from Sigma-Aldrich (St. Louis, MO, USA). 3-deoxyglucosone (3-DG, 3-Deoxy-D-erythro-hexosulose; $\geq 95\%$) was from Cayman Chemical (Ann Arbor, MI, USA). SPE tC-18 Sep-Pak Vac 6 cc columns were from Waters (Milford, MA, USA). Filtration membranes 0.22 μm were from Millipore (Billerica, MA, USA). Difco Luria-Bertani (LB) media was obtained from BD Difco (Mississauga, ON, Canada). Ultrafiltration membranes (Minimate TFF Capsule) were from PALL Life Sciences Corporation (Montreal, QC, Canada). Lectin affinity column 5 mL (HiTrap Con A 4B from *Canavalia ensiformis*) was from GE Healthcare Life Sciences (Mississauga, ON, Canada).

2.2. Experimental setup

This study started with fractionation of Alcalase-hydrolyzed fish gelatin peptides based on the molecular weight (MW), where peptides smaller than 3 kDa were glycosylated with GlcN by the Maillard (-TGase) or TGase-mediated reaction (+TGase). Identification and quantitation of the main α -DCs, was followed by testing of the antimicrobial activity. This was followed by lectin-based affinity chromatography to obtain enriched GlcN-derived glycopeptides. Next, the fluorescent fraction of enriched glycopeptides from both -TGase and +TGase glycation methods was separated from the non-fluorescent fraction and their microbial inhibitory activities were determined toward heat-resistant *E. coli* AW 1.7.

2.3. Enzymatic hydrolysis of fish gelatin

Fish skin gelatin hydrolysate was prepared as described by [Hong et al. \(2014\)](#). Fish skin gelatin at 5% w/v was dissolved in 50 mM ammonium bicarbonate buffer (pH 7.8 ± 0.2), heated at 80 °C for 10 min and cooled down to 50 °C before the addition of Alcalase. Hydrolysis was carried out for 3.5 h at 50 °C at an enzyme-to-gelatin ratio of 1:10. Proteolysis was terminated by enzyme inactivation by heating at 80 °C for 15 min. The resulting hydrolysates were filtered through Whatman No. 1 filter paper. Alcalase-hydrolyzed gelatin peptides were further fractionated by using MW cut off membrane of 3 kDa (5,000 \times g, 35 min, 4 °C) Amicon ultra centrifugal tubes 15 mL (Millipore, Cork, Ireland) to obtain a fraction of peptides with MW of less than 3 kDa. This fraction is referred to the native hydrolysate throughout the manuscript.

2.4. Determination of degree of hydrolysis

Degree of hydrolysis (DH) was determined according to Nielsen et al., 2001. The DH was defined as the percentage of peptide bonds cleaved. The reaction was based on derivatization of primary amino groups with *o*-phthaldialdehyde (OPA) and measuring absorbance at 340 nm (Spectramax Molecular Devices, CA, USA). The control consisted of water and OPA solution. The calculations were performed by using following equations:

$$DH = h/h_{\text{tot}} \times 100$$

$$h = \left[\frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}})}{(\text{Abs}_{\text{standard}} - \text{Abs}_{\text{control}})} \times L \times V \times 100 / (M \times P) \right] - \beta / [\alpha]$$

where DH = degree of hydrolysis (%), Abs = absorbance at 340 nm, h = amount of hydrolyzed bonds measured, h_{tot} = total number of peptide bonds per protein (8.6 mequiv/g), L = leucine concentration, α and β = constants (1.00 and 0.40, respectively), M = mass of sample (g), P = protein content (%), and V = sample volume (L).

2.5. Peptide mass fingerprinting by matrix-assisted laser desorption/ionization (MALDI)

Analyses were performed on a MALDI time of flight (MALDI-ToF/ToF) mass spectrometer (Bruker Daltonics, Bremen, Germany). The mass spectra were acquired in linear positive mode over a mass range of 600–2000 kDa. Gelatin hydrolysates were dissolved in 50% ACN/water and 0.1% TFA and mixed with a matrix solution (α -cyano-4-hydroxycinnamic acid; 4-HCCA, 10 g/L in 50% ACN/water and 0.1% TFA) at 1:1 ratio. After brief mixing of the analyte and the matrix, 1 μ L of the mixture was added on the MALDI target and allowed to air-dry. Samples co-crystallized with matrix on the probe were desorbed and ionized by a nitrogen laser pulse (337 nm) and accelerated under 25 kV.

2.6. Glycation of gelatin hydrolysate with glucosamine

The optimized experimental conditions for gelatin glycation in absence or presence of TGase were used as described by Hong et al. (2014) with some modifications. To glycate (-TGase) gelatin hydrolysate, its lyophilized powder was mixed with GlcN at a weight ratio of 1:1. The powders were dissolved in 50 mM ammonium bicarbonate buffer and incubated at 25 °C for 3.5 h.

To produce glycated (+TGase) hydrolysates, 2 unit/g of TGase was added to the hydrolysate-GlcN mixtures prepared as reported above and incubated at 25 °C for 3.5 h. The enzyme was activated with 5 mM CaCl₂ prior to the addition to the reaction mixture. Followed incubation, TGase was removed by ultrafiltration (3900 \times g, 20

min, 10 °C) using Amicon ultra centrifugal filters (Millipore, Cork, Ireland) with MW cutoff of 10 kDa. Sample resulted from both, -TGase and +TGase treatments were dialyzed exhaustively for 48 h (100–500 Da MW cutoff; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) to remove unreacted GlcN. All the samples were passed through 0.2 µm PVDF syringe filter (Mandel, ON, Canada). Samples were further lyophilized and stored at -20 °C. Native hydrolysate (MW < 3 kDa) was treated under the same conditions to produce experimental control.

