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Original Research Article

Anti-oxidative potential of bioactive peptides released during fermentation of bovine milk with Lactic Acid Bacteria

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Abstract

Antioxidants function by preventing the formation of radicals or by scavenging radicals or hydrogen peroxide and other peroxides. Bioactive-peptides generated from the digestion of milk proteins are reported to have antioxidative activities. This study has been carried out to see the effect of Lactic Acid Bacteria for the production of Anti-oxidative activity through the fermentation of bovine milk. In present research antioxidant activity of milk protein hydrolysates has also been shown. We used three different cultures of Lactic Acid Bacteria for the fermentation of bovine milk from different sources. Out of three one was pure culture of Lactobacillus helveticus NCDC 292, second one was curd with unknown strain and third was combination of Lactococcus and Lactobacilli. Bioactive peptides generated during fermentation demonstrated antioxidative effect on the inhibition of lipid per-oxidation. The Antioxidative effect of peptides was higher for the culture used in combination i.e. 58-89ppm butylated hydroxytoulene (BHT), then of Lactobacillus helveticus i.e. 55-75ppm butylated hydroxytoulene (BHT), & least for local curd i.e. 46-60ppm butylated hydroxytoulene (BHT). This indicate that mixed strain of dairy demonstrate excellent antioxidative activity, Thus that antioxidative activity increases with the combination of specific Lactic acid Bacteria in comparison to the single Lactic Acid Bacteria.

Keywords: *Bovine milk peptides, Fermented milk peptides, Free radicals oxidation, Casein bioactive peptides, Anti-oxidative activity*

1. Introduction

Health care sector is likely to grow around very fast in coming years. Milk is nearly a perfect food and is known to exhibit a range of biological activities (Meisel et al, 1997). Intact milk protein has specific function and physiological importance such as its role in uptake of trace elements and vitamins. Milk protein in their native form is fragmented into smaller peptides, which in turn exhibit biological activities in different physiological systems. Quality deteriotation and shorten of shelf life of food product depends on lipid or fatty acid oxidation. The main cause is that oxidation can generate some free radicals that lead to fatty acid decomposition and development of undesirable rancid ordurs and falvours (Nawar W. W. *et. al.* 1996). The peptides formed in the digestive system are limited, with the help of microorganisms especially Lactic Acid Bacteria, number of such bioactive peptides can be increased many folds (Singh, B., and Chand R.. 2006). Casein peptides have been shown to effectively inhibit lipid peroxidation in ground beef homogenates and mechanically deboned poultry meat, thereby serving as naturally occurring antioxidant in preventing off-flavour formation in meat products (Rossini *et.al.* 2009). Relatively greater antioxidant activity was observed for buffalo milk casein compared to cow milk casein (Rajesh Kumar et. al. 2010). Oxidation is a major risk factor for the coronary heart disease; oxidation and ageing are closely related. Milk lipids can undergo auto oxidation, which may lead to changes in food quality. Lipid oxidation turns into biologically active compounds such as active radicals or low molecular carbonyl compound which has relation with ageing.

There is strong evidence that reactive oxygen species (ROS) and free radicals play an important role in many degenerative diseases like cancer, atherosclerosis and diabetes (Beckman and Ames 1998). Foods containing antioxidative may be useful for the prevention of these diseases. Formation of free radicals, such as super oxide anion radical and hydroxyl radical, is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with other groups or substances in body, leading to cell or tissue injury. The body has its own defense system against ROS based on antioxidant enzymes, as well as low-molecular mass non-enzymatic antioxidant compounds. These defence systems are not effective enough to totally prevent the damage, and therefore, food supplements containing antioxidants may be used to help the human body to reduce oxidative damage (Oxman *et al.* 2000;

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Terahara *et al.* 2001; Kullisaar *et al.* 2003). The addition of adjunct culture of *L. casei ssp. casei* 300 increased the antioxidant activity of Cheddar cheese after ripening for 4 months (Aparna Gupta *et.al.* 2010)

2. Materials and Methods

2.1 Activation of Culture

Activation of *Lactobacillus helveticus* NCDC 292, culture was done using MRS broth which is specific for Lactic Acid Bacteria. Five ml of MRS broth was taken in 15 ml test tube in duplicate. After sterilization, it was cooled at room temperature. The lyophilized ampoule of *L. helveticus* was broken aseptically in laminar air flow and small amount (one loop full) of the dried culture was transferred into tube containing MRS broth. It was mixed properly using vortex shaker and then incubated at 30°C in an incubator for 24 to 48 hours. After 48 hours the tubes were observed for the growth and purity of culture was tested. Active culture of local curd was also collected; another inoculums was prepared by using equal amount of lactococcus and Lactobacillus.

