

Original Research Article

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***In vitro* Assessment of Antimicrobial Activity of A1 and A2 Milk Derived Casein Hydrolysate**

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ABSTRACT

Keywords

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The present study was designed to test the antimicrobial activity of A1 and A2 milk derived casein hydrolysate on *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25924, *Salmonella typhi* MTCC3917 and *Pseudomonas aeruginosa* MTCC1688. Milk was collected from cattle belonging to A1A1 and A2A2 genotyped from Cattle and Buffalo Farm, ICAR-IVRI, Izatnagar. The cows used for the study belonged to definite genotype as designated by AGB Division, ICAR-IVRI, Izatnagar. Results showed no antimicrobial activity for A1 and A2 casein hydrolysate; instead it was acting as a bacterial growth promoter. No significant difference ($p>0.05$) was observed between A1 and A2 casein hydrolysates in bacterial growth of *Staphylococcus aureus* ATCC25924, *Salmonella typhi* MTCC3917 and *Pseudomonas aeruginosa* MTCC1688 at 0 h, 2 h, 4 h and 6 h of hydrolysis except in case of *Listeria monocytogenes* ATCC19115 at 4 h only. No antimicrobial activity was observed for casein hydrolysate instead it was acting as a bacterial growth promoter, which can be used as supplement in growth media.

Introduction

Casein is deemed as the major source of essential amino acids and has high biological value. Casein consists of four fractions, namely α_1 , α_2 , β and κ -casein. β -casein has two variants A1 and A2 on the basis of the presence of amino acid at 67th position. Enzymatic cleavage of A1 β -casein lead to the production of seven amino acid peptide, known as β -casomorphin7 (BCM-7), whereas no BCM-7 is produced upon digestion of A2

milk. Milk is considered as a major source of bioactive peptides with the positive impact on the promotion of health and prevention of disease through enzymatic hydrolysis (Kitts and Weiler, 2003). Bioactive peptides reside within parent protein and are inactive but get released and biologically active upon enzymatic hydrolysis (FitzGerald *et al.*, 2004). The activities of peptides are based on their inherent amino acid composition and sequences. Milk-derived bioactive peptides have the potential to be used as an ingredient

in functional foods and nutraceuticals. Antimicrobial peptides consist of 12-100 amino acids, comparatively short and have a net positive charge. Antimicrobial peptides did not get much recognition due to their low efficiency and high cost of production against traditional antibiotics (Lahov and Regelson, 1996). Currently, there is a growing interest in the use of these antimicrobial peptides as food preservatives or as healthy dietary supplements. Cationic component of antimicrobial peptides interacts with an anionic component of gram positive and gram negative bacteria, resulting in neutralization of charge on the microbial surface, leading to disruption of the cellular envelope and increase the permeability of cell (Oren, 1999). In some cases, antimicrobial peptides lead to the formation of pores in the cell envelope, leading to leakage of cell contents and ultimately resulting in cell death (He *et al.*, 1996). Antimicrobial peptides distort the microbial cell membrane via negatively charged electrostatic interaction, inhibiting the synthesis of protein, DNA and ribonucleic acid (RNA) or interacting with intracellular targets (Bahar and Ren, 2013). Casein-derived bioactive peptides have shown antimicrobial activity against *Salmonella*, *Escherichia* and *Listeria* (Haque and Chand, 2008).

Materials and Methods

Milk was collected from cattle belonging to A1A1 and A2A2 genotyped from Cattle and Buffalo Farm, ICAR-IVRI, Izatnagar. The cows used for the study belonged to definite genotype as designated by AGB Division, ICAR-IVRI, Izatnagar.

Preparation of A1 and A2 casein hydrolysates by enzymatic hydrolysis

The cow milk casein powder was prepared according to the method as narrated by Salami *et al.*, (2011) with slight modification. Cow

milk whole casein solutions (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 °C and 8.0 respectively. The hydrolysis was achieved by incubating the samples at 37 °C 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 °C 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 at 10,000 rpm for 20 min, supernatants were collected and stored at -20 °C until further use and samples were coded as A1 and A2 casein hydrolysate.

Bacterial growth inhibition assay

The four tested bacterial strains (*Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25924, *Salmonella typhi* MTCC3917 and *Pseudomonas aeruginosa* MTCC1688) were cultured in brain heart infusion (BHI) broth as described by Kouadio *et al.*, (2011). Undigested and digested samples were sterilized by filtration into a 0.22-µm-pore-size membrane filter. The antimicrobial test was performed in a sterile 96-well microplate, which was incubated at 37 °C for 24 h. Optical density at 600 nm (OD_{600nm}) was recorded after 24 h using a Microplate reader (Thermo Fisher Scientific). Volumes of 100 µl of overnight cultures *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC25924,

Salmonella typhi MTCC3917 and *Pseudomonas aeruginosa* MTCC1688 previously diluted in BHI broth, used to inoculate each well containing 100 µl of digested or undigested casein sample. In addition to the positive controls, negative controls were also included. The percentage of inhibition of bacterial growth after 24 h of incubation at 37 °C was estimated by comparing OD_{600nm} values measured with the mean values of the positive controls.

Results and Discussion

Although most of the studies had shown antimicrobial effect of casein hydrolysates, but in present study no antimicrobial activity was observed from A1 and A2 casein hydrolysates against all targeted bacteria by well diffusion, disc diffusion and micro-dilution method.

