# Antioxidant and angiotensin-converting enzyme inhibitory activity of yoghurt fortified with sodium calcium caseinate or whey protein concentrate

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ORIGINAL PAPER

# Antioxidant and angiotensin-converting enzyme inhibitory activity of yoghurt fortified with sodium calcium caseinate or whey protein concentrate

**Gülfem Unal · A. Sibel Akalın**

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**Abstract** In this study, the effect of fortification of yoghurt with sodium calcium caseinate (SCC) and whey protein concentrate (WPC) on the antioxidant and angiotensin-converting enzyme (ACE) inhibitory activity was assessed. Milk was fortified with skim milk powder (SMP) as control, SCC, and WPC at 2% and 4% ratios. The antioxidant activity was determined using 2,2,-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging, hydrogen peroxide ($H_2O_2$) scavenging, and $Fe^{2+}$ chelating activity methods. Yoghurt fortified with 4% WPC had the highest $DPPH^{\bullet}$ scavenging activity, whereas there were no significant differences between yoghurt samples containing SMP and SCC at both fortification levels. In the $Fe^{2+}$ chelating activity method, increase in incubation time (from 0 to 60 min, at room temperature) significantly improved the chelating effect of all yoghurt types. Yoghurts supplemented with WPC and 4% SMP showed higher $Fe^{2+}$ chelating activity than those fortified with SCC for all incubation times. Experimental yoghurts also possessed antioxidant activity towards $H_2O_2$ except yoghurts fortified with WPC at a concentration of 0.2 g $mL^{-1}$. Yoghurt containing 4% WPC showed higher $H_2O_2$-scavenging activity than those supplemented with 4% SCC at 0.05 and 0.1 g $mL^{-1}$ concentrations, at 0 and 40 min. Addition of SCC or WPC caused an increase in the ACE inhibitory activity of yoghurt when compared to supplementation with SMP. Fortification of yoghurt with WPC was more effective than that of with SCC in terms of ACE inhibitory activity.

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**酪蛋白酸钙钠和乳清浓缩蛋白强化酸奶的抗氧化性和血管紧张素转换酶抑制活性**

**摘要**： 本文评价了强化酪蛋白酸钙钠(SCC)和乳清浓缩蛋白(WPC)的酸奶对抗氧化活性和血管紧张素转换酶(ACE)抑制活性的影响。将强化脱脂奶粉(SMP)的酸奶作为对照组，强化2% SCC和4% WPC的酸奶作为实验组。抗氧化活性用DPPH•(2,2-二苯基-1-苦基肼)自由基清除率，过氧化氢($H_2O_2$)清除率和$Fe^{2+}$螯合能力的方法来测定。强化4% WPC的酸奶具有最高的DPPH•自由基清除率。然而在强化相同含量的SMP和SCC的酸奶中DPPH•自由基清除率差异不显著。在$Fe^{2+}$螯合能力方面，延长接种时间能够显著提高所有酸奶的$Fe^{2+}$螯合能力。补充了WPC和4% SMP的酸奶与强化SCC的酸奶相比在任何接种时间都具有更高的$Fe^{2+}$螯合能力。强化0.2 g·mL$^{-1}$ WPC的酸奶除外，所有酸奶都具有抗氧化活性。强化4% WPC与补充浓度为0.05 和0.1 g·mL$^{-1}$的 4% SCC、接种时间为0 和40 min相比都具有更高的过氧化氢清除率。添加、SCC和WPC的酸奶与添加SMP的酸奶相比，前者造成了血管紧张素转换酶抑制活性的增加。强化WPC的酸奶在抑制血管紧张素转换酶的活性方面比强化SCC酸奶更有效。

**Keywords** Yoghurt · Sodium calcium caseinate · Whey protein · Antioxidant activity · ACE inhibitory activity

**关键词** 酸奶· 酪蛋白酸钙钠· 乳清蛋白· 抗氧化活性· 血管紧张素转换酶抑制活性

## 1 Introduction

Milk proteins are regarded as a source of energy and essential amino acids, which are needed for growth and maintenance of physiological functions. Bioactive peptides are considered as specific protein fragments, which are inactive within the sequence of the parent protein. They are liberated from milk proteins by enzymatic hydrolysis with digestive enzymes or by the fermentation of milk by proteolytic starter cultures and the action of enzymes derived from these microorganisms. After they are released during gastrointestinal digestion or food processing, they may exert various functions on the metabolism (Sarmadi and Ismail 2010). Two important functions of these peptides derived from milk proteins are antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities. These two activities are generally due to small peptides derived from casein and whey proteins (Suetsuna et al. 2000; Peña-Ramos et al. 2004; Hernández-Ledesma et al. 2005; Donkor et al. 2007; Quirós et al. 2007; Papadimitriou et al. 2007; Miguel et al. 2009). The possible antioxidant activities of the peptides have been observed as chelation of transition metals and scavenging free radicals (Karadag et al. 2009). On the other hand, they exert the antihypertensive effect by inhibition of angiotensin-I-converting enzyme so that they can reduce blood pressure.

