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Original article

Hydrolytic degree and antioxidant activity of purified casein characterised by different haplotypes (α_{s1}-, β- and k-casein) after enzymatic hydrolysis with pepsin and enzymatic extract from *Pleurotus eryngii*

Annamaria Perna,[1] Amalia Simonetti,[1]* Vita Acquaviva,[1] Rocco Rossano,[2] Giulia Grassi[1] & Emilio Gambacorta[1]

1 School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, Potenza 85100, Italy

2 Department of Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, Potenza 85100, Italy

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Summary The aim of this study was to estimate the hydrolytic degree and antioxidant activity of purified casein characterised by different haplotypes (α_{s1}-, β- and k-casein) after *in vitro* digestion with two different enzymatic systems: pepsin from porcine gastric mucosa (EP) and a crude enzymatic extract from the edible mushroom *Pleurotus eryngii*. The used enzymes showed a different mode of casein catalysis with a consequent production of peptides of different antioxidant activity. The CN haplotype significantly influenced peptides production; in fact, the amino acid substitutions caused by genetic polymorphisms at the α_{s1}-, β- and k-CN loci influenced the sites of enzymatic cleavage and therefore the produced peptides. The above is evidenced by the different antioxidant activity found in the hydrolysates depending on the used enzymatic system, the CN haplotype, and the CN haplotype × enzymatic treatment interaction. The findings of this study are a perspective for the production of specific foods that exert a biological effect in addition to the nutritional one.

Keywords Antioxidant activity, bioactive peptides, casein polymorphism, hydrolysis degree, pepsin, *Pleurotus eryngii* proteases.

Introduction

It is known that the bioactive potential of caseins is due to their amino acid sequence which in turn is conditioned by genetic polymorphism. In fact, milk caseins exist in different allelic forms controlled by codominant genes (*CSN1S1*, *CSN2*, *CSN1S2* and *CSN3*) closely linked in a 250-kb cluster on chromosome 6 (Ferretti *et al.*, 1990; Caroli *et al.*, 2009). Today, due to the tight genetic linkage among CN loci, several authors suggested to study whole allelic combination instead to analyse effects of single alleles within a breed (Boettcher *et al.*, 2004; Gambacorta *et al.*, 2005; Secchiari *et al.*, 2009). Variations in the primary structure can influence the bioactive potential of proteins, for example, by altering the sites of enzymatic cleavage, modifying the protein structure or modifying the behaviour of the released peptides (Kamiński *et al.*, 2007; Caroli *et al.*, 2009). Thus, the antioxidant activity of caseins, due both to their high ability to chelate metals and to donate electrons (Tong *et al.*, 2000; Rival *et al.*, 2001), is influenced by the degree of casein heterogeneity that can affect the release of peptides during proteolysis (Minervini *et al.*, 2003; De Noni *et al.*, 2009). Perna *et al.* (2013) observed, in cow milk yogurt, that antioxidant activity was significantly influenced by CN haplotype. Enzymatic hydrolysis by proteases or peptidases is the most common method to obtain bioactive peptides, and the set of peptides generated by a protein depends on the specificity of proteolytic enzymes and on the structure of the protein itself. The proteolytic enzymes can be classified according to their origin (animal, plant and microbial); the catalysed reaction (position of the cleavage site and length of the substrate chain); mechanism of action; and molecular structure (Tavano *et al.*, 2018). In the last few years, several commercial protease preparations, namely Alcalase®, Neutrase® and Flavourzyme® based on bacterial and fungal peptidases are widely used for the synthesis of bioactive peptides (De Castro & Sato, 2014; Bhat *et al.*, 2015; Silva, 2017). Among the mushrooms, those

*Correspondent: Fax: +39 0971 205099;
e-mail: amalia.simonetti@unibas.it

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of the *Pleurotus* genus produce different types of proteases; in particular, *Pleurotus eryngii*, one of the most popular edible mushrooms worldwide, is an excellent producer of aspartic and serine proteases (Inácio *et al*., 2015). Moreover, *Pleurotus eryngii* contains various beneficial bioactive compounds such as polyphenols, polysaccharides, peptides and dietary fibres (Ferreira *et al*., 2009); thus, it is widely utilised in the food industry for developing dairy functional foods (Miao *et al*., 2011).

The aim of this study was to estimate the hydrolytic degree and antioxidant activity of purified casein characterised by different haplotypes (α_{s1}-, β- and k-casein) after *in vitro* digestion with two different enzymatic systems: from porcine gastric mucosa (EP) and an crude enzymatic extract from the edible mushroom *Pleurotus eryngii* (EM). This study for the first time evaluated the interaction between specific proteases and specific casein substrates highlighting the antioxidant activity of hydrolysed caseins.