2.2. Fermentation of milk

Bovine milk was sterilized 250ml in each flask, the milk was then cooled up to room temperature and then flask were inoculated with all three combinations respectively one was pure culture of *Lactobacillus helveticus* NCDC 292, second one was local curd with unknown strain and third one was combination of Lactococcus & Lactobacilli. @ 1.0%, 2.0% , 3.0%, 4.0%, and 5.0%, was incubated at 37°C for a period of 24h respectively. After incubation the curd was mixed properly to break large curd particles and poured in to 50ml autoclaved plastic tubes and centrifuged at 10,000 rpm /10 min at 4^0C using Kubota High speed centrifuge (Japan). The supernatant obtained after centrifugation was lyophilized and used when required.

2.3. Gel filtration

Ready made Gel filtration column (5ml) was purchased from Banglore Geni. The sample 2-5% of bed volume was loaded and eluted with double glass-distilled water. Double the bed volume number of fractions 1ml each was collected (discarded the first 5ml). The presence of peptide determined by taking the absorbance at 340nm.The peaks obtained in G-25 chromatogram were again lyophilized and dissolved in minimal amount of double glass distilled water and inhibition of linolic acid per-oxidation was determined.

2.4. HPLC conditions

The reverse phase HPLC (Waters, USA) with Spherisorb C-18 column (4.6 X 250mm) with 20µl loop was used for the separation of the peptides. Gradient solvent delivery was achieved using two Water's pumps at the flow rate of 0.75 ml/min. Solvent A was 0.1% Trifluoroacetic acid (TFA) in HPLC (Milli-Q) grade water. Solvent B was 0.09% TFA, 90% acetonitrile. Both the solvents were filtered using 0.45µm membrane filters and degassed before use. The C-18 column was thoroughly washed with solvent until the base line was obtained. Twenty µl of the sample was injected. Detection was monitored with (Water's dual detector) at 220 nm and 280 nm for all the fractions. The fractions of the respective peaks was pooled and lyophilized. These fractions were tested for anti-oxidative activity

2.5. Measurement of anti-oxidative activity

The measurement of antioxidative activity of peptide fraction was performed by the Thiobarbituric acid (TBA) method based on the monitoring of inhibition of Linoleic Acid peroxidation by peptide fraction. Linoleic Acid was chosen as the source for unsaturated fatty acid.

Procedure

Linoleic Acid emulsion (20 milliliter) was made up of 1ml of Linoleic Acid, 0.2 ml of Tween 20 and 19.7 ml of de-ionized water. Phosphate buffer solution (0.02M, pH 7.4) was mixed with 1ml of Linoleic Acid emulsion, 0.2ml of $FeSO_4$ (0.01%), 0.2ml of Ascorbate (0.01%) and 0.4ml of peptide fraction and then incubated at 37°C. De-ionized water was substituted for blank samples. After 12 hours of incubation 2ml of reaction solution was mixed with TCA (4%), 2ml of TBA (0.8%) and 0.2ml of Butylated Hydroxytoulene (0.4% BHT). This mixture was incubated at 100°C for 30 min. and allowed to cool. Two milliliter of chloroform was added for extraction. The extraction was obtained and absorbance was measured at 532 nm (A532) the percentage of inhibition of Linoleic Acid peroxidation was determined as follows:

$$\text{Percentage of Inhibition} = \frac{(1\text{-A532 sample})}{(\text{A532 blank})} \times 100$$

The capability of peptide to inhibit Linoleic Acid peroxidation is expressed as the equivalent BHT with the same inhibitory effect.