Oxidation is essential to many living organisms for the production of energy to fuel biological processes (Unal and Akalın 2006). Free radicals and active oxygen species are physiological metabolites formed as a result of respiration in aerobic organisms. Any excessive amount of radicals can damage all types of cellular macromolecules, including proteins, carbohydrates, lipids, and nucleic acids, leading to cell death and tissue damage (Unal and Akalın 2006). This cellular damage can initiate the risk of several diseases including atherosclerosis, arthritis, diabetes, and cancer (Sarmadi and Ismail 2010). It is also well-known that lipid oxidation occurring in food products causes deterioration in food quality, such as the formation of rancid flavor, unacceptable taste, and shortening of shelf life.

Milk proteins have been identified as a source of ACE inhibitory peptides and are currently the best-known class of bioactive peptides (Unal and Akalın 2006). ACE is a key enzyme in the regulation of peripheral blood pressure, and inhibition of ACE helps to reduce hypertension by lowering blood pressure (Meisel et al. 2006).

Antioxidant activity has been reported for milk proteins (Woo et al. 2009), sodium caseinate and whey protein concentrate (Sugiarto et al. 2009), yoghurt (Farvin et al. 2010; Hekmat and McMahon 1998), and lactic acid bacteria (Lin and Yen 1999). In addition, ACE inhibitory activity of peptides derived from milk proteins (Otte et al. 2007; Pan and Guo 2010), fermented milks (Pan et al. 2005; Muguerza et al. 2006), and some other dairy products (Donkor et al. 2005; Papadimitriou et al. 2007) has been determined. Although skim milk powder is mostly used in the standardization of the nonfat dry matter of yoghurt, in recent years, caseinates and whey protein concentrates have been preferred in order to improve both the texture and the functional properties of yoghurt. These milk-protein-based ingredients are good vehicles to increase the antioxidant and ACE inhibitory activities in yoghurt. However, to the best of our knowledge, there is a lack of information on the effect of these ingredients on the antioxidant and ACE inhibitory activity of yoghurt. In addition, there is no study on using sodium calcium caseinate in the manufacture of yoghurt and also its influence on the mentioned biological activities of yoghurt.

The objective of this study was to investigate the effect of fortification of yoghurt with different ratios of sodium calcium caseinate or whey protein concentrate on the antioxidant and ACE inhibitory activities of yoghurt. This will be helpful to evaluate the potential use of these ingredients in the fortification of yoghurt in order to obtain a multifunctional food.

## 2 Materials and methods

### 2.1 Strains and ingredients

The commercial yoghurt starter culture containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (YC X-11) were in a freeze-dried direct vat set form containing $10^{11}$ cfu/g, respectively, and were obtained from Chr. Hansen A/S, Hørsholm, Denmark. Cultures were stored after procurement according to the recommendation of the manufacturer.

Skim milk powder (SMP) and whole milk were kindly provided from Pınar Dairy Products, Izmir, Turkey. Other dairy ingredients, whey protein concentrate (WPC, Oragel DY 101 XP), and sodium calcium caseinate (SCC) were obtained from Armor Protéines, Saint Brice en Cogles, France. The composition of dairy ingredients is shown in Table 1. In addition, Na and Ca contents of sodium calcium caseinate were given as approximately 0.6% and 1%, respectively.