2.7. Analysis of free α -dicarbonyl compounds

The extraction, identification and quantification of α -DCs in glycated gelatin hydrolysates were performed according to Papetti et al. (2014). Briefly, 6 mL of samples aliquots were passed through a pre-conditioned SPE tC-18 Sep-Pak cartridges (Waters, Milford, MA, USA) and washed with 2 mL of water. Eluted polar compounds (8 mL) were spiked with 6 mg of 1,2-diaminobenzene and the pH adjusted to 3.00 ± 0.02 with 4 N HCl, followed by incubation at 37 °C for 1 h protected from light. The quinoxaline derivatives were eluted from another SPE cartridge with 4 mL of a MeOH/H₂O mixture (90/10, v/v). Alpha-DCs were identified using reversed-phase ultra-high performance liquid chromatography with photodiode array detection (RP-UHPLC-PDA) following the conditions described by Hrynets et al. (2015a, b). The elution of α -DCs was performed using Ascentis Express ES-C18 column (Sigma-Aldrich, MO, USA) with the gradient mixture of 0.1% formic acid in water (eluent A) and 100% methanol (eluent B) at a flow rate of 0.3 mL/min and injection volume of 5 µL. Identification of extracted α -DCs was based on comparison with the retention time and absorption spectra of authentic α -DCs standards (Hrynets et al., 2015a, b).

Quantification was performed by using calibration curves, where each quinoxaline derivative was diluted to final concentrations ranging between 0.5 – 16.5 (G), 0.12 – 2 (3-DG), 0.2 – 0.002 (GO), 0.05 – 0.001 (MGO), and 0.05 – 0.005 mM (DA). Each point was analyzed in triplicate. For all five α -DCs, the correlation coefficient (R^2) of a standard curve for quantitative analysis was in the range from 0.9985 to 0.9992. The average limits of detection (LODs) were calculated as 2.15 ± 0.07 (G), 0.27 ± 0.00 (3-DG), 0.13 ± 0.00 (GO), 0.09 ± 0.00 (MGO) and 0.18 ± 0.00 µM (DA). The average limits of quantification (LOQs) was determined as 6.52 ± 0.21 (G), 0.81 ± 0.01 (3-DG), 0.39 ± 0.01 (GO), 0.27 ± 0.01 (MGO), and 0.55 ± 0.02 µM (DA) by assuming a signal-to-noise ratio (S/N) of 3:1 for LODs and S/N of 10:1 for LOQs.

2.8. Isolation of glycopeptides by lectin affinity chromatography

Lectin affinity chromatography according to Alvarez-Manilla et al. (2010) with some modifications was used to isolate the glycopeptides. All chromatographic

steps were conducted on an Agilent 1100 series LC system using lectin affinity columns (HiTrap Concanavalin A, 4B, 5 mL). The mobile phases consisted of binding buffer (20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂) and elution buffer (0.1 M sodium acetate (pH 6), 1 M NaCl). Affinity column was equilibrated by passing 10 column volumes (CV) of binding buffer. Hundred microliter of sample was injected and column was washed with 5CV of binding buffer at a flow rate of 0.5 mL/min or until the baseline reading at $\lambda = 214$ nm was reached. The retentate (the bound fraction with expected glycopeptides) were eluted from the column by 5CV of elution buffer. These two collected fractions corresponded to Con A-permeate- and Con A-retentate for each sample. Fractions were desalted by using ultrafiltration membranes with a MW cut-off of 650 Da. Desalting was monitored by conductivity using an Oakton Acorn CON 6 conductivity meter (Vernon Hills, IL, USA). The desalted fractions were freeze-dried and stored at -20 °C.

2.9. Characterization of the collected fractions

2.9.1. Size exclusion chromatography (SEC)

Con A-permeate and Con A-retentate fractions were subjected to size exclusion chromatography using a 120 mL HiLoad 16/60 Superdex 200 pg column (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) attached to a fast protein liquid chromatography (GE Healthcare Life Sciences). Fractions (1 mg/mL) were eluted isocratically with water at a flow rate of 0.5 mL/min and the injection volume of 100 μ L. Detection was carried out at 214 nm. The mass calibration was performed using a standard compounds mixture with a MW range from 0.2 to 12 kDa.

2.9.2. RP-UHPLC profiles

The RP-UHPLC analyses of Con A fractions were performed on a Shimadzu UHPLC system equipped with an Ascentis Express ES-C18 column (150 \times 4.6 mm, 2.7 μ m particles; Sigma-Aldrich, MO, USA). Solvent A was 0.05% TFA in water, and solvent B was 0.01% TFA in ACN. Separations were performed with a following gradient: 0–3 min (0% B), 3–120 min (0–30% B), and 120–129 min (100% A). The flow rate was 0.5 mL/min, column temperature 60.0 ± 0.5 °C and UV absorption was monitored at 214 nm.

2.9.3. RP-UHPLC purification of Con A-retentate by fluorescence

The Con A-retentate fraction was further purified by fluorescence. The separation was performed at the same conditions as described in the section above with an exception of using fluorescence detector operating at excitation/emission wavelengths of 350/430 nm (Henle et al., 1999). The purification was achieved

by collecting the sample after 6 min of the run to avoid fluorescent compounds, inter alia, intermediate and late products of glycation. Accordingly, the fluorescent fraction was also collected within 6 min of the retention time. Both fluorescent and non-fluorescent (purified) fractions were freeze-dried and stored at $-20\text{ }^{\circ}\text{C}$.

