ISSN 1330-9862
(FTB-2915)

original scientific paper

The Impact of Extrinsic Amino Acids and Solvent Fractionation on the *in vitro* Antioxidant Activity of Plastein Reaction-Stressed Casein Hydrolysates

Nan Yue[1], *Tie-Jing Li*[1,2] *and Xin-Huai Zhao*[1,2]*

[1]Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, 150030 Harbin, PR China

[2]Department of Food Science, Northeast Agricultural University, 150030 Harbin, PR China

Received: September 1, 2011
Accepted: April 24, 2012

Summary

Papain was used to prepare a casein hydrolysate with a degree of hydrolysis of 9.4 %. The hydrolysate had the *in vitro* antioxidant activity with a DPPH radical scavenging activity of 38.7 % and an EC_{50} of 1.63 mg/mL. Extrinsic phenylalanine or tyrosine was added to the hydrolysate for a papain-catalyzed plastein reaction. The temperature, substrate mass per volume fraction, and the levels of enzyme and amino acid addition during the reaction were optimized using response surface methodology with a fixed reaction time of 5 h, and were found to be 30 °C, 50 %, 3 kU per g of peptides and 0.74 mol per mol of the free amino groups of the hydrolysate, respectively. Some modified hydrolysates were prepared and their antioxidant activity was evaluated in terms of DPPH radical scavenging activity and reducing power. The results revealed that all prepared modified hydrolysates had significantly higher ($p<0.05$) scavenging activity and reducing power than the original hydrolysate, and among them one showed the lowest EC_{50} of 1.09 mg/mL against DPPH radical. When the modified hydrolysate with the highest activity was fractionated using ethanol/water solvents in volume ratios of 3:7, 4:6, 5:5 and 6:4, the supernatant or precipitate fraction exhibited an enhanced or decreased activity or reducing power, especially with the solvent of lower polarity (*e.g.* 6:4 by volume). The obtained supernatant with the highest activity thus exhibited an EC_{50} of 0.69 mg/mL. The results show that extrinsic phenylalanine or tyrosine addition in the plastein reaction of casein hydrolysate and further solvent fractionation of the modified hydrolysate is applicable to improve the antioxidant properties of products.

Key words: casein hydrolysate, antioxidant activity, phenylalanine, tyrosine, papain, plastein reaction, solvent fractionation

Introduction

It has been reported that the disease and ageing of the body are related to the formation of free radicals in the body (*1*,*2*). The intake of some antioxidants including antioxidant proteins or peptides has a positive effect on the body health as they can protect the body against the damage induced by the radicals (*3*). Food proteins can be hydrolyzed into antioxidant peptides by enzymatic hydrolysis, digestion or microbial fermentation, which can effectively scavenge free radicals (*4*,*5*), protect the normal structure and functions of the cells and mitochondria with potential physiological regulatory role (*6*,*7*). In recent years, antioxidant peptides have become a hot topic in food science. For example, tuna backbone proteins have been hydrolyzed by Alcalase, α-chymotrypsin,

*Corresponding author; Phone: ++86 451 5519 1813; Fax: ++86 451 5519 0340; E-mail: zhaoxh@mail.neau.edu.cn, xhzhao63@sina.com.cn

Neutrase, papain, pepsin or trypsin to produce antioxidant peptides (*8*). Other studies have also reported that the enzymatic hydrolysates derived from milk proteins (*i.e.* casein or whey) had antioxidant activity (*9–11*).

Plastein reaction is a protease-catalyzed reaction. Food proteins can be treated with the plastein reaction to modify some of their important qualities, *e.g.* to increase the added value, to improve the functional properties or to incorporate some essential amino acids for better nutritive value (*12–14*). It was also reported that the plastein reaction could be applied to elevate the antioxidant activity of some protein hydrolysates (*15,16*). The chemical nature of the peptides might be important for their antioxidant activity. One study has indicated that because of their hydrophobic nature, peptides can have better antioxidant activity (*17*). Another work also revealed that higher antioxidant activities of the peptides were presumed mainly due to the presence of hydrophobic amino acids in their sequence (*18*). In a recent study, antioxidant properties of the soluble peptide extract from a fermented soybean food were impacted by fermentation time and particularly by the polarity of ethanol/water extraction solvent used (*19*). Unfortunately, it is unknown whether the plastein reaction of protein hydrolysates in the presence of hydrophobic amino acids or the solvent fractionation of protein hydrolysates by ethanol/water solvent has an influence on their antioxidant properties.

In the present study, a casein hydrolysate has been prepared by hydrolysis of casein with papain, and then modified by papain-catalyzed plastein reaction in the presence of tyrosine or phenylalanine. Four ethanol/water solvents in the volume ratios of 3:7, 4:6, 5:5 or 6:4, respectively, were used to fractionate the prepared casein hydrolysate and the modified casein hydrolysate. Some antioxidant properties of the casein hydrolysate, the modified hydrolysate and the fractionated hydrolysate were evaluated *in vitro*. The aim of the present work is to investigate the impact of the two added amino acids and the coupled ethanol/water solvent fractionation on the antioxidant activity of casein hydrolysate.

Materials and Methods

Chemicals and apparatus

Papain (22.5 kU/g) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, PR China). Casein was purchased from Beijing Aoboxing Bio-Tech Co. Ltd. (Beijing, PR China) and its protein content was 86.1 % on dry mass basis. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Phenylalanine and L-tyrosine methyl ester hydrochloride were purchased from Aladdin-Reagent Co. Ltd. (Shanghai, PR China). Other chemicals used were of analytical grade. Highly purified water prepared with Milli-Q PLUS (Millipore Corporation, New York, NY, USA) was used to prepare all buffers and solutions.