### 2.2 Yoghurt manufacture

Set-type yoghurt was prepared using whole milk containing 36.5 $g.L^{-1}$ milk fat standardized with SMP to obtain 100 $g.L^{-1}$ of nonfat milk solids. The milk was

**Table 1** Composition of skim milk powder (SMP), sodium calcium caseinate (SCC), and whey protein concentrate (WPC)

Data from Pınar Dairy Products, Izmir, Turkey and Armor Proteinés, Saint Brice en Coglés, France

| | Concentration (g/100 g powder) | | |
|---|---|---|---|
| | SMP | SCC | WPC |
| Protein | 33.7 | 88 | 84 |
| Ash | 6.2 | 6.0 | 5.0 |
| Lactose | 57.2 | 0.5 | 1.0 |

divided into six lots and supplemented with 2% and 4% SMP, SCC, and WPC. After they were mixed properly, each milk base was heated at 85 °C for 30 min, by circulation in a hot water bath, and cooled to 43 °C in an ice bath. All cultures were used according to the manufacturer's instructions. Yoghurt starter culture was poured into 1 L sterilized milk at 40 °C and mixed throughly, and then 4 mL of the mix was added for each 1 L of mixture. The mixtures were then put into 200-mL plastic containers and incubated at 42 °C until a pH 4.70 was reached. After fermentation, the yoghurt samples were cooled and transferred to a refrigerator and stored at 4 °C for analysis. Protein contents ranged between 4.17–5.16% and 4.85–6.29% in yoghurt samples fortified with 2% and 4% dairy ingredients, respectively (data not shown). Analyses to determine the antioxidant and ACE inhibitory activities of the samples were performed within the first week of yoghurt production.

## 2.3 Chemicals and equipment

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2,-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-diphenyl-1, 2,4-triazine-4″, 4″-disulfonic acid sodium salt (ferrozine), ACE (from rabbit lung), and substrate (*N*-hippuryl-histidyl-leucine hydrate) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All other reagents and solvents commercially obtained were of analytical grade. All spectrophotometric data were acquired using a Cary, 100 Bio/visible spectrophotometer (Varian Australia Pty. Ltd., Melbourne, Australia).

## 2.4 Determination of antioxidant activity

### *2.4.1 DPPH$^{\bullet}$ radical scavenging activity*

The DPPH$^{\bullet}$ scavenging activity of the yoghurt samples was estimated according to the procedure described by McCue and Shetty (2005) with minor modifications. A 0.1 mmol.L$^{-1}$ DPPH$^{\bullet}$ radical solution in 95% ethanol was prepared. Eight milliliters of ethanolic DPPH$^{\bullet}$ solution was placed in a 50-mL centrifuge tube and mixed with 2 mL of sample or 95% ethanol (as control), vortexed well, and then incubated for 30 min at room temperature. The samples were then centrifuged for 5 min at 13,500 rpm at room temperature. Supernatants were filtered using Whatman no. 40 filter paper. Absorbance of each sample was measured at 517 nm. Trolox was used as a reference antioxidant at a concentration of 0.25 mg.mL$^{-1}$.

DPPH$^{\bullet}$ scavenging activity percent was calculated as follows:

$$\text{DPPH}^{\bullet}\text{ scavenging activity}(\%) = [(\text{control absorbance} - \text{extract absorbance})/(\text{control absorbance})] \times 100$$

### 2.4.2 $Fe^{2+}$ chelating activity

The chelating activity of the samples on $Fe^{2+}$ was measured according to the method described by El and Karakaya (2004) with minor modifications. One milliliter of sample (1 g.mL$^{-1}$) was mixed with 3.7 mL deionized water. Each sample was incubated with 0.1 mL $FeCl_24H_2O$ (2.0 mmol.L$^{-1}$) for 0, 10, 30, and 60 min at room temperature. After incubation, the reaction was initiated by addition of 0.2 mL ferrozine (5.0 mmol.L$^{-1}$). The mixture was then shaken vigorously and left at room temperature for 10 min. The absorbance of the mixture (formation of the ferrous iron–ferrozine complex) was measured at 562 nm. The control was performed in the same way using water instead of sample. EDTA (0.1 mg.mL$^{-1}$) was also run in the same way for comparison. The chelating activity was calculated as follows:

$$Fe^{2+}\text{ chelating activity}(\%) = [1 - (\text{absorbance of sample}/\text{absorbance of control})] \times 100$$

### 2.4.3 $H_2O_2$-scavenging activity

Yoghurt samples at 0.05, 0.1, and 0.2 g.mL$^{-1}$ concentrations, centrifuged at 9,500 rpm for 10 min at room temperature. The supernatants were then filtered using Whatman no. 40 filter paper.

