Short communication: Inhibition of angiotensin 1-converting enzyme by peptides derived from variants of bovine β -casein upon apical exposure to a Caco-2 cell monolayer

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ABSTRACT

This study investigated the consequence of genetically contingent amino acid substitutions in bovine β -casein (CN) genetic variants A¹, A², B, and I on the structure and bioactive potential of peptides following in vitro digestion. The β -CN variants were digested in vitro using pepsin and pancreatin, and a peptide profile was obtained by liquid chromatography tandem mass spectrometry, revealing among others, the β -casomorphin precursor peptides VYFPFGPIHN and VYFPFGPIPN, derived from variant A¹/B and from A²/I, respectively. These 2 peptides were synthesized and assessed for angiotensin 1-converting enzyme (ACE) inhibitory capacity before and after incubation with a monolayer of Caco-2 intestinal cells. The VYFPFGPIHN was a stronger ACE inhibitor than VYFPFGPIPN, with the concentration needed to reach half-maximal inhibition (IC₅₀) of $123 \pm 14.2 \mu\text{M}$ versus $656 \pm 7.6 \mu\text{M}$. Exposure to a Caco-2 intestinal cell monolayer did not affect ACE inhibition by VYFPFGPIHN, but resulted in an almost 2-fold increase in inhibition by VYFPFGPIPN after incubation. Subsequent tandem mass spectrometric analysis identified the truncated peptide VYFPFGPIP, suggesting hydrolysis by a cell membrane associated peptidase. Thus, genetic variation in bovine β -CN results in the generation of peptides that differ in bioactivity, and are differently affected by intestinal brush border peptidases.

Key words: angiotensin 1-converting enzyme, beta-casein, bioactive peptide, genetic variant, in vitro digestion

Short Communication

Bioactive peptides are protein fragments that are encrypted within the sequence of a parent protein, and can be liberated by the action of proteolytic enzymes

(e.g., in the controlled processing of raw materials) or in the gastrointestinal tract (Phelan et al., 2009). Approximately one-third of the protein in bovine milk is β -CN, and so far 15 genetic variants carrying amino acid substitutions have been described (Caroli et al., 2009; Gallinat et al., 2013), exemplified in a substantial variation in milk from Danish dairy cows (N. A. Poulsen, H. B. Jensen, H. S. Møller, M. Hammershøj, and L. B. Larsen, Department of Food Science, Aarhus University, unpublished data). Bovine β -CN has been shown to be an outstanding source of bioactive peptides that may exert physiologically beneficial effects, such as immune modulation, mineral binding, opioid agonism, thrombin inhibition, antioxidant capacity, and reduction of blood pressure through angiotensin 1-converting enzyme (ACE) inhibition (Meisel, 1998; Korhonen and Pihlanto, 2006). The World Health Organisation assessed the global prevalence of hypertension to be approximately 40% for adults 25 yr or older (WHO, 2009), and it is estimated to cause close to 10 million deaths annually (Lim et al., 2012). This has led to much research into ACE inhibitory effects of food-derived peptides during recent decades, and a large number of milk-derived ACE inhibitory peptides have been identified, including many from β -CN (FitzGerald et al., 2004). The inhibitory capacity of a peptide is determined by its sequence, and the amino acid residues at the C-terminal in particular. Cheung et al. (1980) published the results of one of the first systematic investigations into this particular subject more than 3 decades ago. Since then, many studies have followed, showing that ACE inhibitory peptides can possess quite diverse structural characteristics. Most of these peptides have been cataloged in online databases, such as Biopep (Minkiewicz et al., 2008) and ACEpepDB (<http://www.cftri.com/pepdb/index.php>). From this, it follows that modifications to the amino acid sequence of proteins could affect their potential for generation of ACE inhibitory peptides. Such modifications can be the result of genetic polymorphisms manifested in amino acid substitutions, which have been shown to be of consequence for the digestion pattern of β -CN

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variants (Lisson et al., 2013; Petrat-Melin et al., 2015). This issue has been the matter of extensive scientific debate as genetic polymorphism in β -CN has been suggested to underlie the pathogenesis of noncommunicable diseases, such as type-1 diabetes, autism, and cardiovascular disease (Bell et al., 2006; Kaminski et al., 2007). Nevertheless, no clear causal relationship has been established, and mechanistic evidence is still lacking (Noni et al., 2009). This issue is beyond the scope of the present work and will thus not be discussed further.