Preparation of casein hydrolysate with papain

Casein (5.8 g) was dissolved in 100 mL of water to obtain an original solution with protein mass per volume fraction of 5 %. The solution was adjusted to pH= 6.5 with 1 mol/L of NaOH. Papain premixed with water was added with stirring to the casein solution to give an enzyme/substrate (E/S) ratio of approx. 1.5 kU per g of proteins. The hydrolysis was carried out at 45 °C for 1, 2, 3, 4 and 5 h. After the hydrolysis, the solution was adjusted to pH=4.6 with 1 mol/L of HCl and heated to 100 °C for 20 min to inactivate the papain. Then, the solution was cooled and centrifuged at 3000×*g* for 20 min. The supernatant was separated and lyophilized. The obtained powder (casein hydrolysate) was stored at –20 °C, analyzed for its degree of hydrolysis, protein recovery and scavenging activity against DPPH radical. Based on the analysis results, the casein hydrolysate with higher protein recovery and scavenging activity was bulk prepared as the substrate for the papain-catalyzed plastein reaction, lyophilized and stored.

Modification of casein hydrolysate by papain-catalyzed plastein reaction

The plastein reaction was carried out separately by adding phenylalanine (Phe) or tyrosine (Tyr) and papain to the prepared casein hydrolysate. Design Expert v. 7.0 software (Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental design, data analysis and quadratic model building to find the optimal conditions (*20*). The designed experiment consisted of 30 runs and four independent variables including reaction temperature (°C), substrate mass per volume fraction (%), papain addition (E/S ratio, U per g of peptides) and amino acid addition (mol of Phe per mol of free amino groups of the hydrolysate) at five levels. The detailed design is listed in Table 1. The casein hydrolysate, papain and Phe (or Tyr) were dissolved in water separately and mixed to obtain the selected mixtures as described in Table 1. The mixtures were kept at the selected reaction temperature for 5 h with continuous stirring. After the plastein reaction, the mixtures were heated at 100 °C for 20 min to inactivate papain. The decreased amount of free amino groups of the mixture was taken as the dependent variable or response, which was calculated by subtracting the content of free amino groups of the modified hydrolysate after the plastein reaction from that of the reaction mixture before plastein reaction and expressed as μmol per g of peptides.

Table 1. Ranges and levels of independent variables selected for the optimization of plastein reaction of casein hydrolysate by response surface methodology

Independent variables	Levels				
	–1.682	–1	0	+1	+1.682
Reaction temperature/°C	13.2	20	30	40	46.8
(*m*(substrate)/*V*(mixture))/%	23.2	30	40	50	56.8
(E/S)/(kU per g of peptides)	0.32	1	2	3	3.68
(*n*(amino acid)/*n*(free amino group of hydrolysate))/(mol/mol)	0.06	0.20	0.40	0.60	0.74

With the optimized reaction conditions, some modified casein hydrolysates were prepared by changing the reaction time or/and the level of amino acid addition. Their scavenging activities against DPPH radical and reducing power were evaluated *in vitro*. The modified hydrolysate with the highest activity was thus bulk prepared, lyophilized and subjected to solvent fractionation.

Solvent fractionation of the original or modified casein hydrolysate

The prepared original or modified casein hydrolysate (CH or MCH) was mixed with ethanol/water solvents in the volume ratios of 3:7, 4:6, 5:5 or 6:4, respectively, to obtain a final peptide mass per volume fraction of 50 %. The mixture was centrifuged at 9000×*g* for 30 min to obtain two fractions (supernatant and precipitate). The two fractions were lyophilized, reconstituted in water and analyzed for their peptide recovery, content of free amino groups and antioxidant activity *in vitro.*

Analysis of protein or peptide content, degree of hydrolysis and enzyme activity

The activity of papain was assayed by a method described by Sarath *et al.* (*21*). Nitrogen content of the casein and hydrolysates was determined by the Kjeldahl procedure (*22*) and multiplied by 6.38 to give the protein or peptide content.

The content of free amino groups of the casein, hydrolysate or modified hydrolysate on protein or peptide basis was measured by *o*-phthaldialdehyde (OPA) assay (*23,24*) with some modifications. The OPA reagent was prepared daily by combining the following reagents: 75 mL of 0.2 mol/L of sodium borate buffer (pH=9.5), 5 mL of 400 g/L of sodium dodecyl sulphate (SDS), 80 mg of OPA (in 1 mL of methanol) and 0.4 mL of β-mercaptoethanol, and distilled water was added to obtain a final volume of 100 mL. The assay was carried out by adding 3 mL of analysis sample to 3 mL of OPA reagent. The absorbance of the mixed solution was measured at 340 nm in a spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan) after 5 min. L-Leucine solution (6–30 μg/mL) was used as standard. The degree of hydrolysis (DH) of the casein hydrolysate was determined by assaying the content of free amino groups of the hydrolysates by OPA method and calculated by using the equation given by Adler-Nissen (*25*):

$$DH=(h/h_{tot})\times 100 \qquad /1/$$

where h was the number of broken peptide bonds per mass unit of the hydrolysate, and h_{tot} was total number of bonds per mass unit of casein (8.2 meq per g of proteins).

Assaying of DPPH radical scavenging activity and reducing powder

A method reported by Nsimba *et al.* (*26*) was used with slight modifications to assay the DPPH radical scavenging activity of the analyzed sample. An aliquot (2 mL) of sample solution at peptide concentration of 1 mg/mL was mixed with 1 mL of DPPH (20 μmol/L). The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of the resulting solution was measured at 571 nm in the spectrophotometer. Ethanol was used as a control. DPPH radical scavenging activity was measured as a decrease in the absorbance and expressed as inhibition percentage by the following equation:

$$\text{Scavenging activity}=(A_C-A_E)/A_C\times 100 \qquad /2/$$

where A_E and A_C are the absorbance of the sample and the control, respectively.