The ability of the samples to scavenge $H_2O_2$ was determined spectrophotometrically (El and Karakaya 2004). One milliliter (0.05, 0.1, and 0.2 g.mL$^{-1}$) of sample was mixed with 3.4 mL of 0.1 mol.L$^{-1}$ phosphate buffer (pH 7.4), and then 0.6 mL of a 43 mmol.L$^{-1}$ solution of $H_2O_2$ in the same buffer added. Absorbance values were measured at 0 and 40 min at 230 nm. The values of the reaction mixtures were recorded against a blank solution without $H_2O_2$ for each sample. The concentration (mmol.L$^{-1}$) of $H_2O_2$ in the assay medium was determined using a standard curve. For the standard curve, 3.4 mL phosphate buffer was added to 0.6 mL of 10, 15, 25, 43, and 50 mmol.L$^{-1}$ solutions of $H_2O_2$. The equation was determined by linear regression as follows:

$$A_{(230\text{nm})} = \left(9.4 \times 10^{-3}\right) \times \mathrm{C}(\mathrm{H_2O_2},\ \mathrm{mM}) + 1.13 \times 10^{-2}\left(r^2 = 0.9993\right).$$

Trolox was used as the reference antioxidant at a concentration of 50 ppm. $H_2O_2$-scavenging activity of the samples was calculated using the following equation:

$$\text{Scavenging activity}(\%) = 100 - (H_2O_2\text{ concentration of medium}/H_2O_2\text{ concentration of control}) \times 100$$

## 2.5 Determination of ACE inhibitory activity

The whey fraction from the yoghurt was used for testing the ACE inhibitory effect. Approximately 25 g of yoghurt sample was centrifuged at 4,000×*g* for 15 min at 4 °C.

The supernatant was collected, and the pH was subsequently adjusted to 8.3 using 1 mol.$L^{-1}$ NaOH. The suspension was then centrifuged for 5 min at 14,000×$g$ at 4 °C. The supernatant was collected and used to determine its ACE inhibitory activity.

The ACE inhibitory activity was measured by a spectrophotometric assay according to the method of Donkor et al. (2005), Pan et al. (2005), Muguerza et al. (2006) with some modifications. A volume of 100 μL of buffered substrate solution (5 mmol.$L^{-1}$ Hip–His–Leu in 100 mmol.$L^{-1}$ sodium borate buffer containing 300 mmol.$L^{-1}$ NaCl, pH 8.3, 37 °C) was mixed with 40 μL of the whey fraction and preincubated for 3 min at 37 °C. The reaction was initiated by adding 20 μL of ACE (0.1 unit.$mL^{-1}$), and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 150 μL of 1 mol.$L^{-1}$ HCl and mixed with 1 mL of ethyl acetate. After vigorously stirring for 20 s, the samples were centrifuged at 8,000×$g$ for 15 min, and 750 μL of organic phase was transfered to a glass tube. The ethyl acetate was evaporated to dryness on a water bath for 25 min at 100 °C. The residue containing hippuric acid was dissolved in 800 μL of distilled water, and the absorbance of the solution was measured spectrophotometrically at 228 nm against water as a blank. The ACE inhibitory activity was calculated using the formula:

$$\%\ \text{Inhibitory activity} = [(A - C)/(A - B)] \times 100$$

where $A$ is the absorbance without the whey fraction, $B$ is the absorbance without ACE, and $C$ is the absorbance in the presence of both ACE and the whey fraction. ACE inhibition was also expressed in terms of $IC_{50}$, defined as the concentration of protein (mg.$mL^{-1}$ of whey fraction) required to inhibit 50% of the ACE activity under these conditions. For each whey fraction, inhibition activity was measured at three concentrations for which triplicate analyses were performed. A nonlinear adjustment of the data obtained was performed to calculate the $IC_{50}$ values with the program PRISM version 5.01 for Windows (GraphPad Software, Inc., San Diego, CA, USA). This program gives the estimated value of the $IC_{50}$ together with the standard error. For this purpose, protein concentration of the whey fractions was determined by the Kjeldahl method (IDF 1993).

### 2.6 Statistical analysis

The experiments, including yoghurt making, were performed in triplicate. Six values for each sample were averaged ($n$=6). The data obtained was processed by one-way ANOVA using the general linear model procedure of the SPSS version 11.05 (SPSS Inc., Chicago, IL, USA). The means were compared with the Duncan test at $P$<0.05 level.

## 3 Results and discussion

### 3.1 Antioxidant activity

The antioxidative mechanism of antioxidants can result from metal chelation, free radical scavenging, or cooperative effects of these properties (Karadag et al. 2009).

