Dairy Sci. Technol. 90 (2010) 521–535

DOI: 10.1051/dst/2009054

Available online at:
www.dairy-journal.org

Original article

Preparation and radical scavenging activity of papain-catalyzed casein plasteins

Xin Huai ZHAO*, Dan WU, Tie Jing LI

Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, P.R. China

Received 27 May 2009 – Revised 3rd November 2009 – Accepted 12 November 2009
Published online 19 January 2010

Abstract – The aim of this study was to prepare papain-catalyzed casein plasteins and to demonstrate the influence of the plastein reaction on the radical scavenging activity. Casein was initially hydrolyzed with papain under selected conditions to give a degree of hydrolysis of 9.6%. The casein hydrolysates were then subjected to papain-catalyzed plastein reaction to prepare casein plasteins. The formation of plasteins in the prepared casein plasteins was confirmed by size exclusion chromatography. The optimal conditions for the plastein reaction, determined by response surface methodology using the decrease of free amino groups in the casein plasteins as the response variable, were as follows: papain addition level was fixed at 500 $U \cdot g^{-1}$ proteins, reaction temperature 30 °C, concentration of casein hydrolysates 50% and reaction time 5.6 h. Six different casein plasteins were prepared and the radical scavenging activity on two radicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), was evaluated by the Trolox equivalent antioxidant capacity (TEAC). Analysis showed that all casein plasteins had improved radical scavenging activities. Compared to casein hydrolysates, the scavenging activity of the casein plasteins on DPPH radical had a seven- or ninefold increase, as TEAC increased from 10.45 to 73.41 or 90.31 $mmol \cdot g^{-1}$ proteins, while a onefold increase was detected on $ABTS^+$ (TEAC increased from 8.33 $\mu mol \cdot g^{-1}$ to 15.09 or 16.45 $\mu mol \cdot g^{-1}$ proteins). The results of this study clearly showed that the papain-catalyzed plastein reaction could be applied to prepare casein antioxidant peptides with higher activity, and that the plastein reaction may have a new application in dairy science.

casein / plastein reaction / papain / radical scavenging activity / antioxidant

摘要 – 酪蛋白类蛋白物的木瓜蛋白酶法制备及其自由基清除活性。本研究利用木瓜蛋白酶催化制备酪蛋白类蛋白物，证明类蛋白反应影响酪蛋白类蛋白物的自由基清除活性。首先，在选定的条件下用木瓜蛋白酶水解酪蛋白，制备出水解度为 9.6% 的酪蛋白水解物；然后，酪蛋白水解物进行类蛋白反应制备酪蛋白类蛋白物，并且用排阻色谱证实所制备的酪蛋白类蛋白物中有类蛋白形成。优化的类蛋白反应条件，通过以酪蛋白类蛋白物的游离氨基含量降低为指标、利用响应面法而确定，它们分别是：木瓜蛋白酶添加量固定为每克蛋白质 500 U，反应温度为 30 °C，酪蛋白水解物质量分数为 50%，反应时间为 5.6 h。制备 6 个反应程度不同的酪蛋白类蛋白物，并评价它们对 2 个自由基 1,1-二苯基-2-苦基肼 (DPPH)、2,2'-连氮-二 (3-乙基-苯并噻唑啉-6-磺酸) (ABTS) 的 Trolox 等价抗氧化能力 (TEAC)。分析结果显示，所有的酪蛋白类蛋白物的自由基清除活性得到改善。与酪

*Corresponding author (通讯作者): zhaoxh@mail.neau.edu.cn

蛋白水解物相比，酪蛋白类蛋白物对 DPPH 自由基的清除活性增加 6 ~ 7 倍，因为它们的 TEAC 从10.45 mmol·g^{-1} 增加至 73.41 或 90.31 mmol·g^{-1}，而对 ABTS 自由基的清除活性则增加1倍 (TEAC 从 8.33 μmol·g^{-1} 增加至 15.09 或 16.45 μmol·g^{-1})。

