Food Chemistry 146 (2014) 443–447

Contents lists available at ScienceDirect

Food Chemistry

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

Antimicrobial activity of antihypertensive food-derived peptides and selected alanine analogues

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ARTICLE INFO

Article history:
Received 22 May 2013
Received in revised form 29 August 2013
Accepted 16 September 2013
Available online 25 September 2013

Keywords:
Food derived peptides
Antimicrobial
Mass spectrometry
Peptide synthesis

ABSTRACT

This study evaluated four food-derived peptides with known antihypertensive activities for antimicrobial activity against pathogenic microorganisms, and assessed structure–function relationships using alanine analogues. The peptides (EVSLNSGYY, barley; PGTAVFK, soybean; TTMPLW, α-casein; VHLPP, α-zein) and the six alanine substitution peptides of PGTAVFK were synthesised, characterised and evaluated for antimicrobial activity using the bacteria, *Escherichia coli*, *Staphylococcus aureus*, and *Micrococcus luteus* and the yeast, *Candida albicans*. The peptides TTMPLW and PGTAVFK inhibited growth of all four microorganisms tested, with activities of a similar order of magnitude to ampicillin and ethanol controls. EVSLNSGYY inhibited the growth of the bacteria, but VHLPP showed no antimicrobial activity. The alanine analogue, PGAAVFK showed the highest overall antimicrobial activity and PGTAVFA showed no activity; overall, the activities of the analogues were consistent with their structures. Some peptides with antihypertensive activity also show antimicrobial activity, suggesting that food-derived peptides may exert beneficial effects via a number of mechanisms.

1. Introduction

Peptides from a diverse range of food sources including milk products, cereals, soybeans and fish have shown a very wide spectrum of bioactive properties, including antimicrobial, anticarcinogenic, insulinotropic, immunomodulatory and antihypertensive effects (FitzGerald, Murray, & Walsh, 2004; Kitts & Weiler, 2003; Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008; Pellegrini, 2003).

The potential of some bioactive peptides has been exploited, for example in the production of functional foods with antihypertensive, hypocholesterolemic and other beneficial effects (Mills, Stanton, Hill, & Ross, 2011), and in the development of food preservation and packaging which may exploit antimicrobial effects (Perez Espitia et al., 2012). Furthermore, it is increasingly recognised that food-derived peptides may exert multiple functions. For example, lactoferricin, a 25 amino acid peptide derived from milk, exhibits antibacterial, antifungal, antiviral, antitumour, antiinflammatory, and immunoregulatory properties (Vogel et al., 2002), and a more recent study has shown that peptides released from the caseinate of ovine milk have antioxidant, antihypertensive and antimicrobial properties (Correa et al., 2011).

Alanine substitution studies sequentially replace each amino acid within a peptide to provide new alanine-substituted peptide structures and allowing the contribution of amino acid residues within a peptide towards a particular bioactivity to be examined. This procedure is widely applied in the field of bioactive peptide engineering to determine sites of activity (Alana et al., 2006) and has been used very recently to assess the contribution of amino acids to the activity of the peptide antibiotic feglymycin (Hänchen et al., 2013).

Thus, the aims of this study are to evaluate the antimicrobial activity (using three pathogenic bacteria and one pathogenic fungus) of four diverse food-derived peptides, which have previously been shown to exert hypotensive properties. Furthermore, putative structure–function relationships are evaluated and assessed using the six alanine substitution analogues of one peptide.

2. Materials and methods

2.1. Peptide selection

The peptides selected originated from α-casein (TTMPLW), α-zein (VHLPP), barley (EVSLNSGYY) and soybean (PGTAVFK) and have previously been shown to have antihypertensive properties (Kitts & Weiler, 2003; Migliore-Samour, Floc'h, & Jolles, 1989; Yamamoto, 1997). These peptides are very short (<10 residues) and include three from seed crop sources that, unlike milk, have not been the subject of extensive study. Alanine substitution analogues of

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http://dx.doi.org/10.1016/j.foodchem.2013.09.094

PGTAVFK were also prepared (AGTAVFK, PATAVFK, PGAAVFK, PGTAAFK, PGTAVAK and PGTAVFA) to investigate structure–function relationships. Table 1 shows the food-derived and the alanine substitution analogues tested, with details of relative molecular mass (http://www.peptidesynthetics.co.uk/tools/), source, net charges, and previously reported bioactive properties.

2.2. Peptide synthesis

All peptides in the study were prepared using standard FMOC (9-fluorenyl methoxy carbonyl) solid phase peptide synthesis on an Applied Biosystems 433A automated peptide synthesizer (Applied Biosystems, Warrington, UK). Peptides were extracted from the amide resin and the side-chain protecting groups were removed using Reagent K, a mixture of trifluoroacetic acid (TFA)/water (H_2O)/phenol/thioanisol/ethanedithiol in ratio 82.5/5/5/5/2.5 (Hobba et al., 1998).

2.3. Peptide characterisation and identification

The purity of the synthesised peptides was assessed by matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-ToF MS) using a PerSeptive Biosystems Voyager- DE Mass Spectrometer (PerSeptive Biosystems, Herefordshire, UK). Alpha-cyano 4-hydroxycinnamic acid, was obtained from Sigma Aldrich (Poole, Dorset, UK), and PepMix 2 calibration standard was purchased from LaserBio Labs, (Sophia-Antipolis Cedex, France).

