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EXPOSURE OF RAW-264.7 MACROPHAGE CELL LINE TO WATER-SOLUBLE EXTRACT OF CHEDDAR CHEESE: ASSESSMENT OF ANTIOXIDANT ACTIVITY

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ABSTRACT

Oxidative stress is the most critical factor implicated in various degenerative ailments. The present study was designed to evaluate the *in vitro* radical scavenging potential of water-soluble peptides (WSPs) extract derived from buffalo and cow milk cheddar cheeses during ripening. Moreover, the protective role of WSPs extract on cell viability and production of reactive oxygen species (ROS) in RAW-264.7 macrophages (Lipopolysaccharide- stimulated) cell line was also assessed. The WSPs extracts were potent scavenger of DPPH radical and activity increased progressively with cheese ripening period. Comparatively, highest activity was noticed in the peptide extract obtained from buffalo milk cheddar cheese. The intracellular ROS production in RAW-264.7 macrophages decreased significantly ($P<0.05$) upon treatment with WSPs extract. However, the WSPs fraction collected at 150th day of ripening showed maximum reduction in ROS generation. Additionally, dose-dependent response of WSPs extract was noticed for antioxidant activity. The promising antioxidant potential of cheddar cheese can offer a perspective to reduce the risk of disorders associated with oxidative damage.

Key words: Cheddar cheese, Peptides, Antioxidant, Macrophages.

INTRODUCTION

Oxidative stress is allied with excessive production of free radicals within the body, cellular damage and modification of DNA, proteins and lipids (Gülçin *et al*., 2010). The resultant reactive oxygen species (ROS) may lead to the progression of cardiovascular diseases, atherosclerosis, diabetes, arthritis and inflammation (Graham Espey, 2013). Additionally, ROS are major factors affecting the foods quality contributing rancidity, toxicity and destruction of biomolecules (Heo *et al.*, 2005). Although, synthetic antioxidants are cost-effective and efficient, but may also impose potential risks to human health. Therefore, foods comprising natural antioxidants with chemotherapeutic and preservation properties have greater demand in the current marketplace (Pownall *et al.*, 2010). In this perspective, the utilization of milk protein hydrolysates or peptides in functional foods perceived a novel interest to reduce the risk of various chronic ailments associated with oxidative stress (Carocho and Ferreira, 2013). Multifunctional character of peptides have potential to maintain ROS concentrations at low levels (Power *et al*., 2013).

The bioactive peptides with specific amino acid composition and sequence exhibit numerous functions as anti-hypertensive (Ong *et al*., 2007), anti-oxidative (Suetsuna *et al*., 2000), anti-inflammatory, anti-cancer, anti-bacterial and anti-thrombotic (Minervini *et al*., 2003). Cheeses being rich in proteins show diverse proteolytic systems leading to the production of numerous water- soluble peptides (WSPs) during ripening through the action of coagulants, bacterial proteases and peptidases (Meyer *et al*., 2009). The production and functional role of peptides is related with the increase of proteolysis index in ripened cheese (Lopez-Fandino *et al*., 2006). Therefore, cheese ripening is an important factor affecting the bioactivities of peptides.

The cellular anti-oxidant assays are biologically more representative methods demonstrating a better prediction of antioxidant behavior in biological systems. Moreover, the use of cell culture models permit for fast and economical screening of anti-oxidative components for their bioavailability, metabolism and functionality. The anti-oxidant properties of water- soluble extracts from cheeses have investigated in some studies (Gupta *et al*., 2009; Pritchard *et al*., 2010; Meira *et al*., 2012). Only few studies have been conducted for evaluating the anti-oxidative capacity of protein hydrolysates or isolated peptides using cell cultures (Hernández-Ledesma *et al*., 2007; García-Nebot *et al*., 2014) and animal models. However, to date, no information about the role of WSPs extract from cheddar cheese against cellular oxidative stress has been reported. The present manuscript aimed to assess the *in vitro* anti-oxidant activity of WSPs extract through free radical scavenging activity (DPPH) and cell culture (RAW-264.7) model.