酪蛋白 / 酪蛋白类蛋白物 / 类蛋白反应 / 木瓜蛋白酶 / 自由基清除活性

Résumé – Préparation de plastéines à partir de la catalyse de caséines par la papaïne et leur activité anti-radicalaire. L'objectif de cette étude était de préparer des plastéines à partir de la catalyse de caséines par la papaïne et de démontrer l'influence de la réaction de formation des plastéines sur l'activité anti-radicalaire. La caséine a été préalablement hydrolysée par la papaïne dans des conditions sélectionnées afin d'obtenir un degré d'hydrolyse de 9,6 %. Les hydrolysats de caséine ont ensuite été soumis à une réaction de catalyse par la papaïne pour préparer les plastéines. La formation des plastéines a été confirmée par chromatographie d'exclusion de taille. Les conditions optimales de la réaction, déterminées par la méthode de surface de réponse avec comme variable la diminution des groupements amine libres dans les plastéines, étaient les suivantes : niveau d'addition de papaïne fixé à 500 U·g^{-1} de protéines, température de réaction 30 °C, concentration en hydrolysats de caséine 50 % et temps de réaction 5,6 h. Six plastéines ont été préparées avec des étendues de réaction différentes et leurs activités anti-radicalaires sur le 2,2-diphenyl-1-picrylhydrazyl (DDPH) et l'acide azino -2,2′ bis(ethyl-3-benzothiazoline sulfonique-6) ($ABTS^+$) mesurées par la capacité antioxydante en équivalent Trolox (TEAC). Les résultats ont montré que toutes les plastéines obtenues avaient des activités anti-radicalaires améliorées. Comparée à celle des hydrolysats de caséine, l'activité anti-radicalaire des plastéines était sept à neuf fois plus importante pour le DDPH (TEAC passant de 10,45 mmol·g^{-1} à 73,41 ou 90,31 mmol·g^{-1} protéines) tandis qu'elle avait doublé pour $ABTS^+$ (TEAC passant de 8,33 μmol·g^{-1} à 15,09 ou 16,45 μmol·g^{-1} protéines). Ces résultats indiquent clairement que la réaction de formation des plastéines catalysée par la papaïne pourrait être appliquée pour préparer des peptides antioxydants avec des activités améliorées à partir de caséines, et que cette réaction pourrait trouver une nouvelle application en science laitière.

caséine / plastéine / papaïne / activité anti-radicaux / antioxydant

1. INTRODUCTION

Milk proteins, widely applied as functional and nutritional ingredients, are also considered as particularly good sources of bioactive peptides [16]. In recent years, many researchers have paid more attention to the biological activities of milk proteins such as antioxidant activity, angiotensin-converting enzyme (ACE)-inhibitory activity, antibacterial activity and others [22]. Numerous studies showed that the digestion of native milk proteins would yield physiologically important bioactive peptides [9]. Enzymatic hydrolysis is widely used to hydrolyze food proteins in order to obtain milk protein hydrolysates and to investigate their biological activity [23, 25]. Proteases used were found to play an important role in the bioactivity of protein hydrolysates. Bougatef et al. [3] used five proteases to hydrolyze muscle protein of smooth-hound (*Mustelus mustelus)*, and found that the value of IC_{50} (the concentration needed to decrease the initial concentration of radical by 50%) of hydrolysates obtained by Alcalase or trypsin-like protease was 0.6 ± 0.01 mg·mL^{-1} or 0.73 ± 0.012 mg·mL^{-1} on DPPH radical, while other hydrolysates showed lower radical scavenging activity.

It is well known that natural antioxidants can act as radical scavengers [4]. Antioxidant activity is one of the most important biological properties of protein hydrolysates. The peptides that were obtained by the enzymatic hydrolysis of casein had antioxidant activity [14, 27]. Kitts [15] presented

a review about the antioxidant properties of casein phosphopeptides. Rival et al. [26] concluded that casein-derived peptides, as preferred target over fatty acid radicals, inhibited enzymatic and non-enzymatic lipid peroxidation. Moreover, Hernández-Ledesma et al. [12] studied the antioxidant activity of whey protein hydrolysates (α-lactalbumin and β-lactoglobulin) prepared by commercial proteases and found a moderate ABTS radical scavenging activity in fermented milk. Egg-yolk protein hydrolysates were prepared by the hydrolysis of yolk protein with proteinase from *Bacillus* sp., and the scavenging activities of hydrolysates on DPPH and hydroxyl radical in the concentration of 0.5% (w/v) were 74.2% and 91.7%, respectively [28]. Suetsuna et al. [30] reported that a peptide Tyr-Phe-Tyr-Pro-Glu-Leu from casein protein hydrolysates had a potent superoxide radical scavenging activity with the IC_{50} about 79.2 μmol·L^{-1}.

