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In vitro digestion of purified β-casein variants A[1], A[2], B, and I: Effects on antioxidant and angiotensin-converting enzyme inhibitory capacity

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ABSTRACT

Genetic polymorphisms of bovine milk proteins affect the protein profile of the milk and, hence, certain technological properties, such as casein (CN) number and cheese yield. However, reports show that such polymorphisms may also affect the health-related properties of milk. Therefore, to gain insight into their digestion pattern and bioactive potential, β-CN was purified from bovine milk originating from cows homozygous for the variants A[1], A[2], B, and I by a combination of cold storage, ultracentrifugation, and acid precipitation. The purity of the isolated β-CN was determined by HPLC, variants were verified by mass spectrometry, and molar extinction coefficients at $\lambda = 280$ nm were determined. β-Casein from each of the variants was subjected to in vitro digestion using pepsin and pancreatic enzymes. Antioxidant and angiotensin-converting enzyme (ACE) inhibitory capacities of the hydrolysates were assessed at 3 stages of digestion and related to that of the undigested samples. Neither molar extinction coefficients nor overall digestibility varied significantly between these 4 variants; however, clear differences in digestion pattern were indicated by gel electrophoresis. In particular, after 60 min of pepsin followed by 5 min of pancreatic enzyme digestion, one ≈4 kDa peptide with the N-terminal sequence ^{106}H-K-E-M-P-F-P-K- was absent from β-CN variant B. This is likely a result of the ^{122}Ser to ^{122}Arg substitution in variant B introducing a novel trypsin cleavage site, leading to the changed digestion pattern. All investigated β-CN variants exhibited a significant increase in antioxidant capacity upon digestion, as measured by the Trolox-equivalent antioxidant capacity assay. After 60 min of pepsin + 120 min of pancreatic enzyme digestion, the accumulated increase in antioxidant capacity was ≈1.7-fold for the 4 β-CN variants. The ACE inhibitory capacity was also significantly increased by digestion, with the B variant reaching the highest inhibitory capacity at the end of digestion (60 min of pepsin + 120 min of pancreatic enzymes), possibly because of the observed alternative digestion pattern. These results demonstrate that genetic polymorphisms affect the digestion pattern and bioactivity of milk proteins. Moreover, their capacity for radical scavenging and ACE inhibition is affected by digestion.

Key words: milk protein, β-casein, genetic polymorphism, bioactive peptide

INTRODUCTION

Bioactive peptides are defined as protein fragments that interact with, or have an effect on, bodily tissues or functions and thus may influence health positively; milk proteins are an excellent source of such peptides (Meisel, 1998; Shah, 2000; Nagpal et al., 2011). The 4 caseins (α_{S1}, α_{S2}, β, and κ) constitute approximately 80% of the protein in bovine milk. Casein-derived peptides have been shown to have a range of effects, such as antihypertensive, antithrombotic, antimicrobial, opioid, immune-modulating, and mineral binding (Silva and Malcata, 2005; Phelan et al., 2009) and are therefore suitable candidates for the development of novel functional foods. Bioactive peptides are encrypted within the primary structure of proteins and may be released through various types of enzymatic hydrolysis; that is, the targeted action of microbial or plant-derived enzymes, the action of microbial enzymes during fermentation, or the action of digestive enzymes in vitro or in the gastrointestinal tract (Korhonen and Pihlanto, 2006; Phelan et al., 2009). The set of peptides generated from any given protein depends on the specificity of the proteolytic enzymes and, consequently, on the structure of the protein itself. Variations in primary structure may therefore influence the bioactive potential of proteins; for example, by altering enzyme cleavage sites, modifying protein structure, or changing the behavior of the liberated peptides (Kamiński et al., 2007; Caroli et al., 2009). These and other variations can be the result of genetic polymorphisms and may suggest that some variants of proteins behave differently from others with regard to certain health effects

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(Kamiński et al., 2007). About 40% of the CN in bovine milk is β-CN (Bobe et al., 1998) and, until recently, at least 12 different variants carrying AA substitutions had been identified, designated A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2, and I, with the most common being the A^1, A^2, and B variants (reviewed by Caroli et al., 2009). The number of described variants was increased to 15 in 2013 when the novel variants J, K, and L, described by Gallinat et al. (2013), were identified in *Bos indicus* breeds using DNA sequencing techniques. In a recent study involving approximately 800 Danish dairy cattle (Danish Holstein and Danish Jersey), the B variant was found to be slightly less common in Danish Holstein than the I variant (Poulsen et al., 2013).