2.10. LC-MS/MS analysis of concanavalin isolated fractions

Liquid chromatography-tandem mass spectrometry (nLC-MS/MS) of compounds isolated by fluorescence were analysed by using a nanoAcquity UPLC system (Waters Corp., Milford MA, USA) and a quadrupole-time of flight (Q-ToF) mass spectrometer (Premier, Waters Corp.) equipped with a nano-ESI source. An autosampler was used to inject $5\text{ }\mu\text{L}$ of samples into Symmetry C18 trapping column ($180\text{ }\mu\text{m} \times 20\text{ mm}$, particle size $5\text{ }\mu\text{m}$; Waters Corp.) for desalting and concentration for 4 min at a flow rate of $5\text{ }\mu\text{L}/\text{min}$. Glycopeptides were then separated at a flow of $350\text{ nL}/\text{min}$ on nano analytical column ($75\text{ }\mu\text{m} \times 150\text{ mm}$, Atlantis dC18, particle size $1.7\text{ }\mu\text{m}$; Waters Corp.). Water/ACN gradients containing 0.1% formic acid in both solvents were used for elution, with ACN increasing from 2 to 75% in over 55 min. All analyses were performed using positive mode ESI nano-spray source. Mass spectrometer settings were as follows: capillary voltage 3.5 kV, sampling cone voltage 30 V, extraction cone 3.0 V, source temperature $110\text{ }^{\circ}\text{C}$, and collision gas $0.45\text{ mL}/\text{min}$. The MS scan range was 70–900 m/z . CID spectra were acquired of the most abundant multiply charged ion from each MS survey with collision energy adjusted automatically according to the charge state and m/z value of the ion selected. Dynamic exclusion was also enabled; exclusion time was 60 s. Data were manually evaluated to ascertain that the samples contained glycopeptides.

2.10.1. Bacterial strain and antibacterial assay

Extremely heat-resistant *E. coli* AW 1.7, isolated from a beef processing plant, was grown at conditions described previously (Dlusskaya et al., 2011; Hrynets et al., 2016). *In vitro* antibacterial activity of native and glycated fish gelatin hydrolysate and fractionated treatments against heat resistant *E. coli* AW 1.7 was tested by critical dilutions assay (Gänzle et al., 1999). Samples were sterilized by filtration through sterilized $0.22\text{ }\mu\text{m}$ membrane filters (Millipore, MA, USA). *E. coli* was subcultured twice in liquid media under conditions at $37\text{ }^{\circ}\text{C}$. One hundred microliters of sample and $100\text{ }\mu\text{L}$ of LB broth were mixed, followed by 2-fold serial dilutions on 96-wells microtitre plates (Corning Inc., Corning, NY, USA). Fifty microliters of inoculated media was added to microtitre wells. The initial cell count was 1×10^6 CFUs/mL. Microtitre plates were incubated for 24 h. After incubation, bacterial growth was determined by the development of turbidity (optical density) at 630 nm. After minimum inhibitory concentration (MIC) readings, $10\text{ }\mu\text{L}$ bacteria suspension from wells in which the sample concentrations

were higher than MIC₉₀ were plated on LB agar and incubated at 37 °C for 24 h. MBC was the lowest concentration which could achieve 99.9% killing. The 90% MIC (MIC₉₀) and 90% minimum bactericidal concentration (MBC₉₀) were calculated using GraphPad Prism 7 (GraphPad Software, Inc. San Diego, CA) (Cheng et al., 2017).

2.11. Data analysis

Three independent experiments were performed. Data were expressed as the mean \pm standard deviation. At least two injections were performed to obtain LC profiles of UV and fluorescence detections. For antimicrobial assays, analysis of variance (ANOVA) was used for comparison among experimental groups. When differences were significant, the means were separated using the posthoc Tukey's Honest Significant Difference (HSD) test ($p < 0.05$).

3. Results and discussion

3.1. Degree of hydrolysis and mass distribution of gelatin hydrolysate

Alcalase was chosen based on the previous studies when its high hydrolytic ability on relatively short times was reported in addition to the ability to release peptides with bioactive properties (Gómez-Guillén et al., 2011; Zhu et al., 2008). Alcalase is an industrial food grade enzyme with a low cost. This enzyme has broad specificity, hydrolyzing most peptide bonds, preferentially those containing aromatic amino acid residues (Doucet et al., 2003). Hydrolysates with the DH of $27 \pm 2.4\%$ were obtained after hydrolysis with Alcalase. Fig. 1 presents MALDI-ToF peptide mass fingerprints of Alcalase-hydrolyzed fish gelatin. The use of Alcalase for 3.5 h resulted in MW distribution from 560 to 1900 Da. When hydrolysates were analyzed by RP-LC the elution pattern (0–30% ACN) suggested that peptides were mainly of hydrophilic nature (Fig. 2).

3.2. The effect of GlcN glycation in presence or absence of TGase on antimicrobial activity

To understand whether glycation in presence or absence of TGase provides the antibacterial activity to gelatin hydrolysates the effect of treatments was compared against Gram (–) *E. coli* AW 1.7. It is important to note that despite the use of TGase to catalyze the covalent attachment of GlcN to glutamine-containing peptides, it is still expected the Maillard reaction occur (Hrynets et al., 2014) and thus +TGase treatments are a mixture of peptides produced by the both Maillard and TGase-aided reactions.

