Short communication: Measuring the angiotensin-converting enzyme inhibitory activity of an 8-amino acid (8mer) fragment of the C12 antihypertensive peptide¹

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ABSTRACT

An 8-AA (8mer) fragment (PFPEVFGK) of a known antihypertensive peptide derived from bovine α_{S1} -casein (C12 antihypertensive peptide) was synthesized by microwave-assisted solid-phase peptide synthesis and purified by reverse phase HPLC. Its ability to inhibit angiotensin-converting enzyme (ACE) was assessed and compared with that of the parent 12mer peptide (FFVAPFPEVFGK) to determine the effect of truncating the sequence on overall hypotensive activity. The activity of the truncated 8mer peptide was found to be almost 1.5 times less active than that of the 12mer, with ACE-inhibiting IC_{50} (half-maximal inhibitory concentration) values of 108 and 69 μM , for the 8mer and 12mer, respectively. Although the 8mer peptide is less active than the original 12mer peptide, its overall activity is comparable to activities reported for other small proteins that elicit physiological responses within humans. These results suggest that microbial degradation of the 12mer peptide would not result in a complete loss of antihypertensive activity if used to supplement fermented foods and that the stable 8mer peptide could have potential as a blood pressure-lowering agent for use in functional foods.

Key words: antihypertensive peptide, bovine milk, solid-phase peptide synthesis, 8-amino acid peptide

Short Communication

Being able to increase the nutritional and health-promoting properties of food using bovine milk-derived ingredients is a challenge that has attracted a great

deal of attention recently. Many bioactive compounds derived from milk proteins exhibit potential as functional food ingredients aimed at combating various conditions that can be controlled through diet, such as cardiovascular disease, type II diabetes, and obesity (Korhonen, 2009). These active peptides are hydrolytically released from the parent protein upon treatment with digestive enzymes, bacteria-associated proteases, or other proteases (Korhonen and Pihlanto, 2006). The resulting peptides display a wide variety of in vitro biological activities, including antihypertensive activities (Nagpal et al., 2011). Based on this broad spectrum of activities, there is great potential for the application of these peptides in the production of functional dairy foods.

Several studies have illustrated the correlation between dairy consumption and lowering of blood pressure (Lopez-Fandino et al., 2006; Jauhainen and Korhonen, 2007) and have identified specific peptides with angiotensin-I-converting enzyme (ACE) inhibitory activity (Korhonen and Pihlanto, 2006). Specifically, an α_{S1} -casein-derived 12-AA peptide f(23–34) with the sequence FFVAPFPEVFGK (12mer) has an inhibitory effect on ACE (Maruyama et al., 1987; Karaki et al., 1990; Townsend et al., 2004) and has been reported to reduce systolic and diastolic blood pressure in human studies (Sekiya et al., 1992; Townsend et al., 2004; Cadée et al., 2007). The health effects of this peptide (also called C12 antihypertensive peptide) have been confirmed by the European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies (EFSA, 2010). Some food products have been developed that have utilized milk-derived ACE-inhibitory peptides, including Ameal S (Calpis Co. Ltd., Tokyo, Japan), a tablet-form sour milk product on the market in Japan (Nakamura et al., 1995; Hayes et al., 2007). To date, however, antihypertensive compounds such as the 12mer have not been incorporated into any available fermented dairy products such as cheese or yogurt.

Because it is derived from bovine milk proteins, dairy foods such as yogurt may be an ideal delivery vehicle for

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the 12mer antihypertensive peptide. The major limitation in this application is the presence of the *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* starter cultures used for milk fermentation. Cell-associated peptidases of these lactic acid bacteria (**LAB**) may inactivate the bioactive peptides by hydrolyzing them to AA (Gilbert et al., 1996; Siezen, 1999; Fernandez-Esplá et al., 2000; Courtin et al., 2002). Previous work (Paul and Somkuti, 2009) has shown that the 12mer peptide is significantly degraded in the presence of various *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* cultures, resulting in an 8-AA (**8mer**) by-product with the sequence PFPEVFGK, which is relatively resistant to further proteolysis. Therefore, this study characterized and compared the antihypertensive properties of the 8mer peptide and the parent 12mer peptide to determine if it would be feasible to use either peptide to improve the functional value of fermented dairy foods.

The 8mer degradation product was generated by microwave-assisted solid-phase synthesis using a CEM Liberty 1 synthesizer (CEM Corporation, Matthews, NC). General peptide synthesis and cleavage protocols were followed (Cavalluzzo et al., 2013), with the crude samples analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The sample was further purified by semi-preparative C18 reverse-phase column chromatography (Alltech, Columbia, MD; 10 × 250 mm column with 10- μ m particle size) using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) and a linear gradient from 5 to 100% B (95–0% A) over 60 min at a flow rate of 4.7 mL/min, where A was water with 0.1% trifluoroacetic acid (**TFA**) and B was acetonitrile with 0.1% TFA, and shown to contain a single peak (Figure 1). Reverse phase (**RP**)-HPLC fractions were further analyzed by MALDI-TOF MS and showed the presence of a peptide with the expected mass of 920.5 atomic mass units (amu; Figure 2).

