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Estimation of the antioxidant properties of milk protein preparations hydrolyzed by *Lactobacillus helveticus* T80, T105 and B734

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Abstract: The investigation determined the influence of *Lactobacillus helveticus* strains (T80, T105, B734 and DSMZ 20075) and *Lactobacillus acidophilus* La-05 (probiotic strain) on the antioxidant activity of whey and milk protein preparations. In order to indicate possible mechanism of antioxidant activity of the obtained hydrolysates, the reducing power (*RP*) assay, ability to neutralize free radicals and Fe^{2+} chelating activity analysis were employed. The results suggest that antioxidant activity of tested hydrolysates depends on the type of protein preparation (substrate) and the applied bacterial strain. The strongest antioxidant activity was exhibited by whey protein isolate hydrolysate obtained by using *L. helveticus* T80. A majority of fermented products obtained with the use of the probiotic strain were characterized by higher antioxidant properties than those obtained by application of *L. helveticus*. The solution of caseinoglicomacropeptide fermented by strain T105 exhibited the highest *RP* values, while α-lactalbumin hydrolysed by probiotic strain (La-5) was characterized by the strongest Fe^{2+} chelating activity. The analysed protein preparations and their hydrolysates obtained with using tested bacteria might potentially be applied in food products in order to inhibit oxidation processes.

Keywords: antioxidant activity; fermentation; lactic acid bacteria; LAB

A majority of pathogenic changes initiating diseases are related to the activity of free radicals. The imbalance between free radicals and the efficiency of antioxidant systems leads to oxidative stress that is correlated to central nervous system diseases (Rashad *et al.* 2011). Oxidative stress is one of the main factors responsible for development and progression of such neurodegenerative diseases as amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and Alzheimer's disease (Li *et al.* 2013; Niedzielska *et al.* 2016). Moreover, free radicals are also involved in carcinogenesis, tumor cell progression, and enhancement of their metastatic potential (Ríos-Arrabal *et al.* 2013).

Antioxidants prevent formation of radicals, scavenge free radicals, and enhance production of peroxides. However, some synthetic antioxidants may pose a potential risk in vivo and cause side effects. Therefore, in some countries, the addition of these synthetic substances in food products is limited or even prohibited (Osuntoki & Korie 2010; Abubakr *et al.* 2012). For this reason, antioxidant compounds

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derived from natural sources are extremely valuable. Therefore, research focusing on individual food components as well as their complex compositions is particularly important and can contribute to reduction of the negative impact of free radicals on human health.

The growing consumer awareness of the impact of diet on health status contributes to the fact that food is no longer perceived only in the nutritional context. Nowadays, food products are increasingly required to exert beneficial health-promoting effects. Moreover, consumers' requirements and needs lead to quick development of the manufacture of new value-added food products, which exhibit some desired functional activities (Shiby *et al.* 2013). The functionality of this food consists in maintenance of good health and well- being as well as prevention of certain diseases or support of treatment thereof (Cho *et al.* 2015; Ozcan *et al.* 2016).

Milk is a valuable resource of nutrients and active substances. Whey and milk proteins exhibit a well-balanced amino acid profile and high digestibility, which strongly emphasizes their biological importance. Whey proteins are regarded as a valuable food component with a wide range of nutritional and functional properties (Cho *et al.* 2015). Milk and whey protein preparations are used in dairy manufacture, e.g. to improve the texture characteristics of milk products like yogurt and fermented beverages (Jeewanthi *et al.* 2015; Mahomud *et al.* 2017). Furthermore, milk and whey proteins are also precursors of biologically active peptides, which enclosed in the native structure of protein sequences, are inactive. Bioactive peptides can be generated through the hydrolysis process, for example during food processing, by digestive enzymes in the gastrointestinal tract, and during fermentation and some ripening processes conducted by starter bacterial cultures (Korhonen & Pihlanto 2006).