Materials and methods

Chemicals

All the reagents used were of the highest grade and were purchased from Sigma-Aldrich (Milan, Italy), Bio-Rad Laboratories (Richmond, CA), Carlo Erba (Milan, Italy) and GE Healthcare (Buckinghamshire, UK). Pepsin from porcine gastric mucosa (674 U mg^{-1} - cod. P7000) was purchased from Sigma-Aldrich (Milan, Italy).

Materials

This study was carried out on seventy-two Italian Holstein cows raised on an intensive farm in Potenza province (Southern Italy). Before starting the test, approximately 400 animals in lactation were identified to define their haplotypes by isoelectric focusing (IEF; Perna *et al*., 2013). The cows were grouped by haplotype, which was formed by the combination of the individual allelic loci aggregated by α_{S1}-, β- and κ-CN. Each haplotype was formed by 12 animals. All cows were in the same stage of lactation (70–120 days postpartum), birth order (third calving) and season (spring). From each animal, individual milk was collected to extract and purify the caseins.

Casein extraction and purification

The CN extraction and purification was carried out as described by Perna *et al*. (2016). Briefly, individual milk samples after centrifugation at 6000 ***g*** for 20 min at 4 °C were diluted with distilled water (1:4, v/v) and brought to pH 4.6 with 1N HCl. Then, the precipitated casein was centrifuged (3000 ***g*** for 10 min at 4 °C), the pellet was resuspended in distilled water, and the pH of solution was brought to 7.5 with 1N NaOH. Each precipitation was repeated three times. The purified caseins were dialysed, freeze-dried, lyophilised and stored at −20 °C until analysis.

Identification of the genetic variants of CN by IEF

The purified CN preparation for IEF analysis was carried out as described by Perna *et al*. (2013). The genetic variants of CN were determined by IEF (Multiphor II Electrophoresis System; Pharmacia LKB, Uppsala, Sweden) according to the method of Trieu-Cuot & Gripon (1981) with some modifications as described by Perna *et al*. (2013). Briefly, the polyacrylamide gel (5% acrylamide and 0.15% bisacrylamide) of 1 mm thickness and 2% carrier ampholytes (gradient of pH 2.5–10) was prefocused at a constant value of 0.35 W mL^{-1} of gel and at the maximum limit of 1200 V. The gel was stained in Coomassie Brilliant Blue G-250. Haplotype frequencies at CSN1S1-CSN2-CSN3 loci were calculated as the number of each haplotype divided by the total number of haplotypes [% $= (n_{i,\text{haplotype}}/n_{tot,\text{haplotype}}) \times 100$]. The purity (≥92.3%) of the purified caseins was determined by densitometric analysis (UltroScan XL, Pharmacia LKB, Uppsala, Sweden) of the IEF electrophoretic bands.

Extraction of enzymes from edible mushroom *Pleurotus eryngii*

Fifty grams of frozen mushroom was lyophilised and pulverised. Then, the mushroom powder was extracted for 24 h at 20 °C in a conical flask at 225 r.p.m. with 40 mM of Tris-HCl, pH 7.0 (powder to solvent ratio: 1/16, w/v). The extract (EM) was recovered after centrifugation (6000 ***g*** for 4 min at 20 °C), filtered through a Whatman No.3 filter paper (Sigma-Aldrich, Milan, Italy) and stored at 4 °C.

Assessment of protein content and proteolytic activity of *Pleurotus eryngii* extract

Protein concentration was determined according to the Bradford protein assay (Bradford, 1976), using BSA as standard protein. Total proteolytic activity was assessed by using the spectrophotometric method based on azocasein (Rossano *et al*., 2011). Briefly, 50 μL of the enzymatic extract was added to 0.5 mL of 0.7 % azocasein in 40 mM Tris-HCl, pH 7.0 at 30 °C. The reaction was stopped after 30 min by the addition of 0.55 mL 10% trichloracetic acid (TCA) and centrifuged for 6 min at 6500 ***g*** (Amicon microcentrifuge MC-13; Amicon, Beverly, MA, USA). The supernatants were separated from the undigested substrate, and the

absorbance at 440 nm (UV-VIS Spectrophotometer 1204; Shimadzu, Kyoto, Japan) of the released dye was recorded. One unit of total proteolytic activity (U) was defined as the amount of enzyme yielding 0.01 unit of absorbance per min at 440 nm under the assay conditions. The assay included an appropriate blank, in which TCA was added before the substrate.

