LWT - Food Science and Technology 86 (2017) 530–537

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Simulated gastrointestinal digestion of nisin and interaction between nisin and bile

Ronan Gough [a, b], Paula M. O'Connor [a, c], Mary C. Rea [a, c], Beatriz Gómez-Sala [a], Song Miao [a], Colin Hill [b, c], André Brodkorb [a, *]

[a] Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
[b] School of Microbiology, University College Cork, College Road, Cork, Ireland
[c] APC Microbiome Institute, University College Cork, College Road, Cork, Ireland

ARTICLE INFO

Article history:
Received 22 June 2017
Received in revised form
9 August 2017
Accepted 11 August 2017
Available online 14 August 2017

Keywords:
Nisin
In vitro digestion
Bile
Antimicrobial peptide
Surfactant

ABSTRACT

Nisin, an antimicrobial peptide showing activity against many Gram positive bacteria, is widely used as a food preservative. The simulated gastrointestinal digestion of nisin (variant A) was studied using the *in vitro* INFOGEST digestion method. Following oral, gastric and small intestinal digestion, there was no intact nisin in the system and the nisin was primarily digested by pancreatin. After digestion, six nisin fragments (1–11, 1–12, 1–20, 1–21, 1–29 and 1–32) were identified by reversed phase high performance liquid chromatography and mass spectroscopy and four of these nisin fragments (1–20, 1–21, 1–29 and 1–32) demonstrated low antibacterial activity against *Lactococcus lactis* HP in agar diffusion activity assays. Additionally, it was observed that bile salts form a complex with nisin. This was examined by atomic force microscopy, turbidity and dynamic light scattering, which showed that this interaction resulted in significantly larger bile salt micelles. The presence of bile salts at physiological levels significantly altered the relative amounts of the nisin fragments 1–12, 1–20 and 1–29 produced during an *in vitro* digestion. This study highlights the importance of including bile in simulated digestions of antimicrobial peptides in order to obtain a more accurate simulation of the *in vivo* digestion products and their activity.

1. Introduction

Nisin is a 34 amino acid antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis* that is active against many Gram-positive bacteria and is widely used as a food preservative (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016). Nisin is extremely stable at pH 3 and can be autoclaved at this pH with <5% loss of activity (Davies et al., 1998), whereas above pH 6 it is unstable even at room temperature (Kelly, Reuben, Rhoades, & Roller, 2000).

The discovery that nisin is inactivated by pancreatin (Heinemann & Williams, 1966), primarily due to its chymotrypsin component (Jarvis & Mahoney, 1969), was a factor in nisin being awarded GRAS status by the FDA (U. S. Food and Drug Administration, 1988) and the European Food Safety Authority declaring that nisin is safe for use in food (European Food Safety Authority, 2006) with its assigned E number being E 234 (European Commission, 2011). It has been demonstrated more recently that nisin is also cleaved by the trypsin component of pancreatin (Chan et al., 1996). However these studies focused on pancreatic enzymes and did not take into account the other components of the digestive system such as bile.

Bile salts, the major functional component of bile, are biological surfactants which are involved in the digestion and absorption of lipids in the small intestine; in particular they transport the products of lipolysis in bile salt micelles to the sites of absorption (Bauer, Jakob, & Mosenthin, 2005). For the most common human and porcine bile salts, micelle formation takes place in two stages; hydrophobic interactions between bile salts results in primary micelles, which then interact via hydrogen bonding to form secondary micelles (Kandrac et al., 2006; Partay, Jedlovszky, & Sega, 2007; Small, 1968). The minimum bile salt concentration required for micelle formation is termed the critical micelle concentration (CMC). As the concentration of sodium ions affects the CMC, experiments with bile salts are commonly performed in 0.15 mol/L Na^+ solutions to simulate physiological conditions (Hofmann &

* Corresponding author.
E-mail address: andre.brodkorb@teagasc.ie (A. Brodkorb).

http://dx.doi.org/10.1016/j.lwt.2017.08.031
0023-6438/

Hagey, 2008). In a 0.15 mol/L Na^+ solution, most bile salts have a CMC below 10 mmol/L (Hofmann & Roda, 1984); 10 mmol/L is also the bile salt concentration recommended for simulating physiological conditions during *in vitro* digestion (Minekus et al., 2014).

Previous digestion studies on nisin have focussed on pancreatic enzymes from the small intestine and those that investigated the nisin fragments produced by digestion used enzymes individually and often used digestions in excess of 20 h (Chan et al., 1996; Heinemann & Williams, 1966; Jarvis & Mahoney, 1969; Slootweg, Liskamp, & Rijkers, 2013). In order to study how nisin is digested under more physiologically relevant conditions, the INFOGEST method, a recently developed standardized static method for the digestion of food (Minekus et al., 2014) was utilised. This method is the consensus of an international network of scientists and is based on physiological conditions with each digestion comprising an oral, gastric and intestinal stage (Minekus et al., 2014). This approach would establish which nisin fragments are produced under physiological conditions and also their biological activity. In addition, by performing versions of the digestion without individual digestion components, the importance of non-proteolytic components such as bile on the digestion profile of nisin could be established.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma-Aldrich (Arklow, Ireland) unless otherwise stated. For the simulated digestions the specific Sigma-Aldrich products used were: salivary amylase (A1031), pepsin (P6887), bile (B8631) and pancreatin (P7545). Tween® 80 was obtained from Merck Millipore (Darmstadt, Germany). The nisin preparation used was Nisaplin® (DuPont, Beaminster, UK) (nisin variant A; referred to as 'nisin' throughout this text). This was enriched by salting out as previously described (Gough et al., 2017).

