Role of surface charge of hydrolysed bovine caseins in their iron(II)-binding affinity and antioxidative capacity in iron(II)-facilitated β-carotene and glutathione oxidation

CHIBUIKE CHINEDU UDENIGWE – MIN GONG – AISHWARYA MOHAN – MARYANN CHINONYE UDECHUKWU

Summary

Surface properties of peptides can affect their functionality, including metal-binding capacity. Casein hydrolysed with five proteases had varying surface hydrophobicity (from 56.4 ± 3.1 to 157.0 ± 16.8) and surface charge (ζ-potential, from –43.5 mV ± 0.5 mV to –32.5 mV ± 0.6 mV). The hydrolysates had varying Fe^{2+}-chelating capacity (from 0.049 mg·ml^{-1} ± 0.005 mg·ml^{-1} to 0.134 mg·ml^{-1} ± 0.027 mg·ml^{-1}); the best activities were observed for hydrolysates with the highest surface hydrophobicity. Maximum specific ligand binding (B_{max}) of the samples appeared to be independent of surface charge and hydrophobicity. However, ligand dissociation constant (K_d) of the peptide-Fe^{2+} complex strongly correlated with ζ-potential (r_s = 0.90). This suggests that the negatively charged surface of the particles facilitated peptide-Fe^{2+} chelate complex formation via electrostatic interaction. Casein hydrolysates with the lowest K_d and ζ-potential displayed the maximum capacity for inhibiting Fe^{2+}-facilitated oxidation of β-carotene, suggesting that strong Fe^{2+}-binding by the peptides made the pro-oxidant metal less available for participation in oxidation. However, this relationship was not observed for their inhibition of Fe^{2+}/H_2O_2-induced glutathione oxidation, likely due to peptide inaccessibility to the anionic glutathione. The findings show that surface chemistry of hydrolysed caseins affects their interactions with pro-oxidant iron, which is an important consideration for their use as antioxidants in foods.

Keywords

casein hydrolysate; iron chelation; β-carotene oxidation; glutathione oxidation; binding affinity; surface charge

Oxidative damage of biological molecules has been associated with the etiology and pathogenesis of human health conditions, and also in food quality deterioration [1]. Pro-oxidant transition metals are known to play a critical role in facilitating oxidation. In particular, Fe^{2+} participates in Fenton's reaction leading to the production of highly reactive hydroxyl radical [1]. This reaction has become a target for use in designing food-based antioxidants. For instance, peptides are derived from food proteins by enzymatic hydrolysis [2] and are thought to be antioxidative partly based on their ability to chelate pro-oxidant transition metals [3–5]. Several peptides derived from hydrolysed milk, especially caseinopeptides, are known to be antioxidative based on their Fe^{2+}-chelating capacity [6]. This bioactivity enables peptides to be used as antioxidant ingredients for formulating health-promoting food products and for food preservation. To date, there is a lack of information on the binding affinity of food peptides to divalent metals, particularly the ligand dissociation constant (K_d), and also the maximum ligand binding (B_{max}) parameter. The metal-binding parameters provide information on the capacity of the peptide to bind pro-oxidant metals and the stability of the peptide-metal complexes on binding. Furthermore, binding parameters also help in determining the protective effects of peptides against Fe^{2+}-mediated oxidative damage of biological molecules.

Metal-chelating capacity of protein hydrolysates and peptides has been attributed to the

Chibuike Chinedu Udenigwe, School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, 25 University Private, Ottawa, Ontario K1N 6N5, Canada; Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, 50 Pictou Road, Truro, Nova Scotia B2N 5E3, Canada.

Min Gong, Aishwarya Mohan, Maryann Chinonye Udechukwu, Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, 50 Pictou Road, Truro, Nova Scotia B2N 5E3, Canada.

Correspondence author

Chibuike Udenigwe, e-mail: cudenigw@uottawa.ca

presence of certain amino acid residues including histidine, glutamic acid, aspartic acid, threonine, serine and phosphor-serine [3, 4, 7, 8]. Nevertheless, it is important to understand the role of the peptide surface charge and hydrophobicity in their interaction with oxidants in hydrophobic and hydrophilic matrices. Therefore, the objective of this study was to evaluate the surface charge and hydrophobicity, and Fe^{2+}-binding affinity capacity of casein hydrolysed with five commercial proteases of different specificities in relation to their antioxidative capacities against Fe^{2+}-facilitated oxidation of β-carotene and glutathione.