The concentration of the sample needed to scavenge the DPPH radical by 50 % (EC_{50}) under these conditions was determined by assaying variously diluted samples and plotting the inhibition percentage of the radical as a function of peptide concentration.

A method by Oyaizu (*27*) was used to measure the reducing power of the analysis sample. An aliquot of 2.0 mL of sample solution in phosphate buffer (0.2 mol/L, pH=6.6) was mixed with 2.0 mL of the same buffer and 2.0 mL of 1 % potassium ferricyanide to yield the final peptide concentration of 2 mg/mL. After incubation at 50 °C for 20 min, 2.0 mL of 10 % (by mass) trichloroacetic acid were added, and a 2.0-mL aliquot was mixed with 2.0 mL of distilled water and 0.4 mL of 0.1 % (by mass) ferric chloride. The absorbance of the final solution was measured at 700 nm after 10 min and used as an indication of the reducing power.

Statistical analysis

All experiments were carried out in triplicate. All data obtained were expressed as mean value±standard deviation (S.D.). Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan's multiple range tests. All the tests were considered statistically significant at $p<0.05$. SPSS v. 13.0 software (SPSS Inc., Chicago, IL, USA) and MS Excel 2003 software (Microsoft Corporation, Redmond, WA, USA) were used to analyze and display the data.

Results and Discussion

Preparation of casein hydrolysate and its antioxidant activity in vitro

To prepare casein hydrolysate with higher DPPH radical scavenging activity and peptide recovery, casein was independently hydrolyzed by papain with a hydrolysis period of 5 h. The DH, protein recovery and scavenging activity of the obtained casein hydrolysate were measured and are given in Table 2. The DH of the prepared

Table 2. Degree of hydrolysis (DH), peptide recovery and scavenging activity against DPPH radical of the casein hydrolysate prepared at different hydrolysis times

Hydrolysis time	DH	Peptide recovery*	Scavenging activity against DPPH*
h	%	%	%
1	$(8.9\pm0.1)^{A}$	$(79.4\pm0.9)^{A}$	$(38.5\pm0.4)^{B}$
2	$(9.4\pm0.1)^{B}$	$(80.8\pm1.0)^{AB}$	$(38.7\pm0.5)^{B}$
3	$(9.8\pm0.2)^{C}$	$(81.7\pm1.0)^{BC}$	$(37.5\pm0.7)^{A}$
4	$(10.2\pm0.1)^{D}$	$(82.9\pm1.0)^{C}$	$(37.6\pm0.3)^{AB}$
5	$(10.3\pm0.1)^{D}$	$(83.0\pm1.0)^{C}$	$(37.4\pm0.4)^{A}$

*Final peptide concentration used in the assay was fixed at 1 mg/mL. Different capital letters in the superscripts after the values in the same column indicate that one-way ANOVA of the mean values is significantly different ($p<0.05$)

casein hydrolysate increased with hydrolysis time from 8.9 to 10.3 %, with a scavenging activity of about 37.4–38.7 % (peptide concentration of 1 mg/mL) or a protein recovery of about 79.4–83.0 %. The casein hydrolysate prepared with a hydrolysis time of 2 h had a DH of 9.4 %. Although the hydrolysate had a higher DPPH radical scavenging activity and peptide recovery, it showed no significantly different ($p>0.05$) activity from the casein hydrolysate prepared with a hydrolysis time of 1 or 3 h. The casein hydrolysate with a DH of 9.4 % was thus bulk prepared and used as the substrate for the plastein reaction.

In the study of Bougatef *et al.* (*28*), muscle protein was hydrolyzed by pepsin. When peptide concentration was fixed at 1 mg/mL, the DPPH radical scavenging activity of the prepared hydrolysate was less than 40 %. If the porcine skin collagen was hydrolyzed by different proteases, the DPPH radical scavenging activity of the prepared hydrolysate at 1.38 % (by mass) was about 13.44 % (*29*). The yak casein hydrolysates prepared with five proteases displayed DPPH radical scavenging activity lower than 80 % when the hydrolysate concentration was set at 2.5 mg/mL (*30*). Compared to these reported hydrolysates, the casein hydrolysate prepared in the present work showed better scavenging activity. The casein hydrolysate had a scavenging activity of 38.7 % at peptide concentration of 1 mg/mL, and an estimated EC_{50} value of 1.63 mg/mL.

Modification of casein hydrolysate and its influence on the antioxidant activity in vitro

Design-Expert software was used to design the plastein reaction fixed at a reaction time of 5 h and to fit the relation between the decreased amount of free amino groups (response, Y) and the variables, including reaction temperature (X_1), substrate concentration (X_2), E/S ratio (X_3) and Phe (or Tyr) addition (X_4). From the experimental results of 30 runs, a two-order regression equation was thus obtained as follows:

$$Y=-474.55506+22.60965X_1-0.4576X_2+0.14361X_3-95.2216X_4-0.11187X_1X_2-5.94719\times10^{-4}X_1X_3+6.35172X_1X_4-2.45531\times10^{-4}X_2X_3+8.80141X_2X_4+0.039345X_3X_4-0.3159X_1^2+0.078907X_2^2-2.22423\times10^{-5}X_3^2-247.64259\ X_4^2 \quad /3/$$

The results of the analysis using the software showed that the obtained equation, with $p<0.0001$ and $R^2_{Adj}=0.9053$, could reflect the changing profiles of the decreased amount of free amino groups of the modified hydrolysate during the plastein reaction. Fig. 1 shows the response surface graphs plotted by the software. The detailed impact of each studied variable (*i.e.* reaction condition) on the response is not discussed here. The suitable conditions given by the software were reaction temperature of 30 °C, substrate mass per volume fraction of 50 %, E/S ratio of 3 kU per g of peptides and Phe (or Tyr) addition of 0.74 mol per mol of free amino groups of hydrolysates.