The plastein reaction was first described almost 100 years ago and three different mechanisms were classified as condensation, transpeptidation and physical forces [2, 8, 34]. It had been applied to modify the functional properties of proteins and to improve their nutritional value [2, 32]. In a recent study, plastein reaction was used to modify the antioxidant activity of hydrolysates from squid hepatopancreas with Alcalase [20]. However, the effect of plastein reaction on the antioxidant activity of other protein hydrolysates, such as casein hydrolysates, remains unknown.

In this study, casein was hydrolyzed and then subjected to plastein reaction in order to prepare casein plasteins. The protease used in casein hydrolysis and plastein reaction was papain. Plastein reaction parameters, including substrate concentration, reaction temperature and time, were optimized at the fixed ratio of enzyme to substrate by response surface methodology (RSM), in which the decrease of free amino groups in casein plasteins on protein basis was used as the response variable. The formation of casein plasteins was confirmed by analyzing and comparing the peptide profiles of casein hydrolysates and the casein plasteins prepared with size exclusion chromatography. The radical scavenging activity of casein plasteins was evaluated for two radicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS). The results could indicate the impact of plastein reaction on the radical scavenging activity of casein plasteins and show its potential application in the preparation of antioxidant peptides from proteins.

2. MATERIALS AND METHODS

2.1. Materials

Papain (18000 U·g^{-1}) used was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Casein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), DPPH, ABTS, bovine serum albumin, cytochrome *c*, insulin, oxidized L-glutathione and L-tyrosine were purchased from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade. Water used was distilled water or deionized water prepared with Milli-Q PLUS (Millipore Corporation, USA).

2.2. Preparation of casein hydrolysates

Casein (5 g, on dry basis) was dissolved in 100 mL water to give an original protein concentration of 5% (w·v^{-1}). The solution was adjusted to pH 6.5 with 1 mol·L^{-1} NaOH. Papain (addition level 500 U·g^{-1} proteins) was premixed with water and added to the casein solution while stirring. The hydrolysis reaction was controlled at 45 °C and casein was hydrolyzed for 1–5 h, respectively. After hydrolysis, the solutions were adjusted to pH 4.6 with 1 mol·L^{-1} HCl and

Table I. Factors and levels of the variables in coded units for RSM study.

Factors	Levels				
Code	−1.682	−1	0	+1	+1.682
Temperature (X_1; °C)	13.2	20	30	40	46.8
Substrate concentration (X_2; %)	29.8	38	50	62	70.2
Reaction time (X_3; *h*)	1.3	3	5.5	8	9.7

heated to 100 °C for 20 min to inactivate papain. The solutions were cooled and centrifuged for 20 min at 5000 rpm. The supernatant was separated and lyophilized. The obtained powder, casein hydrolysates, was stored at –20 °C until analysis and the plastein reaction was carried out.

2.3. Plastein reaction of casein hydrolysates and optimization of reaction conditions

Casein hydrolysates were dissolved in water to give an original substrate concentration as described in Table I. Papain dissolved in water was added, while stirring the casein hydrolysates at a fixed addition level of 500 $U{\cdot}g^{-1}$ proteins. Other condition parameters studied are listed in Table I. After the plastein reaction, solutions were heated to 100 °C for 20 min to inactivate papain. The casein plasteins prepared were stored at –20 °C with few drops of methylbenzene added until analysis of the content of free amino groups and ACE-inhibitory activity were carried out. The decrease of free amino groups in casein plasteins was calculated and expressed as μmol $-NH_2{\cdot}g^{-1}$ proteins, which equals the difference of free amino groups in casein hydrolysates from that in casein plasteins.

Design Expert 7.0 software was used in experimental design, to generate a three-factor, five-level Box-Behnken design combinations and for data analysis. The decrease of free amino groups in casein plasteins on protein basis was used as response. The second-order polynomial model generated by the Box-Behnken design was as equation (1):

$$\begin{aligned} Y = B_0 + B_1X_1 + B_2X_2 \\ + B_3X_3 + B_4X_1X_2 \\ + B_5X_2X_3 + B_6X_1X_3 \\ + B_7{X_1}^2 + B_8{X_2}^2 \\ + B_9{X_3}^2, \end{aligned} \quad (1)$$

where Y is the measured response associated with each factor-level combination; X_1, X_2 and X_3 are the factors studied; B_0 is an intercept; and B_1 to B_9 are the regression coefficients.