MATERIALS AND METHODS

Enzymatic hydrolysis of bovine caseins

Caseins were isolated from commercial bovine milk by isoelectric precipitation at pH 4.5 as previously reported [9]. The casein isolate powder (50 $g \cdot l^{-1}$) was hydrolysed with five commercial proteases (Sigma-Aldrich, St. Louis, Missouri, USA) of different specificities at their respective optimum conditions as follows: pancreatin from porcine pancreas (Panc, 37 °C, pH 7.0), thermolysin from *Bacillus thermoproteolyticus* Rokko (Therm, EC 3.4.24.27, 65 °C, pH 8.0), bromelain from pineapple stem (Brom, EC 3.4.22.33, 37 °C, pH 6.5), papain from papaya latex (Pap, EC 3.4.22.2, 65 °C, pH 7.0) and Alcalase from *Bacillus licheniformis* (Alc, 55 °C, pH 8.3) at enzyme-to-substrate ratio of 1 : 100 (w/w) for 5 h using the Metrohm pH-Stat system (Herisau, Switzerland). After hydrolysis, the reaction was terminated by heating the mixture in a water bath at 95 °C for 15 min, followed by cooling to room temperature, centrifugation at 10 000 ×*g* for 10 min, and freeze-drying of the supernatant to obtain the casein hydrolysate (CH) powders.

Zeta (ζ)-potential measurement

Zeta (ζ)-potential, a measure of the surface charge, of aqueous suspension of the five hydrolysed casein samples was determined by electrophoretic light scattering of the particles using Zetasizer Nano Series Nano-ZS (Malvern Instruments, Malvern, United Kingdom). Samples were dispersed in deionized water and the measurements were made in a disposable capillary cell after equilibration for 120 s at 25 °C.

Surface hydrophobicity determination

Surface hydrophobicity (S_o) was determined by a fluorescence method using a hydrophobic probe, 8-anilino-1-naphthalenesulphonic acid, as previously reported [10]. Samples were diluted with 0.01 $mol \cdot l^{-1}$ phosphate buffer (pH 7.0) to concentrations ranging from 0.009 $g \cdot l^{-1}$ to 0.15 $g \cdot l^{-1}$. Fluorescence was then measured at the excitation and emission wavelengths of 390 nm and 470 nm, respectively, using Nova Spectrofluorometer (SPEX Industries, Metuchen, New Jersey, USA). The slope of the fluorescence vs concentration plot was taken to be the surface hydrophobicity.

Fe^{2+} chelation and binding affinity assays

The Fe^{2+}-chelating capacity of the hydrolysed casein products was determined using the Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt) assay as previously reported [11]. A metal chelator, ethylenediaminetetraacetic acid (EDTA), was used as the standard, the samples were measured at various concentrations ranging from 0.001 $mg \cdot ml^{-1}$ to 2 $mg \cdot ml^{-1}$, and their Fe^{2+}-chelating capacity was expressed as half maximal effective concentration (EC_{50}). Saturation binding experiment was conducted by varying Fe^{2+} concentration from 0.0002 μmol to 0.02μmol at constant concentration (30μg·ml^{-1}) of hydrolysed casein products in deionized water at 37 °C for 10 min, and then determining unbound Fe^{2+} in the assay using the Ferrozine assay. A standard curve was obtained with 1–500 μmol·l^{-1} $FeCl_2$ and fractional binding was calculated as bound Fe^{2+} (expressed in micromoles per milligrams of protein). The binding curves were fitted into one-site specific ligand binding (non-linear regression) equation and used to determine B_{max} and K_d using the "simple ligand binding" function of SigmaPlot 12.1 (Systat Software, San Jose, California, USA).