By using the selected conditions, ten modified hydrolysates (MCH 4–13) with different reaction times (1, 5 and 7 h) and two Phe (or Tyr) addition levels (0.25 and 0.74 mol per mol of free amino groups of hydrolysate) were prepared, and the decrease of free amino group content and the two antioxidant properties were evaluated. The original hydrolysate (CH), three modified hydrolysates (MCH 1–3) prepared without the addition of amino acids and four mixtures of the hydrolysate and Phe (or Tyr) (mixture 1–4) were selected as controls and their antioxidant properties were evaluated. The evaluation results are listed in Table 3.

Compared to the original hydrolysate, all modified hydrolysates (MCH 1–3 and MCH 4–13) had higher DPPH radical scavenging activities (42.0–49.3 *vs.* 38.7 %) or reducing power (0.557–0.662 *vs.* 0.497). This leads to an important conclusion that the carried plastein reaction (with or without the addition of Phe or Tyr) can enhance significantly ($p<0.05$) the two investigated antioxidant properties of the hydrolysate.

When extrinsic Phe or Tyr was mixed into the hydrolysate, the obtained mixtures (mixtures 1–4) exhibited unchanged ($p>0.05$) or improved ($p<0.05$) antioxidant properties, compared to the original hydrolysate. Mixtures 1–4 also showed much lower ($p<0.05$) antioxidant properties than MCH 4–13. This result means that the addition of extrinsic Phe or Tyr into the hydrolysate could enhance the antioxidant properties of the hydrolysate in some extent, but it is the plastein reaction that mostly accounted for the improved antioxidant properties of the modified hydrolysate.

Compared to MCH 1–3 (prepared without Phe or Tyr addition), MCH 4–13 had much higher antioxidant properties ($p<0.05$), implying that the addition of extrinsic Phe or Tyr in the plastein reaction system had beneficial effect on the two properties of the modified hydrolysate. Regarding the DPPH radical scavenging activity, the addition of Phe or Tyr at 0.74 mol per mol of free amino groups of hydrolysates resulted in the higher activity of the modified hydrolysate. Regarding the reducing power, the addition of Tyr at 0.74 mol per mol of free amino groups of hydrolysates also resulted in the higher reducing power of the modified hydrolysate. Regarding the types of the added amino acids, Phe resulted in the highest DPPH radical scavenging activity of the modified hydrolysate (*e.g.* MCH 4), while Tyr resulted in the highest reducing power of the modified hydrolysate (*e.g.* MCH 9). MCH 1, MCH 4 and MCH 9 had EC_{50} values of 1.60, 1.09 and 1.46 mg/mL, respectively, indicating that MCH 4 and MCH 9 had better antioxidant activity than MCH 1. Thus, MCH 4 and MCH 9 were used in the later solvent fractionation, with the original hydrolysate and MCH 1 as two controls.

Reaction time of the performed plastein reaction also showed some impact on the two investigated properties of the modified hydrolysate, but in an irregular mode. Totally, a reaction time of 1 or 5 h was suitable for obtaining the modified hydrolysate with higher DPPH radical scavenging activity or reducing power. A longer reaction time (*e.g.* 7 h) showed adverse impact on the two properties.

A peptide separated from casein protein hydrolysate had an EC_{50} of 2.45 mg/mL against DPPH radical (*31*), *i.e.* the peptide at 2.45 mg/mL could scavenge DPPH radical by 50 %. Samaranayaka and Li-Chan (*32*) prepared a Pacific hake protein hydrolysate, which showed maximal DPPH radical scavenging activity (61.3 %) and reducing power (0.603) at peptide concentration of 9 mg/mL. In

