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Comparative Evaluation of Antioxidant Potential of *In-Vitro* Digested Cow Milk Derived A1 and A2 β Casein Variants Using Different Proteases

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ABSTRACT

This study was designed to investigate the antioxidant activity of A1 and A2 β-casein variant with time by applying different enzyme. Pepsin, Trypsin, Alcalase and combination of Pepsin-Trypsin were used for hydrolysis of A1 and A2 β-casein for the duration of 1, 2, 3 and 24 h. All antioxidant parameter including DPPH, ABTS radical-scavenging activity and reducing power assay increasing gradually with time. Enzyme Pepsin-Trypsin combination followed by Alcalsae display comparatively higher antioxidant activity. Among β-casein variant A2 showed relatively higher antioxidant potential over all the entire duration of time but the difference among the A1 and A2 variants was not significant to arrive at a substantial scientific conclusion. It can be concluded from the study that antioxidant potential of the milk depends upon factors such as duration of hydrolysis and enzyme used, during hydrolysis and not alone on the fact that whether the milk is A1 or A2 in nature.

Keywords: A1 and A2 beta casein, enzymatic hydrolysis with time, antioxidant activity

Bioactive peptides (BAPs) are 3–20 amino acid compounds (Manikkam *et al.,* 2016), gaining spotlight now days for their health promoting properties and useful for food industries in the development of functional foods. In the past few decades, a keen interest perceived by food-derived bioactive peptide and wide varieties of BAPs with several healths beneficial activities have been recognized as well. Generally these BAPs are functionally inactive within the native proteins and become active after release from their native protein by proteolysis (in vivo digestion, in vitro enzymatic hydrolysis, or bacterial fermentation) and become "bioactive" by displaying specific roles such as Opioid agonist, ACE inhibitory and immunomodulatory properties shown by β-casein (Park and Nam, 2015). Many of these food-derived BAP show health beneficial properties like antihypertensive, anti-inflammatory, antidiabetic, and antioxidant properties under experimental conditions (Erdmann *et al*., 2008; Hartmann and Meisel, 2007; Sousa *et al*., 2012).

Caseins are the most abundant proteins in bovine milk and they are important and well known source of bioactive peptides (BAP). In bovine milk β-casein represents about 35% of total casein (Huppertz and Kelly, 2018). Among the casein, β-casein is the most polymorphic milk protein. Till now fifteen different genetic variants of bovine have been reported (Caroli *et al.*, 2009; Gallinat *et al.,* 2013). The main difference in between A1 and A2 variant is presence of histidine at 67th position of sequence in A1,

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whereas the variants A2 contain proline at this position (Sodhi *et al.,* 2018).

This little difference produces different BAP with different activity during proteolytic digestion of A1 and A2 milk. Presence of different genetic variants of β-casein produces different bioactive peptides during proteolytic digestion. BAPs released during digestion can interact with receptors on target cells in the human body and induce physiological responses including antioxidant, antihypertensive, opioid, antimicrobial and immunomodulatory (Nongonierma and FitzGerald, 2015). β-Casomorphin-7 (BCM7) is a BAP encrypted in the mature β-casein sequence, which can be released through enzymatic hydrolysis during digestion. A number of studies have described the release of BCM7 after A1 β-casein ingestion (Barnett *et al*., 2014; Boutrou *et al.,* 2013; De Noni, 2008; Jinsmaa and Yoshikawa, 1999; Haq *et al.,* 2015).

Under consideration of above concept this study was designed to investigate whether the generation of different bioactive peptides from genetic variants of β-casein provides different antioxidant potential or not.

MATERIALS AND METHODS

Preparation of β-casein

Collection of milk

Cow milk belonging to A1A1 genotype and A2A2 genotype respectively, was collected from the Cattle and buffalo farm, Indian Veterinary Research Institute, Izatnagar.

Preparation of casein

Isoelectric casein was prepared from both A1 and A2 milk using the method of Davies and Law (1977) with some modification. The fresh cow milk was pre-heated to 37 °C and skimmed. The skim milk was warmed to 37°C and the pH was adjusted slowly to 4.6 using 1.0 N HCl by constant stirring. The clear casein precipitate was allowed to stand at room temperature for 30 min. The precipitate so formed was filtered through four layers of muslin cloth. The casein curd was allowed to stand at room temperature for 30 min over muslin cloth. Finally, the wet casein obtained was air-dried in a food dryer. Dry casein was stored at -20°C till further use.

