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Whey protein hydrolysate induced modulation of endothelial cell gene expression

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ABSTRACT

Whey protein concentrate (WPC) hydrolysates generated using Neutrase®, Alcalase® and Flavourzyme® and their associated ultrafiltration fractions inhibited angiotensin-1-converting enzyme activity (71.14 ± 1.05, 73.22 ± 0.99 and 51.52 ± 5.80% inhibition when assayed at 14.3 μg/mL). Incubation of human umbilical vein endothelial cells with 5 kDa permeates of Neutrase®- and Alcalase®-hydrolysed WPC for 48 h resulted in the beneficial differential expression of genes relevant to blood pressure control, as measured by microarray. Furthermore, real-time reverse transcriptase polymerase chain reaction demonstrated an upregulation of endothelial nitric oxide synthase (+2.14 ± 0.45 and 2.36 ± 0.27-fold) and down-regulation of endothelin-1 (−0.58 ± 0.09 and −0.82 ± 0.11-fold) following incubation with the 5 kDa permeates of Alcalase®- and Neutrase®-hydrolysates, respectively. Peptide sequences within the 5 kDa permeate of the Alcalase®-hydrolysed WPC were identified, many of which have previously been demonstrated as having ACE-inhibitory and/or other bioactivities. These WPC hydrolysates potentially represent sources of bioactive peptides for the beneficial regulation of endothelial cell function.

1. Introduction

Cardiovascular disease (CVD) was associated with 31% of all global deaths in 2015 (WHO, 2017). Hypertension is one of the major risk factors for CVD. Diet plays a major role in the control of hypertension and CVD. Therefore, there is interest in developing foods having anti-hypertensive properties. Food proteins contain peptides that, when released by, e.g., enzymatic hydrolysis, fermentation, processing etc., have bioactive effects. Milk protein-derived bioactive peptides have been associated with a range of bioactivities including anti-microbial, anti-oxidative, anti-thrombotic, anti-hypertensive and immunomodulatory effects (Korhonen & Pihlanto, 2003; Nongonierma, O'Keeffe, & FitzGerald, 2016). The renin-angiotensin system (RAS) is an important regulator of hypertension. Inhibition of angiotensin 1 converting enzyme (ACE), an important component of the RAS, has a number of effects in lowering blood pressure, including the reduced production of the vasoconstrictor, angiotensin 1 and the decreased breakdown of the potent vasodilator, bradykinin. ACE inhibition has been extensively investigated in terms of the potential blood pressure lowering effects of food protein derived peptides (Norris & FitzGerald, 2013). Two of the best known milk protein-derived ACE inhibitory peptides (Val-Pro-Pro and Ile-Pro-Pro) have been identified in a sour milk drink (Ameal S) fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* strains (Nakamura, Yamamoto, Sakai, & Takano, 1995). Numerous studies have shown Val-Pro-Pro and Ile-Pro-Pro, or products containing these peptides, to have beneficial effects on blood pressure in spontaneously hypertensive rats and hypertensive humans (Cicero, Aubin, Azais-Braesco, & Borghi, 2013; Norris & FitzGerald, 2013, for review).

Much attention has also focused on antihypertensive effects of food protein-derived peptides through pathways other than ACE inhibition. These include inhibition of renin and endothelin, blocking of calcium channels and angiotensin receptors, as well as modulation of the nitric oxide pathway (Udenigwe & Mohan, 2014).

The nitric oxide (NO) system is a major controller of vasodilation. NO regulates vascular tone at rest, mediates changes in blood flow to meet metabolic demand of tissue, and dilates vessel diameter in response to increased blood flow (Kelm, 2003). NO is synthesised from L-arginine and oxygen by nitric oxide synthase (NOS). There are three forms of NOS; neuronal (nNOS), inducible (iNOS) and that produced by endothelial cells (eNOS). Elevations in eNOS activity may result in increased NO secretion by endothelial cells in turn leading to vasorelaxation. Consumption of a novel whey protein-derived extract (NOP-47) which increases endothelial NO was reported to improve brachial artery flow mediated dilation in healthy (Ballard et al., 2009) and overweight (Ballard et al., 2013) adults. However, the peptide sequence

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(s) of NOP-47 are not reported in the literature. Some milk protein-derived peptides may exert their antihypertensive effects through inhibition of the potent vasoconstrictor, endothelin-1 (ET-1). Incubation of the whey protein-derived lactokinin peptide, Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR), with endothelial cells resulted in a 29% reduction in basal ET-1 (Maes et al., 2004). However, in general, there is a lack of information on the mechanism of action of many antihypertensive hydrolysates/peptides.