Several methods have been developed to assess antioxidant activity; however, none of them can be used as an official standardized method. Hence, evaluation of antioxidant activity is usually carried out by various methods of measurement. In the present work, antioxidant activity of the samples has been determined by three methods: DPPH$^{\bullet}$ radical scavenging, $Fe^{2+}$ chelating, and $H_2O_2$ inhibition assays.

### *3.1.1 DPPH$^{\bullet}$ radical scavenging activity*

The DPPH$^{\bullet}$ radical is long-lived organic nitrogen radical, and DPPH$^{\bullet}$ method is based on the ability of a sample to scavenge the free stable radical DPPH$^{\bullet}$ by hydrogen donation (Karadag et al. 2009).

The DPPH$^{\bullet}$ scavenging activity of the yoghurt samples ranged from 87.90% to 93.16% (Fig. 1). Trolox at a concentration of 0.25 mg.mL$^{-1}$ showed a DPPH$^{\bullet}$ scavenging activity of 84.83%. The yoghurt fortified with 4% WPC had the highest ($P<0.05$) scavenging activity. There were no significant differences among yoghurt samples supplemented with 2% of any ingredient ($P>0.05$). This shows that the effect of fortification with WPC on the DPPH$^{\bullet}$ scavenging activity of yoghurt is dose-dependent. High scavenging effect of the whey proteins can be attributed to lactoferrin, which has been reported as a key component for high scavenging activity (Lindmark-Månson and Åkesson 2000). In addition, α-lactalbumin (Sadat et al. 2011) and β-lactoglobulin (Hernández-Ledesma et al. 2005; Del Mar Contreras et al. 2011) may also contribute to enhancing the scavenging activity of yoghurt. Le Tien et al. (2001) has shown that whey protein powder was a better antioxidant than calcium caseinate in order to prevent oxidative browning of apples and potatoes.

Similar results have been reported in some yoghurt types. Farvin et al. (2010) studied the antioxidant activity of different fractions of yoghurt and found the DPPH$^{\bullet}$ radical scavenging activity of crude yoghurt (0.2 mg.mL$^{-1}$) to be 94.47%. The authors suggested that the oxidative stability of yoghurt might be due to antioxidant peptides released during the fermentation of milk by lactic acid bacteria. They also concluded that these peptides act as electron donors and could react with free radicals to convert them to more stable products. McCue and Shetty

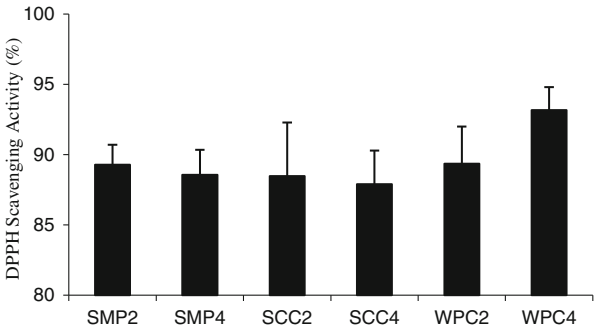


**Fig. 1** DPPH$^{\bullet}$ scavenging activity (%) of yoghurt samples within the first week of manufacture and storage at 4 °C. *SMP2* control yoghurt containing 2% skim milk powder, *SMP4* control yoghurt containing 4% skim milk powder, *SCC2* yoghurt containing 2% sodium calcium caseinate, *SCC4* yoghurt containing 4% sodium calcium caseinate, *WPC2* yoghurt containing 2% whey protein concentrate, *WPC4* yoghurt containing 4% whey protein concentrate

(2005) also investigated the DPPH$^{\bullet}$ scavenging activity of soy yoghurt produced by Kefir cultures and reported the activity as 92.3% after 48 h of production.

### *3.1.2 $Fe^{2+}$ chelating activity*

Iron is essential for life because it is required for oxygen transport, respiration, and the activity of many enzymes. However, it may work as a catalyst for the generation of reactive oxygen species in pathological conditions. The reduced form of iron causes oxygen toxicity by converting, via the Fenton reaction, the less reactive hydrogen peroxide to the more reactive oxygen species, the hydroxyl radical (OH$^{\bullet}$), and the ferryl ion. Therefore, minimization of the $Fe^{2+}$ concentration in the Fenton reaction affords protection against oxidative damage (Karadag et al. 2009).