Recently, it was demonstrated that the in vitro digestion of β-CN genetic variants A^1, A^2, and B generated different arrays of peptides, and the authors suggested that these peptide variations could have an effect on immunoglobulin-E binding activity (Lisson et al., 2013). β-Casein also exhibits antioxidative capacity (Pihlanto, 2006), and studies have shown that this capacity is enhanced by digestion of the protein (Gómez-Ruiz et al., 2008; Kumar et al., 2010). Weimann et al. (2009) published the results of an in silico digestion study of κ-CN, wherein variations in the generation of angiotensin-converting enzyme (**ACE**) inhibitory peptides from different genetic κ-CN variants were characterized. These effects are of relevance with regard to ailments such as arthritis, neurodegenerative disease, cardiovascular disease, and cancer (Halliwell, 2007; Valko et al., 2007). Together, these studies indicate that genetic polymorphisms may indeed influence the bioactive potential of proteins upon digestion.

To investigate the digestion and bioactive potential of β-CN variants and their digestion products, the different variants need to be available in purified form. The basis for the traditional method of separating β-CN from the other CN was described more than half a century ago by Hipp et al. (1952). That method relies on the different solubilities of the caseins in a urea solution, and often involves some type of chromatography, as reviewed by Imafidon et al. (1997). In the last 2 decades, methods have been developed, using reverse-phase HPLC (Bobe et al., 1998; Bonfatti et al., 2009), that are highly effective and able to separate variants of β- and κ-CN, as well as β-LG. However, these methods still use denaturing conditions that disrupt the native secondary structure of the proteins. For some applications, such as in vitro digestion, the presence of urea can be problematic as it can cause carbamylation of Lys and Arg side chains as well as protein amino terminals (Stark et al., 1960; Stark, 1965; Kollipara and Zahedi, 2013). It is therefore preferable to avoid urea in the purification process, because carbamylation of proteins may lead to changes in the pattern or extent of digestion, as well as altered chromatographic retention times. Moreover, protein modifications induced during the purification procedure could modify the behavior of the purified variants relative to the native forms (Mun and Golper, 2000). If denaturing is required in other downstream applications, an alternative chaotrope that does not cause protein modifications, such as guanidine hydrochloride, could be considered.

The aim of this work was to develop a method for purification of β-CN from milk of cows homozygous for the genetic variants A^1, A^2, B, and I, without the use of urea in the purification protocol. Moreover, to the best of our knowledge, the molar extinction coefficients of isolated β-CN or its variants have not been reported previously. Therefore, these were determined to facilitate rapid protein quantification of the different variants purified using the reported method. Susceptibility to digestive enzymes and the bioactivity of peptides is largely determined by AA sequence. Consequently, we investigated the in vitro digestion pattern of each of these 4 variants, as well as their bioactive potential in relation to antioxidant and ACE inhibitory capacities, upon in vitro digestion.

MATERIALS AND METHODS

Reagents and Chemicals

Angiotensin-converting enzyme (EC 3.4.15.1), pepsin (EC 3.4.23.1), pancreatin, bovine CN, fluorescamine, Coomassie brilliant blue G-250 (**CBB** G-250), *o*-phthaldialdehyde, AA standards, captopril, and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (**ABTS**) were all from Sigma (St. Louis, MO). 2-Aminobenzoylglycyl-*p*-nitrophenylalanyl-proline (ACE substrate) was purchased from Bachem (Bubendorf, Switzerland). Polypeptide molecular weight markers (3.5 to 26.6 kDa) were from BioRad Laboratories (Hercules, CA). All other reagents and chemicals were of analytical grade.

Purification of β-CN Variants

β-Casein was purified from milk samples collected by the Danish-Swedish Milk Genomics Initiative as previously described (Jensen et al., 2012a). In brief, morning milk samples from more than 800 individual cows in mid-lactation were collected, analyzed for fat and protein composition by Milkoscan (Foss Analytical, Hillerød, Denmark), and stored at −20°C until use. Genotyping of all cows was carried out as described previously (Poulsen et al., 2013), enabling the identification of milk samples from cows that were homozygous for