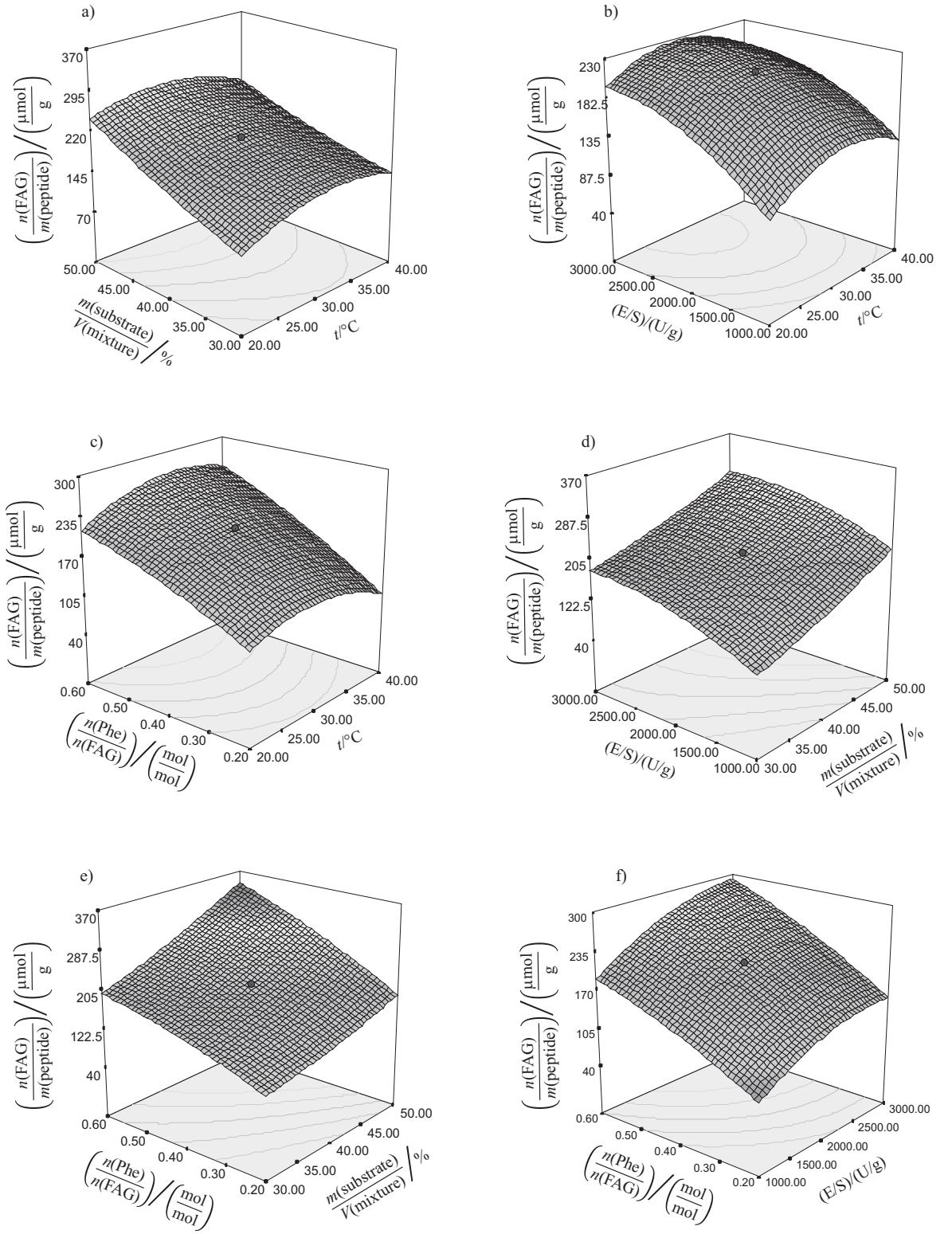

Fig. 1. Response surface graphs showing the impact of reaction temperature, substrate mass per volume fraction, papain addition per g of peptidase (E/S) and phenylalanine (Phe) addition on the decrease of free amino groups (FAG) of the casein hydrolysate modified by plastein reaction

the study of Chang *et al.* (*33*), porcine haemoglobin hydrolysate was prepared by Alcalase-catalyzed hydrolysis followed by Flavourzyme-catalyzed hydrolysis, and the prepared hydrolysate had a reducing power less than 0.25 and a DPPH radical scavenging activity of 41.9 % at peptide concentration of 5 mg/mL. Compared to these reported hydrolysates, the modified hydrolysates (MCH 1–3 or MCH 4–13) showed a DPPH radical scavenging activity larger than 42 % (peptide concentration of 1 mg/mL) and a reducing power larger than 0.557 (pep-

Table 3. Antioxidant properties of casein hydrolysate, mixture of casein hydrolysate and two extrinsic amino acids or some plastein reaction-modified casein hydrolysates*

Sample	$\frac{n(\text{amino acid})}{n(\text{amino group of hydrolysate})}$ $\frac{\text{mol}}{\text{mol}}$	Reaction time / h	$\frac{n(\text{free amino group})}{m(\text{peptide})}$ $\frac{\mu\text{mol}}{\text{g}}$	DPPH scavenging activity / %	Reducing power
CH	0	0	0	$(38.7\pm0.5)^{AB}$	$(0.497\pm0.002)^{A}$
mixture 1	Phe 0.25	0	0	$(37.8\pm0.4)^{AB}$	$(0.506\pm0.002)^{AB}$
mixture 2	Phe 0.74	0	0	$(39.7\pm0.4)^{B}$	$(0.521\pm0.004)^{BC}$
mixture 3	Tyr 0.25	0	0	$(36.9\pm0.9)^{A}$	$(0.509\pm0.005)^{AB}$
mixture 4	Tyr 0.74	0	0	$(39.7\pm0.4)^{B}$	$(0.529\pm0.002)^{C}$
MCH 1	0	1	76.4±4.1	$(42.9\pm1.3)^{C}$	$(0.557\pm0.011)^{D}$
MCH 2	0	5	103.7±6.1	$(42.6\pm1.2)^{C}$	$(0.564\pm0.008)^{D}$
MCH 3	0	7	97.7±1.6	$(42.0\pm2.4)^{C}$	$(0.565\pm0.002)^{D}$
MCH 4	Phe 0.74	1	328.46±39.1	$(49.3\pm0.2)^{F}$	$(0.634\pm0.008)^{G}$
MCH 5	Phe 0.74	5	450.0±12.4	$(47.7\pm0.6)^{DE}$	$(0.620\pm0.002)^{EFG}$
MCH 6	Phe 0.74	7	621.7±5.6	$(46.2\pm0.1)^{D}$	$(0.629\pm0.009)^{FG}$
MCH 7	Phe 0.25	5	155.7±26.9	$(45.7\pm0.7)^{D}$	$(0.619\pm0.013)^{EFG}$
MCH 8	Phe 0.25	7	174.2±11.0	$(47.0\pm2.0)^{DE}$	$(0.621\pm0.012)^{EFG}$
MCH 9	Tyr 0.74	1	224.0±13.6	$(46.9\pm0.9)^{DE}$	$(0.662\pm0.007)^{H}$
MCH 10	Tyr 0.74	5	300.9±11.0	$(48.5\pm2.1)^{EF}$	$(0.632\pm0.014)^{G}$
MCH 11	Tyr 0.74	7	423.7±24.5	$(47.4\pm1.2)^{DE}$	$(0.629\pm0.007)^{FG}$
MCH 12	Tyr 0.25	5	115.5±26.4	$(45.6\pm1.1)^{D}$	$(0.608\pm0.007)^{E}$
MCH 13	Tyr 0.25	7	130.9±13.9	$(46.2\pm1.3)^{D}$	$(0.613\pm0.025)^{EF}$