All yoghurt samples showed more than 75% chelating activity on ferrous ions for an incubation time of 30 min (Table 2). The iron-chelating activity of the yoghurt samples ranged from 75.04% to 88.65% at 30 min of incubation time. However, EDTA showed more effective chelation capacity (99.54%) than samples at a lower concentration (0.1 mg.mL$^{-1}$) at an incubation time of 30 min. As expected, the iron-chelating activity significantly improved in all samples depending on the increase in incubation time. The chelating activity of all samples varied 10.89–30.53% at zero time. The chelating activity of a yoghurt fraction (10–30 kDa) was determined as 28.15% in the study of Farvin et al. (2010) at an incubation time of 3 min.

There were no significant differences ($P>0.05$) in iron-chelating activity among the experimental samples supplemented with 4% of any of the ingredients at the begining of the incubation. Yoghurt samples fortified with WPC (2% and 4%) showed the highest chelating activity at all incubation times. This high chelating ability of WPC can be attributed to the protein fractions (α-lactalbumin, β-

**Table 2** Iron-chelating activity (%) of yoghurt samples at room temperature

| Sample | Incubation time | | | |
|---|---|---|---|---|
| | 0 min | 10 min | 30 min | 60 min |
| SMP2 | $13.21\pm5.42^{cBC}$ | $42.61\pm19.64^{bC}$ | $75.04\pm4.69^{aB}$ | $75.61\pm6.55^{aC}$ |
| SMP4 | $19.07\pm5.05^{cABC}$ | $75.79\pm5.61^{bAB}$ | $81.48\pm1.90^{abB}$ | $86.30\pm3.88^{aAB}$ |
| SCC2 | $10.89\pm1.78^{cC}$ | $65.26\pm8.81^{bB}$ | $75.06\pm13.04^{abB}$ | $77.92\pm9.98^{aC}$ |
| SCC4 | $23.46\pm14.28^{cAB}$ | $70.20\pm11.39^{bB}$ | $77.68\pm5.47^{abB}$ | $81.04\pm5.80^{aBC}$ |
| WPC2 | $26.20\pm7.81^{cA}$ | $81.98\pm5.08^{bA}$ | $88.65\pm3.67^{aA}$ | $90.79\pm2.08^{aA}$ |
| WPC4 | $30.53\pm9.01^{cA}$ | $83.04\pm3.92^{bA}$ | $88.63\pm2.63^{aA}$ | $88.79\pm0.87^{aA}$ |

Means in the same row with different superscripted lowercase letters are significantly different ($P<0.05$). Means in the same column with different superscripted uppercase letters are significantly different ($P<0.05$)

*SMP2* control yoghurt containing 2% skim milk powder, *SMP4* control yoghurt containing 4% skim milk powder, *SCC2* yoghurt containing 2% sodium calcium caseinate, *SCC4* yoghurt containing 4% sodium calcium caseinate, *WPC2* yoghurt containing 2% whey protein concentrate, *WPC4* yoghurt containing 4% whey protein concentrate

lactoglobulin, lactoferrin, and serum albumin) in it. The chelation of prooxidant transition metals by proteins such as lactoferrin (Lindmark-Månson and Åkesson 2000) and serum albumin (Meucci et al. 1991) has been reported. This positive contribution of whey proteins to the iron-chelating activity of yoghurt probably originates from lower molecular weight fractions (Peña-Ramos et al. 2004; Hernández-Ledesma et al. 2005). Farvin et al. (2010) detected higher iron-chelating activity (approximately 99%) in lower molecular weight fractions (3–10 and <3 kDa) than the higher molecular weight fractions in yoghurt. The authors concluded that the lower molecular weight fractions of yoghurt contained some peptides or amino acids that can chelate metal ions such as $Fe^{2+}$. The chelating activity of yoghurt supplemented with 4% SMP was as high as that of yoghurts fortified with 4% WPC for all incubation times with the exception of 30 min. This is probably due to the increase in the whey protein fraction content in sample SMP4. On the other hand, yoghurt supplemented with 4% SCC showed lower chelating activity than when supplemented with 4% WPC with the exception of 0 min. In contrast, Sugiarto et al. (2009) obtained results, reporting that sodium caseinate showed more iron binding sites than whey protein isolate. This controversy may depend on the difference among products, assay medium, and methods used in the studies. The reseachers used sodium caseinate and whey protein isolate, whereas sodium calcium caseinate and whey protein concentrate were used in our study. It was also stated by the authors that the differences in the protein structures and the binding abilities of the different milk proteins result in different binding characteristics. Moreover, we determined the iron-chelating activity in yoghurt, but they investigated the binding of iron to proteins directly.