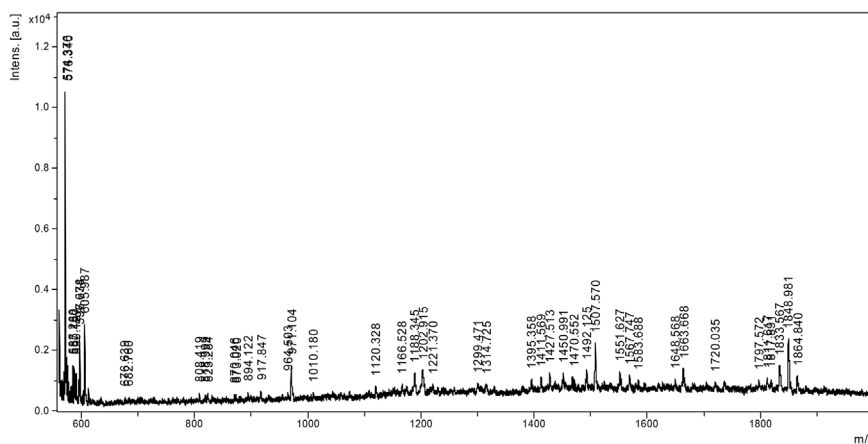


Fig. 1. MALDI-ToF/ToF mass spectra of Alcalase hydrolysates of fish gelatin. Spectra were acquired in positive ion mode on a Bruker Daltonics Ultraflex MALDI-ToF/ToF with a 4-HCCA as matrix.

The native hydrolysate showed an MIC₉₀ at 423 g/L, while the hydrolysates glycosylated in absence (–) TGase or presence (+TGase) of enzyme significantly reduced MIC₉₀ as compared to native hydrolysate, with the values on average of 345 g/L. These concentrations are significantly greater to those reported for glycosylated fish gelatin peptides, where MIC and MBC was attained at 40 g/L (Hong et al., 2014). Unlike Hong et al. (2014) who reported high specificity of glycosylated (+TGase) hydrolysates toward *E. coli*'s inhibition, in the current study MIC₉₀ values for glycosylation in presence or absence of TGase did not differ. The reasons for such differences may be due to different in gelatin quality or difficulty to proper replicate the experiment. However, despite these differences in the value of MIC, more evidences were obtained that GlcN-glycation treatment at this moderate temperature increases the antimicrobial activity of the hydrolysate. In order to understand the reason behind this phenomenon, we did try to fractionate and evaluate the compounds responsible for such activity.

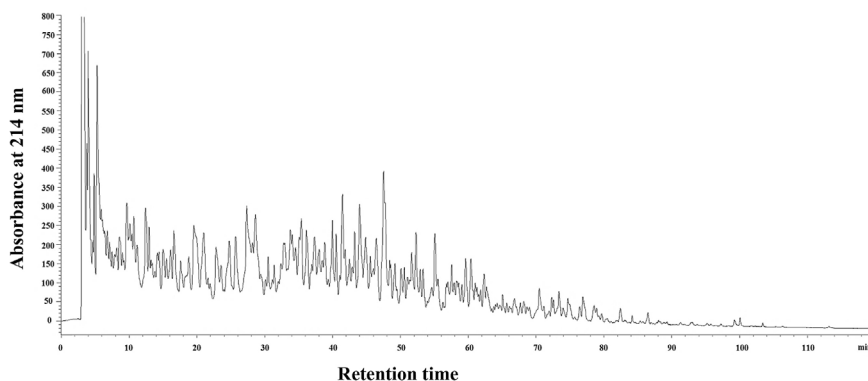


Fig. 2. Reversed-phase UHPLC profile of Alcalase-digested fish gelatin.

3.3. Alpha-dicarbonyl compounds in glycated hydrolysates

Alpha-DCs, including, 3-DG, methylglyoxal and diacetyl, are known to have antimicrobial properties (Daglia et al., 2007; Mavric et al., 2008; Hrynets et al., 2016). Furthermore, Hrynets et al. (2016) and Hong et al. (2016) already demonstrated that GlcN glycation or non-enzymatic browning can produce significant amount of α -DCs at moderate temperatures of 25 and 37 °C. Hence evaluating their concentration in the glycation treatments is of primary importance to correctly elucidate the source of the antimicrobial activity of the glycation treatments.

The retention time of each of the separated α -DCs was compared to a commercially available standard (Fig. 3). Fig. 4 shows the concentrations of the major α -DCs found in glycated gelatin hydrolysates. There was no difference ($p > 0.05$) in concentration of α -DCs between hydrolysates glycated by the Maillard (-TGase) or enzymatic (+TGase) reactions except for 3-deoxyglucosone, with greater ($p < 0.05$) amount in hydrolysates glycated in the presence of TGase. Native gelatin peptides did not contain any of the tested α -DCs (data not shown).

In a previous study (Hrynets et al., 2016) an MIC₅₀ of methylglyoxal, glyoxal, diacetyl, 3-deoxyglucosone and glucosone produced in GlcN/Fe²⁺ system was 0.05, 0.4, 0.1, 1.0 g/L at pH 5 and increased by 4.3, 2.0, 3.0, 1.7 and 1.3 times at pH 7 for the respective α -DCs. Hence, in this study it seems that only the 3-deoxyglucosone is the range of exerting antimicrobial activity. Despite the concentration of 3-deoxyglucosone in both -TGase and +TGase treatments is in the range to potentially exert an effect, the overall MIC₉₀ of the glycated hydrolysates without or with TGase were 350 and 340 g/L, respectively. This means that the efficacy of 3-deoxyglucosone is mitigated by the matrix effect, in this case the peptides in the hydrolysates.

