Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2021; 9(1): 1575-1578

Received: 19-10-2020
Accepted: 16-12-2020

Lalita Garg
Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Geeta Chauhan
Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

SK Mendiratta
Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Kamal Kumar
Division of Extension Education, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Corresponding Author:
Lalita Garg
Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

In vitro assessment of ACE inhibitory activity of A1 and A2 cow milk casein hydrolysate

Lalita Garg, Geeta Chauhan, SK Mendiratta and Kamal Kumar

DOI: https://doi.org/10.22271/j.ento.2021.v9.i1v.8363

Abstract
The aim of the present study was *in vitro* assessment of ACE inhibition property of A1 and A2 cow milk casein digested with trypsin. Casein powder was reconstituted (1%, 3% and 5% concentration) and trypsin was added at 1:100 (E:S), hydrolysis was carried out for 6 h at 37 ºC and samples were drawn at 2 h intervals from both A1 and A2 casein hydrolysates. Bioactive peptides are encrypted within parent protein and are inactive, upon hydrolysis it become biologically active and shows positive health impact. Both A1 and A2 casein hydrolysates manifested ACE inhibition, whereas improved activity was recorded with progress in time and degree of hydrolysis. The results stipulate that hypertension was partially control by ACE inhibitory properties of casein-derived peptides.

Keywords: A1/A2 cow milk, enzymatic hydrolysis by casein, A1/A2 casein hydrolysate, ACE inhibition

Introduction
Hypertension can lead to cardiovascular disease complexes such as myocardial infarction, stroke, and heart failure; almost one-third of the western population is suffering from hypertension. Numerous lifestyle changes (low salt intake, weight reduction, increased physical activity, and cessation of alcohol consumption) and pharmacological treatments are applied to treat hypertension [1]. In clinical practice, vasodilators, diuretics, calcium channel blockers, angiotensin II receptor blockers, and angiotensin I-converting enzyme (ACE) inhibitors were utilized [2]. ACE inhibitors interfere with the renin-angiotensin and kinin-kallikrein pathway in the body. ACE inhibitory peptides are present in many food protein sources, and among them, milk protein is an immeasurable source of bioactive peptides [3]. Both casein and whey derived peptides have shown ACE inhibitory activity [1]. A product named "casein DP peptio drink" made up of casein hydrolyzed with trypsin was prepared to control the blood pressure [4]. Intake of 20 g of casein hydrolysate per day for 4 weeks would decrease the blood pressure in hypertensive patients [5]. Another product named "C12 Peption" in the Netherlands is composed of tryptic hydrolyzed casein, prescribed in hypertensive animals and humans to control the blood pressure [6, 7]. Calpis, fermented milk, cultured using *Saccharomyces cerevisiae* and *Lactobacillus helveticus*, contained two well-known ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro from β-casein and κ-casein, respectively [8].
Cow milk is deemed as a major source of functional peptides with the positive repercussions on the promotion of health and prevention of disease [9]. Upon hydrolysis, the bioactive peptides get released from its native protein [10] and perform multi-functional role like antioxidant [11], antihypertensive [12, 13], antidiabetic [14], antithrombotic [15], and immunomodulation [16]. Milk-derived bioactive peptides have the potential to be used as an ingredient in functional foods and nutraceuticals. Casein is considered as the major source of indispensable amino acids and has high biological value. Casein consists of four fractions, namely αs1, αs2, β and κ-casein. β-casein consists of 209 amino acids and has two variants A1 and A2 on the basis of the presence of amino acid at 67th position. A1 type consists of histidine at the 67th position whereas A2 consists of proline at the same position.
Therefore, this research designed to investigate the *in vitro* health-promoting benefits namely ACE inhibition of A1 and A2 cow milk casein digested with trypsin at different casein concentrations, and at a various time of hydrolysis and also the comparison between the casein variant derived from the A1 and A2 cow milk.

Materials and Methods

Milk

Milk was collected from cattle belonging to A1A1 and A2A2 genotyped from Cattle and Buffalo Farm, ICAR-IVRI, Izatnagar. The cattle belonging to Vrindavani breeds (crossbred) and Tharparkar breeds (indigenous) were taken as a source of A1 and A2 milk respectively. The cows herd designated as A1A1 and A2A2 was genotyped by AGB Division, ICAR-IVRI, Izatnagar.