The main aims of this study therefore were (i) to assess the ACE-inhibitory properties of UF fractions of WPC hydrolysates (produced using Alcalase®, Neutrase® and Flavourzyme®) and (ii) to investigate the potential antihypertensive effects of WPC-derived peptides though pathways other than ACE inhibition, by assessing differential expression of vasomodulatory genes in HUVECs exposed to 5 kDa permeates of Alcalase® and Neutrase® WPC hydrolysates.

2. Materials and methods

2.1. Materials

Whey protein concentrate (WPC80, 75.8% protein) manufactured from sweet whey was purchased from a commercial supplier (Carberry Milk Products, Ballineen, Ireland). Alcalase® 2.4L, Neutrase® and Flavourzyme® 500L were generously donated by Novozymes A/S (Bagsvaerd, Denmark). MCDB-131, L-glutamine, foetal bovine serum (FBS), trypsin and trizol® were all from Invitrogen (CA, USA). Epidermal growth factor (EGF), insulin growth factor (IGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) were from Peprotech (London, UK). O-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-nitro-Pro-OH) and o-aminobenzoylglycine (Abz-Gly) were from Bachem (79576 Weil am Rhein, Germany). Rabbit-lung acetone powder and sodium tetraborate decahydrate (borax) were from Sigma (Wicklow, Ireland). Human umbilical vein endothelial cells (HUVECs) were from Lonza Biologics (Slough, UK). The QuantiTect reverse transcription kit was from Qiagen (Crawley, West Sussex, UK). The LightCycler® FastStart DNA master plus sybr green I kit and the LightCycler® capillaries were from Roche Diagnostics (West Sussex, UK).

2.2. Enzymatic hydrolysis of whey protein concentrate, membrane processing and spray-drying of hydrolysates

WPC hydrolysates (WPHs) were generated, membrane processed and spray dried as previously described by O'Keeffe and FitzGerald (2014). In short, WPC (10% w/v, protein) was allowed to hydrate at room temperature for 1 h. Hydrolysis was carried out using Neutrase®, Alcalase® and Flavourzyme® at an enzyme: substrate ratio of 0.3% (v/w) at 50 °C for 4 h and using a pH Stat (842 Stat Titrino, Methrom, Herisau, Switzerland) to maintain the pH at 7.0. Hydrolysates were subsequently heated to 80 °C for 20 min to inactivate the enzyme. Cooled hydrolysates were processed through UF membranes having nominal molecular mass cut-offs of 0.2 µm, 5 kDa and 1 kDa using a bench-scale ultrafiltarion system (Sartoflow Alpha, Sartorius AG, Goettingen, Germany). A 650 Da permeate fraction was generated by passing the 1 kDa permeate through a 650 Da membrane using a tangential flow filtration (TFF) in a Minimate™ TFF Capsule with an Omega™ 65D membrane (Pall Life Sciences, Ann Arbor, MI, USA). Hydrolysates and UF fractions were spray-dried using a bench-top B290 mini spray dryer (Buchi Labortechnik AG, Switzerland). The inlet and outlet temperatures were 140–160 and ≤70 °C, respectively.

2.3. Angiotensin converting enzyme (ACE) inhibition assay

The ACE activity was from rabbit lung acetone powder and ACE inhibition of UF permeates of hydrolysates was determined as previously described (Norris, Casey, FitzGerald, Shields, & Mooney, 2012; Sentandreu & Toldra, 2006) with some modifications. The amount of ACE enzyme employed was 3 mU/mL. UF permeates of hydrolysates were investigated for ACE inhibitory activity at a final concentration in the assay of 14.3 µg/mL.