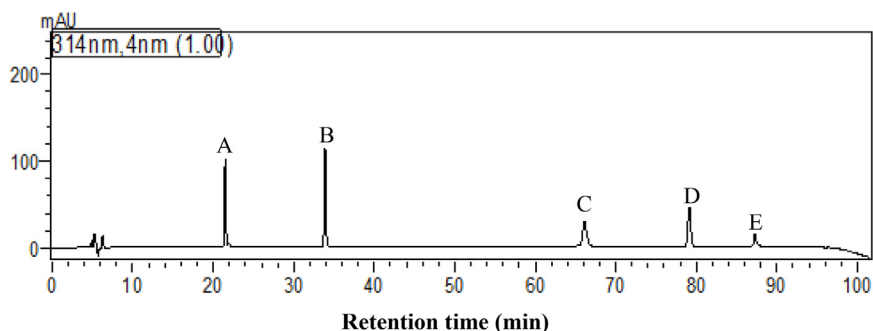


Fig. 3. Reversed-phase UHPLC profile of α -dicarbonyl compounds standards: (A) glucosone, (B) 3-deoxyglucosone, (C) glyoxal, (D) methylglyoxal and (E) diacetyl.

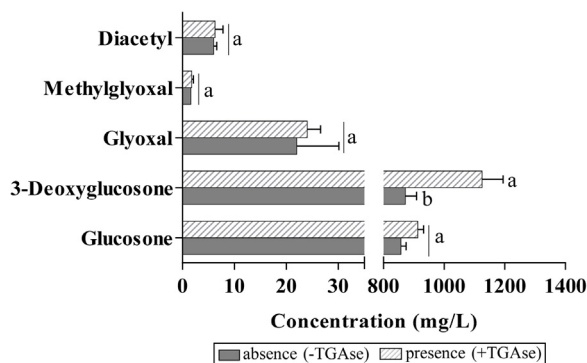


Fig. 4. Concentration (mg/L) of free α -dicarbonyl compounds generated from 150 g/L of fish gelatin hydrolysate glycated in absence (-TGase) or presence (+TGase) of TGase. The results are presented as mean \pm SD ($n = 3$). Means with different letters within the same α -dicarbonyl compound are significantly different ($p < 0.05$).

3.4. Concanavalin A affinity chromatography of glycated hydrolysates

To further understand the compounds responsible for antimicrobial activity, Con A lectin affinity chromatography was used to capture and enrich the glycospecies from both -TGase and +TGase reaction systems; we were hoping to isolate the “simple” glycopeptides containing GlcN moieties (i.e. Amadori glycopeptides or TGase-induced glycopeptides). Con A specifically binds to non-reducing terminal α -D-mannosyl or α -D-glucosyl moieties (Zhang et al., 2016). Con A was chosen due to structural similarities among 2-amino-2-deoxy-D-glucopyranose, D-mannopyranosyl and D-glucopyranosyl, being free hydroxyl groups at C-3, C-4 and C-5. Previous study (Bessler and Schindler, 1979) showed possible to purify 2-substituted derivatives of glucose and sterically related sugars by affinity chromatography on Con A-sepharose. Fig. 5 indicates that adsorption/desorption stages of enrichment using Con A produced two main fractions: the retentate (bound fraction) and the permeate (non-bound fraction). The bound fraction possibly containing the glycopeptides eluted near 10 min, and the non-bound fraction were washed out at 5 min. One gram of hydrolysate yielded \sim 30–50 mg of bound fraction, accounting for a yield of 3–5%. Native hydrolysate was also passed through the affinity column however no interaction with the concanavalin A was found. Followed desalting, bound and not bound fractions were characterized by using size exclusion and RP-UHPLC chromatography.

3.5. Characterization of affinity chromatography fractions using size-exclusion and RP-UHPLC chromatography

Fig. 6A shows the representative SEC pattern of the affinity fractions collected from the glycated (+TGase) hydrolysates. Five major peaks were separated in Con

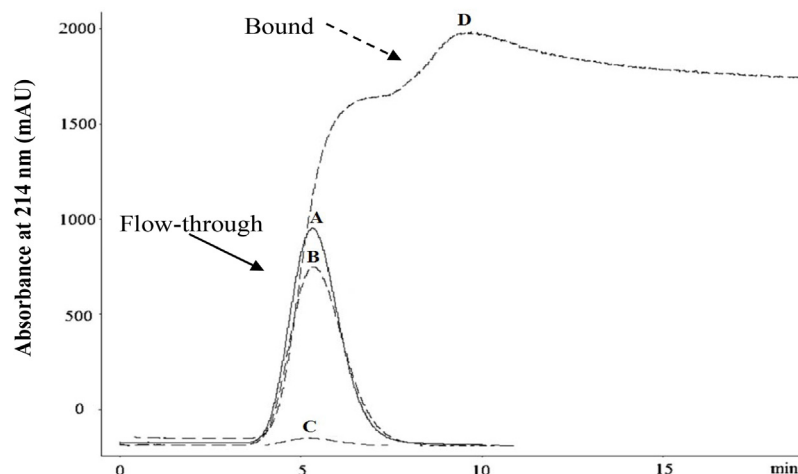


Fig. 5. Representative affinity chromatography profile of glycosylated (+TGase) treatment. Permeate fractions (A) Con A-first wash, (B) Con A-second wash, (C) Con A-third wash; (D) Con A-retentate. The arrows show flow-through (thick arrow) and bound (dashed arrow) fractions.

A-permeate fraction, while two peaks with lower MW range from 0.5 to 2 kDa were resolved in Con A-retentate fraction. No major difference in SEC profile of hydrolysates glycosylated by the TGase-induced (Fig. 6A) and the Maillard (-TGase) reactions (data not shown) was found.

The characterization by RP-UHPLC also showed the difference between the fractions, where more peaks eluted Con A-permeate fraction (Fig. 6B). Around 20 peaks eluted in the Con A-retentate fraction (Fig. 6C). Similar results were obtained for affinity-enriched fractions of glycosylated (-TGase) hydrolysates, where greater number of peaks eluted in the Con A-permeate fraction as compared to the Con A-retentate (Fig. 7). These results showed the existing differences in the peptide distribution profiles of Con A fractions, indicating that concanavalin A affinity chromatography may selectively bind specific peptide sequences and possibly the glycopeptides.