Preparation of freeze-dried casein from A1 and A2 genotyped cows milk

The cow milk casein powder was prepared according to the method as narrated by Salami *et al.* (2011) with slight modification [17]. The fresh cow milk was warmed up to 37 ºC and skimming was done by using a manual cream separator at Animal By-Product lab, Division of LPT. The pH of skim milk was reduced to 4.6 with 2% citric acid solution. Then the solution was mixed properly at 37 ºC for 30 min and casein was precipitated and separated from whey proteins by centrifugation (10,000 rpm, 60 min, and 4 ºC) and then lyophilized (Scan Vac, Cool Safe Freeze Dryers) and stored at -20 °C until use.

Preparation of A1 and A2 casein hydrolysates by enzymatic hydrolysis

Cow milk whole casein solutions (1%, 3% and 5% w/v on total solid basis) were made by reconstituting the casein in distilled water. The casein solution was heated in boiling water bath for 5 min to destroy the microorganisms, if present, which may produce proteolytic enzymes during hydrolysis process and to denature the native enzymes of the milk, if present, as well as to heat denature the proteins, which increase its susceptibility to proteolytic enzymes. Enzyme/substrate ratio of 1:100 (w/w) was kept constant for trypsin. The temperature and pH of trypsin were adjusted to 37 ºC and 8.0 respectively. The hydrolysis was achieved by incubating the samples at 37 ºC for trypsin in stirred water bath and samples were drawn after definite time interval i.e. 2 h, 4 h and 6 h of incubation. Each hydrolyzed sample was instantly warmed to 85 ºC for 15 min. in water bath to inactivate the enzymes left in the hydrolysates. Then the samples were cooled and centrifuged in the refrigerated centrifuge (Hermle, High-Speed Universal Refrigerated Centrifuge) at 10,000 rpm for 20 min, supernatants were collected and stored at -20 ºC until further use and samples were coded as A1 and A2 derived casein hydrolysate.

Angiotensin I-converting enzyme (ACE) inhibition

ACE inhibitory activity of casein hydrolysates, were determined as per Cushman and Cheung (1971) [18]. 20 µl of hydrolysate samples mixed with 20 µl of an ACE solution. Following preincubation at 37 ºC for 15 min, 110 µl of Hippuryl-L-histidyl-L-leucine (HHL) substrate solution in 0.1 M sodium borate buffer comprising 0.3 M NaCl, and pH 8.3 was added. After incubating at 37 ºC for 60 min, the reaction was stopped, by adding 60 µl of 1 N HCl. To extract hippuric acid, add 1ml of ethyl acetate to the mixture and was centrifuged at 3000 rpm for 10 min. Then, 750 µl of the upper organic phase was evaporated by heating at 95 ºC for 30 min in a water bath. The dried material was dissolved in 1 ml of distilled water, and the absorbance was measured at 228 nm using a spectrophotometer. The control sample was prepared by replacing the test sample with distilled water. The inhibition (%) was calculated as below:

% ACE inhibition= $(Absorbance_{control}-Absorbance_{sample})/Absorbance_{control} \times 100$

Statistical analysis

Data are presented as mean±standard error (SE) of nine independent experiments. Data were analyzed using the SPSS package (SPSS 20, Version 20, IBM, USA). The difference between the mean was compared using a t-test. Further, Significant differences were determined using Tukey tests ($p<0.05$). Data obtained from the experiments were pooled. Firstly A1 and A2 samples were compared within the group based on the time of hydrolysis and then based on casein concentration furthermore a comparison was made between A1 and A2 at the same time period of hydrolysis.

Results and Discussion

Determination of *In vitro* antihypertensive activity of casein hydrolysate

Angiotensin I-converting enzyme (ACE) inhibition

It is clear from Table 1 that the ACE inhibitory activity of 1%, 3%, and 5% casein hydrolysate ranged from 47.54-66.79%, 52.25-86.26%, and 53.56-87.94%, respectively. The comparison of ACE inhibitory activity within the group showed that, at 1% casein concentration, there was a significant ($p<0.05$) increase in activity of A1 and A2 casein hydrolysates at 2 h, 4 h, and 6 h of hydrolysis. In the case of 3% casein, A1 and A2 casein hydrolysate showed a significant ($p<0.05$) increase in activity at 2 h and 6 h of hydrolysis. A1 and A2 casein hydrolysate at 5% casein level showed a significant ($p<0.05$) increase at 2 h and 4 h followed by a non-significant ($p>0.05$) increase thereafter. Results indicated that the ACE inhibitory activity of casein hydrolysates increased with an increase in hydrolysis time and protein concentration (Fig. 1.).