*Peptide concentration used in the assay of scavenging activity on DPPH or reducing power was 1 or 6 mg/mL. Different capital letters in the superscripts after the values in the same column indicate that one-way ANOVA of the mean values is significantly different ($p<0.05$). The mixtures 1–4 were casein hydrolysate mixed with Phe or Tyr at different levels.
CH=casein hydrolysate, MCH=plastein reaction-modified casein hydrolysate

tide concentration of 6 mg/mL), implying that the modified hydrolysates had better antioxidant properties.

Solvent fractionation and its influence on the antioxidant activity of casein hydrolysate

Ethanol/water (E/W) solvents at four volume ratios were applied in the present study to fractionate the original hydrolysate and three modified hydrolysates (MCH 1, MCH 4 and MCH 9). The obtained fractionated products were analyzed for their peptide recovery and the two investigated antioxidant properties. The data listed in Table 4 show that the fractionation treatment had clear impact on the antioxidant properties of each fractionated product. To summarize, fractionation treatment led to the supernatant (or precipitate) with higher (or lower) scavenging activity and reducing power. Also, the composition of the used solvent exhibited some influence on these antioxidant properties. For example, higher ethanol content in the solvent (*e.g.* E/W=3:7 *vs.* 6:4) was able to induce much higher (or lower) scavenging activity or reducing power ($p<0.05$) of the supernatant (or precipitate). Three comparisons are given here for the obtained supernatant fraction. When casein hydrolysate was fractionated with E/W=3:7, the scavenging activity and reducing power of the supernatant were 39.7 % and 0.503, respectively; if it was fractionated with E/W=6:4, the scavenging activity and reducing power of the supernatant were enhanced to 42.6 % and 0.557. When MCH 9 (an EC_{50} of 1.46 mg/mL) was fractionated with E/W=3:7, the scavenging activity and reducing power of the supernatant were 47.7 % and 0.683. If MCH 9 was fractionated with E/W=6:4, the scavenging activity and reducing power of the supernatant were increased to 52.0 % (an EC_{50} of 0.99 mg/mL) and to 0.722. The MCH 4 supernatant (an EC_{50} of 1.09 mg/mL) obtained using E/W=6:4 also showed an EC_{50} of 0.69 mg/mL. These results indicate that the polarity of fractionation solvent is a key factor to mediate the antioxidant properties of the fractionated product.

Hydrophobic amino acids are important to the antioxidant activity of protein hydrolysates (*17*,*18*). The hydrolysate with higher hydrophobic amino acids exhibited a higher DPPH radical scavenging activity (*34*). It was also confirmed that hydrophobic peptides contributed more to the antioxidant activities of protein hydrolysates than the hydrophilic peptides (*35*). In the present study, when more ethanol was incorporated into the fractionation solvent, the solvent had lower polarity and thus preferred to extract the peptides with more hydrophobic amino acids from the treated hydrolysates. This meant that the obtained supernatant or the precipitate should have more or less hydrophobic amino acids, and reasonably higher or lower antioxidant properties. A similar result was also found in a recent report (*19*), in which extraction solvent with higher ethanol content was able to extract soluble peptide fraction from a fermented soybean

Table 4. Effects of ethanol/water solvent fractionation of casein hydrolysate or plastein reaction-modified casein hydrolysate on some characteristics investigated