β-Carotene oxidation assay

β-Carotene (100 μl of 30 mmol·l^{-1} in acetone) was mixed with 50 μl of the hydrolysed casein products, at 1 $mg \cdot ml^{-1}$ final concentration, and 50 μl of $FeCl_2$ (15 mmol·l^{-1}) followed by incubation at 37 °C. Absorbance of β-carotene was then measured at 450 nm at 20 min intervals for 2 h using Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek Instruments, Winooski, Vermont, USA). The rate of β-carotene oxidation, represented by decrease in absorbance during incubation (expressed as reciprocal minutes), was determined and used to calculate the percentage of inhibition of oxidation relative to a control assay that did not contain the hydrolysed caseins.

Glutathione oxidation assay

The ability of the hydrolysed caseins to inhibit Fe^{2+}/H_2O_2-facilitated oxidation of the sulf-

hydryl group of reduced glutathione (GSH) was evaluated in vitro as previously reported [11]. Briefly, the samples (1 mg·ml^{-1} in 50 mmol·l^{-1} sodium phosphate buffer, pH 7.2) was mixed with 20 mmol·l^{-1} $FeCl_2$ and incubated at 37 °C for 30 min. GSH (83 μg·ml^{-1} in the phosphate buffer) was then added to the mixture and equilibrated for 10 min followed by the addition of 1 mmol·l^{-1} H_2O_2 and further incubation at 37 °C for 1 h. Control experiments containing only GSH or GSH with Fe^{2+}/H_2O_2 were also conducted. The assay mixture was then analysed for reactive sulfhydryl content using 5,5'-dithiobis(2-nitrobenzoic acid). Antioxidative capacity of the samples was then expressed as percentage of GSH sulfhydryl content conserved by the treatments after oxidation.

Statistical analysis

Assays were conducted in triplicate and results expressed as mean ± standard deviation. Statistical significance of difference ($P < 0.05$) between treatments was analysed by one-way analysis of variance followed by the Holm-Sidak multiple comparison test. Relationships between the surface properties, binding affinity and antioxidative capacities were analysed by Spearman's Rank Order Correlation. The statistical analyses were done using SigmaPlot 12.1.

RESULTS AND DISCUSSION

Surface charge and hydrophobicity of the hydrolysed caseins

Enzymatic hydrolysis of casein isolate with the commercial proteases of different specificities yielded hydrolysate products with significantly different ($P < 0.001$) surface properties. The peptides are expected to carry negative surface charges at the hydrolysis pH due to their negatively charged carboxyl groups. The extent of the negative charge however depends on the composition of each hydrolysate and particular arrangements of the peptides in the aqueous solution. As shown in Fig. 1, the hydrolysed caseins had net negatively charged surfaces with ζ-potential exceeding –30 mV. This indicated that the peptides had moderate stability in aqueous suspension. Notably, pancreatin and Alcalase produced hydrolysates with the lowest ζ-potential, i.e. the highest negative surface charge, whereas hydrolysates from bromelain and papain reactions had the least magnitude of ζ-potential. Moreover, S_o was found to be highest for the casein hydrolysate produced with thermolysin. This can be attributed to the protease specificity in cleaving proteins at *N*-terminal (P1') position of hydrophobic residues, leucine, phenylalanine, isoleucine, valine, methionine and alanine [2, 12], leading to the release of peptides containing accessible hydrophobic amino acid residues. The low S_o values of the hydrolysed caseins, compared to other protein sources, can be due to the highly anionic surfaces of casein phosphoserine residues. Variation in S_o and ζ-potential of the samples (Fig. 1 and Fig. 2) can affect molecular interactions and influence the formation of peptide secondary structures and aggregates. To date, antioxidative capacity of peptides has been often attributed to their composition of antioxi-

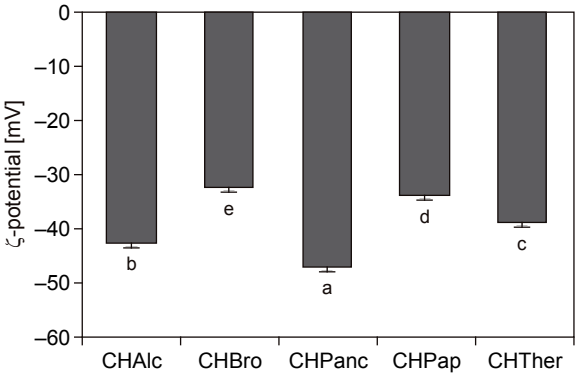

Fig. 1. Zeta (ζ)-potential of the casein hydrolysate products.