The active amino acid sequences liberated by bacterial enzymes exhibit multifunctional activities within the antioxidant properties that can support natural cell defense against the harmful effects of oxidative stress (Pihlanto 2006; Abubakr *et al.* 2012; Griffiths & Tellez 2013). Therefore, the aim of this investigation was to determine the influence of *Lactobacillus helveticus* strains and a probiotic *Lactobacillus acidophilus* strain La-5 on the antioxidant activity of whey and milk protein preparations. Moreover, the reducing power, ABTS radical scavenging activities, and ferrous ion-chelating activity were also analysed in order to indicate the possible mechanism of antioxidant activity of protein hydrolysates.

MATERIAL AND METHODS

Bacterial strains and culture conditions. The strains of *L. helveticus*: T80, T105, and B734 isolated from fermented Polish milk products were kindly provided by Prof. Łucja Łaniewska-Trokenheim (University of Warmia and Mazury in Olsztyn, Poland). The strains have not been industrially used yet. *L. helveticus* DSMZ 20075 (DSMZ, Germany) was used as a reference strain. Moreover, a probiotic strain *Lactobacillus acidophilus* LA-5 (Christian Hansen, Denmark) was also used in the research.

All bacterial strains were maintained in 15% glycerol stock and stored at –80°C. Before beginning the experiments, each strain was regularly transferred into fresh sterile medium cultured (2% v/v) in Man-Rogosa-Sharpe broth (BTL, Poland). The culture medium for *L. helveticus* strain was additionally supplemented with 0.05% L-cysteine (pH of MRS broth has been adjusted to 6.3) and incubated (42°C/16 h) under anaerobic conditions (Waśko *et al.* 2014). While, the tested probiotic strain (*L. acidophilus* La-5) was incubated at 37°C/16 hours.

Preparation of protein hydrolyzates. Aqueous solutions (1% w/v) of whole milk powder (WMP) (OSM Krasnystaw, Poland), α-lactoalbumin (L-la) (Arla Food, Denmark), caseinoglycomacropeptide (CGMP) (Arla Food, Denmark), whey protein isolate (WPI) (Milei GmbH, Germany) and whey protein concentrate: WPC30, WPC40, WPC60, WPC80 (POLSERO, Poland) were prepared according to the method described previously (Skrzypczak *et al.* 2017). Briefly, an appropriate amount of each protein preparations has been dissolved in water and after sterilization (121°C/15 min) cooled down to 40°C. Samples of protein preparation solutions were inoculated (2% v/v) with the strain cell suspensions (OD_{600} = 0.7). The bacterial inoculums were prepared according to Beganović *et al.* (2013) with some modifications. Briefly, overnight cultures of the *L. helveticus* strains (grown in MRS broth) were used to inoculate 100 ml of MRS broths to an optical density OD_{600}= 0.50 nm. Then, the strain cultures were incubated at 37°C until the exponential phase of growth reached OD_{600} = 0.8. Subsequently, bacterial cells were harvested by centrifugation (8000 *g*, 4°C for 10 min). The pellets were washed twice in a sterile saline solution (0.9%

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NaCl) and finally re-suspended (in saline) in order to receive the optical densities of all the analysed strain cell suspensions equal to OD_{600} = 0.7. The bacterial suspensions obtained were used for inoculation of previously prepared protein preparations. The inoculated samples and control samples (non-inoculated solutions of protein preparations) were incubated for 24 h at 37°C in anaerobic conditions. Then, in order to inactivate protease and lactic acid bacteria, the reaction was inhibited by heating the samples at 100°C for 5 minutes.

Subsequently, the samples were centrifuged (13 000 *g*, 5°C for 10 min). The supernatants were immediately collected, filtered through 0.45 µm syringe filters, and subjected to further analysis as hydrolysates of protein preparations (together with their control samples).

