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Bioactivity of cheddar cheese during ripening

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Abstract
Bioactivity in Cheddar cheese is mainly developed during its ripening period. The peptides formed during ripening results the bioactivity. Anti-oxidant, ACE (angiotensin converting enzyme) -inhibitory, anti-microbial, opioid, anti-inflammatory *etc*. are the various bioactivity found in the Cheddar cheese. In the current study the antioxidant and ACE-inhibitory activity of Cheddar cheese during ripening was studied. Ripening was conducted at 6-9 ˚C for 4 months. The anti-oxidant activity was found to be increased during ripening up to 60th day and thereafter started decreasing, whereas the ACE-inhibitory activity was found highest on 30th day of ripening and thereafter started decreasing. The statistical analysis had shown a significant (P<0.05) effect of ripening period on anti-oxidant activity and ACE-inhibitory activity. The increase followed by decrease in bio- activity can be explained as follows, formation of peptides responsible for increasing bioactivity in cheese and further splitting of the bioactive peptides resulting in formation of smaller ones with no or less bio- activity resulting a decrease in bio-activity.

Keywords: Cheddar cheese, ripening, anti-oxidant activity, ACE-inhibitory activity

Introduction
Proteolysis in cheese results in formation of bioactive peptides. Bioactive peptides are specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. They affect numerous biological processes including evoking behavioural, neurological, hormonal, gastrointestinal and nutritional responses. This peptides are inactive within the sequence of the parent protein and can be released bydigestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing. Anti-hypertensive-food-derived peptides act by inhibiting the angiotensin converting enzyme (ACE), which is associated with the renin-angiotensin system regulating peripheral blood pressure. The antioxidant peptides present in the food system play a vital role in the maintenance of antioxidant defence systems by preventing the formation of free radicals or scavenging free radicals and active oxygen species, which induce oxidative damage to biomolecules and cause ageing, cancer, heart diseases, stroke, arteriosclerosis, etc. These two bio-active peptides i.e. anti-oxidative (Pritchard *et al*., 2010, Gupta *et al*., 2009) [16,5] and ACE-inhibitory (Ong *et al*., 2008, Gupta *et al*., 2009, Korhonen and Pihlanto 2006) [14,5,8] are mainly present in ripened Cheddar cheese.

Cheeses specially ripened cheese shows bio-activity like anti-microbial, anti-oxidant, ACE-inhibitory activity etc. due to formation of small sized peptides formed during proteolysis. Many researchers had identified bioactive peptides from various varieties of cheeses. Anti-oxidant, anti-microbial, anti-hypertensive (Pritchard *et al*., 2010) [16], ACE-inhibitory (Ong *et al*., 2008) [14] was identified inn Cheddar cheese. ACE-inhibitory activity was found in Asiago d'allevo cheese (Lignitto *et al*., 2010), Manchego cheese (Gomez, 2002), Italian varieties cheese (Mozzarella, Crescenza, Italico, Gorgonzola) (Smacchi and Gobbetti, 1998) and Finnish varieties cheese (Edam, Emmental, Turunmaa, Cheddar) (Korhonen and Pihlanto, 2006) [8]. Anti-hypertensive peptides majorly had been found in Gouda cheese (Saito, 2000). Ovine cheese has Antioxidant, ACE-inhibitory, anti-microbial, immunomodulatory activity (Meira, 2012) [12]. Australian varieties cheese (Cheddar, Edam, Swiss, Feta, Camembert, Blue vein) has immunomodulatory, ACE-inhibitory, anti-amnesic, opioidagonist activity (Dionysius *et al*., 2000) [2]. Emmental cheese has immunostimulatory, phosphopeptides, antimicrobial activity (Gagnaire *et al*., 2001), Angiotensin-converting enzyme (ACE)-inhibitory activity (Parrot *et al*., 2003) [15].