Bars with different letters represent significantly different mean values ($P < 0.001$).
CHAlc – casein hydrolysed with Alcalase, CHBro – casein hydrolysed with bromelain, CHPanc – casein hydrolysed with pancreatin, CHPap – casein hydrolysed with papain, CHTher – casein hydrolysed with thermolysin.

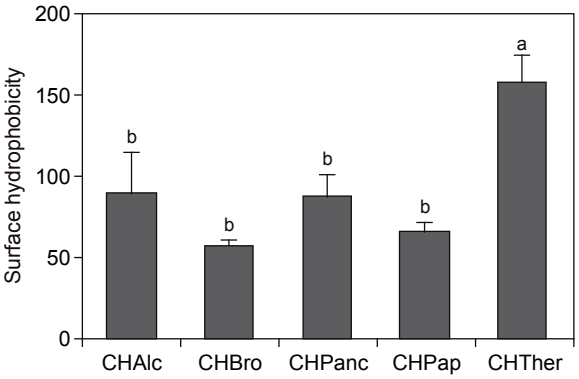

Fig. 2. Surface hydrophobicity of aqueous suspension of the enzymatic casein hydrolysate products.

Bars with different letters represent significantly different mean values ($P < 0.001$).
CHAlc – casein hydrolysed with Alcalase, CHBro – casein hydrolysed with bromelain, CHPanc – casein hydrolysed with pancreatin, CHPap – casein hydrolysed with papain, CHTher – casein hydrolysed with thermolysin.

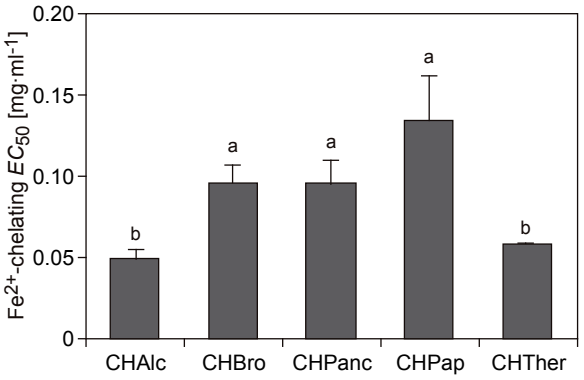

Fig. 3. Half maximal effective concentration of the hydrolysed casein products in chelating Fe^{2+}.

Bars with different letters represent significantly different mean values ($P < 0.001$).
CHAlc – casein hydrolysed with Alcalase, CHBro – casein hydrolysed with bromelain, CHPanc – casein hydrolysed with pancreatin, CHPap – casein hydrolysed with papain, CHTher – casein hydrolysed with thermolysin.

dative and oxidatively-labile amino acid residues [3–5]. However, the accessibility of these antioxidant amino acid residues to solvents in assay matrices, or their location relative to the surface of peptide aggregate structures, have yet to be reported. Knowledge of the surface chemistry enhances our understanding of the interaction of antioxidative peptides with their targets in physiological or food matrices.