2.2. Simulated digestion

Simulated oral, gastric and small intestinal digestions were performed as described in the INFOGEST method (Minekus et al., 2014). Five variations of the digestion were performed: (i) nisin with all digestion components, (ii) nisin with all digestion components except bile, (iii) nisin with all digestion components except pancreatin, (iv) nisin with all digestion components except pepsin, bile and pancreatin, (v) all digestion components but no nisin. A minimum of three replicates were performed of each of these five digestion setups. The initial nisin concentration was chosen so that the nisin concentration in the digestion product would be sufficient for quantification by reversed phase - high performance liquid chromatography (RP-HPLC). The digestion containing nisin and all digestion components was performed as follows: for the oral stage 5 mL of an 8.7 mg/mL nisin solution was combined with simulated salivary fluid (SSF) and salivary amylase (75 U/mL in final oral solution) to a final total volume of 10 mL; this was incubated at 37 °C for 2 min. For the gastric stage, the sample pH was adjusted to 3 using dilute HCl and combined with simulated gastric fluid (SGF) and pepsin (2000 U/mL in final gastric solution) to a final total volume of 20 mL; this was incubated at 37 °C for 2 h. For the small intestinal stage the pH was adjusted to 7 using dilute NaOH and combined with simulated intestinal fluid (SIF) and bile (10 mmol/L bile salts in final intestinal solution) and pancreatin (100 TAME U/mL in final intestinal volume) to a final total volume of 40 mL, this was incubated at 37 °C for 2 h. The digestion products were snap-frozen in liquid nitrogen.

2.3. Determination of the effect of the presence of bile during digestion on the activity of the digestion products

To determine the effect of the presence of bile during digestion on the activity of the digestion products a simplified digestion method based on Minekus et al. (2014) was used; nisin was incubated with pancreatin in a MOPS buffer at pH 7 and 37 °C for 2 h with bile added either before or after digestion, with an equivalent volume of water added to samples that did not receive bile. The final constituents in each sample, in a total volume of 0.5 mL, were 100 μg/mL nisin, bile at a bile salt concentration of 0.3 mmol/L, pancreatin at a concentration such that its trypsin activity was 100 TAME units per mL, 50 mmol/L MOPS, 0.15 mol/L NaCl and the pH was 7. The digestion products were analysed by activity assay as described in section 2.6.

2.4. Reversed phase - high performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on a Jupiter, 5 μm, C18, 300 Å, 250 mm × 4.6 mm column from Phenomenex (Macclesfield, UK) with an acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) gradient as described previously (Gough et al., 2017). In the case of digested nisin, fractions were collected throughout the gradient to determine the nisin fragments produced by digestion.

2.5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS)

The molecular mass of the RP-HPLC peaks were determined using MALDI TOF MS using an Axima TOF^2 mass spectrometer (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).

2.6. Activity assay

Biological activity was estimated by agar diffusion activity assays (Ryan, Rea, Hill, & Ross, 1996) in agar plates seeded with *Lactococcus lactis* subsp. *cremoris* HP which were prepared as described previously (Gough et al., 2017). Serial two-fold dilutions of the samples were performed in 0.15 mol/L NaCl, 50 mmol/L MOPS, pH 7. In specific cases a surfactant (0.3 mmol/L bile salts, 8 mmol/L Tween® 80 or 0.2 mmol/L Triton™ X-100) was included in the diluent. The samples (50 μL) were dispensed into the wells and the plates incubated overnight at 30 °C. The activity of nisin resulted in zones of inhibition surrounding the wells. Activity is expressed as the minimum inhibitory concentration (MIC) in terms of μg/mL (Chan et al., 1996). MIC was calculated by plotting the area of the zone of inhibition at each dilution stage against the log of the nisin concentration (Bernbom et al., 2006); these had a linear relationship and the MIC was calculated from the equation of the line.

2.7. Atomic force microscopy (AFM)

For AFM, samples comprised 10 mmol/L bile salts, 0.15 mol/L NaCl, and 50 mmol/L MOPS at pH 7, with or without 0.5 μg/mL nisin. The nisin concentration of 0.5 μg/mL was chosen as this is within the range that could occur in the small intestine after consumption of a nisin containing foodstuff (Delves-Broughton, 2005; Minekus et al., 2014). Aliquots (5 μL) were deposited onto freshly cleaved mica surfaces, dried in a desiccator and subsequently stored at ambient conditions to ensure equilibrated hydration. AFM images were obtained with an Asylum Research MFP-3D-AFM (Asylum Research UK Ltd., Oxford, UK) using AC-mode in ambient