2.4. Chemical analysis and evaluation of radical scavenging activity

2.4.1. Papain activity and protein content

The activity of papain was spectrophotometrically assayed by a method described by Sarath et al. [29] with some modifications. Nitrogen contents of casein hydrolysates and casein plasteins were determined by the Kjeldahl procedure according to FIL-IDF 20B:1993 [13] and multiplied by 6.38 to achieve the value for protein content.

2.4.2. The content of free amino groups and degree of hydrolysis

The content of free amino groups of casein hydrolysates or casein plasteins

on protein basis was measured by the *o*-phthaldialdehyde (OPA) assay used by Church et al. [7] and Hernández et al. [11] with some modifications. The final solution of OPA was made by combining the following reagents and diluting them to 100 mL with boric-borate buffer: boric-borate buffer of pH 9.5 consisting 0.4 mol·L^{-1} boric acid and 0.3 mol·L^{-1} NaOH, 7 mg of OPA (dissolved in 1 mL of ethanol) and 40 μL of β-mercaptoethanol. The reagent was prepared daily and protected from light. The OPA assay was carried out by the addition of 3 mL casein hydrolysates (or standard leucine or casein plasteins) solution to 3 mL of the OPA reagent. The solution was mixed well and incubated for 6 min at ambient temperature. The absorbance of the solution was measured at 334.5 nm in a 3 mL quartz cuvette against water in an UV spectrophotometer (UV-2401PC, Shimadzu, Japan) and the reading was taken after 5 min. All analysis was carried out at least in triplicate.

L-Leucine standard solution was prepared as follows: L-leucine (0.3000 g) was dissolved in 1 mol·L^{-1} HCl (5 mL) and diluted to a final concentration of 600 μg·mL^{-1} with water. A serial of diluted solutions in the range of 0 to 30 μg·mL^{-1} was prepared by further dilution and used for the determination of a standard curve.

The degree of hydrolysis (DH) of casein hydrolysates was determined by assaying the content of free amino groups of casein hydrolysates by the OPA method and calculated using the equation (2) given by Adler-Nissen [1]:

$$\mathrm{DH}(\%) = \frac{h}{h_{\mathrm{tot}}} \times 100, \qquad (2)$$

where h is the number of broken peptide bonds per unit weight, and h_{tot} is the total number of bonds per unit weight, which equals 8.3 meq·g^{-1} proteins.

2.4.3. *Assay of size exclusion chromatography*

The procedure described by Chu and Ng [6] was applied with some modifications. Casein hydrolysates and the plasteins prepared were dissolved in 0.05 mol·L^{-1} sodium acetate-0.1 mol·L^{-1} NaCl buffer to give a final protein concentration of 10 mg·mL^{-1}. Half of milliliter of the samples was applied to a 10 × 300 mm Amersham Pharmacia Superdex-75 10/300 GL column (GE Amersham, USA) and analyzed in an AKTA Explorer 100 (GE Amersham, USA). The assay was operated with a fixed pressure of 1.80 MPa and an elution rate of 0.9 mL·min^{-1} at 4 °C, and the elution was monitored with an UV detector at 280 nm. Bovine serum albumin (66.2 kg·mol^{-1}), cytochrome *c* (12.4 kg·mol^{-1}), insulin (5.7 kg·mol^{-1}), oxidized L-glutathione (0.6 kg·mol^{-1}) and L-tyrosine (0.2 kg·mol^{-1}) were used to determine molecular weight distribution of the peptides in casein hydrolysates and a prepared casein plasteins.

2.4.4. *Assay of radical scavenging activity*

The scavenging activity of casein hydrolysates or casein plasteins on DPPH radical was determined by the method of Nsimba et al. [19] with some modifications. Two milliliters of sample solution or Trolox solutions (final concentration 0, 4, 8, 12, 16 and 20 μmol L^{-1}) in ethanol were mixed with 1 mL of ethanolic solution containing DPPH radical (20 μmol·L^{-1}). The mixture was shaken vigorously and left for 30 min in the dark before measuring the absorbance at 517 nm against a blank. Triplicate tests were carried out at each dilution of the standard, and the percentage inhibition of absorbance was calculated and plotted as a function of Trolox concentration. The antioxidant activity of the samples was estimated at a minimum of three different

concentrations within the range of the dose-response curve, and the mean value was derived as the TEAC (Trolox equivalent antioxidant capacity) and expressed as mmol·g^{-1} proteins. The half-inhibition (IC_{50}), defined as the concentration of sample required to decrease the initial concentration of DPPH radical by 50%, was calculated.