Determination of antioxidant activity. Antioxidant activity was assayed by determination of the radical scavenging activity with the method described by Namdari and Nejati (2016). In order to prepare the working solution of free radicals, equal quantities of 4.88 mM potassium persulphate (Merck, Germany) and 14 mM ABTS (Sigma-Aldrich, Germany) stock solution, were mixed. The mixture was allowed to react overnight in the dark at ambient temperature. Then, the solution was diluted with 0.1 M phosphate buffer (pH 7.4) to obtain a working solution with the final absorbance equal to 0.70 ± 0.05 at 734 nm. The analysed sample (50 µl) or blank (phosphate buffer) was added to 1 ml of the working $ABTS^{\bullet+}$ radical solution. Then, the samples were mixed vigorously and, after 5 min of incubation (in ambient temperature), the absorbance was measured at 734 nm. The scavenging activity was calculated as follows (Equation 1):

$$\text{Inhibition (\%)} = (A_0 - A_s)/A_0 \times 100 \quad (1)$$

where: A_0 – absorbance of the $ABTS^{\bullet+}$ working solution (absorbance of uninhibited radical cation solution); A_s – absorbance of test sample measured 5 min after the addition of sample.

Polyacrylamide gel electrophoresis. Protein concentrations in the hydrolysates (and control samples) were measured with the method of Bradford (1976) using bovine serum albumin as a standard. The hydrolysates and control samples of WMP (~15 µg) were analysed by SDS-PAGE according to the Laemmli (1970) on vertical slab gels using a stacking gel containing 4% acrylamide and 10% resolving gel. Electrophoresis was carried out at 120 V for 60 min using the MiniProtean system (Bio-Rad). Proteins profiles were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Germany).

Reducing power activity assay (RP). The reducing power was measured according to the method described by El-Fattah *et al.* (2016). The analysed samples (1 ml) were mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide (Merck, Germany). Then, the mixtures were incubated at 50°C for 20 minutes. Afterwards, 1 ml of 10% trichloroacetic acid (Sigma-Aldrich, Germany) was added to each sample. After centrifugation (1500 *g*, 25°C for 10 min), 2 ml of the supernatant were collected and mixed with 2 ml of distilled water and 0.4 ml of 0.1% ferric chloride (Sigma-Aldrich, Germany). Then, after 10 min of incubation at ambient temperature, the absorbance was measured at 700 nm. A solution of 0.2 mM L-ascorbic acid (POCH, Poland) was used as a positive control. Increased absorbance of the reaction mixtures corresponds to increased reducing power.

Iron (II) chelating activity (FCA). The ferrous ion-chelating ability of the hydrolysate supernatants was determined according to El-Fattah *et al.* (2016). The samples of the hydrolysates of each protein preparation (1 ml) were diluted with distilled water (2.7 ml). Then, 0.1 ml of 2 mM/l $FeCl_2$ (Merck, Germany) was added and the reaction was stopped after 3 min by addition 0.2 ml of 5 mM/l ferrozine (Merck, Germany). After vigorous shaking, the mixtures were incubated for 10 min at ambient temperature. Absorbance was measured at 562 nm. Distilled water (1 ml) was used as a blank instead of the sample, while 0.1 mg/ml EDTA (POCH, Poland). was a positive control. The chelating capacity was calculated as follows (Equation 2):

$$\text{Chelating activity (\%)} = (A_b - A_s)/A_b \times 100 \quad (2)$$

where: A_b – absorbance of the blank; A_s – absorbance of the analysed sample of the protein preparation hydrolysate measured at 562 nm.

Statistical analysis. Statistical analysis was carried out using the Statistica ver.13.1 program (StatSoft Inc., USA). All data were presented as mean values with their standard deviations (mean ± s.d.). The analysis of variance (ANOVA) was performed using Tukey's HSD test in order to estimate the significance of the differences between the mean values. The results were discussed based on a significance level set at $P < 0.05$.

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RESULTS AND DISCUSSION

The search for suitable enzymes and sources of protein that allow generation of hydrolysates and peptides exhibiting functional properties is highly relevant due to their wide potential of applications in food technology and effects on human health (Phelan *et al.* 2009; Sarmadi & Ismail 2010). Therefore, the research was focused on analysis of the antioxidant activities of various milk and whey protein preparations and their hydrolysates obtained with the use of probiotic lactic acid bacteria *L. acidophilus* La-5 or selected strains of *L. helveticus.*

The examined products exhibited a great variability in terms of their capacity of $ABTS^{\bullet+}$ radical scavenging (Table 1). The observed differences can be explained by the fact that the bioactive properties of peptides and protein hydrolysates strongly depended on the protein substrate used and enzymes involved in the hydrolysis process (Corrêa *et al.* 2011, 2014).