3.6. Antimicrobial activity of concanavalin fraction

The effect of Con A lectin chromatography on the antimicrobial activity against *E. coli* is shown in Fig. 8. In both glycation treatments, -TGase and +TGase, affinity chromatography with Con A produced retentate fractions with MIC₉₀ of 36 and 24 g/L, respectively; while the MBC₉₀ values for these treatments were double to the MIC₉₀. The MIC₉₀ of Con A retentate fractions were significantly lower compared to the samples that were not passed through Con A (glycosylated hydrolysate + TGase and glycosylated hydrolysate - TGase). However, no statistically significant difference ($p > 0.05$) was observed between the Con-A retentate with and without TGase indicating that the presence of TGase was not effective in improving the

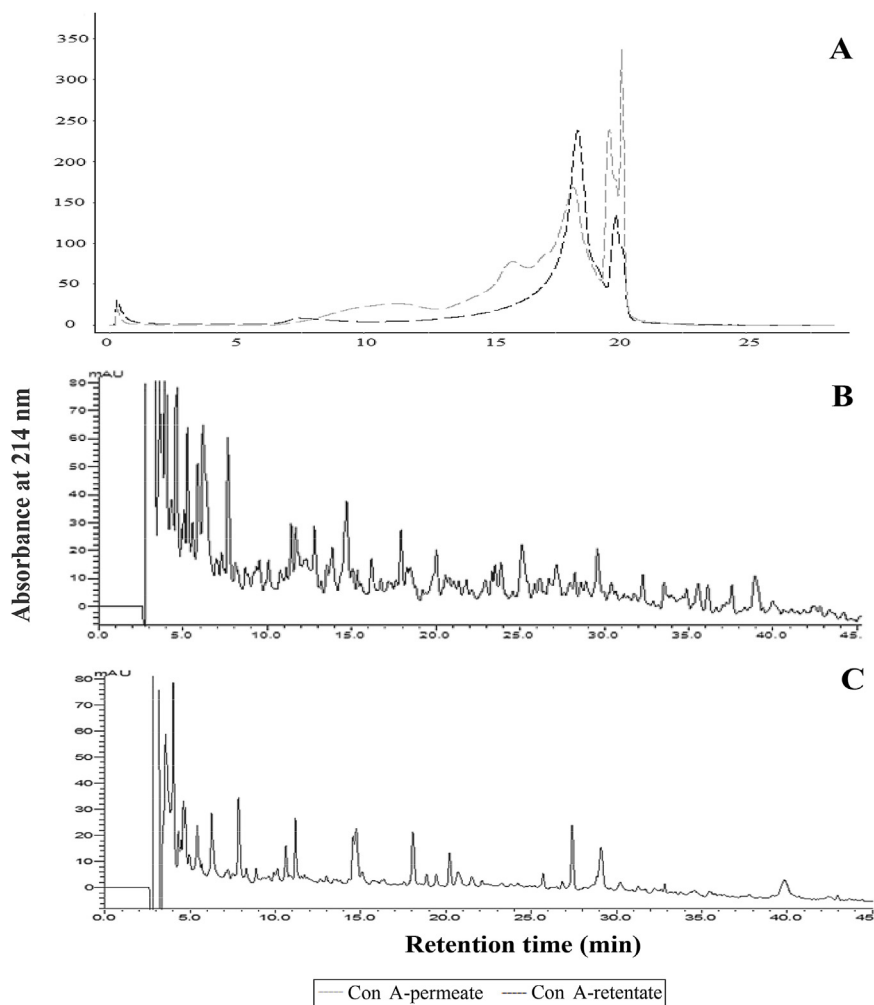


Fig. 6. Characterization of the Con A isolated fractions from glycosylated (+TGase) sample. (A) Representative size exclusion chromatogram of Con A-permeate and Con A-retentate; Reversed-phase UHPLC profiles of (B) Con A-permeate and (C) Con A-retentate detected at 214 nm obtained from TGase-mediated glycosylation.

antimicrobial activity. The Con A-permeate fractions of $-$ TGase and $+$ TGase treatments were also tested to inhibit *E. coli* and no MIC was obtained up to a concentration of 120 g/L (data not shown). Con A affinity chromatography was able to reduce the MIC₉₀ of *E. coli* AW 1.7 by around 14 and 11 times compared to the non-enriched native and glycosylated hydrolysates, respectively.

Con A fraction was also subjected to RP-UHPLC with fluorescence detection in order to understand if free of peptide-bound fluorophores were present. The Amadori compounds that are formed in the initial stages of the Maillard reaction are not fluorescent (Bosch et al., 2007). Even though temperature and pH influence the

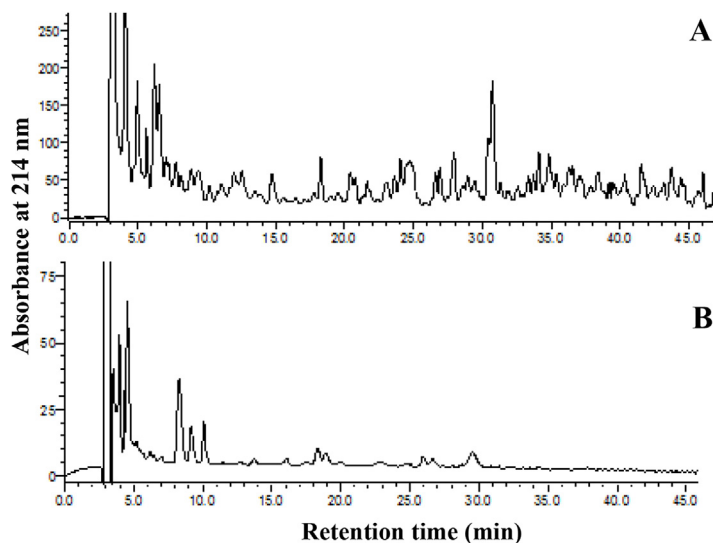


Fig. 7. Reversed-phase UHPLC profiles of lectin affinity chromatography enriched glycosylated (-TGase) hydrolysates: (A) Con A-permeate and (B) Con A-retentate detected at 214 nm; detected at 214 nm.

