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Glycation and transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine enhance bioactivity

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ABSTRACT

A mixture of novel glycopeptides from glycosylation between cold water fish skin gelatin hydrolysates and glucosamine (GlcN) *via* transglutaminase (TGase), as well as glycation between fish gelatin hydrolysate and GlcN were identified by their pattern of molecular distribution using MALDI-TOF-MS. Glycated/glycosylated hydrolysates showed superior bioactivity to their original hydrolysates. Alcalase-derived fish skin gelatin hydrolysate glycosylated with GlcN in the presence of TGase at 25 °C (FAT25) possessed antioxidant activity when tested in a linoleic acid oxidation system, when measured according to its 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and when tested at the cellular level with human hepatocarcinoma (HepG2) cells as target cells. In addition, Alcalase-derived glycosylated hydrolysates showed specificity toward the inhibition of *Escherichia coli* (*E. coli*). The Flavourzyme-derived glycopeptides prepared at 37 °C (FFC37 and FFT37) showed better DPPH scavenging activity than their native hydrolysates. The glycated Flavourzyme-derived hydrolysates were found to act as potential antimicrobial agents when incubated with *E. coli* and *Bacillus subtilis*.

1. Introduction

Protein post-translational modification (PTM) is a crucial step in altering the biological activity of cells as it regulates the interaction between the cellular molecules, such as proteins, peptides and lipids. PTMs may occur in several ways; among the identified main processes are phosphorylation, glycosylation, methylation, lipidation, and proteolysis. Protein glycosylation is regarded as one of the major PTMs in cells, it is an enzyme-driven process that facilitates the production of biopolymers, such as the production of DNA, RNA, proteins and glycoproteins.

In contrast to glycosylation, glycation is a non-enzymatic reaction between amino acids and reducing sugars. It can occur in the body (endogenous) and in food systems (exogenous). Exogenous glycation, otherwise known as the Maillard reaction, has been studied extensively in food as it produces browning effects and flavour compounds. The accumulation of advanced glycation end-products (AGEs) from Maillard reaction in human cells is regarded as detrimental to health. Often, it is associated with diseases, such as diabetes, ageing, renal failure and inflammation. Interest in the study of glycoproteins and glycopeptides has grown due to their potential biological applications in areas such as inflammatory response, reproductive system, immune system and neuronal development (Gamblin, Scanlan, & Davis, 2009).

The potential of glycosylated/glycated protein and peptides is vast and can be further exploited in food systems. Incorporation of carbohydrate into protein has shown promising improvements in several aspects of functionality in glycosylated vegetable protein as well as in animal protein, compared to the native protein form. There have been a handful of studies on determining the bioactivities of peptides but these have not fully examined the potential of glycopeptides in food systems. These bioactive compounds have been successfully isolated from insects and plants.

In order to produce glycosylated/glycated peptides, the occurrence of a browning process and sugar degradation as part of the Maillard reaction in the reaction system seemed to be a requirement. On top of that, the kinetics of the reaction depended on the type of sugar used (Van Boekel, 2001). As the sugar plays a pivotal role in the Maillard process, selection of a suitable candidate

Abbreviations: Alc, Alcalase; *E. coli*, *Escherichia coli*; *B. subtilis*, *Bacillus subtilis*; DH, degree of hydrolysis; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Flv, Flavourzyme; GlcN, glucosamine; Gln, glutamine; HepG2, human hepatocarcinoma cells; MALDI-TOF-MS, Matrix-assisted laser desorption ionisation-time of flight-mass spectrometry; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; MRP, Maillard reaction product; MW, molecular weight; SEC, size exclusion chromatography; TGase, transglutaminase.

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has to be carefully considered. A recent study conducted by Hrynets, Ndagijimana, and Betti (2013) demonstrated that the amino sugar glucosamine (GlcN) was able to accelerate protein glycation at moderate temperatures (25 and 37 °C) as compared to glucose; therefore it could be a potential candidate for peptide glycation.

Transglutaminase (TGase, EC 2.3.2.13) is a group of calcium-dependent enzymes that is involved in the post-translational modification of proteins. The use of TGase in food processing is popular in the dairy and meat processing sectors, as it modifies the functional properties in proteins/peptides by introducing cross-links. It is more commonly referred to as an enzyme that catalyses acyl-transfer between the γ-carboxyamide group of peptide-bound glutamine (Gln) residues, which act as acyl donors, with primary amines, which are the acyl acceptors. In the absence of the amine substrate, TGase will undergo Gln deamidation where water is used as an acyl acceptor. Furthermore, aminosugars such as GlcN could be used as a substrate for creating a link between sugar and peptide/protein, which is referred to as glycosylation. Since GlcN possesses a free amino group, it could serve as an amine donor that will allow TGase to bind its α-amino group to a Gln-containing peptide. Thus, GlcN is an ideal candidate for such an interaction to modify the functionality and bioactivity of some common food protein hydrolysates. However, there is no literature reported thus far on producing glycosylated fish gelatin peptide *via* TGase using GlcN.