### *3.1.3 $H_2O_2$-scavenging activity*

Hydrogen peroxide is regarded as poorly reactive because of its weak oxidizing capability, but it can form a highly reactive hydroxyl radical through the Fenton reaction (Karadag et al. 2009). The yoghurt samples were capable of scavenging $H_2O_2$ depending on the sample concentration. None of the concentrations of the yoghurts assayed could completely remove $H_2O_2$ from the assay medium. Figure 2 gives the scavenging activity of the samples at three concentrations and two incubation times. The SCC2 yoghurt had higher scavenging activity than those fortified with 2% SMP or 2% WPC at the concentration of 0.05 $g.mL^{-1}$. Kitts (2005) reported that the sequestering of ferrous ion by casein phosphopeptides corresponded to reducing the Fenton reaction generated hydroxyl radical ($OH^{\bullet}$) formation. On the other hand, fortification of yoghurt with 4% WPC was more effective on the $H_2O_2$-scavenging activity when compared to the fortification with 4% SCC at both sample concentrations. No significant differences ($P>0.05$) were found in the scavenging activity among the samples with the exception of sample WPC4, which showed the highest value ($P<0.05$) at the concentration of 0.1 $g.mL^{-1}$. SMP and SCC showed similar $H_2O_2$-scavenging activities at the concentration of 0.2 $g.mL^{-1}$ for both incubation times. In our study, Trolox, at a concentration of 50 ppm, showed 28% and 29% $H_2O_2$-scavenging activity at 0 and 40 min, respectively. Lindmark-Månson and Åkesson (2000) have reported the role of the whey protein fraction, lactoferrin, in the scavenging activity of $H_2O_2$. Lactoferrin has been reported by

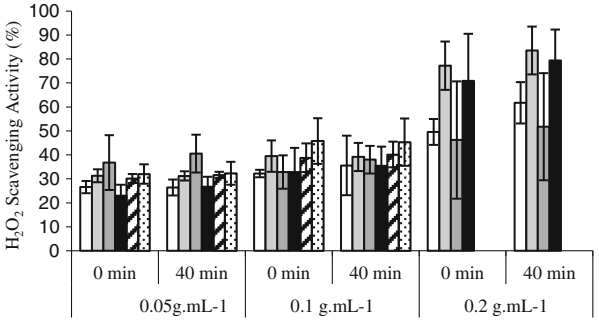


**Fig. 2** $H_2O_2$-scavenging activity (%) of yoghurt samples at different (0.05, 0.1, and 0.2 g.mL$^{-1}$) concentrations within the first week of manufacture and storage at 4 °C. *SMP2* control yoghurt containing 2% skim milk powder (*white bar*), *SMP4* control yoghurt containing 4% skim milk powder (*light gray bar*), *SCC2* yoghurt containing 2% sodium calcium caseinate (*dark gray bar*), *SCC4* yoghurt containing 4% sodium calcium caseinate (*black bar*), *WPC2* yoghurt containing 2% whey protein concentrate (*hashed bar*), *WPC4* yoghurt containing 4% whey protein concentrate (*dotted bar*)

the latter authors to have an influence on the conversion of hydrogen peroxide into the hydroxyl radical, which can be controlled by the availability of iron ions. However, no previous study has investigated the $H_2O_2$-scavenging activity of yoghurt and also the influence of fortification with milk-based ingredients on this activity.

### 3.2 ACE inhibitory activity

The percent ACE inhibitory activity and the corresponding $IC_{50}$ (mg.mL$^{-1}$) values of the experimental yoghurts are shown in Fig. 3. The ACE inhibitory activity (%) and $IC_{50}$ values of the samples changed between 61.31–96.61% and 0.32–2.10 mg.mL$^{-1}$, respectively. The percent inhibitory activity had an inverse relationship with the $IC_{50}$ value of the yoghurt samples. The ACE inhibitory activities of all samples were found to be significantly ($P<0.05$) different from each other. This shows the importance of the nature of protein used in the production of yoghurt and also the fortification ratio. The yoghurt fortified with 4% WPC showed the highest inhibitory activity whereas, the lowest value was found in the sample SMP2, with an $IC_{50}$ value of 2.10 mg.mL$^{-1}$, indicating that a large amount of medium would be required to inhibit 50% of the enzyme activity.