rate of fluorescent species formation, usually the fluorescent products formed during the intermediate stage of the Maillard reaction and are the active precursors of more advanced browning (Morales et al., 1996). To further purify glycopeptides from the intermediate-to-late stages products formed during glycation, fluorescence compounds were separated and a non-fluorescent fraction was collected. The RP-HPLC profiles of the latter fractions deriving from glycosylated and enriched hydrolysates are shown in Fig. 9. The formation of fluorescent reaction products

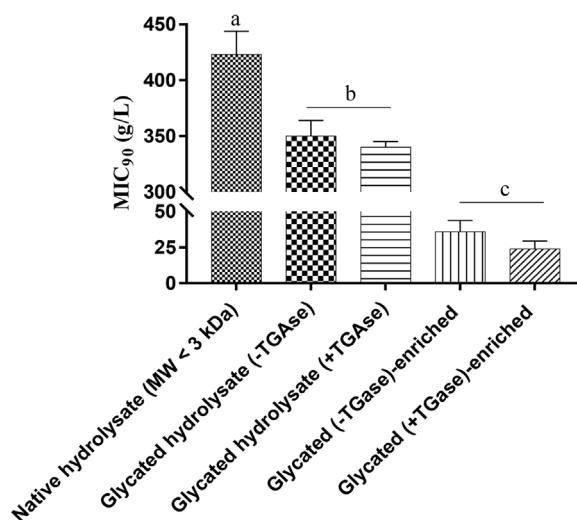


Fig. 8. Minimum inhibitory concentration (MIC₉₀) against *E. coli* AW 1.7 of native gelatin hydrolysate (MW < 3 kDa); gelatin hydrolysates glycosylated in absence (-TGase) or presence (+TGase) of TGase; affinity enriched Con A-retentate fractions from gelatin hydrolysates glycosylated in absence (-TGase) or presence (+TGase) of TGase. Means with different letters are significantly different ($p < 0.05$).

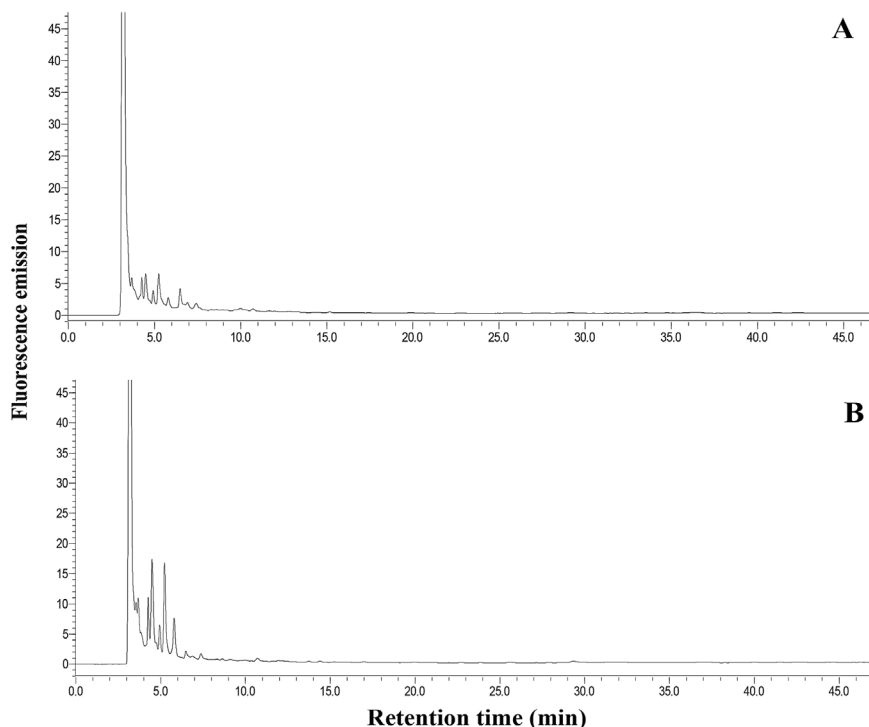


Fig. 9. Reversed-phase UHPLC profiles of lectin affinity chromatography enriched glycosylated fish gelatin hydrolysate: (A) Con A-retentate from glycosylated treatments in absence (A) or presence (B) of TGase detected by fluorescence at excitation/emission at 350/430 nm.

was found in both $-$ TGase and $+$ TGase glycosylated, however the number of peaks and their intensity were greater in those conjugates produced by the TGase-mediated reaction. When the antibacterial activity of fluorescent and non-fluorescent fractions was compared, the fluorescent fraction did not inhibit bacterial growth, while non-fluorescent fraction had an antimicrobial activity comparable to those of enriched glycopeptides (data not shown). This means that the antimicrobial activity of the glycosylated ($-/+$ TGase) hydrolysates was derived from the non-fluorescent reaction products. Although several studies reported the