Fractionation of β-casein

β-casein was fractionated from isoelectric casein precipitate by following the protocol of Fox and Guiney (1972). The sediment was dissolved in urea to 6.6 M concentration. The pH of the solution was adjusted to 4.5 and diluted with distilled water to get 3.3 M concentration of urea. The solution was allowed to stand for 30 min and centrifuged at 5000 rpm for 15 min. Supernatant was collected and diluted to get 1 M urea concentration. The pH of the solution was adjusted to 4.9 and centrifuged at 5000 rpm for 15 min. The pellet so formed was crude β-casein. The pellet was re-suspended in 6.6 M urea solution, and pH was adjusted to 4.5 followed by dilution up to 3.3 M urea. The solution was centrifuged at 5000 rpm for 15 min. Again supernatant was diluted up to 1M urea and pH was adjusted to 4.9 following centrifugation at 5000 rpm for 15 min. Sediment was collected, dispersed in distilled water and pH was adjusted to 7.0. Dialysis was done to remove traces of urea from purified β-casein.

Preparation of enzymatic hydrolysates

Fractionated β-casein of A1 and A2 milk was mixed with distilled water at a ratio of 2:100 (w/v), the proteins were hydrolyzed using proteases at an enzyme/substrate ratio of 1:100 (w/w). Optimum temperature and pH for hydrolysis were adjusted to the optimal values for each enzymes i.e. Pepsin, 37° and pH 2; Trypsin, 37° and pH 8; and Alcalase 55° and pH 8. Optimum temperature and pH for enzymes hydrolysis was stopped by heating at 95° in water bath for 15 min to ensure enzyme deactivation. Samples were drawn after 1, 2, 3 and 24 h of hydrolysis. Then β-casein hydrolysates were centrifuged at 10000 rpm for 20 min and the supernatant was collected to measure antioxidant activity.

Experiment design

A1 and A2 β-casein variants of milk were hydrolysed by different enzymes and eight groups were design includes Pepsin (A1P and A2P), Trypsin (A1T and A2T), Alcalase (A1Alc and A2Alc) and combination of pepsin and trypsin (A1PT and A2PT). Four different durations i.e. 1, 2, 3

and 24 h of hydrolysis provided to each eight groups. In combination of pepsin and trypsin the duration was 1:1 for both enzyme in which first half duration kept under pepsin hydrolysis and half duration under trypsin hydrolysis. Supernatants were prepared after definite period of enzymatic hydrolysis for determining the antioxidant activity. In this study, three methods were used to evaluate the antioxidant activity of the β- casein hydrolysate by the proteolytic enzymes including DPPH radical scavenging, ABTS radical-scavenging activity and reducing power assay.

pH measurement

The pH of each β- casein hydrolysate samples were measured after a definite period of proteolytic hydrolysis using combined glass electrodes of Hanna Instruments Edge Digital pH Meter. The Ph of the samples was measured just before heating the samples.

ABTS radical scavenging activity

ABTS radical-scavenging activity of β- casein hydrolysate samples were determined according to method described by Re *et al.* (1999). The ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulphate. The solution was incubated in the dark at room temperature for 16 h. The stock solution was diluted with ethanol just before use and the absorbance of the solution was adjusted to 0.70 and equilibrated at 30°C exactly 6 min. after initial mixing. ABTS working standard solution (1 ml) was mixed with 10 µL of extract/ hydrolysate; absorbance was measured after 20 min at 734 nm in a 96 well ELISA plate using a spectrophotometer and thereafter calculated using the formula:

$$ABTS\ activity\ (\%) = [(At_0 - At_{20})/At_0]*100$$

DPPH radical-scavenging activity

The radical scavenging activity of β- casein hydrolysate against stable 2, 2-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Zhidong *et al.* (1995) with slight modifications. Briefly, an aliquot of 4.9 ml of 0.1 mM of DPPH reagent in methanol was mixed with 100 µl hydrolyate samples in test tube. The contents were mixed gently and incubated in dark at room temperature and absorbance was measured at 517 nm after 30 minutes of incubation (At_{30}). Methanol was used as blank. The decrease in absorbance at 517 nm showed a reduction of the DPPH radical to 2, 2-diphenyl-1-picrylhydrazine. The results were expressed as:

$$Scavenging\ rate\ (\%) = [A_C - A_S/A_C)] \times 100$$

Where A_C is absorbance of control and A_S is absorbance of test sample.