This study aimed to demonstrate a new approach for the production of glycosylated/glycated hydrolysates (glycopeptides) derived from cold water fish skin gelatin and GlcN at moderate temperatures *via* TGase. The variations in bioactivity due to selected conditions of glycosylated/glycated hydrolysates were characterised.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Gelatin (from cold water fish skin), Alcalase (Alc, EC 3.4.21.62, from *Bacillus licheniformis*) and Flavourzyme (Flv, from *Aspergillus oryzae*), GlcN hydrochloride (GlcN), TGase from guinea pig liver, Folin–Ciocalteu reagent, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and Hanks' Balanced Salt Solution (HBSS) were purchased from Sigma–Aldrich (St. Louis, MO). All chemicals used in size exclusion chromatography (SEC) and MALDI-TOF were of HPLC grade supplied by Sigma–Aldrich, whereas other chemicals were of analytical grade. 2,2′-Azobis(2-amidinopropane)dihydrochloride (ABAP) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). Foetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

2.1.2. Bacterial strains and human HepG2 cells

Strains used for antimicrobial analyses were *Escherichia coli* AW1.7 and *Bacillus subtilis* FAD 110 (both from University of Alberta). HepG2 cells used in this study were purchased from ATCC (Manassas, VA).

2.2. Production of hydrolysates

Exactly 5% w/v of gelatin was dissolved in 0.05 M $(NH_4)HCO_3$/NH_4OH buffer, the final pH of the mixture was pH 7.5–8. It was heated at 80 °C for 10 min. The gelatin mixture was cooled to 50 °C before adding Alc and Flv sequentially at 1:10 enzyme:gelatin ratio followed by incubation in a shaker (50 °C, 3.5 h, 200 rpm). Incubation was completed at 80 °C for 10 min, the hydrolysates were centrifuged at 10,000*g* (10 °C) for 15 min and filtered using Whatman No. 1 filter paper. The filtrate was collected, lyophilised and stored at −18 °C for further use.

2.3. Preparation of glycosylated peptides

Samples (1.5 g) obtained from the lyophilised hydrolysate powders (from Alc and Flv) were added to GlcN at a weight ratio of 1:1. Each of the weighed powders was dissolved in 30 mL of 0.05 M $(NH_4)HCO_3$/NH_4OH buffer (pH 7.0 ± 0.5) and each incubated at 25 °C and 37 °C for 3.5 h. The experimental conditions were selected based on a preliminary study (data not shown). Samples with GlcN were subjected to incubation with or without TGase (2 unit/g gelatin) at pH 7.5 (Fig. 1). TGase was activated with 5 mM calcium chloride prior to use. Controls that consisted of the lyophilised hydrolysate were prepared without GlcN (Fig. 1, FAH and FFH). At the end of incubation, all the mixtures were passed through a 0.2 μm nylon syringe filter (13 mm, Mandel, Guelph, ON, Canada). TGase was removed by ultrafiltration with a molecular weight cut-off (MWCO) membrane of 10 kDa (3900*g*, 20 min, 10 °C, Amicon Ultra centrifugal filters (Millipore, Cork, Ireland)). Excess of GlcN was removed by dialysis membrane with a MWCO of 100–500 Da (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Each of the retentates was collected and lyophilised; samples were then stored at −18 °C for further analysis.

2.4. Degree of hydrolysis (DH)

The measurement of DH was carried out according to *o*-phthaldialdehyde (OPA) method as stated by Nielsen, Petersen, and Dambmann (2001), by using serine as a standard for hydrolysis determination. Protein contents of gelatin samples were assessed. The percentage of DH was calculated according to Adler-Nissen (1986).

2.5. Size exclusion chromatography (SEC)

Gelatin and hydrolysate samples were subjected to size exclusion chromatography using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Life Sciences, Baied'Urfe, QC, Canada) connected to a fast protein liquid chromatography (GE Healthcare Life Sciences). A sample volume of 500 μL (1 mg/mL) was injected and eluted isocratically at a flow rate of 1.2 mL/min with 50 mM phosphate buffer containing 0.15 M NaCl. Eluted peptides were detected at 215 nm. The mass calibration was performed using a protein mixture (MW 1332 Da to 669 kDa). As for glycosylated/glycated hydrolysates, each of the 100 μL (1 mg/mL) samples was eluted with a Superdex Peptide 10/300 GL column (GE Healthcare Life Sciences) at a flow rate of 0.5 mL/min with the same mobile phase. Eluted peptides were detected at 215 and 280 nm.

2.6. Determination of glycation and glycosylation by matrix-assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS)

For analysis by MALDI-TOF-MS, the samples were diluted tenfold in 50% acetonitrile/water + 0.1% trifluoroacetic acid. One microlitre of each sample was mixed with 1 μL of α-cyano-4-hydroxycinnamic acid (4-HCCA, 10 mg/ml in 50% acetonitrile/water + 0.1% trifluoroacetic acid). 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/TOF (Bruker Daltonic, GmbH, Rheinstetten, Germany). Ions were analysed in positive mode after acceleration from the ion source by 25 kV. External calibration was performed by use of a standard peptide mixture.