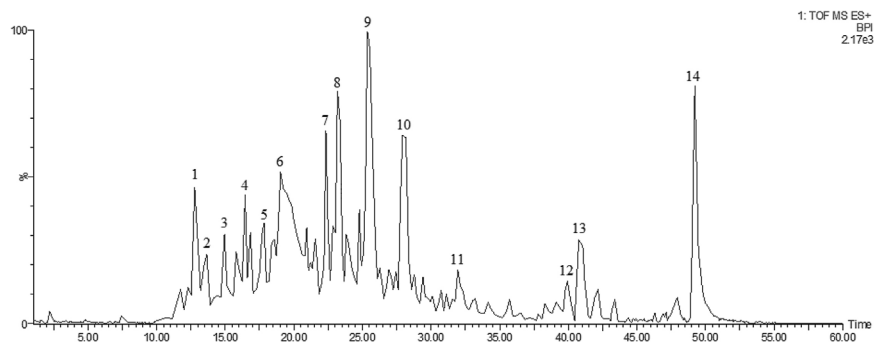


Fig. 10. LC-MS chromatogram of non-fluorescent Con A-retentate fraction.

potent antimicrobial activity of fluorophores from the Maillard reaction (Stecchini et al., 1991; Rurian-Henares and Morales, 2008; Wu et al., 2014) the reaction conditions of 3.5 h at 25 °C used in this study were likely not severe enough to produce adequate amounts of fluorescent products to inhibit *E. coli* AW 1.7.

3.7. Tentative glycopeptides identification by LC-MS

The non-fluorescent fraction from the Con A-retentate treatments (+TGase) was subjected to LC-MS/MS to confirm if the glycopeptides are responsible for the antimicrobial activity of glycated hydrolysates. Fig. 10 shows the LC-MS chromatogram where 14 major peaks were identified and eluted mainly from 10 to 55 min. Mass spectrometry-based identification of glycopeptides was performed by computer analysis using the MASCOT software. All data from the CID scans were inspected manually to verify their identification. Individual MS/MS fragmentation spectra were analyzed in order to identify the loss of a GlcN moiety (m/z 162 and 163). In addition to the abovementioned ions, repetitive losses of up to four water molecules (72 Da) could be observed in MS spectra due to structural alterations during the MS analyses (Frolov et al., 2006; Zhang et al., 2008; Yamaguchi et al., 2014). Surprisingly, only one peak 13 (Fig. 11) contained glycated peptides. The failure to identify glycated peptides in other peaks is likely not only due to a faulty approach in identification, but also due to the great complexity of the system. In contrast to our original hypotheses, we could not verify that the antimicrobial effect is fully attributed to “simple” TGase-induced and Amadori-glycopeptides. Nevertheless, we think that the observed antimicrobial effect could be due to enzyme and/or Maillard-reacted peptides, but not those at the initial stage of glycation, identified by a mass addition of 162 (or 163) or a cleavage resulting in a presence of GlcN residue in the MS/MS spectra. It is very likely that α -DCs can glycate the peptides in a hydrolysate and also provide an antimicrobial effect through formation of more complex structures, however at this

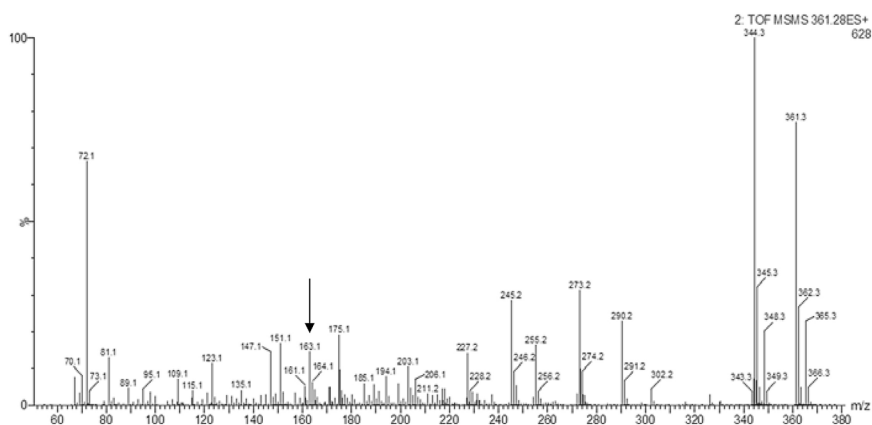


Fig. 11. MS/MS spectra of peak 13 showing the presence of GlcN moiety at m/z 163.

point of time we cannot provide a full identification of the resulting glycospecies. Considering the large activity of GlcN in glycation reactions, it is possible that within 3 h, some of the glycated species passed the point of “Amadori” peptide and would be recognized as a AGE- or “melano”- peptide, thus preventing their identification as “simple” glycopeptides. Since the nature of the compounds responsible for the antimicrobial activity is unclear, it is difficult to speculate a mechanism of action.

4. Conclusion

This study demonstrated that the glycation of gelatin hydrolysate in presence or absence of TGase increases its antimicrobial activity. Concanavalin affinity chromatography enrichment improved the antibacterial activity as compared to the non-enriched counterpart, with no difference between two glycation methods. Surprisingly, LC-MS results showed that the concanavalin fraction contained only one peak associated with glycated peptide, whereas the majority of peaks were unidentified, possibly representing modified peptides the result of GlcN reactivity. Even though the identification of “simple” glycated peptides was not fully successful, these results are still relevant and of practical importance. It demonstrates that glucosamine can be used to glycate food protein-derived peptides at a moderate temperature to produce savory browning products, and at the same time create a preservative, antimicrobial ingredient. More research is needed to fully optimize and characterize the production of chemical compounds responsible for these bioactivities.

Declarations

Author contribution statement

Yuliya Hrynets: Analyzed and interpreted the data; Wrote the paper.

Daylin J H Martinez: Performed the experiments.

Maurice Ndagijimana: Contributed reagents, materials, analysis tools or data.

Mirko Betti: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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