The level of free radical ($ABTS^{\bullet+}$) inhibition ranged from 31.86 ± 0.83% (WPC60 hydrolysed by T105) to 95.86 ± 0.32% (WPI hydrolysed by T80). It was suggested that the sequences of amino acids released from native proteins in the aqueous medium could act directly as primary free radical scavengers (Rossini *et al.* 2009). Probably, this may also be an effect of formation of other compounds in the analysed samples during fermentation and hydrolysis e.g. oligopeptides, peptides, and organic acids.

Generally, among the all analysed types of protein preparations, the WPI samples demonstrated the highest ability to neutralize free radicals, wherein the strongest free radical inhibition was exhibited by hydrolysate obtained using *L. helveticus* T80 (95.86 ± 0.32%).

The antioxidant activities of the WPI samples were comparable to the free radical scavenging capacity (determined using the ABTS method as well) exhibited by milk fermented by *Lactococcus lactis* ATCC 19435 and *Lactobacillus acidophilus* ATCC 4356 (92.8 ± 0.4%) as well as samples of milk fermented by *Leuconostoc cremoris* B and *L. lactis* ATCC 19435 (93.1 ± 0.7%) (Virtanen *et al.* 2007). Moreover, in the research conducted by Namdari and Nejati (2016), the antioxidant activity of milk samples fermented for 24 h by individual isolates of *L. helveticus* exhibited lower values from 57.64 ± 1.42% (strain HY12) to 68.39 ± 3.90% (strain SY41). The findings correspond to the values of free radical scavenging activities obtained for the WMP samples (Table 1).

It was suggested that the use of mixed cultures of lactic acid bacteria resulted in higher antioxidant activity of fermented milk than that in products fermented by a single strain (Virtanen *et al.* 2007). The presented results also indicate that the antioxidant activity of the hydrolysates obtained depended on the type of protein preparation (substrate) and the applied strain. It is also worth noting that a majority of fermented products (WPC 30, WPC 60, WMP, CGMP) obtained with the use of the probiotic strain demonstrated higher antioxidant properties than in the case of *L. helveticus* (Table 1). The research results are in accordance with findings described by Virtanen *et al.* (2007), who suggest, that the radi-

Table 1. Antioxidant activity (%)

Protein preparation	Unfermented*	ABTS radical scavenging activity				
		Lactobacillus helveticus				*Lactobacillus acidophilus* La-5
		B734	DSMZ 20075	T80	T105	
WPC 30	$75.17 \pm 0.62^{b1,2,3}$	35.92 ± 0.01^{f7}	64.58 ± 0.09^{c3}	49.66 ± 0.26^{e7}	52.38 ± 0.15^{d5}	$86.07 \pm 0.20^{a1,2}$
WPC 40	$79.53 \pm 0.09^{a1,2,3}$	57.86 ± 0.14^{d5}	35.11 ± 0.12^{f5}	40.51 ± 0.30^{e8}	60.08 ± 0.11^{c4}	$73.00 \pm 0.08^{b3,4}$
WPC 60	$67.98 \pm 0.19^{a2,3}$	50.00 ± 3.19^{b6}	58.18 ± 0.27^{b4}	53.83 ± 4.16^{b6}	31.86 ± 0.83^{c7}	69.85 ± 2.40^{a4}
WPC 80	$85.29 \pm 0.27^{a1,2}$	74.51 ± 0.19^{d3}	73.86 ± 0.13^{e2}	76.56 ± 0.13^{c3}	55.01 ± 0.11^{f5}	$84.82 \pm 0.05^{b1,2}$
WPI	94.06 ± 2.48^{a1}	84.90 ± 7.09^{b2}	91.36 ± 3.43^{ab1}	95.86 ± 0.32^{a1}	92.91 ± 3.31^{ab1}	$90.17 \pm 3.02^{ab2,3,4}$
WMP	65.61 ± 0.28^{c3}	66.68 ± 0.04^{b4}	61.53 ± 0.25^{d3}	59.55 ± 0.08^{e5}	43.11 ± 0.10^{f6}	78.68 ± 0.08^{a1}
L-la	$78.99 \pm 0.68^{a1,2,3}$	91.90 ± 3.51^{a1}	92.65 ± 2.44^{a1}	88.94 ± 2.14^{a2}	87.93 ± 0.04^{a2}	$80.86 \pm 2.59^{a1,2,3}$
CGMP	$68.84 \pm 3.54^{a2,3}$	68.80 ± 4.26^{a4}	70.77 ± 3.16^{a2}	68.41 ± 3.62^{a4}	71.11 ± 4.05^{a3}	$72.08 \pm 3.79^{a3,4}$