Besides peptide sequence variation, it is also essential to consider the stability of any bioactive peptides when exposed to the intestinal environment. Both animal and human intervention studies with different allegedly anti-hypertensive foods, hydrolysates, or peptides have been reported (FitzGerald et al., 2004; Fekete et al., 2013; Iwaniak et al., 2014). Some of these provide evidence for the survival and intestinal uptake of ACE inhibitory peptides, and even systemic blood pressure lowering effects.

The aim of the present work was to investigate the effect of genetic variation in β -CN on the peptides generated by in vitro gastrointestinal digestion (IVD) of β -CN variants A¹, A², B, and I (Table 1). Two peptides, related to previously described bioactive peptides (Saito et al., 2000; Eisele et al., 2013), derived from different variants of β -CN, were synthesized and assessed for ACE inhibition. Furthermore, the effect of ACE inhibition upon exposure to the human Caco-2 intestinal cell line was investigated.

β -Casein genetic variants A¹, A², B, and I were isolated from milk samples obtained from homozygous cows of known β -CN genotype (Poulsen et al., 2013) and were subjected to IVD using pepsin and pancreatin in 2 consecutive steps, as previously described (Petrat-Melin et al., 2015). A peptide profile of the resulting hydrolysates was obtained by liquid chromatography tandem mass spectrometry (LC-MS/MS). The LC system consisted of a 1200 series capillary pump and autosampler (Agilent Technologies, Waldbronn, Germany) fitted with a Jupiter C18 300 Å micro-column (Phenomenex, Værløse, Denmark) of dimensions 150 mm × 0.5 mm with a particle size of 5 µm. The gradient of solvent A (1% acetic acid) and solvent B (80% acetonitrile, 1% acetic acid) was as follows: 2% B over 5 min increasing to 100% B over 25 min, and keeping at 100% B for 10 min at a flow rate of 10 µL/min. The column was operated at 20°C. The LC system was coupled to an HCT Ultra ion-trap tandem mass spectrometer equipped with an electrospray ion source (Bruker Daltonik, Bremen, Germany). The MS was run in positive mode with an m/z range from 250 to 2,000. Tandem MS spectra were recorded and searched against a custom Mascot database (Matrix Science, Boston, MA) of

bovine milk proteins, including genetic variants. The search was performed with no specific enzyme cleavage sites, an MS mass tolerance of 0.1%, and an MS/MS mass tolerance of 0.5 Da. Peptide hits above the Mascot score significance threshold ($P < 0.05$) were accepted. Fourteen unique peptides from the regions of amino acid substitutions were positively identified (not shown). In the digest from all 4 β -CN variants, the V-betacasomorphin-9 (V- β CM-9) like decapeptide β -CN f59–68 was found, constituting VYPFPGPIHN from variants A¹ and B, and VYPFPGPIPN from variants A² and I. Boutrou et al. (2013) identified also this peptide and 4 additional β CM precursors from jejunal aspirates of healthy human subjects. However, being aspirated from the lumen of the intestine, these peptides will likely not have been in proximity of brush border peptidases (BBP). Peptide VYPFPGPIPN has previously been observed in a system where CN was hydrolysed with *Bacillus lentus* alkaline peptidase, and was found to be an ACE inhibitor with an concentration needed to reach half-maximal inhibition (IC₅₀) of 325 µM (Eisele et al., 2013). Moreover, A²/I derived β CM-9 (i.e., YPFPGPIPN), was shown to be a much stronger ACE inhibitor with an IC₅₀ of 14.8 µM (Saito et al., 2000). However, the ACE inhibition of A¹/B derived V- β CM-9 has not previously been reported. Therefore, to explore the consequence of genetic polymorphism for ACE inhibitory capacity the 2 V- β CM-9 peptides were synthesized by Schafer-N (Copenhagen, Denmark) using solid phase synthesis. Angiotensin 1-converting enzyme inhibition was determined using a modified version of the method of Sentandreu and Toldra (2006) as previously described (Petrat-Melin et al., 2015). The peptides were measured at 7 different concentrations, and the IC₅₀ values were determined by 4 parameter logistic regression using SigmaPlot version 11 (Systat Software, Erkrath, Germany; Table 2). The A¹/B derived V- β CM-9 was a rather potent ACE inhibitor, with an IC₅₀ more than 5-fold lower than the A²/I derived V- β CM-9, suggesting that the H67P substitution in the A² and I variants is unfavorable for ACE inhibition. This is in accordance with Wu et al. (2006) who found H to be one of the more favorable