2.4. Cell culture and microarray analysis

Routine cell maintenance and microarray analysis was carried out as described by O'Keeffe and FitzGerald (2014). In short, HUVECs were maintained in MCDB-131 with FBS (10%, v/v), VEGF (1 ng/mL), FGF (2 ng/mL), IGF (2 ng/mL) and EGF (10 ng/mL). For microarray analysis HUVECs between passages 6 and 10 were grown on 10 cm^2 dishes and were incubated with vehicle control (media)/unhydrolysed WPC/5 kDa permeate of Alcalase®- or Neutrase®-hydrolysed WPC. RNA was isolated using Trizol® following the manufacturer's instructions (Invitrogen, CA, USA). Microarray analysis was carried out by Almac Diagnostics (Craigavon, Northern Ireland) using an Affeymetrix® Human Genome U133 Plus 2.0 array (Affeymetrix, Santa Clara, CA, USA) as described by Knudsen et al. (2012). All RNA samples passed spectrophotometric ($A_{260/280}$ and $A_{260/230}$ ratios of ~2.0) and Bioanalyser quality control analyses at Almac Diagnostics. The Human Genome U133 Plus 2.0 Array analyses the expression levels of over 47,000 transcripts and variants. This includes 38,500 well-characterised human genes. A number of controls are included on the array, e.g., hybridisation controls (bioB, bioC, bioD, cre), Poly-A controls (dap, lys, phe, thr) and housekeeping/control genes (GAPDH, beta-actin, STAT-1). The full list of target genes is available at https://www.thermofisher.com/order/catalog/product/900466. Gene regulation in the three treatment groups (unhydrolysed WPC, 5 kDa permeate of Alcalase® WPH and 5 kDa permeate of Neutrase® WPH) was expressed as a function of gene expression in vehicle-treated HUVECs. The gene expression ratios were generated using an Affymetrix® default ratio builder error model. The data was processed using the Rosetta Error Model with further stringency being applied through the use of specific filters, i.e., subtraction of three times the average standard deviation of the background intensity and a fold-change filter whereby > 1.5-fold change was required for stringent lists. Pathway analysis was carried out using MetaCore™ data mining software (GeneGo, CA, USA) in order to determine the specific molecular pathways that were most regulated.

2.5. Real-time reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from HUVECs that had been incubated with unhydrolysed WPC/5 kDa permeate of the Alcalase® WPH/5 kDa permeate of the Neutrase WPH using the Trizol® procedure according to manufacturer's instructions (Invitrogen, CA, USA). Traces of genomic DNA were eliminated and first strand cDNA was synthesised from 0.8 µg RNA using the QuantiTect® reverse transcription kit as per manufacturer's instructions (Qiagen, Crawley, West Sussex, UK). Thereafter, aliquots (5 µL of 1:10 diluted) of first strand cDNA were used as templates in each real-time PCR reaction (20 µL) using the LightCycler® FastStart DNA master plus sybr green I kit following the manufacturer's instructions (Roche Diagnostics, West Sussex, UK). Oligonucleotide primers were designed using the Roche Universal ProbeLibrary version 2.43 software and included the forward and reverse primers for endothelial nitric oxide synthase (eNOS); 5′-GACCC TCACCGCTACAACAT-3′ and 5′-CCGGGTATCCAGGTCCAT-3′, respectively, forward and reverse primers for endothelin-1 (ET-1); 5′-TCTCT GCTGTTTGTTTGTGGCTTG-3′ and 5′-GAGCTCAGCGCCTAAGACTG-3′, respectively, and forward and reverse primers for hypoxanthine-phosphoribosyl-transferase (HPRT); 5′-CGTGATTAGTGATGATGAACCAG-3′ and 5′-CGAGCAAGACGTTCAGTCCT-3′, respectively. Real-time RT-PCR reactions were carried out on the LightCycler® Carousel system (Roche Diagnostics, West Sussex, UK) and were analysed using the relative quantification function of LightCycler® version 4.0 software. Fold change was determined using the delta Ct model and as a function of