WMP – whole milk powder; L-la – α-lactoalbumin; CGMP – caseinoglycomacropeptide, WPI – whey protein isolate; WPC – whey protein concentrate; $^{a–f}$mean ± mean s.d. (n = 5) followed by different letters within the same row are significantly different $P < 0.05$; $^{1–8}$mean ± mean s.d. (n = 5) followed by different superscripts within the same column for each strain that carried out the proteolysis of the protein preparations are significantly different $P < 0.05$; *control samples

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cal scavenging activity is dependent on the strain (proteolytic enzymes) and is not directly connected with the fermentation time (although the activity increased during the incubation).

Among all hitherto known LAB, *Lactobacillus helveticus* possesses the strongest proteolytic activities (especially, extracellular proteinase activity), which is connected with the highest abilities of the species to generate biologically active peptides during milk fermentation (Griffiths & Tellez 2013; Elfahri *et al.* 2016). However, considerable biodiversity in terms of cell wall-associated proteases (CEPs) has been noticed among different strains of *L. helveticus*, which exhibit from 1 to 4 various types of those enzymes (Sadat-Mekmene *et al.* 2013). Moreover, the CEPs are characterized by a large variety of different casein cleavage sites (Jensen *et al.* 2009; Sadat-Mekmene *et al.* 2011).

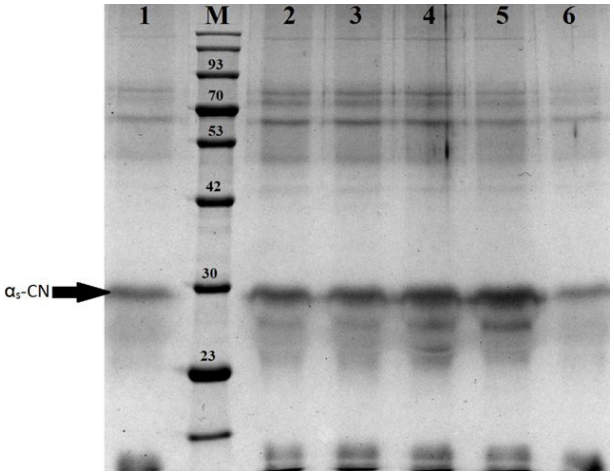

Figure 1. SDS-PAGE analysis of WMP fermented by analysed bacteria

1 – α_{s1}-CN (non-hydrolysed); M – molecular weight protein standards; 2 – casein fraction hydrolysed by *L. helveticus* B734, 3 – reference strain, 4 – *L. acidophilus* La-5, 5 – *L. helveticus* T80; 6 – *L. helveticus* T105

The variability of the profiles detected through electrophoretic separation of the WMP hydrolysates (Figure 1) might indicate differences in the proteolytic activity of the tested bacteria. The diversity of hydrolysis patterns was especially visible in the range of 30–25 kDa. Among all the analysed WMP samples, the lowest amount of products in this range of molecular weight was detected in the hydrolysate obtained using strain T105 (Figure 1). Interestingly, these samples exhibited the lowest ability to inhibit free radical activity (Table 1) and the highest reducing power of all WMP samples (Table 2). Moreover, CGMP fermented by the *L. helveticus* T105 strain exhibited the highest reducing power of all the analysed samples. It has been suggested that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity (Praveesh *et al.* 2013). However, in the study, no clear relationship between both parameters (reducing power and antioxidant activity) has been noticed.