Moreover, among different casein concentrations at the same time interval showed a significant ($p<0.05$) increase in ACE activity of A1 casein hydrolysates at 0 h of hydrolysis in case of 1% and 3% casein, afterward increase was not significant ($p>0.05$). However, in the case of A2 casein hydrolysate a significant ($p<0.05$) increase was marked between different casein concentrations. At 2 h, 4 h, and 6 h of hydrolysis, a significant ($p<0.05$) increase was recorded among different casein concentration in both A1 and A2, with the highest registered in 5% followed by 3% and 1% (Table 1).

In the case of 1%, 3%, and 5% casein, the distinctions between A1 and A2 casein hydrolysates did not vary significantly ($p>0.05$) at 0 h and 2 h thereafter, a significant difference ($p<0.05$) was discerned at 4 h and 6 h of hydrolysis with higher activity toward A2 casein hydrolysate (Table 1).

Peptic hydrolyzed bovine casein had higher ACE inhibitory activity than whole casein, attributed to the presence of lower molecular weight peptides [19]. Proline, tryptophan, tyrosine, and phenylalanine were the most effective at the C-terminal position, with proline unexpectedly binding well to ACE [20], hence, A2 casein hydrolysate showed higher activity than A1 casein hydrolysate. Proline-containing peptides are more resistant to hydrolysis by digestive enzymes, thus sustained for a longer period in the digestive tract and exert *in vivo* bioactivity [21]. Casein is superior to whey protein and showed >80% inhibition since casein is rich in proline that potentiates the activity of casein hydrolysate [22]. Tryptic milk protein hydrolysate showed higher ACE inhibitory activity due to the occurrence of the positively charged amino acid at

the ultimate C-terminal position [23]. Lower activity of A1 variant than the A2 variant of β-casein was reported on trypsin digestion [22]. After oral feeding of tryptic digests of casein in spontaneous hypertensive rats, the decrease in blood pressure was reported [7]. Trypsin digested casein showed maximum inhibition in contrast to the papain and pancreatin digest further, a slower activity rate was observed after 1 h of hydrolysis [24]. IC_{50} value (ACE inhibition) of bovine κ-casein and ovine S2-casein hydrolysates was 9.97 and 41.8 µg/ml, respectively [25]. Several ACE inhibitory peptides were extracted from α-casein and β-lactoglobulin [13, 26]. Petrat-Melin *et al.* (2014) reported maximum ACE inhibition in the A1 and B variant as compared to the A2 and I variant of β-casein hydrolyzed with pepsin and pancreatic enzyme [27].

Table 1: ACE inhibitory potential of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis (Mean±SE)

ACE inhibitory potential of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis (Mean±SE)								
Casein concentration	ACE inhibition (%)							
	Time of hydrolysis							
	0 h		2 h		4 h		6 h	
	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate
1% casein	47.54 ± 0.41^{2d}	48.15 ± 0.39^{3D}	62.14 ± 0.42^{3c}	62.61 ± 0.43^{3C}	63.98 ± 0.29^{3b}	$65.18\pm0.35^{*3B}$	65.85 ± 0.24^{3a}	$66.79\pm0.25^{*3A}$
3% casein	52.25 ± 0.31^{1c}	52.60 ± 0.30^{2C}	82.14 ± 0.34^{2b}	83.24 ± 0.38^{2B}	83.19 ± 0.30^{2b}	$84.64\pm0.60^{*2A\ B}$	84.96 ± 0.36^{2a}	$86.26\pm0.40^{*2A}$
5% casein	53.56 ± 0.42^{1c}	53.96 ± 0.31^{1C}	84.59 ± 0.44^{1b}	84.92 ± 0.26^{1B}	86.50 ± 0.26^{1a}	$87.52\pm0.24^{*1A}$	86.99 ± 0.15^{1a}	$87.94\pm0.24^{*1A}$
(n=9); *significant (p<0.05) difference between A1 and A2 samples Mean±SE with different superscripts row wise (small alphabets show difference among A1 samples; capital alphabets show difference among A2 samples) and column wise (number show difference among concentration) differ significantly (p<0.05)								