Casein hydrolysis

The *in vitro* digestion of lyophilised casein was simulated according to the method of Ao & Li (2013) with some modifications, using two different enzymes: EP and EM. To aliquots of 1 mL of CN (1.5 mg mL^{-1} in 20 mM sodium phosphate buffer; pH 6.5) were added separately 40 μL of EP (corresponding to 40.4 U) and 40 μL of EM (corresponding to 5.4 U), respectively. The different units of enzyme activity correspond to the enzymatic activity of the two preparations able to obtain the same degree of hydrolysis in a standard quantity of casein. After 24 h of digestion at 37 °C and continuous stirring, the enzymes were inactivated by heating for 10 min at 95 °C. Several aliquots were collected during digestion at different times (0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h). Successively, they were centrifuged at 5,000 ***g*** for 20 min at 4 °C, and supernatant was filtered through a 0.2-μm cellulose acetate membrane filter and stored at –20 °C until analysed.

Determination of hydrolysis degree (DH)

The DH of casein was measured by the o-phthaldialdehyde (OPA) method as described by Church *et al.* (1983), with some modifications. The OPA reagent was prepared as following: 25 mL of 0.1 M borate buffer (pH 9.3), 2.5 mL of 20% (w/v) SDS solution, 100 μL of *N*,*N*-dimethyl-2-mercaptoethylammonium chloride, 40 mg of OPA dissolved in 1 mL methanol and brought to 50 mL of total volume with distilled water. For the assay, 100 μL of the each sample was mixed with 1900 μL of the OPA reagent and incubated at 25 °C for 2 min; then, the absorbance was read at 340 nm (UV-VIS Spectrophotometer 1204; Shimadzu, Kyoto, Japan). Results were expressed as μg of lysine per mg of CN, using a standard calibration curve obtained with different concentrations of lysine. Moreover, during enzymatic hydrolysis, for each considered CN haplotype, the percentage variation (Δ%) of proteolytic activity was determined as:

$$\Delta\% = \left(\frac{\text{DH value of EM}-\text{digested CN}-\text{DH value of EP}-\text{digested CN}}{\text{DH value of EP- digested CN}}\right) \times 100.$$

Antioxidant activity of CN hydrolysates

The antioxidant capacity of CN hydrolysates, at 0.5, 2 and 24 h of digestion, was determined by two assays: 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging and ferric-reducing antioxidant power (FRAP). The ABTS assay was carried out according to the method of Re *et al.* (1999), as modified by Russo *et al.* (2018); FRAP assay was determined as described by Benzie & Strain (1996), as modified by Perna *et al.* (2015). All measurements were made in triplicate, and the results for both assays were expressed as milligrams of Trolox equivalent (TE) per milligram of CN.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) using the general linear model procedure of the Statistical Analysis System software (SAS Institute, 1996):

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha * \beta)_{ij} + \varepsilon_{ijk}$$

where y_{ijk} is the kth observation at the ith level of factor α and the jth level of factor β; μ is the overall mean; α_i is the fixed effect of the ith haplotype (i = 1, 2, 3, 4, 5, 6); β_j is the fixed effect of the jth treatment (j = 1, 2); $(\alpha\beta)_{ij}$ is the interaction of CN haplotype × treatment; and ε_{ijk} is the random error. Student's t-test was used for all variable comparisons, and differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

Results and discussion

Proteolytic activity of casein

The six different CN haplotypes ($BB^{\alpha s1\text{-}CN}$-$A_1A_1{}^{\beta\text{-}CN}$-$AA^{k\text{-}CN}$, $BB^{\alpha s1\text{-}CN}$-$A_1A_1{}^{\beta\text{-}CN}$-$BB^{k\text{-}CN}$, $BB^{\alpha s1\text{-}CN}$-$BB^{\beta\text{-}CN}$-$BB^{k\text{-}CN}$, $BB^{\alpha s1\text{-}CN}$-$A_2A_2{}^{\beta\text{-}CN}$-$AA^{k\text{-}CN}$, $BB^{\alpha s1\text{-}CN}$-$BB^{\beta\text{-}CN}$-$AA^{k\text{-}CN}$ and $BB^{\alpha s1\text{-}CN}$-$A_2A_2{}^{\beta\text{-}CN}$-$BB^{k\text{-}CN}$), identified by IEF (Fig. 1), were treated with two different proteolytic systems: EP and EM. In Fig. 2, is reported the trend of DH values of EP- and EM-digested caseins during considered time intervals. Both hydrolysis curves increased during the 24 h; in particular, the rate of CN hydrolysis increased more in the first hour of digestion, mostly maintained stable up to 4 h, to then increase again, thus indicating that the proteases and substrates did not reach a state of saturation. As reported in Table 1, considering both the enzymatic systems, after 24 h of

