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Short communication: Effect of casein haplotype on angiotensin-converting enzyme inhibitory and antioxidant capacities of milk casein from Italian Holstein cows before and following in vitro digestion with gastrointestinal enzymes

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ABSTRACT

The aim of this work was to investigate the effect of casein haplotype (α_{S1}, β, and κ) on antioxidative and angiotensin-converting enzyme (ACE) inhibitory capacities of milk casein from Italian Holstein cows before and following in vitro digestion with gastrointestinal enzymes. The antioxidant capacity was measured using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and ferric-reducing antioxidant power assays, whereas ACE inhibition was determined by ACE-inhibitory assay. The ACE-inhibitory and antioxidant capacities of milk casein increased during in vitro gastrointestinal digestion. Casein haplotype significantly influenced the antioxidative and ACE-inhibitory capacities of digested casein. In particular, $BB\text{-}A^2A^1\text{-}AA$ casein and $BB\text{-}A^1A^1\text{-}AA$ casein showed the highest ACE-inhibitory capacity, $BB\text{-}A^2A^2\text{-}AA$ casein showed the highest antioxidant capacity, whereas $BB\text{-}A^2A^2\text{-}BB$ casein showed the lowest biological capacity. To date, few studies have been done on the effect of casein haplotype on biological capacity of milk casein, thus the present study sets the basis for a new knowledge that could lead to the production of milk with better nutraceutical properties.
Key words: casein haplotype, ACE-inhibitory capacity, antioxidant capacity, Italian Holstein milk

Short Communication

Many studies have highlighted the nutraceutical effect of some milk compounds in the human body (Alberti-Fidanza et al., 2002; Silva and Malcata, 2005; Phelan et al., 2009) such as α_{S1}-, α_{S2}-, β-, and κ-CN (*CSN1S1, CSN1S2, CSN2*, and *CSN3* gene loci, respectively), which are a biologically active peptides source that can have health-promoting properties. These bioactive peptides are released from milk casein sequences mostly during gastrointestinal digestion by gastric and pancreatic enzymes (Chabance et al., 1995, 1998), whereas others can be released in an active form by enzymes during milk processing and are, therefore, naturally present in dairy products (Smacchi and Gobbetti, 2000). Casein-derived peptides have shown a range of biological activities, such as antihypertensive, antioxidant, antithrombotic, antimicrobial, opioid, immune-modulating, and mineral-binding properties (Silva and Malcata, 2005; Phelan et al., 2009). Some peptides can combine more than one of these effects, resulting in multifunctional structures which have broader applications in health maintenance (Corrêa et al., 2011). Maruyama et al. (1987) reported that peptides derived from tryptic hydrolysis of α_{S1}-CN (fragments 23–24, 23–27, and 194–199) and of β-CN (fragments 177–183 and 193–202), known as casokinins, inhibited angiotensin I-converting enzyme (**ACE**; EC 3.4.15.1) activity. Angiotensin I-converting enzyme is a key enzyme in the regulation of blood pressure; in particular, ACE catalyzes the conversion of angiotensin I into angiotensin II, a potent vasoconstrictor, and inactivates bradykinin, a vasodilator (Ondetti and Cushman, 1977). The inhibition of ACE is a method widely used for the treatment of hypertension (Conlin, 2001). It has been reported that the antioxidant capacity of caseins is related to their high tendency to chelate metals (Tong et al., 2000; Rival et al., 2001), and to their ability to donate electrons and atoms (Colbert and Decker, 1991). The bioactive potential of the casein depends on its AA sequence, which is conditioned by genetic polymorphism. The degree of casein heterogeneity may influence the release of peptides formed during proteolysis (Minervini et al., 2003; Hernández -Ledesma et al., 2004; De Noni et al., 2009); this is due to the presence in the casein of specific sequences, known as strategic zones, that have biological activities (Fiat and Jolles, 1989). Milk caseins occur in different allelic forms controlled by codominant genes (*CSN1S1, CSN2, CSN1S2*, and *CSN3*) tightly linked in a 250-kb

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cluster mapped on chromosome 6 (Ferretti et al., 1990; Caroli et al., 2009). Until a few years ago, the association between the genetic forms of a single locus and the milk characteristics was the approach used (Di Stasio and Mariani, 2000; Martin et al., 2002). Currently, it is believed that a better estimation of effects is obtained when considering the whole casein cluster instead of the single casein loci within a single breed (Beja-Pereira et al., 2002; Boettcher et al., 2004; Gambacorta et al., 2005). In this regard, the aim of our study was to estimate the effect of casein haplotype on ACE-inhibitory and antioxidant capacities of milk casein from Italian Holstein cows before and following in vitro digestion with gastrointestinal enzymes.