3. Results

Lactic Acid Bacteria are able to exhibit antioxidative activity in varying ways, and to distinguish only one mechanism or compound behind the antioxidative activity is mostly very difficult. In the present study, altogether 25 samples were screened for their antioxidant activity. All strains exhibited radical scavenging activity. The measured activities differed according to the strain. When two or three selected strains were fermented together, an increase in antioxidant activity was found. To see the comparatively effect in the anti-oxidative activity we had taken three cultures including Local curd, *L. helveticus helveticus* NCDC 292 and mixed culture. The antioxidative activity of research carried out varied from 46% to 89% and was expressed in terms of BHT. When milk was inoculated with different concentrations of inoculums i.e. 1ml, 2ml, 3ml, 4ml, 5ml which were named

as S1, S2, S3, S4, S5. Different cultures have different inhibition percent for the same size of the inoculums. This can be viewed as follows

Table 3.1: Anti-oxidative activity of the local curd prepared

S.No.	Concentration of culture	Absorbance	% of Inhibition
1.	S1	1.068	46.54
2.	S2	1.076	52.02
3.	S3	1.084	57.49
4.	S4	1.089	60.91
5.	S5	1.090	60.92

Table 3.2: Anti-oxidative Activity of the curd prepared from *Lactobacillus helveticus*

S.No.	Concentration of culture	Absorbance	% of Inhibition
1.	S1	0.535	55.16
2.	S2	0.479	61.80
3.	S3	0.595	65.59
4.	S4	0.532	75.80
5.	S5	0.531	75.96

Table 3.3: Anti-oxidative Activity of the curd prepared from mixed culture

S.No.	Concentration of culture	Absorbance	% of Inhibition
1.	S1	0.637	58.72
2.	S2	0.608	63.40
3.	S3	0.427	67.97
4.	S4	0.249	89.08
5.	S5	0.246	89.44

When 1ml amount was used local curd showed inhibition percent of 46.54%, while *Lactobacillus species* show more than of local curd i.e. 55.16% and mixed culture gives out the most highest inhibition percent i.e. 58.72% (Table 3.1). When 2ml amount was used local curd have inhibition percent of 52.02%,while *Lactobacillus species* posses more than local curd i.e. 61.80% where as mixed culture curd again gives out the most highest inhibition percent i.e. 63.40%. When 3ml amount was used local curd posses inhibition percent of 57.49%, while *Lactobacillus species* posses more than local curd i.e. 65.59% where as mixed culture curd gives percent i.e. 67.97%. When 4ml amount was used Local curd have inhibition percent of 60.91%,while *Lactobacillus species* posses more than local curd i.e. 75.80% where as mixed culture gives out the inhibition percent i.e. 89.08%. When 5ml amount was used local curd posses inhibition percent of 60.92%,while *Lactobacillus species* posses more than local curd i.e. 75.96% where as mixed culture curd again gives out the most highest inhibition percent i.e. 89.44%. Comparative graphical representation of inhibition percentage has been shown in figure 3.1. The effectiveness of lipid per-oxidation inhibition was in the order of local curd < *Lactobacillus* < mixed culture.

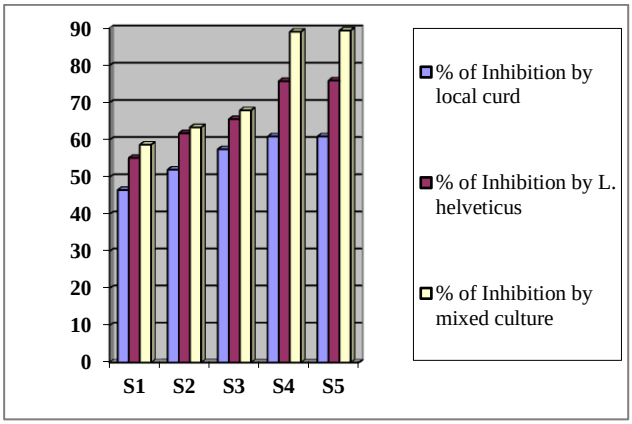

Fig. 3.1 Comparative graphical representation of inhibition percentage

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