Peptide structures and masses were confirmed by MS/MS sequencing with quadrupole time of flight mass spectrometry (QToF Ultima API, Micromass, Manchester, UK) and an LCQ™ 'Classic' quadrupole ion trap (QIT) mass spectrometer (Thermo, San Jose, CA, USA). The ion trap mass spectrometer had an electrospray interface operated in positive ion mode coupled with full scan mode and an ion injection time of 200 ms. The capillary temperature was 220 °C and the spray voltage was 4.5 kV. The nitrogen sheath and the auxiliary gas flows were set at 50 and 5 arbitrary units, respectively, as defined by the software.

De novo MS/MS sequencing was performed on a QToF Ultima API, quadrupole time of flight mass spectrometer (Waters, Manchester, UK). Aliquots of the synthesised peptides were prepared in acetonitrile and directly infused into the nanospray source from a syringe pump at flow rate of 0.1 and 1 μL/min. The spray voltage was 3.5 kV and the source temperature was 80 °C. The QToF analyser was operated in 'V' optics mode while the mass spectrometer was in positive ion mode with the cone voltage set to 100 V. The QToF mass spectrometer produced raw spectral data of the singly charged precursor ion patterns, generated from the synthesised peptides. MS/MS fragmentation of each selected precursor was induced by raising the collision energy from 10–35 eV until a well-populated MS/MS spectrum of product ions was obtained. The data were analysed using MassLynx version 3.5 software incorporating Mass Seq and Pep Seq tools and the MaxEnt 3 deconvolution algorithm (Waters, Milford, MA, USA).

2.4. Antimicrobial assays

The peptides were dissolved in phosphate-buffered saline (PBS) to provide the following stock concentrations: PGTAVFK 2.5 mM; VHLPP 1.4 mM; EVSLNSGYY 0.9 mM and TTMPLW 1.7 mM. Stock concentrations of the alanine analogues of PGTAVFK (AGTAVFK, PATAVFK, PGAAVFK, PGTAAFK, PGTAVAK, PGTAVFA) were approximately 2.9 mM. Cultures of the Gram-negative bacterium, *Escherichia coli* (ATCC No. 11775), the Gram-positive bacteria *Staphylococcus aureus* (ATCC No. 12600) and *Micrococcus luteus* (ATCC No. 49732), and the yeast, *Candida albicans* (ATCC No. 14053-u) came from University of Ulster stocks. Nutrient agar, malt extract agar and PBS tablets were purchased from Oxoid Pty. Ltd. (West Heidelberg, Australia). *E. coli*, *S. aureus* and *M. luteus* were cultured aerobically in nutrient broth at 37 °C, and *C. albicans* was cultured aerobically in malt extract broth at 25 °C.

E. coli, S. aureus and *M. luteus* were cultured initially in nutrient broth for 24 h, while *C. albicans* was cultured in malt extract broth for 48 h. The antimicrobial assay was modified from the critical dilution method as previously described (Conlon et al., 2005). Briefly, twelve serial, threefold dilutions of the peptides and alanine analogues were incubated in 96 well plates with an inoculum (100 μL of 1 $\times 10^6$ colony forming units/mL) from a log phase culture of each of the microorganisms. The dilutions were carried out as follows; 50 μl of a peptide and bacteria solution were pipetted from well one and mixed with the contents of well two (50 μl of PBS and 50 μl of the log phase culture or microorganism broth) and then 50 μl was removed from well two into well three and sequentially until well twelve. Control incubations were carried out in parallel with ampicillin (5 mg/mL; 14.3 mM) and with aqueous ethanol (70% v/v) for the bacteria and yeast respectively. The 96 well plates containing the bacteria were incubated initially for 18 h at 37 °C (*E. coli, S. aureus, M. luteus*) in humidified atmospheric air, while the incubations with *C. albicans* were carried out in malt extract broth initially for 48 h at 25 °C. Viability of the microorganisms was tested after this initial incubation period by adding 40 μL of 0.2 mg/mL iodonitrotetrazolium chloride (INT, Sigma Aldrich, Poole, Dorset, UK) to each well, and incubating for three hours in the initial conditions (37 °C or 25 °C). Iodonitrotetrazolium chloride is a colourless dye which when taken up by viable respiring cells is reduced and turns pink in colour. Therefore, where wells remain colourless this is an indication that bacterial growth was inhibited. Following this, the wells were plated out on nutrient agar and malt extract agar and the colonies were manually counted. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the peptide that inhibited the visible

Table 1
Sources and properties of the food-derived peptides and alanine analogues. All peptides were prepared using solid phase peptide synthesis.

Amino acid sequence	Food source	RMM (Da)	Observed *m/z* ratio (QIT MS)	Net charge	Reported bioactive properties	References
TTMPLW	α-S2 casein	746.9	747.8	0	Antihypertensive, immunostimulatory	Migliore-Samour, Floc'h, and Jolles (1989)
VHLPP	α-Zein	560.7	561.6	+1	Antihypertensive	Yamamoto (1997)
EVSLNSGYY	Barley	1030.1	1031.2	−1	Antihypertensive	Kitts and Weiler (2003)
PGTAVFK	Soybean	717.9	718.9	+1	Antihypertensive	Kitts and Weiler (2003)
AGTAVFK	Synthetic	691.8	692.7	+1	None	–
PATAVFK	Synthetic	731.9	732.8	+1	None	–
PGAAVFK	Synthetic	687.8	688.8	+1	None	–
PGTAAFK	Synthetic	689.8	690.7	+1	None	–
PGTAVAK	Synthetic	641.8	642.7	+1	None	–
PGTAVFA	Synthetic	660.8	661.7	0	None	–

RMM = relative molecular mass; QIT MS – quadrupole ion trap mass spectrometry.