The yoghurt samples fortified with WPC had higher ACE inhibitory activity than those fortified with SCC at both fortification levels. Proteolysis is known to be one of the most important biochemical process occuring during fermentation. In addition, it is accepted that strong proteolytic activity is required to produce ACE inhibitory and antihypertensive peptides (Pihlanto et al. 2010). The greater inhibitory activity of yoghurts supplemented with WPC can be attributed to the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus*, which was higher in terms of viable count, in the samples fortified with WPC in comparison to samples containing SCC (data not shown). The proteolytic system of *L. delbrueckii* subsp. *bulgaricus* is already well characterized. This system consists of cell-wall-bound proteinase and a number of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases, and dipeptidases in order to produce small

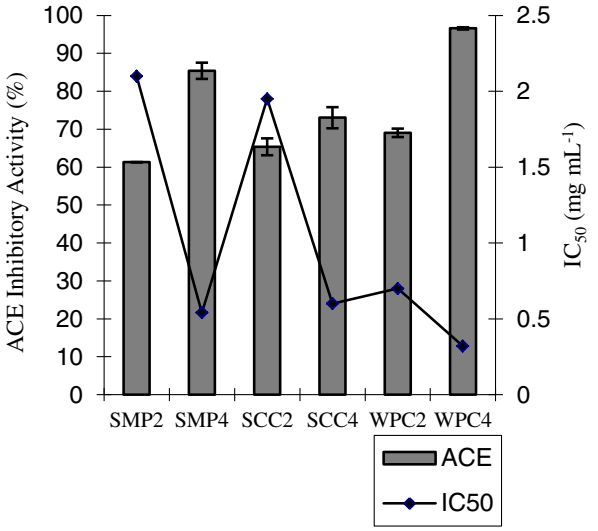


**Fig. 3** ACE inhibitory activity (%) and $IC_{50}$ values ($mg.mL^{-1}$) of yoghurt samples within the first week of manufacture and storage at 4 °C. *SMP2* control yoghurt containing 2% skim milk powder, *SMP4* control yoghurt containing 4% skim milk powder, *SCC2* yoghurt containing 2% sodium calcium caseinate, *SCC4* yoghurt containing 4% sodium calcium caseinate, *WPC2* yoghurt containing 2% whey protein concentrate, *WPC4* yoghurt containing 4% whey protein concentrate

peptides (Christensen et al. 1999). Moreover, Papadimitriou et al. (2007) demonstrated that *L. delbrueckii* subsp. *bulgaricus* possessed a more powerful proteolytic system than *S. thermophilus*. Gobbetti et al. (2004) also reported that the type of lactic acid bacteria starter used is one of the main factors that influences the synthesis of hypotensive peptides in dairy products. The higher inhibitory activity can also be due to the composition of whey protein concentrate. α-lactalbumin, which is a part of the whey proteins, has been shown to cause a high ACE inhibitory activity (Otte et al. 2007). In addition, whey-protein-derived peptides possessing ACE inhibitory activities have been isolated in previous studies (Gauthier and Pouliot 2003; Pan and Guo 2010). The similar relationship between the viable counts of *L. delbrueckii* subsp. *bulgaricus* and ACE inhibitory activity was also detected for our control yoghurt samples. Control yoghurts had the lowest viable counts of *L. delbrueckii* subsp. *bulgaricus* (data not shown), and they also showed the lowest ACE inhibitory activities ($P<0.05$).

In our study, the degree of the ACE inhibition of the yoghurt samples fortified with 2% and 4% SMP (control samples) were found to be 61.31% and 85.43%, respectively. Donkor et al. (2007) and Amirdivani and Baba (2011) obtained similar inhibitory activities for plain yoghurt within the first week of production. On the other hand, a lower (46.6%) inhibitory activity was detected in full-fat yoghurt, which could be caused by different parameters used in the method (Hernández-Ledesma et al. 2003). The $IC_{50}$ value for soy yoghurt was lower (0.35 mg/mL) than those of our control samples, probably due to the difference in product composition (Donkor et al. 2005). An $IC_{50}$ value of 1.3 $mg.mL^{-1}$ was determined for traditional sheep milk yoghurt on fifth day of storage by Papadimitriou et al. (2007), which is in between our values.

## 4 Conclusion

The present study indicated that, in general, fortification of yoghurt with milk-protein-based ingredients increases both the antioxidant and ACE inhibitory activity in comparison to the fortification with skim milk powder. Yoghurt supplemented with 4% WPC showed the strongest antioxidant and ACE inhibitory activity among experimental yoghurts. Therefore, the development of yoghurt containing 4% WPC may deliver health benefits to consumers. Nevertheless, further investigation is needed in order to demonstrate these health benefits via in vivo trails and clinical studies.

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