Fe^{2+} chelation and binding affinity of the hydrolysed casein products

The casein hydrolysates exhibited strong dose-dependent Fe^{2+}-chelating capacity with maximum activity of >90% chelation observed at 0.25 mg·ml^{-1} for casein hydrolysed with Alcalase (CHAlc) and casein hydrolysed with thermolysin (CHTherm), and at 0.5 mg·ml^{-1} for casein hydrolysed with pancreatin (CHPanc). Accordingly, as shown in Fig. 3, CHAlc and CHTherm had the highest chelating capacity (i.e. the lowest EC_{50} values of 0.049 mg·ml^{-1} and 0.058 mg·ml^{-1}, respectively) whereas casein hydrolysed with papain (CHPap) had the least chelating capacity (EC_{50} of 0.134 mg·ml^{-1}). Casein hydrolysates with higher S_o and higher net negative surface charge generally had better chelating capacity. In order to gain further insight on the capacity and strength of the peptide-metal binding, saturation curves (plots of bound Fe^{2+} vs total Fe^{2+}) were obtained for all the hydrolysed caseins. CHTherm had the highest B_{max} indicating that it could hold the most Fe^{2+} compared to the other hydrolysates (Tab. 1). The casein hydrolysates with the best Fe^{2+}-chelating capacity also had the highest B_{max}. Moreover, CHPanc had the lowest K_d, indicating the strongest peptide-Fe^{2+} interaction of all the casein hydrolysates. K_d strongly correlated with ζ-potential ($r_s = 0.90$), with CHPanc showing both the highest net negative surface charge and strongest Fe^{2+} affinity, and casein hydrolysed with bromelain (CHBrom) showing the opposite properties. This indicates that high net negative surface charge is an important contributing factor in establishing strong casein peptide-Fe^{2+} chelate complexes. In addition to the iron-chelating ligands of amino acids, the phosphate groups of caseinophosphopeptides are known to bind iron by electrostatic attraction [13], and this was used for preparing chelates for increasing the bioavailability of dietary iron [7]. Therefore, formation of the iron complexes in this study would be facilitated by electrostatic interaction of the opposite charges on the metal ions and peptide surfaces.

Inhibition of Fe^{2+}-facilitated β-carotene oxidation

Chelation of pro-oxidant metals is expected to prevent or delay their participation in oxidative reactions and subsequent damage to biological structures. In this study, oxidatively-labile β-carotene was used as a hydrophobic oxidation target. The hydrolysed casein samples (CHPanc and CHAlc) with the highest Fe^{2+} affinity (i.e. lowest K_d) and lowest ζ-potential (i.e. highest net negative surface charge) had the maximum capacity for inhibiting Fe^{2+}-facilitated oxidation of β-carotene in the emulsion (Fig. 4). The results demonstrated that tight Fe^{2+}-binding would likely make the pro-oxidant metal less available for

Tab. 1. Fe^{2+}-binding parameters of the hydrolysed casein products.

Binding parameter	CHAlc	CHBro	CHPanc	CHPap	CHTher
B_{max} [μg·mg^{-1}]	0.069 ± 0.001^{ab}	0.071 ± 0.015^{ab}	0.053 ± 0.002^{b}	0.067 ± 0.004^{ab}	0.085 ± 0.013^{a}
K_d [$\times 10^{-3}$ μmol]	3.70 ± 0.04^{b}	14.83 ± 4.21^{a}	2.47 ± 0.23^{b}	4.60 ± 0.42^{b}	6.03 ± 2.91^{b}

Numbers with different letters in each row represent significantly different mean values ($P < 0.001$).
B_{max} – maximum ligand binding (expressed per milligram of protein); K_d – ligand dissociation constant.
CHAlc – casein hydrolysed with Alcalase, CHBro – casein hydrolysed with bromelain, CHPanc – casein hydrolysed with pancreatin, CHPap – casein hydrolysed with papain, CHTher – casein hydrolysed with thermolysin.

participation in the oxidative reaction. Furthermore, peptide hydrophobicity is often thought to enhance their accessibility to hydrophobic oxidative systems [4]. However, the ability of the hydrolysed caseins to inhibit hydrophobic β-carotene oxidation was not related to their S_o. This was likely due to the highly charged peptide surface, which would make the peptides less available for interaction and protection of the hydrophobic β-carotene phase of the emulsion. Consequently, inhibition of β-carotene oxidation could be due to Fe^{2+} chelation in the emulsion aqueous phase.