Table 1. Position of amino acid substitutions¹ within the mature protein of variants of β -CN

Position	Variant ¹			
	A ¹	A ²	B	I
67	His (H)	Pro (P)		Pro (P)
93	Met (M)			
122	Ser (S)	Arg (R)		

¹A¹ is the reference sequence (Caroli et al., 2009).

residues to have at the penultimate position of peptides of 4 to 10 AA in length, concerning ACE inhibition.

The final phase of protein and peptide digestion takes place in the small intestine immediately before the resulting peptides and AA are transported across the intestinal wall. To exert any physiological effect, ACE inhibitory peptides must resist further degradation by the brush border enzymes of the enterocytes. This was evaluated in the present study by exposure to the human intestinal cell line Caco-2, obtained from the American Type Culture Collection (HTB-37, ATCC, Manassas, VA). The cells were propagated using standard cell culture techniques. In brief, they were cultured in complete growth medium (GM, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1% NEAA) in 75-cm² cell culture flasks at 37°C, 95% air, and 5% CO₂ in a humidified incubator. Trypsin-EDTA, Dulbecco's modified Eagle medium, and fetal bovine serum were from Gibco (Naerum, Denmark). Penicillin/streptomycin, Hanks' buffered salt solution (HBSS), HEPES, 2-(*N*-morpholino)ethanesulfonic acid, lucifer yellow, and NEAA were all from Sigma Aldrich (St. Louis, MO). Growth medium was changed 3 times weekly. Subculturing was performed at 50 to 60% confluency by washing twice with PBS, and incubating with 1.25 mg/mL of trypsin and 0.1 mg/mL of EDTA for 5 to 10 min until detachment was observed. The cell suspension was centrifuged at 200 × *g* for 4 min at room temperature. The supernatant was discarded and the cells were resuspended in fresh GM for re-seeding. For the assay, the Caco-2 cells were seeded at a density of 6.5 × 10⁴ cells/cm² on polycarbonate cell culture inserts with a pore size of 0.4 μm and a growth surface area of 0.6 cm² (Merck-Millipore, Darmstadt, Germany) and kept in standard 24-well cell culture plates. They were allowed to proliferate and differentiate into a tight monolayer for 20 to 25 d, with GM changed 3 times weekly. The integrity of the monolayers was assessed by measuring the trans-epithelial electrical resistance (TEER) with an ERS-2 Volt-ohm meter equipped with chopstick electrodes (Merck-Millipore). The TEER values were calculated as ($\Omega_{\text{monolayer}} - \Omega_{\text{blank}}$) × 0.6 cm², and only inserts with values above 200 Ω