Reducing power assay

The reducing power of the β- casein hydrolysate was determined according to the method of Oyaizu (1986). About 0.2 ml of sample was mixed with phosphate buffer (0.2 M, pH 6.6) and 0.2 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated for 20 min at 50°C followed by the addition of 0.2 ml of 10 % TCA and then centrifuged at 700 rpm for 10 min. After that 0.2 ml supernatant was mixed with 0.2 ml of DW and 0.2 ml of ferric chloride (0.1% w/v) and absorbance was measured at 700 nm. An increase in the absorbance of the reaction mixture indicated reducing power of the sample.

Statistical analysis

Experiments were repeated three times and all the parameters were analyzed in triplicate (n=9). Data were expressed as means with standard error. Two-way analysis of variance (ANOVA) was done by comparing the means by using Duncan's multiple range test (DMRT), at 95% confidence level using a SPSS package (SPSS 20.0, SPSS Inc., USA).

RESULTS AND DISCUSSION

Change in pH during hydrolysis

Data (Mean±SE) present in Table 1 showed the changes in pH of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes. A significant ($P<0.05$) decrease in pH values of the β- casein hydrolysate was observed with the progression of hydrolysis time except pepsin hydrolysate where increase in pH was observed. No similar kind of pH difference observed among A1 and A2 milk hydrolysate. Among all the four hydrolysis

Table 1: Change in pH of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes

	pH			
Treatment	**1h**	**2h**	**3h**	**24h**
A2P	2.12 ± 0.02^{dB}	2.17 ± 0.03^{eB}	2.19 ± 0.03^{dB}	2.39 ± 0.02^{fA}
A1P	2.19 ± 0.03^{cB}	2.22 ± 0.04^{eB}	2.26 ± 0.03^{cB}	2.45 ± 0.02^{eA}
A2T	7.92 ± 0.02^{aA}	7.87 ± 0.02^{aA}	7.76 ± 0.02^{aB}	7.61 ± 0.02^{aC}
A1T	7.88 ± 0.02^{abA}	7.79 ± 0.02^{abB}	7.71 ± 0.01^{bC}	7.52 ± 0.02^{bD}
A2ALC	7.87 ± 0.02^{abA}	7.71 ± 0.03^{bcB}	7.60 ± 0.03^{bC}	7.51 ± 0.02^{bD}
A1ALC	7.84 ± 0.02^{bA}	7.61 ± 0.04^{dB}	7.55 ± 0.01^{bB}	7.45 ± 0.02^{cC}
A2PT	7.85 ± 0.01^{bA}	7.65 ± 0.03^{cdB}	7.58 ± 0.00^{bC}	7.37 ± 0.02^{dD}
A1PT	7.86 ± 0.02^{bA}	7.65 ± 0.02^{cdB}	7.59 ± 0.01^{bC}	7.33 ± 0.02^{dD}

Values expressed as Mean±SE; ABC/abcMeans bearing different superscripts in a row (*ABC*) or column (*abc*) differ significantly (P<0.05).