Materials and Methods

Raw materials

Cheddar cheese was manufactured form cow milk provided by the experimental dairy of National Dairy Research Institute, Karnal. The starter culture of strain *Lactococcus lactis* subsp. *lactis* was collected form National Collection of Dairy Cultures, National Dairy Research Institute, Karnal (NCDC, Karnal). Meito rennet produced from *Mucor pusillus* var. *lindt*, was procured from Meigo Sangyo Co. Ltd., Japan. Commercial grade fine sodium chloride salt was procured from M/S Tata chemicals, Mumbai. All the chemicals and reagents used for chemical analysis were of AR grade.

Cheddar cheese manufacturing

Cheddar cheese was manufactured from 600 kg of cow`s milk by the method of Kosikowski (1982). The milk was at first standardized to casein: fat ratio of 0.7. After pasteurization the starter culture was added @ 1L/100L of milk at 30 ˚C. After acidity development the rennet was added @ 1.5g/100 L milk. The set curd was then cut into cubes by cheese knife. The curd was left for some time for healing, after healing the cooking was done to 39 ˚C at rate of 1 ˚C per 5 minute. After cooking the whey was drained. Cheddaring was done by piling and re-piling at 15 minute interval. The cheddar mass then milled and salted @ 1.5% (w/w). The salted curd was then hooped and pressed overnight. After removing from the hoop, the blocks were left for surface drying followed by vacuum packing in 3-layer-nylon packaging material, and stored at 6-9 ˚C for 4 months for ripening. The anti-oxidant and ACE-inhibitory activity was analysed at 0^{th}, 30^{th}, 60^{th} and 120^{th} day of ripening.

Preparation of water-soluble extracts

Water soluble extracts of the peptides formed in Cheddar cheese were prepared based on the method developed by Kuchroo and Fox (1982). 60 ml of glass distilled water was added to 20 g of grated Cheddar cheese, then it was mixed thoroughly. The homogenates were centrifuged using a refrigerated centrifuge at 4000g for 40 min. The upper-fat layer was discarded and the water extract was retained. The extracts were filtered through Whatman No.1 filter paper. The pH of the filtrate was adjusted to pH 4.6 using 1 N HCl. The precipitated proteins were removed by filtering through Whatman No.1 filter paper. To further remove any impurities, the water-soluble extracts were filtered through 0.22 µm pore size filter (Millipore). This water soluble extract were used for further analysis.

Anti-oxidant activity by ABTS method

Water soluble extracts of Cheddar cheeses were used to determine the antioxidant activity. Free radical scavenging activity was determined by ABTS method (Hernandez *et al*., 2005).

Preparation of standard curve

One ml of ABTS working solution was added to micro cuvette and initial absorbance against buffer blank was recorded at 734 nm using a spectrophotometer. Appropriate volumes of Trolox (250 µM) solution (5- 20µl) corresponding to the concentration of 5-20 µM were added to micro cuvette using micropipette. The contents were mixed for 30 sec. and the absorbance at 734 nm was recorded after 10 min reaction time. The standard curve was prepared by plotting concentration (µM) of Trolox (X-axis) v/s % ABTS inhibition (Y-axis).

Trolox equivalent antioxidant capacity (TEAC) & % ABTS inhibition

One ml of ABTS working solution made with PBS (pH 7.4) was added to micro cuvette (1 ml capacity) and absorbance was adjusted to 0.70±0.02 against the phosphate buffer. 10 µl of sample was added to ABTS working solution as well as in the blank. The contents were mixed for 5 seconds and the absorbance was recorded after 10 min at 734 nm using a spectrophotometer.

Calculation

The results were expressed as test sample absorbance as given below:

$$\% \text{ ABTS inhibition} = \frac{(\text{A 734 nm control} - \text{A 734 nm sample})}{\text{A 734 nm control}} \times 100$$

Standard Equation: Y = mX + C
Y is the % ABTS inhibition
X is the µM concentration of Trolox
The results were expressed as % ABTS inhibition &Trolox Equivalent Antioxidant Capacity (TEAC) i.e. µM of Trolox / mg of the protein.

Antioxidant activity assay by DPPH method

The method of Shimada *et al.* (1992) [18] was used to assess the DPPH radical scavenging activity of Water-soluble extracts of Cheddar cheese.