The scavenging activity of $ABTS^{\cdot+}$ radicals was measured by the method of Re et al. [24] with some modifications. $ABTS^{\cdot+}$ was produced by reacting 7 mmol·L^{-1} ABTS aqueous solution with 2.45 mmol·L^{-1} potassium persulfate in the dark for 12–16 h at ambient temperature. Prior to testing, the solution was diluted in ethanol (about 1:89 v·v^{-1}) and equilibrated at 30 °C to give an absorbance at 734 nm of 0.700 ± 0.02. After addition of 1.0 mL of diluted $ABTS^{\cdot+}$ solution to 10 μL of samples or Trolox solutions (final concentration 0–4.5 μmol·L^{-1}) in ethanol or phosphate-buffered saline (PBS), the absorbance was taken at 30 °C exactly 1 min after initial mixing and up to 6 min. Appropriate solvent blanks were run in each test. Triplicate determinations were made for each standard or sample, and the percentage inhibition of absorbance was calculated and plotted as a function of Trolox concentration for the standard reference data. The antioxidant activity of samples was estimated at a minimum of three different concentrations within the range of the dose-response curve, and the mean value was derived as the TEAC and expressed as μmol·g^{-1} proteins. The half-inhibition IC_{50}, defined as the concentration of sample required to decrease the initial concentration of $ABTS^{\cdot+}$ by 50%, was calculated.

2.5. Statistical analysis

All data were expressed as means ± SD from at least three independent trials. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison test with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The level of confidence required for significance was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Preparation of casein hydrolysates

In order to prepare casein hydrolysates with a better radical scavenging activity, casein was independently hydrolyzed by papain with different hydrolysis times. The DH of casein hydrolysates was determined, ranging from 8% to 14%, as shown in Figure 1. The inhibition or scavenging activity of casein hydrolysates prepared on DPPH radical and $ABTS^{\cdot+}$ is shown in Figure 2. Casein hydrolysates prepared with hydrolysis time of 2 h, whose DH was 9.6% and the content of free amino groups on protein basis was about 981.7 μmol·g^{-1} proteins, had the highest scavenging activity on DPPH radical at 100 μg·mL^{-1}(with a inhibition up to 86%). If hydrolysis time of casein increased or decreased, viz. excessive hydrolysis and insufficient hydrolysis of casein, the scavenging activity of casein hydrolysates on DPPH radical would be impaired. The scavenging activity of casein hydrolysates on $ABTS^{\cdot+}$ shared similarity to that of casein hydrolysates on DPPH radical. When casein hydrolysates had a DH of 9.6%, its inhibition on $ABTS^{\cdot+}$ at 800 μg·mL^{-1} was also the highest (up to 87%). Casein hydrolysates with a DH other than 9.6% had lower scavenging activity on both radicals. Based on this fact, it could be expected that if casein hydrolysates of DH 9.6% were hydrolyzed or resynthesized further by papain to higher or lower DH during plastein reaction, the radical scavenging activity of casein plasteins on DPPH radical or $ABTS^{\cdot+}$ would be decreased. Therefore, casein hydrolysates of DH 9.6% were

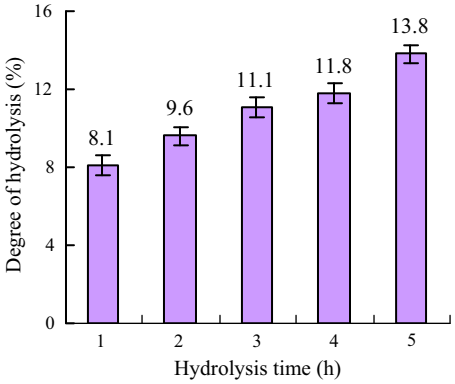

Figure 1. The degree of hydrolysis (DH) of casein hydrolysates at different hydrolysis times. Casein of 5 g was dissolved in 100 mL distilled water. Other hydrolysis conditions were as follows: pH 6.5, enzyme addition level 500 U·g^{-1} proteins and hydrolysis temperature 45 °C. The number of trial times was three.