Inhibition of Fe^{2+}/H_2O_2-facilitated glutathione oxidation

Fe^{2+}-facilitated Fenton's reaction can oxidize GSH and the reactive sulfhydryl group has been measured as an indicator of redox activity [11]. The Fenton's reagent induced GSH oxidation with a 68.6% ± 0.1% decrease in its reactive sulfhydryl group. The hydrolysed caseins each contained less than 2 μmol·l^{-1} sulfhydryl per gram of protein, which were by three orders of magnitude lower than the sulfhydryl group of GSH present in the assay. When added to the GSH oxidation system, the hydrolysed caseins differed in their ability to conserve GSH sulfhydryl group (Fig. 5). Casein hydrolysate generated with papain had the best activity in protecting GSH sulfhydryl group, whereas CHBrom had the weakest activity. No correlation was observed between the surface properties or Fe^{2+}-binding parameters and the activity of the hydrolysates in protecting GSH from oxidation. This indicated that Fe^{2+} chelation was not the sole mechanism for the observed antioxidative capacity, or that the binding affinity was not strong enough to reduce the oxidation of the hydrophilic molecular target. Moreover, GSH exists in the anionic form at the physiological pH used in this study, and is expected to repel the casein peptides due to their negatively charged particle surfaces. This can lead to limited association between the antioxidants and oxidatively-labile target in the aqueous matrix.

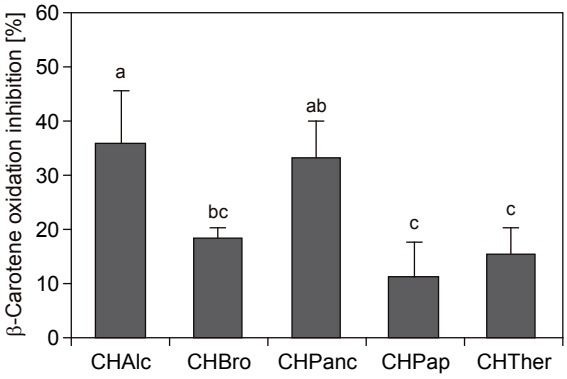

Fig. 4. Effects of the hydrolysed casein products against Fe^{2+}-facilitated β-carotene oxidation.

Bars with different letters represent significantly different mean values ($P = 0.003$).
CHAlc – casein hydrolysed with Alcalase, CHBro – casein hydrolysed with bromelain, CHPanc – casein hydrolysed with pancreatin, CHPap – casein hydrolysed with papain, CHTher – casein hydrolysed with thermolysin.

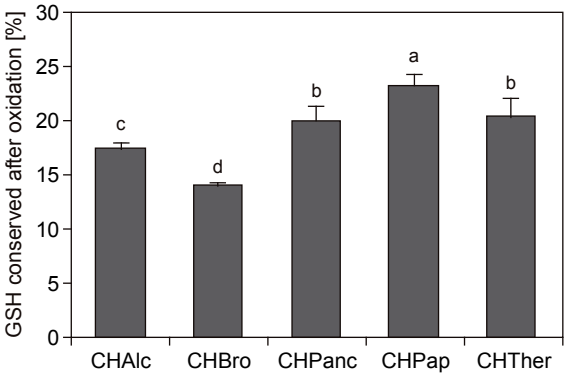

Fig. 5. Effects of the hydrolysed casein products in conserving reduced glutathione sulfhydryl group during Fe^{2+}/H_2O_2-facilitated oxidation.

Bars with different letters represent significantly different mean values ($P < 0.001$).
CHAlc – casein hydrolysed with Alcalase, CHBro – casein hydrolysed with bromelain, CHPanc – casein hydrolysed with pancreatin, CHPap – casein hydrolysed with papain, CHTher – casein hydrolysed with thermolysin.

CONCLUSION

The heterogenity of peptides within protein hydrolysates makes them promising polydentate ligand candidates for the formation of chelate complexes with pro-oxidant metals, and for subsequent inhibition of metal-facilitated oxidative process and damage to cellular and food components. For the first time, this study has reported the relationship of the surface property and Fe^{2+}-binding affinity of enzymatically hydrolysed caseins with their inhibitory effects against Fe^{2+}-facilitated oxidation of β-carotene and reduced glutathione. The findings demonstrate that the highly negative surface charge of the hydrolysates is an important consideration for establishing strong electrostatic interaction with Fe^{2+}, which was relevant in enhancing the inhibition of Fe^{2+}-facilitated oxidation. Understanding the surface properties and Fe^{2+}-binding affinity of the hydrolysed caseins will provide insight into their behaviour and antioxidative capacity in different

food matrices, especially when intended for use in formulating health-promoting food products and in food quality preservation.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant (RGPIN 435865-2013), and Canada Foundation for Innovation (CFI) through an Infrastructure Grant.