The tested samples were also characterized by a wide diversity in terms of the Fe^{2+} chelating activity (FCA) (Table 3). The value of the analysed parameter ranged from 23.23 ± 0.71% (WPI hydrolyzed by *L. helveticus* DSMZ 20075) to 75.31 ± 0.04% (α-lactalbumin hydrolyzed by *L. acidphilus* La-5). It was determined that hydrolysis of whey concentrate and heat-denatured whey concentrate with pepsin and trypsin resulted

Table 2. Reducing power (%)

Ascorbic acid (10 mM)	Protein preparation	Unfermented*	Bacterial inoculum				
			Lactobacillus helveticus				*Lactobacillus acidophilus* La-5
			B734	DSMZ 20075	T80	T105	
0.404 ± 0.04^{ab}	WPC 30	0.346 ± 0.10^{ab1}	0.280 ± 0.05^{b1,2}	0.286 ± 0.05^{b1}	0.427 ± 0.02^{a1}	0.302 ± 0.05^{ab4}	0.371 ± 0.12^{ab1,2}
0.404 ± 0.04^{a}	WPC 40	0.367 ± 0.03^{a1}	0.315 ± 0.04^{a1}	0.392 ± 0.08^{a1}	0.362 ± 0.05^{a1}	0.338 ± 0.03^{a3,4}	0.327 ± 0.07^{a1,2}
0.404 ± 0.04^{a}	WPC 60	0.356 ± 0.05^{abc1}	0.375 ± 0.03^{ab1}	0.346 ± 0.02^{abc1}	0.360 ± 0.03^{ab1}	0.331 ± 0.04^{bc3,4}	0.297 ± 0.02^{c2}
0.404 ± 0.04^{abc}	WPC 80	0.346 ± 0.10^{abc1}	0.280 ± 0.05^{d1,2}	0.342 ± 0.04^{bcd1}	0.334 ± 0.03^{ed1,2}	0.445 ± 0.08^{a2}	0.371 ± 0.12^{ab1}
0.404 ± 0.04^{a}	WPI	0.333 ± 0.12^{a1}	0.284 ± 0.16^{a1,2}	0.374 ± 0.09^{a1}	0.361 ± 0.02^{a}	0.391 ± 0.02^{a2,3}	0.350 ± 0.08^{a1,2}
0.404 ± 0.04^{ab}	WMP	0.265 ± 0.06^{bc1}	0.335 ± 0.04^{abc1}	0.394 ± 0.07^{ab1}	0.202 ± 0.16^{c3}	0.469 ± 0.02^{a2}	0.375 ± 0.03^{ab1,2}
0.404 ± 0.04^{ab}	L-la	0.375 ± 0.15^{ab1}	0.314 ± 0.03^{ab1}	0.290 ± 0.03^{b1}	0.355 ± 0.03^{ab1}	0.382 ± 0.07^{ab2,3,4}	0.424 ± 0.07^{a1}
0.404 ± 0.04^{b}	CGMP	0.345 ± 0.07^{bcd1}	0.182 ± 0.04^{e2}	0.278 ± 0.09^{cde1}	0.231 ± 0.07^{de2,3}	0.581 ± 0.03^{a1}	0.383 ± 0.02^{bc1,2}

$^{a–f}$mean values ± mean s.d. (n = 5) followed by different letters within the same row are significantly different $P < 0.05$; $^{1–8}$mean values ± mean s.d. (n = 5) followed by different superscripts within the same column for each strain that carried out the proteolysis of the protein preparations are significantly different $P < 0.05$; *control samples

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Table 3. Ferrous ion chelating activity (%)