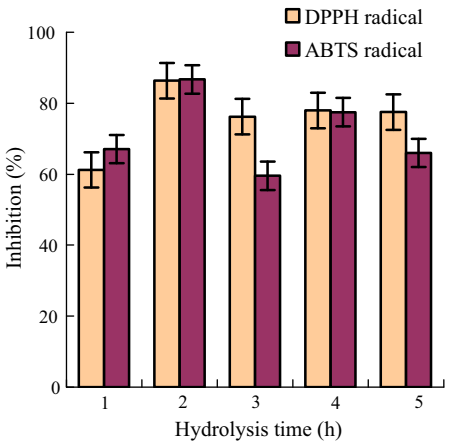

Figure 2. The inhibition of casein hydrolysates on DPPH radicals and ABTS$^{\cdot+}$ at different hydrolysis times. The inhibitions of casein on DPPH radicals and ABTS$^{\cdot+}$ were about 0% and 19.1 ± 1.0%, respectively. The concentrations of casein or casein hydrolysates used in DPPH radical and ABTS$^{\cdot+}$ inhibition were 100 and 800 μg·mL^{-1}, respectively. The number of trial times was three.

chosen as suitable substrates for papain-catalyzed plastein reaction, and the results of the radical scavenging activities of casein plasteins on two radicals could show the impact of papain-catalyzed plastein reaction.

Chiu and Kitts [5] found that the inhibition of casein phosphopeptides on ABTS$^{\cdot+}$ was 91.8% at 1.0 mg·mL^{-1}, 75.1% at 0.50 mg·mL^{-1}, 7.9% at 0.10 mg·mL^{-1} and 1.5% at 0.05 mg·mL^{-1}, respectively. Mao et al. [17] had hydrolyzed yak casein with various enzymes (pepsin, trypsin, Alcalase, flavozyme and papain), and the inhibition of hydrolysates on DPPH radical was found to be < 80% at 2.5 mg·mL^{-1}. It was also reported that when whey protein isolate (WPI) was hydrolyzed by Alcalase for 5 h, the prepared hydrolysates possessed the strongest antioxidant activity, and the inhibition of all WPI hydrolysates or fractions on DPPH radical ranged from 14.7% to 58.7% at 1 mg·mL^{-1} [21]. Compared to these results, casein hydrolysates of DH 9.6% prepared in our study showed a better ability to scavenge DPPH radical or ABTS$^{\cdot+}$, which was characterized by higher inhibition.

3.2. Optimal conditions of plastein reaction for casein hydrolysates

The matrix of the Box-Behnken design has been widely used in RSM and also applied in our study to obtain 20 combinations of reaction conditions for the plastein reaction of casein hydrolysates. The detailed combinations are depicted in Table II. The decrease of free amino groups in casein plasteins, which had been used in our previous study to show the reaction extent of casein plasteins during Alcalase-catalyzed plastein reaction [35], was evaluated and used as response. A second-order polynomial equation was established to show the effects of the variables on the

Table II. Central composite design matrix and results.

Experimental no.	Reaction temperature (X_1)/°C	Substrate concentration (X_2)/%	Reaction time (X_3)/h	The decrease of free amino groups (Y)/μmol·g^{-1} proteins
1	−1	−1	−1	10.87
2	1	−1	−1	−31.91
3	−1	1	−1	−16.23
4	1	1	−1	7.07
5	−1	−1	1	−6.79
6	1	−1	1	14.26
7	−1	1	1	−34.12
8	1	1	1	9.15
9	−1.68	0	0	−9.80
10	1.68	0	0	−20.64
11	0	−1.68	0	−8.60
12	0	1.68	0	18.43
13	0	0	−1.68	−7.74
14	0	0	1.68	−4.64
15	0	0	0	78.95
16	0	0	0	81.01
17	0	0	0	80.50
18	0	0	0	80.50
19	0	0	0	79.98
20	0	0	0	79.46

response in terms of linear, quadratic and cross-product terms. The predictive equation was obtained with coefficients exhibiting an R^2 value of 0.9725 (Tab. III). The equation obtained with the significant terms is given as equation (3):

$$\begin{aligned} Y = & -661.11466 + 12.90736X_1 \\ & + 16.16788X_2 + 49.11057X_3 \\ & + 0.091969X_1X_2 + 0.41895X_1X_3 \\ & - 0.18471X_2X_3 \quad - 0.32692{X_1}^2 \\ & - 0.17759{X_2}^2 - 4.71988{X_3}^2. \quad (3) \end{aligned}$$