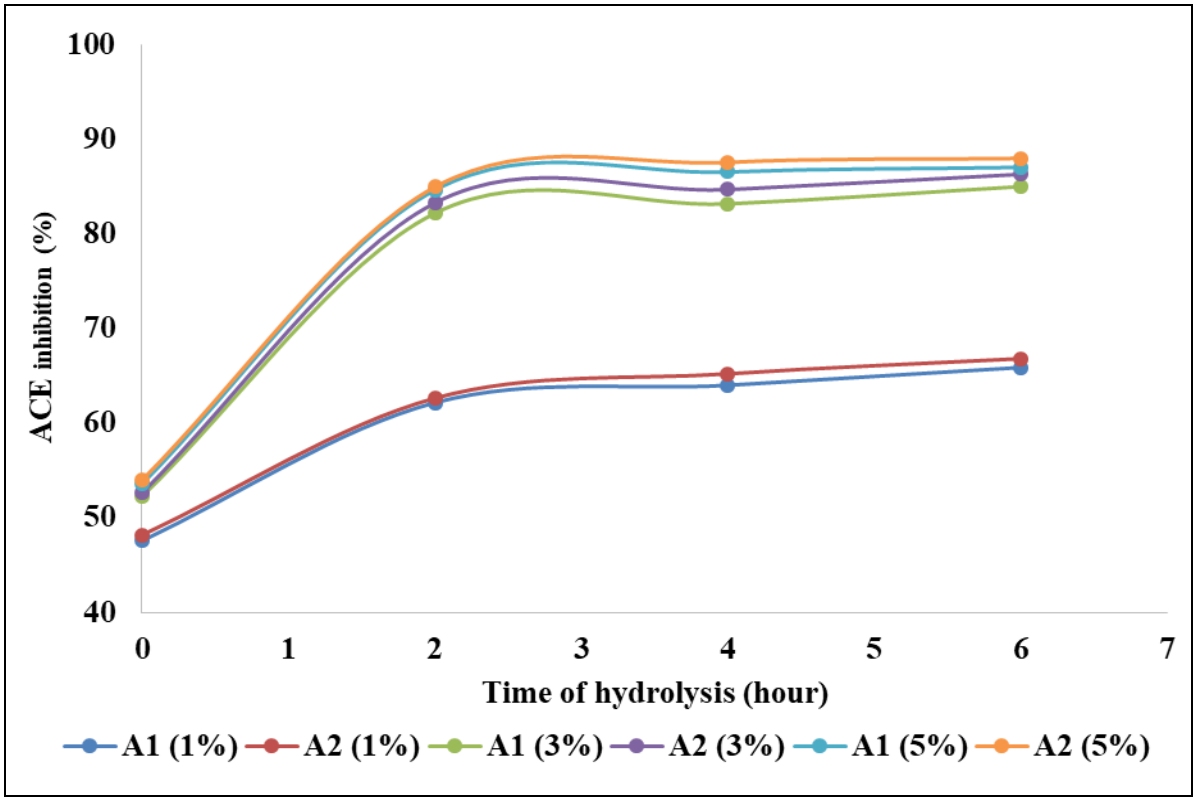

Fig 1: ACE inhibition of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis

Conclusion

The result of present study demonstrates that hydrolysis of A1 and A2 casein by the trypsin resulted in production of peptides with inhibitory properties against ACE and activity get improved with time of hydrolysis. These finding suggested that both A1 and A2 casein hydrolysates with such inhibitory traits may potentially improve the blood pressure regulation by means of their ability to inactivate the ACE enzyme. However, further study is required for a better understanding of the molecular mechanisms of action of the defined peptides. Besides, clinical studies are certain to validate the efficiency and bioavailability of casein-derived peptides in humans.

Acknowledgments

The authors are grateful to the Director of ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, for providing funding and laboratory facilities to carry out this piece of work.

Conflicts of interest

There are none potential conflicts between authors and others that bias our work.