MATERIALS AND METHODS

Manufacture of Cheddar cheese: The buffalo milk Cheddar cheese (BCC) and cow milk Cheddar cheese (CCC) was manufactured (triplicate batches for each) using starter culture (Chr. Hansen Ireland Ltd.) and chymosin (CHY-MAX®, Chr. Hansen, Denmark) following the method described by Ong *et al.* (2006) and kept for ripening at 8°C for 180 days.

Preparation of WSPs extract of Cheddar cheese: The WSPs extract were prepared at different stages of ripening according to the method of Kuchroo and Fox (1982). The grated cheese was homogenized (VELP®Scientifica N˙R171362 OV5) with twice the volume of distilled water. The slurry was centrifuged (30 min at 4000 × g, 4°C), filtered (Whatman No. 41), freeze-dried (CHRIST®, ALPHA 1-4 LD plus, Germany) and stored at -20°C. The concentration of peptides in water-soluble extracts of Cheddar cheese was calculated according to the method of Lowry *et al.* (1951).

Antioxidant activity of WSPs extract: The antioxidant potential of WSPs extract was measured by1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and 2', 7'-dichlorofluorescin-diacetate (DCFH-DA) assay using RAW-264.7 macrophage cell line model.

Measurement of DPPH- radical scavenging activity: The scavenging activity of WSPs extract was carried out (Apostolidis *et al.,* 2007) with some modifications. Briefly, aliquots of 1 mL WSPs extract (250, 500, 750 µg/mL) were transferred to test tubes of freshly prepared 60 µM DPPH (Sigma-Aldrich, St. Louis, MO, USA) methanol solution and homogenized. After incubation (45 min), the absorbance was measured by spectrophotometer (Elx800TM absorbance micro plate reader, BioTek instrument, VT, USA) at 517 nm.

Cell culture: RAW-264.7 macrophage cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modification of eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) and 1% non-essential amino acid solution at 37ºC in a humidified atmosphere of 95% air with 5% CO_2.

Cell viability assay: Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well plates for incubation (37ºC, 24 hrs) in a humidified atmosphere (95%) and 5% CO_2. After that, cells were treated with different concentrations (100, 200, 300, 400 and 500 µg/mL) of WSPs extract in 200 µL media. Then the media were replaced with MTT (Sigma-Aldrich, St. Louis, MO, USA) and incubated (37°C, 2 hrs). Afterwards, containing media were discarded and the formazan dye was solublized by adding 100 µL of DMSO (Fisher scientific CHEMTREC®, USA) to each well. The absorbance was measured at 570 nm by using a plate reader (Elx800TM, BioTek Instrument, VT, USA).

Cellular ROS determination by DCFH-DA assay: The intracellular ROS formation was quantified following the method as described previously by Alía *et al.* (2005) with some modifications, using DCFH (Sigma-Aldrich, St. Louis, MO, USA) as fluorescent probe. The macrophage cells (200 µL) were seeded in black fluorescence microtiter 96-well plates (1.5 x 10^4 cells per well) and incubated in a humidified incubator (5% CO_2, 95% air, 37°C) for 24 hrs. The culture medium was removed and cells were pretreated with 200 µL of 100, 200, 300, 400 and 500 µg/mL WSPs extract and incubated for 24 hrs. Subsequently, 100 µL of 10 µM DCFH-DA in PBS (pH 7.4) was added and incubated in the dark for 30 min. The fluorescence was monitored at the excitation wavelength (Ex, 485 nm) and emission wavelength (Em, 528 nm) by fluorescence microplate reader (Biotek microplate reader, GEN (GEN5.19) software).

Statistical analysis: The analyses were performed in triplicate and resultant data was analyzed statistically by ANOVA using Minitab statistical package and Tukey's test for multiple comparisons (α= 0.05) between means. The results were expressed as mean values ± standard error (SE)**.** Simple linear correlation analysis was used to determine a relationship between the mean values of peptide content and bioactivities.