× cm², measured at 37°C, were used for experiments. The V-βCM-9 were assessed for retention of inhibitory capacity after incubation with the Caco-2 cell monolayer, as described in the following. First, the inserts were washed in HEPES-buffered HBSS (pH 7.4) at 37°C for 10 min while measuring initial TEER values. Then, the inserts were incubated with samples dissolved in 2-(*N*-morpholino)ethanesulfonic acid-buffered HBSS (pH 6.5) in the apical chamber, and with fresh HEPES-buffered HBSS in the basolateral chamber. The initial concentration of the peptides was 500 μM. Lucifer yellow was added at 100 μM to all samples and controls for assessment of monolayer integrity during the assay. The incubation was carried out at 37°C for 120 min on a rocker table set to 30 rpm, and TEER values were measured again at the end of incubation. Only data from inserts where less than 1% of the initial lucifer yellow had leaked into the basolateral chamber, and the loss in TEER value was less than 25%, were used. Experimental solutions from both apical and basolateral chambers were aspirated at the end of incubation, and kept at −20°C until further analysis. The observed changes in inhibition measured in the apical samples were significantly different between the 2 peptides ($P < 0.001$), with no change for VYPFPGPIHN ($P > 0.05$), and an 81% increase for VYPFPGPIP ($P < 0.05$; Figure 1). Interestingly, in addition to the intact peptides, the C-terminally truncated VYPFPGPIH and VYPFPGPIP were detected in the apical chamber following incubation. This likely resulted in a more favorable C-terminal for the A² derived peptide (−PIP) than for the A¹ derived (−PIH). In addition, no significant uptake or transport of these 2 peptides was noted, evidenced by no appearance of activity in the basolateral chamber, and no peptides detected by LC-MS/MS. Nevertheless, it was earlier shown that the hexapeptide LHLPLP [β-CN f(133–138)] was both transported and converted into HLPLP by Caco-2 cells (Quirós et al., 2008). Transport of βCM-7 (YPFPGPI) across a Caco-2 monolayer has also been demonstrated, but this peptide was also extensively hydrolyzed by dipeptidyl peptidase IV (DPP-IV; Iwan et al., 2008). The DPP-IV hydrolyzes βCM-7 because it favors peptide substrates with a P residue in the second position

Table 2. The IC₅₀¹ of angiotensin 1-converting enzyme inhibitory peptides derived from β-CN variants

Peptide	Variant	Position ²	IC ₅₀ (μM)	SEM
VYPFPGPIHN	A ¹ , B	59–68	123 ^a	14.2
VYPFPGPIP	A ² , I	59–68	656 ^b	7.6

^{a,b}Different letters within column denote statistically significant difference ($P < 0.001$).

¹Concentration needed to reach half-maximal inhibition.

²The position of the peptide within the mature β-CN amino acid sequence.

Figure 1. Angiotensin 1-converting enzyme (ACE) inhibition by β -CN-derived peptides, measured at $83 \mu\text{M}$, before and after 120 min incubation with the apical side of a differentiated Caco-2 cell monolayer. Different letters indicate a significant difference ($P < 0.01$) in ACE inhibition before and after incubation. Error bars represent SEM ($n = 3$).

from the N-terminal, from which it cleaves a dipeptide (Chen, 2006). The added V residue at the N-terminal of V- β CM-9 likely prevents the action of DPP-IV. In another study of milk protein digestion, using an in vitro system that included BBP, only 2 β CM were identified [β -CN f(60–66) and f(60–67)] (Picariello et al., 2015). Compared with the work of Boutrou et al. (2013), this highlights the role of BBP in the degradation of ingested proteins before absorption. However, further studies should elucidate whether there is any matrix effect on intestinal digestion by luminal peptidases as well as BBP, and on subsequent absorption, when digesting complete milk samples.

In summary, the present study shows that genetic polymorphisms result in different peptides being generated during IVD of β -CN variants A¹, A², B, and I. As a result, peptides with varying ACE inhibitory capacities were observed. In addition, contrasting effects of proteolysis by intestinal cell associated proteases was shown. However, no apparent transport of ACE inhibitory peptides across the Caco-2 monolayer was

observed. Taken together, this demonstrates that protein variation at the sequence level may influence both gastrointestinal digestion and the bioactive potential of generated peptides.

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