This study was conducted on an intensive farm, consisting of more than 500 Italian Holstein cattle, in the countryside of Potenza, southern Italy. Before starting the test, approximately 250 animals in lactation were identified by isoelectric focusing to define their haplotypes (Perna et al., 2013). Haplotypes were formed by the combination of the individual allelic loci aggregated by α_{S1}-, β-, and κ-CN. After defining the individual phenotypes, the cows were grouped by haplotype. Each group was formed by 10 to 12 animals, an equal stage of lactation (70–120 d postpartum), season (spring), and order of birth (third calving). All animals were fed a commercial standard diet according to milk yield. The individual cow milk of the morning milking was collected once and all milk samples were analyzed within 3 h from collection. Individual milk samples were skimmed by centrifugation (6,000 × *g* for 20 min at 4°C), diluted with distilled water (1:4, vol/vol), and the pH of solution was adjusted to 4.6 with 1 *N* HCl for casein precipitation. After centrifugation at 3,000 × *g* for 10 min at 4°C, the casein pellet was resuspended in distilled water and casein was solubilized by adjusting the pH to 7.5 with 1 *N* NaOH. Each precipitation was repeated 3 times. Then, the purified casein was lyophilized and stored at −20°C until analysis. The in vitro gastrointestinal digestion of the lyophilized casein was simulated using pepsin and trypsin according to the method of Ao and Li (2013), with some modifications. Three hundred milligrams of lyophilized casein was mixed with 27 mL of bidistilled water, the pH was adjusted to 2 with 1 *N* HCl, and the stomach phase was simulated by adding pepsin (Sigma-Aldrich, Milan, Italy) at a 1:200 (enzyme:substrate) ratio. After 2 h of digestion at 37°C and continuous stirring, the enzyme was inactivated by adjusting the pH to 7.5 with 1 *N* $NaHCO_3$. Then, trypsin (Sigma-Aldrich) was added at a 1:25 (enzyme:substrate) ratio to simulated the intestinal phase. After 4 h of digestion at 37°C, enzyme activity was terminated by heating for 10 min at 95°C. Samples were collected before adding the enzymes, after pepsin (120 min) and trypsin (240 min) digestion. Each sample was centrifuged at 5,000 × *g* for 20 min at 4°C to remove large particles, the supernatant was filtered through a 0.2-μm cellulose acetate membrane filter (Sigma-Aldrich), and it was frozen and kept a −55°C until analysis. The ACE-inhibitory capacity was measured by the spectrophotometric assay of Hernández-Ledesma et al. (2005). The IC_{50} values were calculated as the concentration (μ*M*) of the sample required to inhibit 50% of ACE capacity and was tested in triplicate. The antioxidant capacity of casein was determined by 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (**ABTS**) radical scavenging and ferric-reducing antioxidant power (**FRAP**) assays. The ABTS assay was determined according to the method of Re et al. (1999), as modified by Perna et al. (2013); FRAP assay was determined according to the method of Benzie and Strain (1996). Each determination and measurement was made in triplicate and the results for both assays were expressed as milligrams of Trolox equivalent (**TE**) per milligram of casein. Data were analyzed according to the following linear model (SAS Institute, 1996):

$$y_{ik} = \mu + \alpha_i + \varepsilon_{ik},$$

where y_{ik} is the observation; μ is the overall mean; α_i is the fixed effect of the *i*th haplotype (i = 1, 2, 3, 4, 5); and ε_{ik} is the random error. Before setting the values, expressed as a percentage, they were subjected to angular transformation. Student's *t*-test was used to compare all variables. Differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

In our study, unhydrolyzed casein (t = 0 min) did not show ACE-inhibitory capacity (IC_{50} >1,000 μ*M*), whereas the casein samples after in vitro pepsin (t = 120 min) and trypsin (t = 240 min) digestion showed ACE inhibition (Table 1). The ACE-inhibitory capacity is influenced by the casein haplotype ($P < 0.001$); this finding is supported by De Noni et al. (2009), who showed that the genetic polymorphism influenced the type of bioactive peptides released from milk proteins. The ACE inhibition increased during in vitro digestion with gastrointestinal enzymes. The greatest increase of ACE-inhibitory capacity was detected between undigested and pepsin-digested casein, in agreement with what reported by López-Expósito et al. (2007) in hydrolyzed ovine casein with pepsin. With regard to casein haplotype, after pepsin digestion (120 min) *BB*-A^2A^2-*BB* casein showed the lowest ACE-inhibitory capacity ($P < 0.05$), whereas *BB*-A^1A^1-*AA* casein showed the highest capacity ($P < 0.05$). This could be