Table 2: DPPH radical scavenging activity of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes

	DPPH			
Treatment	**1h**	**2h**	**3h**	**24h**
A2P	21.07 ± 0.72^{ef}	22.27 ± 0.95^{ef}	22.73 ± 1.05^{ef}	23.75 ± 1.35^{ef}
A1P	19.74 ± 0.80^{f}	20.86 ± 0.91^{f}	21.58 ± 1.07^{f}	22.44 ± 1.30^{f}
A2T	23.22 ± 0.94^{de}	25.03 ± 1.24^{de}	25.81 ± 1.33^{de}	27.01 ± 1.40^{de}
A1T	21.88 ± 0.71^{ef}	23.76 ± 1.11^{def}	24.55 ± 1.27^{def}	25.66 ± 1.35^{def}
A2ALC	26.97 ± 0.96^{bcB}	29.00 ± 1.10^{bcAB}	30.04 ± 1.16^{bcAB}	31.89 ± 1.47^{bcA}
A1ALC	24.89 ± 1.13^{cdC}	26.80 ± 1.39^{cdBC}	27.78 ± 1.48^{cdAB}	29.41 ± 1.84^{cdA}
A2PT	31.46 ± 1.05^{aB}	33.61 ± 0.82^{aAB}	34.75 ± 0.74^{aA}	36.37 ± 0.71^{aA}
A1PT	28.80 ± 0.98^{abC}	31.15 ± 0.79^{abBC}	32.25 ± 0.67^{abAB}	33.85 ± 0.53^{abA}

Values expressed as Mean±SE; ABC/abc Means bearing different superscripts in a row (*ABC*) or column (*abc*) differ significantly (P<0.05).

Table 3: ABTS activity of A1 and A2 β- casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes

	ABTS			
Treatment	**1h**	**2h**	**3h**	**24h**
A2P	36.09 ± 1.83^{eB}	39.62 ± 2.02^{eAB}	42.00 ± 2.00^{fAB}	46.09 ± 2.21^{eA}
A1P	33.14 ± 1.38^{eC}	37.47 ± 1.74^{eBC}	40.14 ± 1.64^{fAB}	43.53 ± 2.17^{eA}
A2T	50.85 ± 0.46^{cD}	57.43 ± 0.25^{cC}	60.14 ± 0.52^{dB}	64.95 ± 0.19^{cA}
A1T	43.95 ± 0.50^{dD}	50.29 ± 1.08^{dC}	53.38 ± 1.18^{eB}	59.72 ± 0.30^{dA}
A2ALC	58.57 ± 0.68^{bD}	62.15 ± 0.60^{bC}	64.95 ± 0.62^{bcB}	69.67 ± 0.34^{bcA}
A1ALC	55.00 ± 0.72^{bC}	57.71 ± 1.03^{cC}	60.76 ± 0.98^{cdB}	65.71 ± 0.50^{cA}
A2PT	62.48 ± 1.42^{aC}	68.28 ± 0.76^{aB}	71.81 ± 1.67^{aAB}	76.10 ± 2.11^{aA}
A1PT	57.43 ± 1.45^{bB}	63.43 ± 0.79^{bAB}	68.67 ± 2.07^{abAB}	72.95 ± 2.67^{abA}

Values expressed as Mean±SE; ABC/abcMeans bearing different superscripts in a row (*ABC*) or column (*abc*) differ significantly (P<0.05).

duration, Pepsin-Trypsin combination shows higher and significant (P<0.05) reduction in pH values. The order of pH decrease after 24 h of enzymatic hydrolysis recorded was Pepsin-Trypsin> Alcalase >Trypsin hydrolysate.

The decreases in pH with the progress of hydrolysis time as found in the present study were also reported in camel casein hydrolysate (Kumar *et al.*, 2016) and ovine casein hydrolysate (Daroit *et al.*, 2012). Normally protein hydrolysis is normally accompanied by H^+ release, which accumulates and leads to reactant pH decreases with time. Decreases in pH with the progress of hydrolysis time may be due to the release of protons (H+ ion) and/or production of acidic amino acids (Kumar *et al.*, 2016).

DPPH radical-scavenging activity

DPPH has commonly been used in the analysis of antioxidant activity and the test system can be used for the primary characterization of the scavenging potential of peptides. This method based on DPPH radical in ethanol encountering a proton-donating antioxidant and the radical would then be scavenged, reducing the absorbance at 517 nm (Liu *et al.*, 2010).