RESULTS AND DISCUSSION

Peptides content of water-soluble extract: The peptide contents of soluble extract from both cheeses were increased significantly ($p<0.01$) during whole ripening period. However, the concentration of soluble protein of BCC was comparatively higher than CCC (Table 1). The increasing trend in protein contents at different stages of ripening was also reported in many studies (Gupta *et al*., 2009; Meira *et al*., 2012). The peptides and free amino acids are produced upon degradation of caseins by chymosin, plasmin, starters and non-starters proteinases and peptidases (Mcsweeney, 2004; Pihlanto, 2006) during ripening. Relatively higher peptides concentration in BCC could be due to high protein (casein) contents of buffalo milk (Ahmad *et al*., 2013) and also in cheese.

Anti-oxidant activities: The DPPH-free radical scavenging potential signify the capability of antioxidants to donate hydrogen or electrons resulting into a more stable molecules. The scavenging activity (% inhibition) differed significantly between cheeses of different milk species, ripening days and concentration of peptide extract ($p<0.05$). The inhibition (%) of WSPs extract increased progressively throughout the ripening period with maximum activity 30.80 % and 28.14 % at 180th day

in BCC and CCC respectively. However, it is evident (Fig. 1) that relatively higher anti-radical potential was noticed BCC at different stages of cheese maturation. Moreover, results indicated the dose-dependent inhibition of DPPH which increased due to increasing concentration of peptides. Hence, the peptide extract with maximum concentration (750 µg/mL) had higher levels of inhibition.

Ripening of cheeses results in the release of numerous peptides that originate mainly from casein degradation (Kudoh *et al*., 2001) with proteolytic lactic acid bacteria. The scavenging potential of cheddar cheese water-soluble extracts is dependent on ripening stages and it was found to increase with ripening stages (Songisepp *et al*., 2004; Gupta *et al*., 2009) and doses/concentration of peptides (Pritchard *et al*., 2010). Moreover, it was found that degree of proteolysis and the rate of formation of soluble peptides is also related to the antioxidant activity. The correlation was observed from Pecorino Toscano (r=0.98), Pecorino Sardo (r=0.93) and Cerrillano (r=0.93) cheeses (Meira *et al.*, 2012). Hence, the highest radical quenching action of BCC was evidenced with higher contents of soluble peptides in the present investigation (Table 2). The results are also in agreement with Virtanen *et al.* (2007) and Igoshi *et al.* (2008) who reported the significant relationship between anti-oxidative activity and the amount of peptides generated during cheese maturation. The water-soluble peptides extract may act synergistically to exert several physiological roles. Therefore, it would be economically more expedient and feasible to use crude or semi-purified peptide extract in foods.

The distinctive behavior may also be attributed to the operational conditions for peptide's extraction, peptide structure, configuration, size (Kim *et al*., 2009), and amino acid sequence (Corrêa *et al*., 2011) and concentration of milk proteins from different species (Ahmad *et al*., 2013). Peptides comprised of glutamine, lysine, methionine, tyrosine, histidine, cysteine and proline possess strong antioxidant activity. The bioactive peptides reported in water-soluble extract of Roquefort cheese showed proline residues (Gómez-Ruiz *et al*., 2008). Besides, leucine and valine amino acids released from β-CN (211–220) and α_{S1}-CN (121–130) are also capable of quenching the radicals (Farvin *et al*., 2010), respectively. In the fermented milk, DPPH radical scavenging peptide was isolated from k-casein (Kudoh *et al*., 2001) and the peptide (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile) obtained from β-lactoglobulin (Hernández-Ledesma *et al*., 2005).

Cell viability: The MTT assay measures the metabolic action of cells through oxidation– reduction response and is applied to measure the viability of cells during cytotoxicity assays (Mosmann, 1983) using cell culture systems. The results revealed that the sample treatments evoked no changes in cell viability (data not shown), demonstrating that the concentrations selected for the study (100-500 µg/mL) did not damage cell integrity during incubation period.