Sample	ψ(ethanol, water)	Fraction	Peptide recovery %	DPPH scavenging activity* %	Reducing power*
CH	3:7	supernatant	89.7±0.3	$(39.7\pm0.3)^{C}$	$(0.503\pm0.003)^{D}$
		precipitate	8.4±0.4	$(36.7\pm0.5)^{B}$	$(0.484\pm0.003)^{C}$
	4:6	supernatant	87.8±0.7	$(40.0\pm0.6)^{C}$	$(0.508\pm0.001)^{D}$
		precipitate	10.3±0.3	$(35.9\pm1.0)^{AB}$	$(0.481\pm0.002)^{C}$
	5:5	supernatant	65.1±0.4	$(41.3\pm0.6)^{D}$	$(0.556\pm0.004)^{E}$
		precipitate	34.1±0.4	$(35.8\pm1.0)^{AB}$	$(0.474\pm0.003)^{B}$
	6:4	supernatant	58.5±0.7	$(42.6\pm0.7)^{E}$	$(0.557\pm0.003)^{E}$
		precipitate	38.5±1.0	$(34.8\pm1.1)^{A}$	$(0.460\pm0.005)^{A}$
MCH 1	3:7	supernatant	79.2±1.0	$(43.7\pm0.2)^{DE}$	$(0.578\pm0.005)^{D}$
		precipitate	17.2±0.6	$(37.4\pm0.9)^{C}$	$(0.542\pm0.001)^{C}$
	4:6	supernatant	78.3±0.8	$(44.7\pm0.3)^{EF}$	$(0.585\pm0.012)^{D}$
		precipitate	18.5±0. 4	$(35.8\pm0.9)^{B}$	$(0.529\pm0.006)^{B}$
	5:5	supernatant	54.4±0.7	$(45.4\pm0.3)^{G}$	$(0.618\pm0.003)^{E}$
		precipitate	44.3±0.8	$(35.2\pm0.2)^{AB}$	$(0.513\pm0.005)^{A}$
	6:4	supernatant	50.5±0.7	$(46.8\pm0.3)^{H}$	$(0.621\pm0.002)^{E}$
		precipitate	47.1±0.6	$(34.4\pm0.5)^{A}$	$(0.505\pm0.006)^{A}$
MCH 4	3:7	supernatant	73.1±0.5	$(50.0\pm0.5)^{C}$	$(0.644\pm0.006)^{D}$
		precipitate	23.7±0.3	$(44.8\pm0.9)^{B}$	$(0.625\pm0.003)^{C}$
	4:6	supernatant	70.1±0.3	$(52.9\pm0.4)^{D}$	$(0.698\pm0.007)^{E}$
		precipitate	29.0±0.4	$(44.0\pm0.5)^{B}$	$(0.603\pm0.006)^{B}$
	5:5	supernatant	47.8±0.3	$(53.7\pm0.4)^{D}$	$(0.704\pm0.003)^{E}$
		precipitate	48.3±0.8	$(42.5\pm0.5)^{A}$	$(0.603\pm0.005)^{B}$
	6:4	supernatant	44.5±0.6	$(56.6\pm0.3)^{E}$	$(0.725\pm0.004)^{F}$
		precipitate	52.5±0.3	$(41.9\pm0.6)^{A}$	$(0.581\pm0.007)^{A}$
MCH 9	3:7	supernatant	88.4±0.6	$(47.7\pm0.5)^{D}$	$(0.683\pm0.003)^{C}$
		precipitate	7.9±0.2	$(43.9\pm0.3)^{C}$	$(0.650\pm0.005)^{B}$
	4:6	supernatant	78.4±0.4	$(48.7\pm0.5)^{E}$	$(0.686\pm0.008)^{C}$
		precipitate	19.4±0.6	$(43.1\pm0.6)^{BC}$	$(0.647\pm0.005)^{B}$
	5:5	supernatant	51.8±0.4	$(49.9\pm0.5)^{F}$	$(0.700\pm0.005)^{D}$
		precipitate	45.3±1.2	$(42.8\pm0.4)^{B}$	$(0.617\pm0.006)^{A}$
	6:4	supernatant	45.3±1.9	$(52.0\pm0.4)^{G}$	$(0.722\pm0.007)^{E}$
		precipitate	50.4±1.2	$(40.7\pm0.4)^{A}$	$(0.608\pm0.002)^{A}$

*The peptide concentration used in the assay of scavenging activity against DPPH radical or reducing power was 1 or 6 mg/mL. Different capital letters in the superscripts after the values in the same column for the same hydrolysate indicate that one-way ANOVA of the mean values is significantly different ($p<0.05$). For the MCH 1, 4 and 9 and their two properties, see notes in Table 3. CH=casein hydrolysate, MCH=plastein reaction-modified casein hydrolysate

food with a higher antioxidant activity. These results also support the finding that the solvent fractionation has a potential application in the separation of antioxidant peptides with better activity.

Conclusions

A casein hydrolysate with the degree of hydrolysis of 9.4 % and *in vitro* DPPH radical scavenging activity of 38.7 % was prepared by papain-catalyzed hydrolysis of casein. Two antioxidant properties, DPPH radical scavenging activity and the reducing power of the prepared casein hydrolysate, can be enhanced by a papain-catalyzed plastein reaction, especially in the presence of extrinsic amino acid Phe or Tyr. The optimized conditions of the plastein reaction were reaction temperature of 30 °C, substrate mass per volume fraction of 50 %, E/S ratio of 3 kU per g of peptides, the addition of 0.74 mol of Phe or Tyr per mol of free amino groups of hydrolysates and

reaction time of 5 h. The two antioxidant properties of the plastein reaction-stressed casein hydrolysate were further influenced by the conducted fractionation with ethanol/water solvent. Upon this solvent fractionation treatment, the obtained supernatant or precipitate had an enhanced or decreased antioxidant activity. The ethanol/water solvent with higher ethanol content (*e.g.* in a volume ratio of 6:4) resulted in much higher or lower antioxidant activity of the supernatant or precipitate. Plastein reaction catalyzed by papain in the presence of extrinsic amino acids, followed by solvent fractionation treatment, is applicable to obtain casein hydrolysate with better antioxidant activity.

Acknowledgements

This work was supported by the Innovative Research Team of Higher Education of Heilongjiang Province, PR China (No. 2010td11). The authors thank anonymous reviewers and the editors for their constructive and valuable work on the paper.