The Data (Mean±SE) of changes in DPPH radical scavenging activity of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes was given in Table 2. Although the DPPH radical scavenging activity of hydrolysate prepared from each enzymatic hydrolysis gradually increases with the progression of hydrolysis time but significant (P<0.05) increase were observed in Alcalase and Pepsin-Trypsin derived hydrolysate of both A1 and A2 variant. Among all the four hydrolysis duration, Pepsin-Trypsin derived A2 hydrolysate shows significantly (P<0.05) higher value for DPPH radical scavenging activity. Among A1 and A2 milk only Alcalase derived hydrolysate shows significant (P<0.05) difference during whole hydrolysis in which A2 milk derived hydrolysate was significantly (P<0.05) higher.

In cow milk Abd El-Fattah *et al.* (2017) also reported the DPPH radical scavenging activity improved gradually with the increase in the hydrolysis time and pepsin-treated milk exhibited the significant lowest activity after 60 min of hydrolysis. Kumar *et al.* (2016) also supported with the statement, the DPPH radical scavenging activity significantly (P<0.05) increased with increase in hydrolysis time in camel milk casein hydrolysate. DPPH radical scavenging activity increase with hydrolysis time might be due to generation of more and more BAP as hydrolysis progress that exhibit antioxidant activity. These BAP were electron donors that could react with free radicals, converting them into more stable form and terminating the radical chain reaction (Chi *et al.,* 2015).

It suggested that the antioxidant activities of protein hydrolysate depend not only on their amino acid composition, but also on the size and sequence of their

Table 4: Reducing power assay of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes

Reducing Power				
Treatment	**1h**	**2h**	**3h**	**24h**
A2P	0.20 ± 0.02^{e}	0.22 ± 0.02^{c}	0.23 ± 0.02^{cd}	0.26 ± 0.02^{cd}
A1P	0.19 ± 0.02^{e}	0.21 ± 0.02^{c}	0.22 ± 0.02^{d}	0.24 ± 0.02^{d}
A2T	0.34 ± 0.00^{dD}	0.36 ± 0.00^{bC}	0.40 ± 0.00^{bB}	0.48 ± 0.01^{bA}
A1T	0.29 ± 0.00^{dD}	0.31 ± 0.00^{bcC}	0.35 ± 0.00^{bcB}	0.42 ± 0.01^{bcA}
A2ALC	0.61 ± 0.03^{abC}	0.73 ± 0.04^{aBC}	0.89 ± 0.05^{aAB}	0.99 ± 0.08^{aA}
A1ALC	0.48 ± 0.03^{cC}	0.68 ± 0.07^{aBC}	0.85 ± 0.08^{aAB}	0.95 ± 0.08^{aA}
A2PT	0.65 ± 0.04^{aC}	0.75 ± 0.04^{aBC}	0.90 ± 0.05^{aAB}	1.07 ± 0.07^{aA}
A1PT	0.54 ± 0.04^{bcB}	0.69 ± 0.07^{aB}	0.89 ± 0.05^{aA}	1.00 ± 0.08^{aA}

Values expressed as Mean±SE; ABC/abcMeans bearing different superscripts in a row (*ABC*) or column (*abc*) differ significantly (P<0.05).

amino acids. This might also be due to the enzyme specificity to the particular site in the peptide chain. This was the reason behind significantly (P<0.05) higher DPPH radical scavenging value of Pepsin-Trypsin followed by Alcalase derived hydrolysate than other enzyme derived hydrolysate.

A1 and A2 variant on hydrolysis produce different BAP with different antioxidant activity due to breakdown of peptide from different site. This might be the reason for significant (P<0.05) higher value of DPPH radical scavenging activity of Alcalase derived A2 as compare to A1 beta casein. Cleavage of peptide bond from specific position depend upon enzyme therefore enzyme play a key role in generation of BAP.

ABTS radical scavenging activity

The cationic radical scavenging activity of ABTS+ is most frequently utilized to measure antioxidant activity of food ingredients and processed meat/food products. Since, the reagents dissolve well in both aqueous hydrophilic and organic solvent hydrophobic groups, this assay measures both the hydrophilic and lipophilic antioxidants. Its efficiency depends upon the number of aromatic rings, nature of hydroxyl groups and molecular weight (Hagerman *et al.,* 1998).