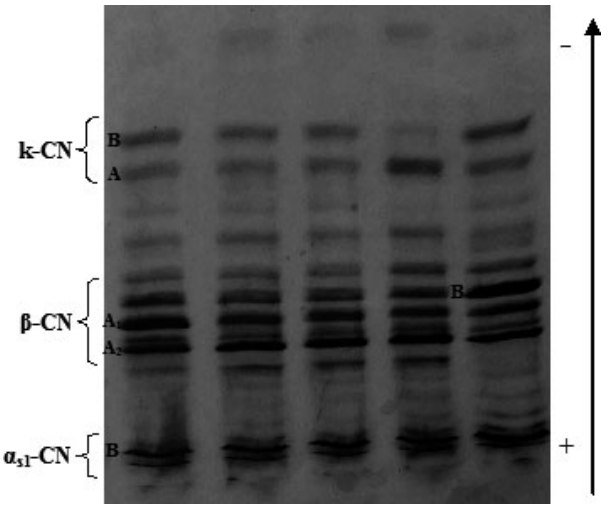

Figure 1 Isoelectric focusing patterns of same purified CN samples separated in a pH range of 2.5 to 10.

hydrolysis the average value of observed DH (224.26 ± 27.81 μg lysine/mg CN) was 3.2 times higher than the starting value (69.86 ± 11.44 μg lysine/mg CN). Among the used proteases, at the end of the trial (24 h), EM-digested casein showed a higher DH compared to EP-digested casein ($P < 0.001$; Fig. 2); in particular, DH values found were 260.45 and 188.06 μg lysine/mg CN for EM- and EP-digested caseins, respectively. These results could be ascribed to the different specificity (number and types of catalytic sites) of the used proteases system. It is known that pepsin has broad specificity with a preference for peptides containing linkages with hydrophobic amino acids and preferentially cleaves C-terminal to Phe and Leu. Whereas, *Pleurotus eryngii* possesses a pool of acid and basic proteases (Inácio *et al*., 2015) belonging to the families of aspartyl (Wang & Ng, 2001), serine (Cha *et al*., 2010) and metalloproteases (Shen *et al*., 2007), characterised by different active sites that hydrolyse the protein in several sites simultaneously, thus leading with a consequent increase in DH value. As shown in Table 1, DH values were significantly influenced by the CN haplotype ($P < 0.001$), in agreement with Petrat-Melin *et al*. (2015) who reported that the haplotype influences positively or negatively the presence of zones resistant to enzymatic hydrolysis. In fact, changes in the casein primary structure may alter enzyme cleavage sites, modifying both the structure of proteins and the behaviour of liberated peptides (Kamiński *et al*., 2007; Caroli *et al*., 2009). In support of this, Lisson *et al*. (2013) reported that the hydrolysis of the A_1, A_2 and B variants of β-CN, and A, B and E variants of k-CN leads to the release of unique peptides from each variant. Comparing different CN haplotypes, BB-A_1A_1-BB and BB-A_2A_2-AA caseins showed the highest DH with values, respectively, of 234.42 and 270.78 μg lysine/mg CN after 24 h ($P < 0.05$; Table 1). On the contrary, BB-BB-AA casein showed the lowest susceptibility to proteases in all considered time intervals ($P < 0.05$). The haplotypes give an indication of the expression encoded by a well-defined allelic combination; thus, the phenotypic effect is due to allele interaction within this combination. The interactions that are established at the level of closely associated *loci* influence the effect of each allele; therefore, a single allele will determine different results depending on the combinations that will make up the haplotype. Within the β-CN locus, for α_{s1}-CN variant BB and k-CN variant AA, BB-A_2A_2-AA casein showed the highest susceptibility to proteases, whereas BB-BB-AA casein resulted the most resistant ($P < 0.05$). The difference between A_2 and B variants is in position 67 and 122 of the chain, where, respectively, the A2 variant

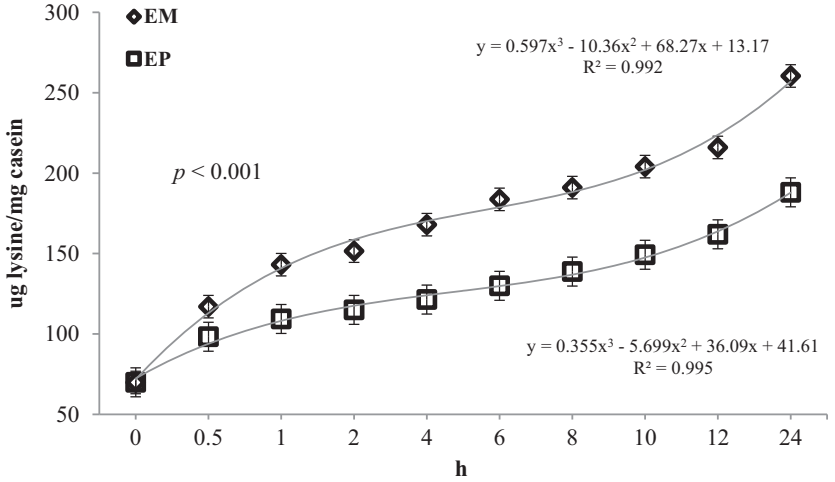

Figure 2 Trend of hydrolysis degree values of EP- and EM-digested caseins during considered time intervals, regardless of considered casein haplotype. EM: *enzymatic extract from the edible mushroom Pleurotus eryngii;* EP: pepsin from porcine gastric mucosa.