In the present study A1 and A2 casein hydrolysates at 5% concentration, was initially tested for antimicrobial activity against each two Gram positive (*Listeria monocytogenes* ATCC19115 and *Staphylococcus aureus* ATCC25924) and Gram negative (*Salmonella typhi* MTCC3917 and *Pseudomonas aeruginosa* MTCC1688) foodborne bacteria by using agar well diffusion and disc diffusion assay on BHI plates. No zone of inhibition was observed against any targeted bacteria by A1 and A2 casein hydrolysates at 0 h, 2 h, 4 h and 6 h of hydrolysis, which might be due to non-diffusibility of peptides.

Then, antimicrobial activity was studied by using much sensitive micro-dilution method. Results in Table 1 showed a clear increase in the OD values of A1 and A2 casein hydrolysates when added to growth media for study purpose. The OD_{600 nm} of both A1 and A2 casein hydrolysates were higher than the control sample. Consequently, it was

suggested that no antimicrobial activity was observed in both A1 and A2 casein hydrolysates at 5% casein concentration at 0 h, 2 h, 4 h and 6 h of hydrolysis. No significant difference ($p>0.05$) was observed between A1 and A2 casein hydrolysates in bacterial growth of *Listeria Staphylococcus aureus* ATCC25924, *Salmonella typhi* MTCC3917 and *Pseudomonas aeruginosa* MTCC1688 at 0 h, 2 h, 4 h and 6 h of hydrolysis. In case of *Listeria monocytogenes* ATCC19115, significant difference ($p<0.05$) was observed between A1 and A2 casein hydrolysates at 4 h only, showing higher for A2.

The differentiation of bacterial growth within the groups showed that significant difference ($p<0.05$) was observed only in case of *Pseudomonas aeruginosa* MTCC1688, where A1 casein hydrolysate showed significant ($p<0.05$) decrease at 2 h followed by further non-significant variation ($p>0.05$).

The results of present study was supported with the finding of St-Gelais *et al.*, (1993), who reported that casein hydrolyzed with trypsin and alcalase was rich in amino acids and peptides that could be used to supplement culture medium and to promote the growth of *Lactococci*, moreover some strains of *Lactococci* grew better in culture media enriched with casein hydrolysates that has high molecular weight peptide, where as other strains preferred culture media with lower molecular weight peptides.

The results are in concurrence with Gray *et al.*, (2008), who reported that pancreatin and HCl digests of casein had shorter generation interval for *Escherichia coli*, *Salmonella* ser. Poona, *Salmonella* ser. Typhimurium and *Staphylococcus aureus* and acting as better peptones than gelatine, meat, soy and yeast extract.

Table.1 Effect of A1 and A2 casein hydrolysates by trypsin (5%) on bacterial growth by micro-dilution method at various time periods of hydrolysis (growth rate in terms of OD_{600nm}) (Mean±SE)

Bacteria	Growth rate (OD _{600nm})								
	Time of hydrolysis								
	Control	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	A2 casein hydrolysate
<i>Staphylococcus aureus</i> ATCC25924	0.30±0.07	0.42±0.05	0.52±0.10	0.45±0.09	0.52±0.10	0.40±0.06	0.47±0.08	0.41±0.05	0.34±0.05
<i>Listeria monocytogenes</i> ATCC19115	0.14±0.01	0.62±0.25	0.59±0.31	0.44±0.02	0.48±0.02	0.39±0.01	0.45±0.01*	0.41±0.01	0.39±0.02
<i>Salmonella typhi</i> MTCC3917	0.49±0.00	0.64±0.06	0.80±0.35	0.62±0.07	0.56±0.07	0.64±0.06	0.62±0.05	0.61±0.06	0.53±0.08
<i>Pseudomonas aeruginosa</i> MTCC1688	0.50±0.03	0.98±0.07 ^a	0.78±0.25	0.69±0.06 ^b	0.59±0.05	0.53±0.09 ^b	0.70±0.02	0.69±0.02 ^b	0.69±0.03

(n=6); *significant (p<0.05) difference between A1 and A2 samples
Mean±SE with superscripts row wise (small alphabets show difference among A1 samples) differ significantly (p<0.05)

Similar to the present finding, Poch and Bezkorovainy (1988) elucidated that bovine casein digests was promoting the growth of *Bifidobacterium* sp. as it serve a source of nitrogen and unknown growth factor for bacteria. Higuchi and Carlin (1957) reported that casein hydrolysate was better growth medium for *Pasteurella pestis*.

In conclusion the no antimicrobial activity was observed for casein hydrolysate instead acting as bacterial growth promoter, which can be used as supplement in growth media. Further, No significant difference ($p>0.05$) was observed between A1 and A2 casein hydrolysates in bacterial growth of *Staphylococcus aureus* ATCC25924, *Salmonella typhi* MTCC3917 and *Pseudomonas aeruginosa* MTCC1688 at 0 h, 2 h, 4 h and 6 h of hydrolysis except in case of *Listeria monocytogenes* ATCC19115 at 4 h only.

However, The present results are not in agreement with Recio and Visser (1999), Bougherra *et al.*, (2017), Lopez-Exposito *et al.*, (2007), which might be due to the utilization of different enzyme i.e. trypsin, production of different pattern of peptides upon hydrolysis or the less amount of antimicrobial peptides as compared to the bacterial growth promoting peptides, which could only be confirmed by further isolation of specific peptide sequence having antimicrobial activity and not opted in the present study.

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