The Data (Mean±SE) of changes in ABTS radical scavenging activity of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes was given in Table 2. ABTS radical scavenging activity of each beta casein hydrolysate treatment increased significantly (P<0.05) as the hydrolysis progress and it was maximum after 24 h of hydrolysis. Trypsin derived A2 beta casein hydrolysate shows significantly (P<0.05) higher ABTS radical scavenging value than A1 beta casein hydrolysate during each four duration of hydrolysis. A2 beta casein hydrolysate also show significantly higher ABTS radical scavenging activity than A1 beta casein hydrolysate after 1, 2 h of Pepsin-Trypsin and 2h of Alcalase derived hydrolysate. ABTS radical scavenging activity is also significantly (P<0.05) higher for Pepsin-Trypsin derived A2 beta casein hydrolysate. Decreasing order of ABTS radical scavenging activity was Pepsin-Trypsin> Alcalase >Trypsin > Pepsin.

Kumar *et al.* (2016) also reported that the ABTS radical-scavenging activity increased significantly (P<0.05) with the increase in time of hydrolysis camel milk casein hydrolysate. As the hydrolysis advanced efficient and more break down of peptide bond happens that generates more and smaller BAP molecules that synergistically show higher ABTS radical scavenging activity.

The accumulated increase in antioxidant capacity up to 1.7-fold for the 4 β-CN variants was observed after 60 min of pepsin + 120 min of pancreatic enzyme digestion (Petrat-Melin *et al.,* 2014). Pepsin-Trypsin derived A2 beta casein hydrolysate shows highest ABTS radical scavenging activity that might be due to cumulative effect of both the enzyme in breakdown of peptide bonds.

Pepsin-Trypsin and Alcalase derived A2 beta casein hydrolysate showed significantly (P<0.05) higher ABTS radical scavenging activity than A1 beta casein hydrolysate was due to breakdown of peptide bonds from specific site leads to dissimilar BAP production with dissimilar ABTS radical scavenging activity.

Reducing power assay

Data (Mean±SE) present in Table 4 showed the changes in pH of A1 and A2 β-casein after 1, 2, 3 and 24 h of hydrolysis with different proteolytic enzymes. Reducing Power was significantly (P<0.05) increasing for Trypsin, Alcalase and Pepsin-Trypsin derived beta casein hydrolysate of both A1 and A2 variant during whole hydrolysis. In each treatment reducing power as similar to other antioxidant parameters was higher after 24 h of hydrolysis. Similar trend of decreasing order was observed in reducing power as shown by the other antioxidant parameters i.e. Pepsin-Trypsin> Alcalase >Trypsin > Pepsin. Trypsin derived A2 beta casein hydrolysate shows significantly (P<0.05) higher Reducing Power value than A1 beta casein hydrolysate during each four duration of hydrolysis. Among all eight treatments value of reducing power for Pepsin-Trypsin derived A2 beta casein hydrolysate was highest.

In the present study, the reducing power increased with the increase of hydrolysis time in cow milk as observed by Abd El-Fattah *et al.* (2017). This is attributed to the increment of availability of protons and electrons resulting from enzymatic hydrolysis (Luo *et al.,* 2014). The differences in the reducing power may be imputed to the composition and sequence of amino acids in the resultant peptides where the hydrolysate with a high level of peptide

possessed a strong antioxidant activity. These peptides react with free radical to form more stable products (Abd El-Fattah *et al.,* 2017).

CONCLUSION

Both the hydrolysate of beta casein variant A1 and A2 display antioxidant potential and with progression of hydrolysis it increases gradually irrespective of the enzymes. In this study Antioxidant activity was higher for Pepsin-Trypsin followed by Alcalase, Trypsin and Pepsin derived beta casein hydrolysate. A2 derived beta casein hydrolysate exhibit slightly higher antioxidant activity than A1 derived beta casein hydrolysate irrespective of the enzymes, but the variation was not significant (P>0.05). On the basis of the findings of this study it is safe to conclude that antioxidant activity of beta casein hydrolysate depends upon sequence of amino acid of intact beta casein, site of cleavage in sequence of amino acid of beta casein, duration of hydrolysis, enzyme used, size and sequence of amino acid produced during hydrolysis and not on the fact that the milk is A1 or A2 in nature.

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