Table 1 Hydrolysis degree (μg lysine/mg CN) of casein hydrolysates during considered digestion time, for each CN haplotypes and regardless of enzymatic treatment

	Haplotype (α_{S1}-CN, β-CN, κ-CN)							
Time (h)	BB-A_1A_1-AA	BB-A_1A_1-BB	BB-BB-BB	BB-A_2A_2-AA	BB-BB-AA	BB-A_2A_2-BB	Overall	SEM
	μg lysine/mg casein							
0 (Unhydrolyse CN)	55.41^{a}	86.31^{e}	75.17^{c}	69.14^{d}	74.44^{c}	58.69^{b}	69.86	1.91
0.5	102.91^{b}	127.78^{d}	104.22$^{b.c}$	104.80$^{b.c}$	106.18^{c}	99.84^{a}	107.62	1.68
1	123.44^{d}	162.74^{f}	120.19^{c}	127.33^{e}	112.97^{b}	110.48^{a}	126.19	3.16
2	130.61^{c}	165.89^{e}	126.63^{b}	138.94^{d}	118.83^{a}	118.49^{a}	133.23	2.96
4	138.78^{d}	174.44^{f}	134.61^{c}	165.87^{e}	123.93^{a}	130.21^{b}	144.64	3.42
6	148.23^{c}	181.67^{e}	151.06^{d}	183.98^{f}	132.85^{a}	143.30^{b}	156.85	3.51
8	158.90^{c}	190.08^{d}	159.67^{c}	190.03^{d}	139.30^{a}	151.44^{b}	164.90	3.47
10	165.53^{b}	200.34^{d}	173.36^{c}	203.95^{e}	149.94^{a}	166.77^{b}	176.65	3.54
12	177.47^{b}	206.87^{c}	177.98^{b}	223.92^{d}	170.13^{a}	177.35^{b}	188.95	3.56
24	219.66^{c}	234.42^{e}	226.69^{d}	270.78^{f}	190.39^{a}	203.62^{b}	224.26	4.63

SEM: standard error of means.

$^{a–f}$Means within a row with different superscripts differ ($P < 0.05$).

has Pro and Ser, while the B variant has His and Arg. Jinsmaa & Yoshikawa (1999) reported that β-CN variants have different susceptibility to enzymatic hydrolysis, since the genetic variant influences the molecule hydrophobicity. Moreover, the allele B in homozygous form of both k-CN and α_{s1}-CN, in the BB-A_2A_2-BB haplotype, characterised a casein with low susceptibility, whereas in BB-A_1A_1-BB casein the proteolysis was more pronounced. DH of casein with different haplotypes during considered time intervals and separately for enzymatic treatment is reported in Table 2. Based on the ANOVA results, a haplotype × treatment interaction was detected ($P < 0.001$): BB-A_1A_1-BB casein digested by EM showed the highest DH values ($P < 0.05$) during all considered time intervals, except for the final time, followed by EM-digested BB-A_2A_2-AA casein which showed the highest DH value at the final time ($P < 0.05$); all EP-digested caseins showed instead a higher resistance ($P < 0.05$); in particular, EP-digested BB-BB-BB casein resulted the most resistant ($P < 0.05$). Evaluating the percentage variation (Δ%) of proteolytic activity, determined as the percentage ratio between the DH values of EM-digested casein and EP-digested casein, separately for considered CN haplotype during enzymatic hydrolysis (Fig. 3), it was possible to detect that the BB-A_1A_1-BB casein showed a greater percentage variation than the other CN haplotypes during all considered time intervals, except for the final time, where the highest percentage variation was found in the BB-BB-BB casein hydrolysate. These findings highlighted that the susceptibility to CN hydrolysis is markedly influenced by the CN haplotype. Genetic modifications can alter the properties of proteins, influence the aggregation state of the micellar system and interfere with the proteolytic action of enzymes (Perna *et al*., 2013).