Trolox equivalent antioxidant capacity (TEAC) & % DPPH scavenging activity

Five ml of the sample solution was mixed with 1 ml of freshly prepared DPPH methanolic solution (1 mM). The resulting solution was then mixed vigorously and incubated for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Under similar conditions, the blank value was determined by using phosphate buffer (0.1 M, pH 7.0) and measured the reduction in absorbance.

Preparation of standard curve

Appropriate standard dilutions (5-20 µM) of Trolox solutions were made in methanol 0.5 ml of the standard Trolox solutions (5-20 µM), which was mixed with 1 ml of freshly prepared DPPH methanolic solution (1 mM) following the same protocol as sample was prepared.

Calculation:

The results were expressed as test sample absorbance as given below:

$$\% \text{ DPPH scavenging activity} = 1 - \frac{\text{A 517 nm Sample}}{\text{A 517 nm Blank}} \text{ x } 100$$

Standard Equation: Y = mX + C
Y is the % DPPH scavenging activity
X is the µM concentration of Trolox
The results were expressed as % DPPH scavenging activity & Trolox Equivalent Antioxidant Capacity (TEAC) i.e. µM of Trolox / mg of the protein.

Angiotensin-converting enzyme (ACE) inhibition assay

Angiotensin-converting enzyme (ACE) inhibitory activity was measured using the method of Cushman and Cheung

(1971) [1] as modified by Hernandez *et al.* (2003). The method is based on the liberation of hippuric acid fromhippuryl-L-histidyl-L-leucine (HHL) catalyzed by ACE.

Twenty µl of sample was added to 110 µl of substrate (5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3). After addition of 20 µl ACE (4mU), the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 250 µl of 1M HCl. The hippuric acid formed was extracted with 1.5 ml of ethyl acetate (by centrifugation at 3000 g for 10 min). An aliquot of one ml of the upper organic layer was collected and dried out completely by heating at 95°C for 20min, re-dissolved in 1 ml distilled water and measured spectro photometrically at 228 nm. The activity of each sample was tested in triplicate. The positive control of the reaction was carried out by adding only substrate, ACE and water (no sample). The blank was prepared by only substrate and water (ACE volume was replaced by the equal amount of water).

Calculation

$$\% \text{ ACE-inhibitory activity} = \frac{(\text{A 228nm Control} - \text{A 228nmSample})}{(\text{A 228nm Control} - \text{A 228nm Blank})} \times 100$$

Results were expressed as % ACE-inhibitory activity.

Statistical Analysis

One way ANOVA was carried out using IBM SPSS 20 for evaluating the effect of independent parameter (ripening time) on the dependent parameters (anti-oxidant activity, ACE-inhibitory activity). Tukey`s HSD post-hoc analysis was carried out using 5% level of significance.

Results and Discussions

In this part the analytical results of anti-oxidant and ACE-inhibitory activity is compared with the existing research published.

Anti-oxidant activity

Anti-oxidant activity was determined by spectrophotometric method. Two standard methods of anti-oxidant activity determination were used *i.e.* ABTS and DPPH method.

In ABTS assay, radical mono cation 2, 2' azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+.) is generated by potassium persulfate oxidation of ABTS which is reduced in the presence of hydrogen donating antioxidant. It is a type of inhibition assay in which the extent of scavenging of a preformed free radical is measured relative to that of a standard antioxidant compound (Trolox), which corresponds to the value of antioxidant activity. The Trolox Equivalent Antioxidant Capacity (TEAC) values are obtained by the capacity of each sample to scavenge the ABTS+. Up to 10 minutes reaction time relative to trolox and the results are expressed in terms of µM of Trolox /mg of protein.

The DPPH assay measured the ability of the test compounds to donate hydrogen which was checked by using the stable free radical DPPH. In the presence of hydrogen donors, DPPH is reduced and stable free radical is formed from the scavenger. The reduction in the concentration of DPPH is monitored by estimating the decrease in the absorbance at a characteristic wavelength when it encountered proton radical scavenger. The Trolox Equivalent Antioxidant Capacity (TEAC) is obtained by the capacity of each sample to donate hydrogen to reduce DPPH after 30 minutes reaction time relative to trolox and the results are expressed in terms of µM of Trolox /mg of protein.