References

1. J.P. Kehrer, Free radicals as mediators of tissue injury and disease, *Crit. Rev. Toxicol. 23* (1993) 21–48.
2. Y. Christen, Oxidative stress and Alzheimer disease, *Am. J. Clin. Nutr. 71* (2000) 621–629.
3. M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, *Int. J. Biochem. Cell Biol. 39* (2007) 44–84.
4. S.J. Bishov, A.S. Henick, Antioxidant effect of protein hydrolyzates in a freeze-dried model system, *J. Food Sci. 37* (1972) 873–875.
5. A. Saiga, S. Tanabe, T. Nishimura, Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment, *J. Agric. Food Chem. 51* (2003) 3661–3667.
6. A. Pihlanto-Leppälä, Bioactive peptides derived from bovine whey proteins: Opioid and ACE-inhibitory peptides, *Trends Food Sci. Technol. 11* (2001) 347–356.
7. J. Živković, Z. Zeković, I. Mujić, V. Tumbas, D. Cvetković, I. Spasojević, Antioxidant properties of phenolics in *Castanea sativa* Mill. extracts, *Food Technol. Biotechnol. 47* (2009) 421–427.
8. G.N. Kim, H.D. Jang, C.I. Kim, Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase, *Food Chem. 104* (2007) 1359–1365.
9. D.D. Kitts, Antioxidant properties of casein-phosphopeptides, *Trends Food Sci. Technol. 16* (2005) 549–556.
10. Y. Thiansilakul, S. Benjakul, F. Shahidi, Antioxidative activity of protein hydrolysate from round scad muscle using Alcalase and Flavourzyme, *J. Food Biochem. 31* (2007) 266–287.
11. H.H. Wasteneys, H. Borsook, The enzymatic synthesis of protein. I. The synthesizing action of pepsin, *J. Biol. Chem. 62* (1924) 15–29.
12. A. Osuntoki, I. Korie, Antioxidant activity of whey from milk fermented with *Lactobacillus* species isolated from Nigerian fermented foods, *Food Technol. Biotechnol. 48* (2010) 505–511.
13. M. Yamashita, S. Arai, S.J. Tsai, M. Fujimaki, Plastein reaction as a method for enhancing the sulfur-containing amino acid level of soybean protein, *J. Agric. Food Chem. 19* (1971) 1151–1154.
14. X.H. Zhao, Y.Y. Li, An approach to improve ACE-inhibitory activity of casein hydrolysates with plastein reaction catalyzed by Alcalase, *Eur. Food Res. Technol. 229* (2009) 795–805.
15. S. Ono, D. Kasai, T. Sugano, K. Ohba, K. Takahashi, Production of water soluble antioxidative plastein from squid hepatopancreas, *J. Oleo Sci. 53* (2004) 267–273.
16. X.H. Zhao, D. Wu, T.J. Li, Preparation and the radical scavenging activity of papain-catalyzed casein plasteins, *Dairy Sci. Technol. 90* (2010) 521–535.
17. H.M. Chen, K. Muramoto, F. Yamauchi, K. Fujimoto, K. Nokihara, Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein, *J. Agric. Food Chem. 46* (1998) 49–53.
18. J. Ren, X.Q. Zheng, X.L. Liu, H. Liu, Purification and characterization of antioxidant peptide from sunflower protein hydrolysate, *Food Technol. Biotechnol. 48* (2010) 519–523.
19. M. Hang, X.H. Zhao, Fermentation time and extraction solvents influenced *in vitro* antioxidant property of soluble extracts of mao-tofu fermented with *Mucor* sp., *Biotechnology, 10* (2011) 60–69.
20. C.J.B. de Lima, L.F. Coelho, J. Contiero, The use of response surface methodology in optimization of lactic acid production: Focus on medium supplementation, temperature and pH control, *Food Technol. Biotechnol. 48* (2010) 175–181.
21. G. Sarath, R.S. De La Motte, F.W. Wangner: Protease Assay Methods. In: *Proteolytic Enzymes, A Practical Approach*, R.J. Beynon, J.S. Bond (Ed.), IRL Press, Oxford, UK (1989) pp. 25–55.
22. Determination of the Nitrogen (Kjeldahl Method) and Calculation of the Crude Protein Content, IDF Standard 20B, International Dairy Federation, Brussels, Belgium (1993).
23. F.C. Church, H.E. Swaisgood, D.H. Porter, G.L. Catignani, Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins, *J. Dairy Sci. 66* (1983) 1219–1227.
24. M.J.M. Hernández, E.B. Domingo, R.M.V. Camañas, M.C.G. Alvarez-Coque, Evaluation of the proteolysis degree with *o*-phthalaldehyde/N-acetyl-L-cysteine reagent, *Fresenius J. Anal. Chem. 338* (1999) 62–65.
25. J. Adler-Nissen, Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid, *J. Agric. Food Chem. 27* (1979) 1256–1262.
26. R.Y. Nsimba, H. Kikuzaki, Y. Konishi, Antioxidant activity of various extracts and fractions of *Chenopodium quinoa* and *Amaranthus* spp. seeds, *Food Chem. 106* (2008) 760–766.
27. M. Oyaizu, Antioxidative activities of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography, *Nippon Shokuhin Kogyo Gakkaishi, 35* (1988) 771–775.
28. A. Bougatef, M. Hajji, R. Balti, I. Lassoued, Y. Triki-Ellouz, M. Nasri, Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases, *Food Chem. 114* (2009) 1198–1205.
29. B. Li, F. Chen, X. Wang, B. Ji, Y. Wu, Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry, *Food Chem. 102* (2007) 1135–1143.
30. X.Y. Mao, X. Cheng, X. Wang, S.J. Wu, Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis, *Food Chem. 126* (2011) 484–490.
31. K. Suetsuna, H. Ukeda, H. Ochi, Isolation and characterization of free radical scavenging activities peptides derived from casein, *J. Nutr. Biochem. 11* (2000) 128–131.
32. A.G.P. Samaranayaka, E.C.Y. Li-Chan, Autolysis-assisted production of fish protein hydrolysates with antioxidant

properties from Pacific hake (*Merluccius productus*), *Food Chem. 107* (2008) 768–776.

33. C.Y. Chang, K.C. Wu, S.H. Chiang, Antioxidant properties and protein compositions of porcine heamoglobin hydrolysates, *Food Chem. 100* (2000) 1537–1543.

34. X.S. Wang, C.H. Tang, L. Chen, X.Q. Yang, Characterization and antioxidant properties of hemp protein hydrolysates obtained with neutrase, *Food Technol. Biotechnol. 47* (2009) 428–434.

35. J. Ren, M. Zhao, J. Shi, J. Wang, Y. Jiang, C. Cui *et al.*, Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry, *Food Chem. 108* (2008) 727–736.