References

1. López-Fandiño R, Otte J, Van Camp J. Physiological,

chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. International Dairy Journal. 2006;16(11):1277-1293.
2. Praveesh BV, Angayarkanni J, Palaniswamy M. Antihypertensive and anticancer effect of cow milk fermented by *Lactobacillus plantarum* and *Lactobacillus casei*. International Journal of Pharmaceutical Science 2011;3(5):452-456.
3. Meisel H. Multifunctional peptides encrypted in milk proteins. Biofactors. 2004;21(1-4):55-61.
4. Sugai R. ACE inhibitors and functional foods [Angiotensin-I Converting enzyme]. International Dairy Federation 1998.
5. Sekiya S, Kobayashi Y, kita E, Imamura Y, Toyama S. Antihypertensive Effects of Tryptic Hydrolysate of Casein on Normotensive and Hypertensive Volunteers. Journal of Japan Society of Nutrition and Food Sciences 1992;45(6):513-517.
6. Cadée JA, Chang CY, Chen CW, Huang CN, Chen SL, Wang CK. Bovine casein hydrolysate (C12 peptide) reduces blood pressure in prehypertensive subjects. American Journal of Hypertension 2007;20(1):1-5.
7. Karaki H, Sugano S, Uchiwa H, Sugai R, Murakami U, Takemoto S. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. Comparative biochemistry and physiology. C, Comparative pharmacology and toxicology. 1990;96(2):367-371.
8. Nakamura Y, Yamamoto N, Sakai K, Takano T. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. Journal of dairy science 1995;78(6):1253-1257.
9. Kitts DD, Weiler K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Current Pharmaceutical Design. 2003;9(16):1309-1323.
10. FitzGerald RJ, Murray BA, Walsh DJ. Hypotensive peptides from milk proteins. Journal of Nutrition 2004; 134(4):980-988.
11. Suetsuna K, Ukeda H, Ochi H. Isolation and characterization of free radical scavenging activities peptides derived from casein. Journal of Nutritional Biochemistry 2000;11(3):128-131.
12. Maruyama S, Suzuki H. A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. Agricultural and Biological Chemistry 1982;46(5):1393-1394.
13. Maruyama S, Mitachi H, Tanaka H, Tomizuka N, Suzuki H. Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. Agricultural and Biological Chemistry. 1987;51(6):1581-1586.
14. Nongonierma AB, FitzGerald RJ. Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. Peptides 2013;39:157-163.
15. Erdmann K, Cheung BW, Schroder H. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. Journal of Nutritional Biochemistry 2008;19(10):643-654.
16. Gill HS, Doull F, Rutherfurd KJ, Cross ML. Immunoregulatory peptides in bovine milk. British Journal of Nutrition 2000;84(1):111-117.
17. Salami M, Moosavi-Movahedi AA, Moosavi-Movahedi F, Ehsani MR, Yousefi R, Farhadi M, Haertlé T. Biological activity of camel milk casein following enzymatic digestion. Journal of dairy research. 2011;78(4):471.
18. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochemical pharmacology. 1971;20(7):1637-1648.
19. Miguel M, Contreras MM, Recio I, Aleixandre A. ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. Food Chemistry 2009;112(1):211-214.
20. Cheung HS, Wang FL, Ondetti MA, Sabo EF, Cushman DW. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. Journal of Biological Chemistry 1980;255(2):401-407.
21. Yang CM, Russell JB. Resistance of proline-containing peptides to ruminal degradation *in vitro*. Applied and Environmental Microbiology 1992;58(12):3954-3958.
22. Otte J, Shalaby SM, Zakora M, Nielsen MS. Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. International Dairy Journal 2007;17(12):1460-1472.
23. Li GH, Le GW, Shi YH, Shrestha S. Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. Nutrition research 2004;24(7):469-486.
24. Luo Y, Pan K, Zhong Q. Physical, chemical and biochemical properties of casein hydrolyzed by three proteases: partial characterizations. Food chemistry. 2014;155:146-154.
25. López-Expósito I, Quirós A, Amigo L, Recio I. Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. Le Lait 2007;87(4, 5):241-249
26. Hernández-Ledesma B, Amigo L, Recio I, Bartolomé B. ACE-inhibitory and radical-scavenging activity of peptides derived from β-lactoglobulin f (19− 25). Interactions with ascorbic acid. Journal of Agricultural and Food Chemistry 2007;55(9):3392-3397.
27. Petrat-Melin B, Andersen P, Rasmussen JT, Poulsen NA, Larsen LB, Young JF. *In vitro* digestion of purified β-casein variants A1, A2, B, and I: Effects on antioxidant and angiotensin-converting enzyme inhibitory capacity. Journal of dairy science 2015;98(1):15-26.