Antioxidant activity of casein

In this study, the antioxidant capacity of EP- and EM-digested caseins, separately for six considered CN haplotypes, during the 24 h of digestion was assessed by two antioxidant assays: ABTS, which shows the activity of both hydrophilic and lipophilic antioxidants, and FRAP, which represents a direct measure of the total reducing power of a solution. Overall, the increase of antioxidant activity was noted already after 30 min of hydrolysis and it was still relevant after 24 h of hydrolysis (Fig. 4). These findings were in agreement with what reported by other authors (Salami *et al*., 2011; Petrat-Melin *et al*., 2015; Shu *et al*., 2018) who detected that the antioxidant activity of casein increased during enzymatic digestion. In particular, both EM- and EP-digested caseins showed antioxidant activity and both trends highlighted a direct relationship between the time of hydrolysis and development of antioxidant activity (Fig. 4) in agreement with several authors (Hernandez-Ledesma *et al*., 2005; Gupta *et al*., 2009; Assem *et al*., 2018). Despite EP-digested caseins showed less DH than EM-digested caseins, we found that the antioxidant activity of EP-digested caseins was higher in all considered times than that obtained with the EM extract ($P < 0.05$). Taking into account that the antioxidant activity during hydrolysis is affected by enzyme specificity that generates peptides and free amino acids with different size, level and composition (Wu *et al*., 2003), the explanation could be ascribed to the pepsin action, specific towards hydrophobic amino acids which, following cleavage, become exposed facilitating the transfer of electrons to the reactive oxygen species (Power *et al*., 2013). EM extract, on the other hand, is characterised by the presence of proteolytic enzymes with different cleavage

Table 2 Hydrolysis degree (μg lysine/mg CN) of casein with different haplotypes during considered time intervals for each considered enzymatic treatment

	Haplotype (α_{S1}-CN, β-CN, κ-CN)												
	BB-A_1A_1-AA		BB-A_1A_1-BB		BB-BB-BB		BB-A_2A_2-AA		BB-BB-AA		BB-A_2A_2-BB		
Time (h)	EM[g]	EP[h]	EM	EP	EM	EP	EM	EP	EM	EP	EM	EP	SEM[i]
0 (Unhydrolyse CN)	55.19[a]	55.63[a]	86.29[b]	86.34[b]	75.33[c]	75.00[c]	69.17[d]	69.11[d]	74.33[c]	74.56[c]	58.73[a]	58.65[a]	0.297
0.5	114.48[d]	91.35[a]	144.67[e]	110.89[c,d]	111.56[d]	96.89[b]	111.33[c,d]	98.26[b]	112.56[d]	99.80[b]	107.33[c]	92.35[a]	0.300
1	144.59[g]	102.28[a]	206.22[h]	119.26[b]	135.11[e]	105.28[f]	136.72[e]	117.93[b,c]	116.58[c]	109.36[d]	119.37[b]	101.58[a]	0.201
2	150.59[g]	110.63[b]	210.89[h]	120.89[c]	143.22[f]	110.03[b]	149.33[g]	128.54[e]	124.53[d]	113.14[b]	130.52[6]	106.46[a]	0.224
4	160.48[f]	117.07[a,b]	225.11[l]	123.78[c]	148.56[e]	120.67[b,c]	197.01[h]	134.74[g]	130.91[d]	116.94[a,b]	145.40[e]	115.02[a]	0.270
6	172.37[g]	124.09[b]	231.56[m]	131.78[d]	176.22[i]	125.89[b,c]	218.38[l]	149.57[f]	137.81[e]	127.89[c]	166.04[h]	120.56[a]	0.212
8	189.04[g]	128.76[a]	235.78[i]	144.39[c]	182.89[f]	136.44[b]	223.31[h]	156.74[d]	144.65[c]	133.94[b]	170.14[e]	132.74[a,b]	0.358
10	194.09[g]	136.96[a]	251.78[l]	148.91[c]	198.06[h]	148.67[c]	238.80[i]	169.09[f]	154.56[d]	145.33[b]	186.95[e]	146.59[b,c]	0.231
12	200.58[d]	154.35[a]	258.08[e]	155.67[a]	204.28[d]	151.68[a]	257.69[e]	190.15[c]	184.89[c]	155.36[a]	190.08[c]	164.63[b]	0.419
24	252.57[f]	186.74[c]	259.94[f]	208.89[d]	296.44[g]	156.94[a]	314.05[h]	227.50[e]	210.33[d]	170.44[b]	229.37[e]	177.87[b]	0.612

[a–f]Means within a row with different superscripts differ ($P < 0.05$).
[g]EM: *enzyme extract from the edible mushroom Pleurotus eryngii;* [h]EP: enzyme pepsin; [i]SEM: standard error of means.