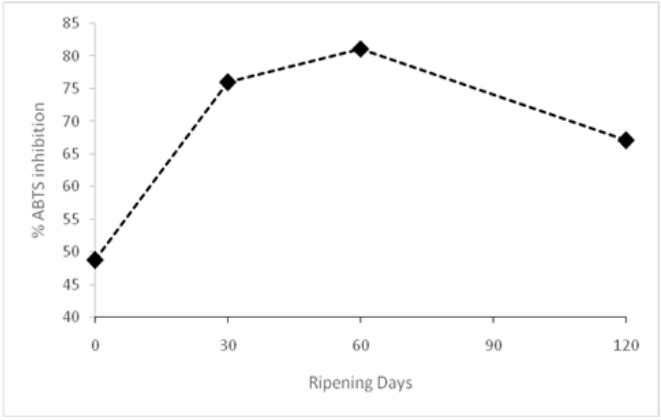

Fig 1: Activity is shown as % ABTS inhibition of Cheddar cheese water soluble extract (N=4).

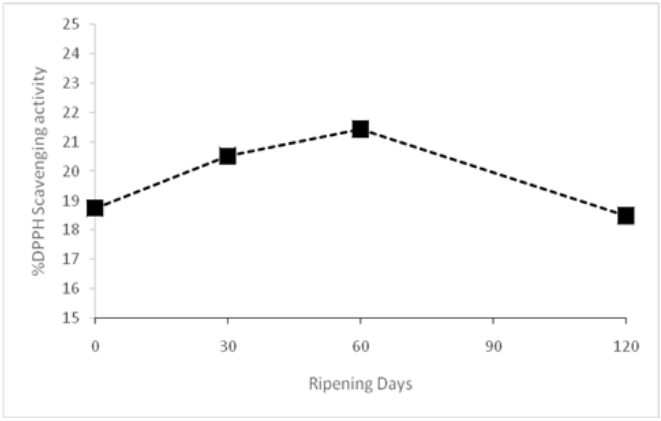

Fig 2: Activity is shown as % DPPH scavenging activity of Cheddar cheese water soluble extract (N=4).

The effect of ripening period on antioxidant activity of Cheddar cheese is shown in Figure 1 (in the form of % ABTS inhibition) and in Figure 2 (in the form of % DPPH scavenging activity), respectively.

Analysing the two figures, it can be observed that during ripening all cheese sample got highest antioxidant activity at 60th day & after that antioxidant activity started decreasing. This result is not supporting the result reported by Gupta *et al.*, (2009), as they reported that up to 4th month of ripening the antioxidant activity increased followed by a decrease in activity. This variation may be due to use of different starter culture, rennet or processing parameters, which causes difference in ripening pattern and speed. But these two studies were same at this point *i.e.* after reaching to the peak value the activity got decreased. We have discussed earlier that the antioxidant activity is dependent on the peptide formation by plasmin or other proteolytic enzymes, we had seen that throughout the ripening periods the plasmin activity got increased as a result the proteolysis. The decrease in antioxidant activity can be explained as follows, after formation of peptides responsible for bioactivity of plasmin and other proteolytic enzymes further splitting of the bioactive peptides resulting in formation of smaller ones with no or less antioxidant activity. The study of determination of the point of the highest antioxidant activity is very crucial to have a Cheddar cheese with higher antioxidant activity.

Table 1: ANOVA table showing the effect of ripening period on antioxidant activity (ABTS) of Cheddar cheese

	Sum of Squares	df	Mean Square	F-value	Significance
Between Groups	2429.217	3	809.739	372.217	.000
Within Groups	26.105	12	2.175		
Total	2455.322	15			

Table 2: ANOVA table showing the effect of ripening period on antioxidant activity (DPPH) of Cheddar cheese

	Sum of Squares	df	Mean Square	F-value	Significance
Between Groups	23.932	3	7.977	4.907	.019
Within Groups	19.508	12	1.626		
Total	43.440	15			

From the Table 1 and Table 2 it can be concluded that, the ripening period of Cheddar cheese had significant (P<0.05) effect on antioxidant activity.