REFERENCES

1. Aruoma, O. I.: Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American Oil Chemists' Society, *75*, 1998, pp. 199–212. DOI: 10.1007/s11746-998-0032-9.
2. Tavano, O. L.: Protein hydrolysis using proteases: An important tool for food biotechnology. Journal of Molecular Catalysis B: Enzymatic, *90*, 2013, pp. 1–11. DOI: 10.1016/j.molcatb.2013.01.011.
3. Elias, R. J. – Kellerby, S. S. – Decker, E. A.: Antioxidant activity of proteins and peptides. Critical Reviews in Food Science and Nutrition, *48*, 2008, pp. 430–441. DOI: 10.1080/10408390701425615.
4. Sarmadi, B. H. – Ismail, A.: Antioxidative peptides from food proteins: A review. Peptides, *31*, 2010, pp. 1949–1956. DOI: 10.1016/j.peptides.2010.06.020.
5. Samaranayaka, A. G. P. – Li-Chan, E. C. Y.: Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. Journal of Functional Foods, *3*, 2011, pp. 229–254. DOI: 10.1016/j.jff.2011.05.006.
6. Pihlanto, A.: Antioxidative peptides derived from milk proteins. International Dairy Journal, *16*, 2006, pp. 1306–1314. DOI: 10.1016/j.idairyj.2006.06.005.
7. Guo, L. – Harnedy, P. A. – Li, B. – Hou, H. – Zhang, Z. – Zhao, X. – Fitzgerald, R. J.: Food protein-derived chelating peptides: Biofunctional ingredients for dietary mineral bioavailability enhancement. Trends in Food Science & Technology, *37*, 2014, pp. 92–105. DOI: 10.1016/j.tifs.2014.02.007.
8. Udechukwu, M. C. – Collins, S. A. – Udenigwe, C. C.: Prospects of enhancing dietary zinc bioavailability with food-derived zinc-chelating peptides. Food & Function, *7*, 2016, pp. 4137–4144. DOI: 10.1039/C6FO00706F.
9. Mohan, A. – Udenigwe, C. C.: Towards the design of hypolipidaemic peptides: Deoxycholate binding affinity of hydrophobic peptide aggregates of casein plastein. Journal of Functional Foods, *18*, 2015, pp. 129–136. DOI: 10.1016/j.jff.2015.06.064.
10. Horax, R. – Hettiarachchy, N. S. – Chen, P. – Jalaluddin, M.: Preparation and characterization of protein isolate from cowpea (*Vigna unguiculata* L. Walp.). Journal of Food Science, *69*, 2006, pp. fct114–fct118. DOI: 10.1111/j.1365-2621.2004.tb15500.x.
11. Udenigwe, C. C. – Udechukwu, M. C. – Yiridoe, C. – Gibson, A. – Gong, M.: Antioxidant mechanism of potato protein hydrolysates against in vitro oxidation of reduced glutathione. Journal of Functional Foods, *20*, 2016, pp. 195–203. DOI: 10.1016/j.jff.2015.11.004.
12. Keil, B.: Essential substrate residues for action of endopeptidases. In: Keil, B.: Specificity of proteolysis. Berlin, Heidelberg : Springer, 1992, pp. 43–228. ISBN: 9783642483820. DOI: 10.1007/978-3-642-48380-6_5.
13. Bouhallab, S. – Cinga, V. – Aít-Oukhatar, N. – Bureau, F. – Neuville, D. – Arhan, P. – Maubois, J. L. – Bouglé, D.: Influence of various phosphopeptides of caseins on iron absorption. Journal of Agricultural and Food Chemistry, *50*, 2002, pp. 7127–7130. DOI: 10.1021/jf025554v.

Received 2 December 2016; 1st revised 16 February 2017; accepted 11 March 2017; published online 3 May 2017.