Protein preparation	Unfermented*	Bacterial strain used as inoculum				
		Lactobacillus helveticus				*Lactobacillus acidophilus* La-5
		B734	DSMZ 20075	T80	T105	
WPC 30	70.27 ± 0.62^{a2}	42.53 ± 0.04^{d6}	39.40 ± 0.43^{e4}	39.06 ± 0.11^{e6}	59.04 ± 0.03^{b1}	53.69 ± 0.03^{c4}
WPC 40	64.36 ± 0.81^{c4}	44.62 ± 0.09^{e5}	33.73 ± 0.60^{f5}	70.46 ± 0.15^{a2}	55.53 ± 0.23^{d2}	68.58 ± 0.39^{b2}
WPC 60	68.68 ± 1.06^{a3}	46.42 ± 0.42^{d4}	50.35 ± 0.08^{c2}	46.69 ± 0.81^{d5}	45.71 ± 0.45^{d4}	60.49 ± 2.81^{b3}
WPC 80	48.11 ± 1.06^{a7}	46.14 ± 0.41^{a4}	23.23 ± 0.71^{c7}	47.92 ± 0.73^{a5}	27.44 ± 0.10^{b7}	48.30 ± 2.7^{a5}
WPI	25.79 ± 0.06^{d8}	30.83 ± 0.04^{c7}	32.27 ± 0.18^{a6}	23.96 ± 0.23^{e7}	31.63 ± 0.11^{b6}	31.92 ± 0.28^{b6}
WMP	58.06 ± 0.01^{b5}	51.76 ± 0.02^{d2}	32.25 ± 0.04^{f6}	64.11 ± 0.02^{a3}	38.21 ± 0.02^{e5}	53.10 ± 0.21^{c4}
L-la	74.61 ± 0.81^{b1}	73.26 ± 0.08^{c1}	63.09 ± 0.19^{d1}	73.30 ± 0.11^{c1}	49.55 ± 0.04^{e3}	75.31 ± 0.04^{a1}
CGMP	49.48 ± 0.04^{bc6}	49.21 ± 0.13^{bc3}	47.80 ± 0.41^{cd3}	51.56 ± 3.38^{b4}	45.40 ± 1.99^{d4}	54.69 ± 0.04^{a4}

Fe^{2+} chelating activity of the positive control (0.1 mg/ml EDTA solution) was 99.93 ± 0.01%; $^{a–f}$mean ± mean s.d. (n = 5) followed by different letters within the same row are significantly different $P < 0.05$; $^{1–8}$mean ± mean s.d. (n = 5) followed by different superscripts within the same column for each strain that carried out the proteolysis of the protein preparations are significantly different $P < 0.05$; *control samples

in an increased ability to chelate iron (Conway *et al.* 2013). However, the results obtained showed that some of the tested hydrolysates exhibited a lower value of FCA than the control samples. This is presumably related to the difference in the activity and specificity of bacterial proteolytic enzymes leading to formation of various hydrolysis products. It was also suggested that the higher capacity of Fe^{2+} chelation exhibited by the skimmed milk protein hydrolysates was a consequence of the presence of casein-derived phosphopeptides containing amino acid residues such as Cys, Trp, Ser, or Tyr, which are more active in interacting and binding with metal ions. This was noted in research on casein derived from sheep milk analysed after hydrolysis conducted with the use of a protease preparation from *Bacillus* sp. (Corrêa *et al.* 2014). Moreover, Khantaphant *et al.* (2011) suggested that lower chelating activities might be caused by the increasing hydrolysis degree, where generated shorter peptides might lose their properties to build complexes with Fe^{2+}. The observed decline in the chelating activity (noted in some of the analysed protein preparations) after the hydrolysis process might indicate that peptides possessing Fe^{2+}-chelating ability were further hydrolysed resulting in formation of products with a lower capability of iron chelation (Corrêa *et al.* 2014)

CONCLUSIONS

The analysed hydrolysates exhibited such properties as Fe^{2+} chelating activity, reducing power activities and antioxidant properties suggesting that they might be potentially applied in food products in order to inhibit oxidation processes. This may lead to improvement of the quality of various food products and might be an interesting approach, contributing to rational management of by-products (whey and whey protein fractions). The findings indicate that capacity to free radical scavenging exhibited by tested hydrolysates depends on the type of used protein preparation (substrate) and also the applied strain of bacteria.

Among all tested solutions of protein preparation, WPI characterized by the highest ability to neutralize free radicals, while in case of hydrolysates, the highest anti-oxidative properties was exhibited by samples of WPI hydrolysed by *L. helveticus* T80.

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