ACE-inhibitory activity

ACE-inhibitory activity was determined by spectrophotometric method. Standard method of ACE-inhibitory activity determination *i.e.* HHL method, was used. In HHL (hippuryl-histidyl-leucine) method hippuric acid was formed from HHL by action of the angiotensin-converting enzyme. This hippuric acid was separated by ethyl acetate and concentrated by drying. Spectrophotometric assay of the aqueous solution of dried hippuric acid was done to determine the ACE-inhibitory activity.

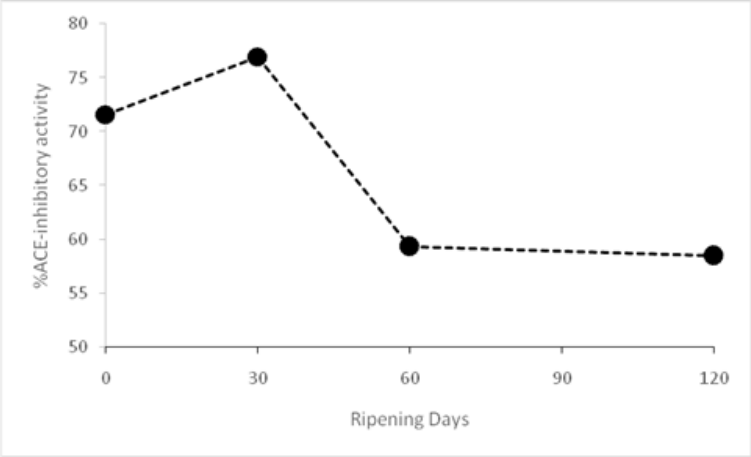

Fig. 3: Activity is shown as % ACE-inhibitory activity of Cheddar cheese water soluble extract (N=4).

The effect of ripening is shown in Figure 3. From the figure we can easily see that the Activity was increased up to 1 month, and after which, it started decreasing. The samples which had higher activity showed more sharp decrease. This can be explained as the samples had higher proteolytic activity & had reached higher activity within 1 month but after that the peptides responsible for ACE-inhibition got fractionated by proteolytic enzymes causing a decreased ACE-inhibitory activity. Meisel (1997) reported that ACE-inhibition in Cheddar cheeses is dependent on proteolysis to a certain extent. The ACE-inhibitory activity increases as proteolysis develops, but the ACE-inhibition index decreases when the proteolysis during cheese maturation exceeds a certain level. Ong *et al.* (2007) [14] also reported that Cheddar cheese had shown highest ACE-inhibitory activity on 6th month followed by a decrease after that. In the current study the ripening period was 4 month, so within 4 month a decrease was found at 30th day, if the study was continued more than 6 month the results may be similar as that of Ong *et al.* It may also be happened due to culture variation. In the current study more proteolytic activity of starter culture as compared to the culture used in the former study caused the variation in result. But the trend was same for these two studies.

Table 3: ANOVA table showing the effect of ripening period on ACE-inhibitory activity of Cheddar cheese

	Sum of Squares	df	Mean Square	F-value	Significance
Between Groups	1004.925	3	334.975	142.710	.000
Within Groups	28.167	12	2.347		
Total	1033.092	15			

From the Table 3 it can be concluded that, the ripening period of Cheddar cheese has significant (P<0.05) effect on ACE-inhibitory activity.

Conclusion

Form the study it can be concluded that the Cheddar cheese develops the bioactivity (anti-oxidant and ACE-inhibitory activity) during the ripening. The antioxidant activity was highest on 60th day, whereas the ACE-inhibitory activity was highest on 30th day. After this they started decreasing. The ANOVA analysis also showed that the ripening period has significant (P<0.05) effect on bio-activity of Cheddar cheese.

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