A preparation of the parent 12mer peptide was previously obtained from SynPep (Dublin, CA) and used as a control for comparison of ACE-inhibitory activity. A stock solution of commercial ACE from rabbit lung (Sigma-Aldrich, St. Louis, MO) was prepared in reaction buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl) to give a final concentration of 0.25 U/mL. Inhibition of ACE activity was determined using the method previously published by Paul and Van Hekken (2011). Varying amounts of the 12mer peptide (final concentrations of 0–0.33 mg/mL) were preincubated with 4 μ L of ACE at 37°C for 30 min, after which 5 μ L of the substrate FAPGG {*N*-3-([2-furylacryloyl]-Phe-Gly-Gly); 100 μ M stock} was added and the reaction was carried out for an additional 40 min. The reaction was stopped by the

Figure 1. Reverse phase-HPLC chromatogram of 8-AA (8mer) peptide. mAU = milli-absorbance units.

addition of 5 μ L of TFA (10%) and FAPGG hydrolysis by ACE was monitored by HPLC (Figure 3). The half-maximal inhibitory concentration (**IC**₅₀) value for the 12mer was calculated from the inhibition data that was collected and found to be 0.095 mg/mL, corresponding to 69.0 μ M (using the peptide molecular mass of 1,384.6 g/mol). This value is consistent with the value of 77 μ M previously reported by Maruyama et al. (1987).

Similar amounts of the 8mer peptide (final concentration 0–0.35 mg/mL) were tested for inhibiting ACE activity (Figure 4). The **IC**₅₀ value for the 8mer was calculated to be 0.099 mg/mL, corresponding to 108 μ M (using the peptide molecular mass of 920.1 g/mol). This value corresponds to an efficacy about 1.5 times less than that of the 12mer. It has been reported,

Figure 2. Matrix-assisted laser desorption/ionization-time-of-flight MS data analysis of purified 8-AA (8mer) peptide (m/z = 920.5 atomic mass units corresponds to 8mer mass).

Figure 3. Angiotensin-converting enzyme inhibition curve of 12-AA (12mer) peptide with an incubation time of 40 min with the calculated half-maximal inhibitory concentration (IC_{50}) value (0.096 mg/mL) indicated.

however, that other antihypertensive peptides derived from food with IC_{50} values in the range of 100 to 500 μM may retain activity and elicit physiological effects in humans following oral administration (Sekiya et al., 1992). This suggests that the stable 8mer peptide may retain enough physiological activity to be considered a functional food ingredient.

There is interest in incorporating bioactive compounds into foods to enhance their overall nutritive or health benefits. Bioactive compounds that are food-derived are considered very attractive as they are intrinsically considered safe for consumption because

Figure 4. Angiotensin-converting enzyme inhibition curve of 8-AA (8mer) peptide with an incubation time of 40 min with the calculated half-maximal inhibitory concentration (IC_{50}) value (0.099 mg/mL) indicated.

they come from foods. Therefore, the ability to fortify foods with these bioactive peptides has become increasingly appealing. This is especially true with milk and dairy products, which have been reported to be rich sources of bioactive compounds (Gobbetti et al., 2002; Pellegrini, 2003; FitzGerald et al., 2004; Zimecki and Kruzel, 2007; Chen et al., 2009; Korhonen, 2009).

Previous studies showing the effectiveness of the C12 peptide in reducing blood pressure in human subjects were carried out by administering the peptide in tablet form (Townsend et al., 2004; Cadée et al., 2007). However, it has been speculated that this peptide could also serve as a functional food ingredient to increase the health benefits of dairy foods such as yogurt. A major concern about its effectiveness as a functional food ingredient centers on its stability within products that contain viable LAB cultures (Paul and Somkuti, 2009). Paul and Somkuti (2009) reported that the C12 antihypertensive peptide was degraded by microbial proteases, and that the resulting 8mer degradation product was fairly robust under conditions that mimic the production of yogurt. Although proteolysis was most efficient under conditions that mimicked the yogurt fermentation step, with 66 to 99% of the peptide degraded within 4 h, peptide degradation was also observed at pH 4.5, with 22 to 34% of the C12 peptide degraded within 60 min (Paul and Somkuti, 2009). These results suggest that the timing of addition of the antihypertensive peptide to the fermented food will influence its stability. For example, the peptide could be added into yogurt during a post-fermentation step, such as when fruit is incorporated into Swiss-style yogurts, or by including it with other supplemental food ingredients such as oatmeal or granola, which are kept separate from the yogurt until just before consumption (e.g., flip-top yogurt packaging). However, once the peptide is exposed to the cultures, the potential for proteolysis exists. The results from our study show that the predominant 8mer degradation product retains a level of antihypertensive activity comparable to that of other antihypertensive peptides that have elicited physiological responses within hosts after oral administration (Sekiya et al., 1992). The results from this study demonstrate the potential for using the C12 peptide as a functional food ingredient because the degradation product retains a physiologically relevant level of antihypertensive activity. Moreover, our results suggest that the 8mer degradation product itself could serve as a bioactive ingredient. Further research is needed on the overall bioavailability of both the 12mer and 8mer peptides after exposure to conditions that mimic the human digestive system to determine their true potential as ingredients for improving the functional value of dairy foods.

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