sites which act simultaneously on the substrate, causing an increase in the DH over time, but also the probable release of low antioxidant capacity peptides and inactive peptides. Moreover, the decrease in the antioxidant activity could be due to the hydrophobic peptides generate during the hydrolysis which favour the formation of peptide aggregates (Adt *et al.*, 2011). The CN haplotype significantly influenced antioxidant activity of hydrolysates ($P < 0.001$), in agreement with what reported by De Noni *et al.* (2009) and Perna *et al.* (2013) who reported that genetic polymorphism influences the type of bioactive peptides that are released from milk proteins. ABTS and FRAP values of CN hydrolysates during considered digestion time separately for CN haplotypes and independently of the enzymatic treatment are reported in Table 3. The findings highlighted that after 2 h of enzymatic digestion, BB-A_1A_1-AA and BB-A_2A_2-BB caseins showed the highest ABTS value (4.00 μg TE/mg CN; $P < 0.05$), whereas BB-BB-AA casein showed the highest FRAP value (0.49 μg TE/mg CN). At the end of enzymatic digestion (24 h), BB-A_1A_1-AA, BB-BB-AA and BB-A_2A_2-BB showed the highest ABTS values ($P < 0.05$), and among these, BB-A_1A_1-AA casein also showed the highest chelating activity (1.12 μg TE/mg CN; $P < 0.05$). The high variability of the results in the studied hydrolysates is due to the existence of genetic variants of proteins characterised by amino acid substitutions due to genetic polymorphism. These substitutions, in fact, are all potentially antioxidant activity-altering substitution. To support, Petrat-Melin *et al.* (2015) reported that ACE-inhibitory activity depends on the specific amino acid sequence of peptides. In addition, a significant CN haplotype × treatment interaction was found for antioxidant activity ($P < 0.01$): for all studied CN haplotypes, EP-digested casein showed the highest antioxidant capacity; also, EP-digested BB-A_2A_2-AA casein showed the highest ABTS and FRAP values at the final time ($P < 0.05$).

Conclusions

Enzymatic hydrolysis is the most common method to obtain peptides with biological functions useful for consumer health. The use of proteolytic enzymes, therefore, represents an important tool to obtain hydrolysates with specific biopeptides. In this study, the used enzymes (pepsin and extract from the edible mushroom *Pleurotus eryngii*) showed a different mode of casein catalysis with a consequent different antioxidant activity in the hydrolysates. These differences are also due to the CN haplotype, as well as the CN haplotype × enzymatic treatment interaction. The findings of this study are a perspective for the production of specific foods that exert a biological effect in addition to the nutritional one.

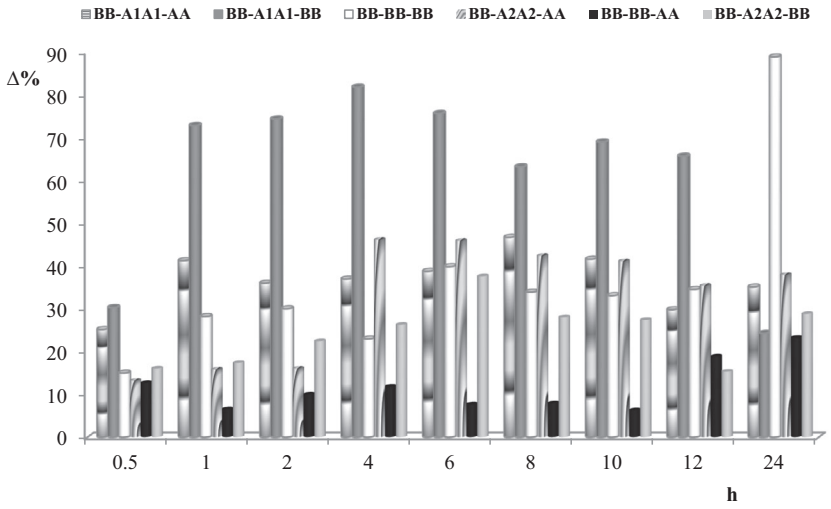

Figure 3 Percentage variation (Δ%) of proteolytic activity during considered digestion time, for each considered casein haplotype. Percentage variation of proteolytic activity was defined as: (DH value of EM-digested CN − DH value of EP-digested CN/DH value of EP-digested CN) × 100.