Intracellular ROS production in RAW-264.7 macrophage: The RAW-264.7 cells are capable to liberate ROS after stimulation with (Liu and Finley, 2005) lipopolysaccharide (LPS). The results depicted that ROS production (%) in RAW-264.7 macrophages decreased significantly ($P<0.05$) upon treatment with WSPs extract. However, the WSPs fraction collected at 150^{th} day of ripening showed maximum reduction in ROS production. The decreasing trend of ROS production in LPS-stimulated RAW-264.7 cells was 12.92% and 12.73% in BCC and CCC respectively, during cheese aging (Fig. 2). Moreover, the dose-dependent response of the WSPs extract was noticed demonstrating the potent cellular anti-oxidant activity at highest dose (500 µg/mL) applied in present investigation. Surprisingly, the water-soluble nitrogen contents of cheeses showed negative relationship with the cellular anti-oxidant activity in macrophage cell model as the increase in activity was noticed till the 150^{th} day of ripening but decreased again at 180^{th} ripening day (Table 2).

Increased production of ROS is considered cytotoxic and associated with various degenerative ailments. In this perspective, the inhibition of ROS levels by antioxidants and radical scavengers can attenuate such disorders. The caseinophospho peptides have demonstrated to preserve viability and protected against oxidative damage at intestinal (Caco-2 cells lines) level (García-Nebot *et al*., 2011). Similarly, the casein and whey protein hydrolyzates were previously reported for protective effects inH_2O_2-induced hepatic (HepG2) and neuronal (PC12) cells, respectively (Xie *et al*., 2013; Zhang *et al.*, 2012). The intracellular free radical scavenging activity on RAW-264.7 cells was evaluated using the treatment with the purified peptide lunasin indicating that this peptide significantly reduced DCF fluorescence intensity in a dose- and time-dependently. Upon cellular uptake, the DCFH-DA probe is hydrolyzed to DCFH by intracellular esterases and oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. The extent of oxidation can therefore be measured by the fluorescence intensity which is a good marker of the overall oxidative stress in cells.

Table 1. Peptide contents of water soluble extract of cheddar cheeses at different ripening stages.

	Ripening (days)			
Cheddar cheese	**1**	**60**	**150**	**180**
BCC	80.98 ± 2.72^{f}	112.32 ± 2.53^{d}	138.97 ± 2.12^{c}	169.65 ± 1.35^{a}
CCC	74.25 ± 2.01^{g}	104.22 ± 1.76^{e}	137.02 ± 2.63^{c}	158.87 ± 2.56^{b}

Results are expressed as means ± standard deviation. Means sharing similar letter (superscript) in a row or in a column are statistically non-significant (P>0.05). Protein content of water soluble extract expressed as mg/mL, BCC= buffalo milk cheddar cheese; CCC= cow milk cheddar cheese.

Table 2. Correlation of bioactivities with peptide contents of cheddar cheese water-soluble extract.

Bioactivities	**BCC**	**CCC**	**Overall**
DPPH- radical scavenging activity	0.83^{**}	0.83^{**}	0.82^{**}
	P<0.01	P<0.01	P<0.01
Intracellular anti-oxidant activity	-0.70^{*}	-0.79^{**}	-0.65^{*}
	P<0.05	P<0.02	P<0.05

Upper values indicated Pearson's correlation coefficient; Lower values indicated level of significance at 5% probability.* = Significant (P<0.05); ** = Highly significant (P<0.01)