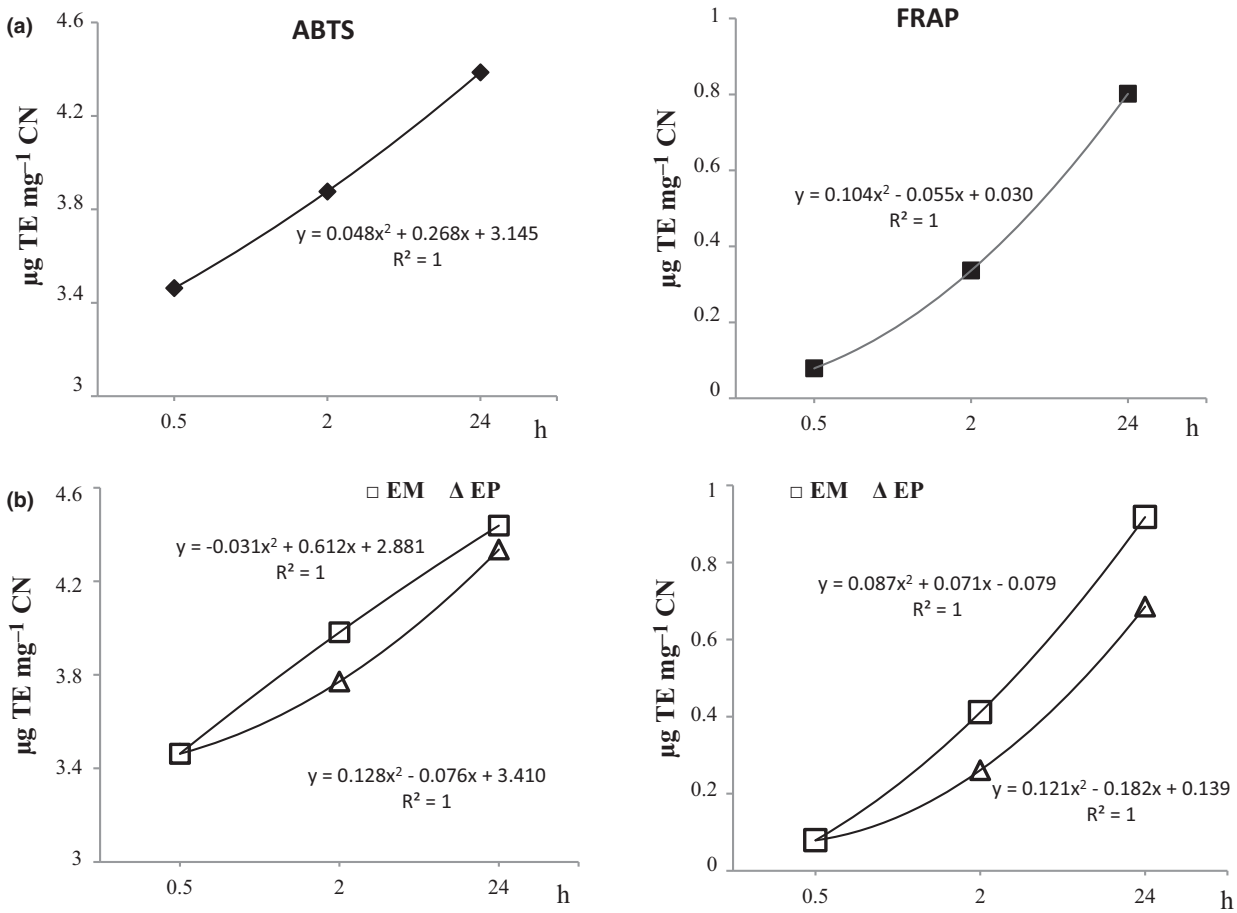

Figure 4 Trend of antioxidant activity of CN hydrolysates during the considered digestion time, regardless of enzymatic treatment and casein haplotype (a) and for each enzymatic treatment (b). EM: *crude enzymatic extract from the edible mushroom Pleurotus eryngii;* EP: pepsin from *porcine gastric mucosa*.

Table 3 Antioxidant activity of casein hydrolysates during considered digestion time, regardless of enzymatic treatment

	Haplotype (α_{S1}-CN, β-CN, κ-CN)							
Time (h)	**BB-A_1A_1-AA**	**BB-A_1A_1-BB**	**BB-BB-BB**	**BB-A_2A_2-AA**	**BB-BB-AA**	**BB-A_2A_2-BB**	**Overall**	**SEM[e]**
ABTS (μg Trolox equivalent/mg CN)								
0.5	3.33[c]	3.44[c,b]	3.41[c,b]	3.63[a]	3.43[c,b]	3.54[a,b]	3.46	0.008
2	4.00[b]	3.77[a]	3.82[a]	3.85[a]	3.83[a]	4.00[b]	3.88	0.005
24	4.51[b]	4.34[c]	4.13[a]	4.33[c]	4.49[b]	4.52[b]	4.39	0.013
FRAP (μg Trolox equivalent/mg CN)								
0.5	0.04[a]	0.08[c,b]	0.11[d]	0.09[c]	0.07[b]	0.08[c,b]	0.08	0.001
2	0.28[a,b]	0.26[a]	0.32[b]	0.38[c]	0.49[d]	0.29[a,b]	0.34	0.013
24	1.12[d]	0.66[a]	0.74[b]	0.78[b,c]	0.85[c]	0.66[a]	0.80	0.009

[a-d]Means within a row with different superscripts differ ($P < 0.05$).
[e]SEM: standard error of means.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Guidelines

Ethics approval was not required for this research.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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