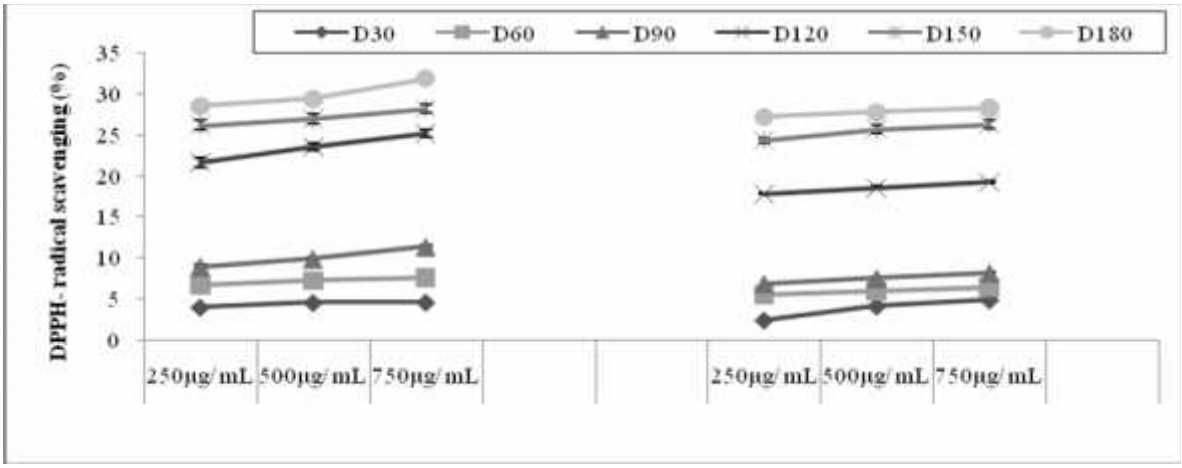

Figure 1. DPPH-radical scavenging (%) of WSPs extract of cheddar cheeses at different ripening stages. Values are expressed as the mean ± standard error of triplicate experiments.

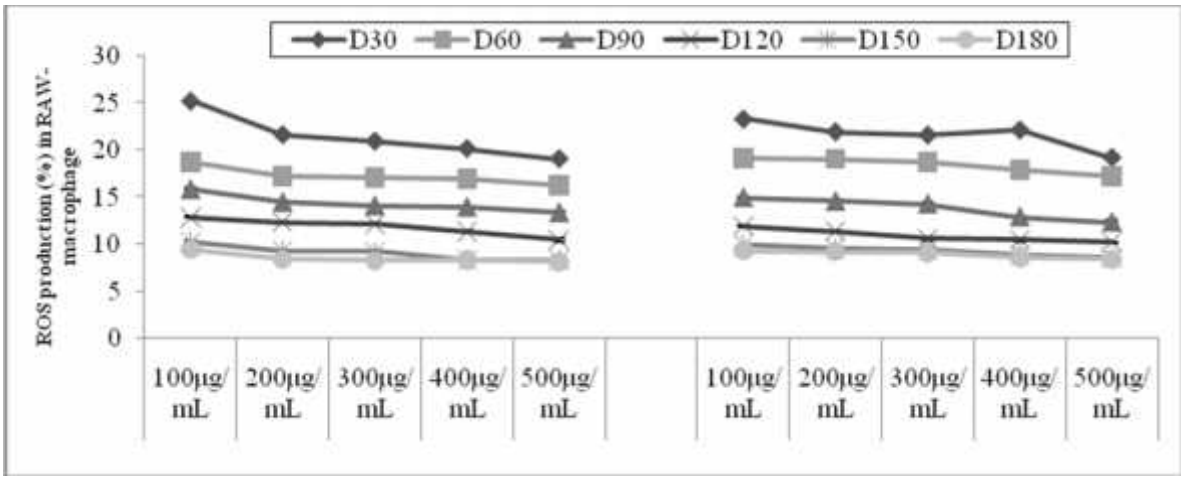

Figure 2. Effect of concentration of water soluble peptides (WSPs) extract on ROS production (%) in LPS-induced RAW-264.7 macrophage cells at different ripening stages in buffalo and cow cheddar cheeses. Values are expressed as the mean ± standard error of triplicate experiments.

Conclusions: The results confirmed that the WSPs extract from Cheddar cheese scavenged free radicals and inhibited radical mediated oxidation in RAW-264.7 macrophage cells. It provides great promise that these peptidic extracts may act against oxidative damage and attenuate the associated risk of degenerative ailments. However, animal studies and human clinical trials should be needed to conduct to confirm bioavailability and the desired biological function *in vivo*.

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