According to the responses listed in Table III, response surface plots of papain-catalyzed plastein reaction for casein hydrolysates are shown in Figure 3, which indicates the effects of reaction temperature (X_1), substrate concentration (X_2) and reaction time (X_3) on the decrease of free amino groups in casein plasteins. The optimal conditions of plastein reaction of casein hydrolysates were generated and listed as follows: papain addition level fixed at 500 U·g^{-1} proteins, reaction temperature 30 °C, reaction time 5.6 h and concentration of casein hydrolysates 50% (w·v^{-1}). Under these conditions, the highest decrease of free amino groups in casein plasteins was predicted to be 80.01 μmol·g^{-1} proteins. The actual experimental results showed that the decrease of free amino groups in casein plasteins was 80.06 μmol·g^{-1} proteins (mean of six results), indicating a good fit between predicted and experimental result.

Williams et al. [33] had applied pepsin-catalyzed plastein reaction to mycoprotein

Table III. The one-way ANOVA for the fitted quadratic polynomial model.

Source	Sum of square	*df*	Mean square	*F* value	*P* value[a]
Model	33 495.96	9	3721.77	39.30	< 0.0001
Lack of fit	944.10	5	188.82	326.91	< 0.0001
Pure error	2.89	5	0.58		
Corrected total	34 442.96	19			

R^2 = 0.9725 and R^2_{Adj} = 0.9478.
[a] Coefficients with *P* values < 0.0001 indicate that they are significant.

and found that plastein yield increased over the substrate concentration ranging from 11% to 43% ($w \cdot w^{-1}$), while the rate of plastein formation was higher at 65 °C. Sukan and Andrews [31] found that the decrease of free amino groups in plastein reaction products increased with rising reaction temperature from 10 to 50 °C. Ono et al. [20] reported in a study that substrate concentration that gave the highest plastein yield was 30% ($w \cdot w^{-1}$) when the plastein reaction was performed at 55 °C for 24 h. In comparison with these studies, our study results shared some similarity, such as substrate concentration, but had a lower reaction temperature.

Primary analysis results from size exclusion chromatography showed the peptide profiles of casein hydrolysates and the casein plasteins prepared, and are given in Figure 4. Bovine serum albumin, cytochrome *c*, insulin, oxidized L-glutathione or L-tyrosine was eluted at 10.6, 16.0, 17.1, 20.6 and 22.9 min, respectively. The main peptides of casein hydrolysates were eluted at 16.0 to 22.2 min, with molecular weight calculated in the range of 0.2 to 6.0 $kg \cdot mol^{-1}$ (Fig. 4a). The main peptides of the casein plasteins prepared showed some differences to those of casein hydrolysates (Fig. 4b), with molecular weight calculated in the range of 0.2–9.0 $kg \cdot mol^{-1}$. Meanwhile, it could be seen in Figure 4b that a peak with a molecular weight larger than 66.2 $kg \cdot mol^{-1}$ was monitored, indicating the formation of plasteins in the casein plasteins prepared.

3.3. Antioxidant activity of the casein plasteins

The TEAC and IC_{50} of casein hydrolysates and the six casein plasteins prepared with different reaction extents were evaluated for two radicals, DPPH radical and $ABTS^{\cdot+}$. The results are given in Table IV. All casein plasteins had higher antioxidant activity than casein hydrolysates ($P < 0.05$). The scavenging activity of casein plasteins on DPPH radical had a seven- or ninefold increase compared to that of casein hydrolysates (TEAC increased from 10.45 $mmol \cdot g^{-1}$ proteins to 73.41 or 90.31 $mmol \cdot g^{-1}$ proteins), while the scavenging activity of casein plasteins on $ABTS^{\cdot+}$ doubled (TEAC increased from 8.33 $\mu mol \cdot g^{-1}$ proteins to 15.09 or 16.45 $\mu mol \cdot g^{-1}$ proteins). Further statistical analysis indicated that the six casein plasteins had similar antioxidant activity on DPPH radical because their TEAC were not significantly different, but they showed different antioxidant activity on $ABTS^{\cdot+}$, which needs to be further investigated. The results clearly demonstrated that the plastein reaction catalyzed by papain could improve antioxidant activity of the casein plasteins and suggested the potential application of plastein reaction in the preparation of antioxidant peptides with higher activity.

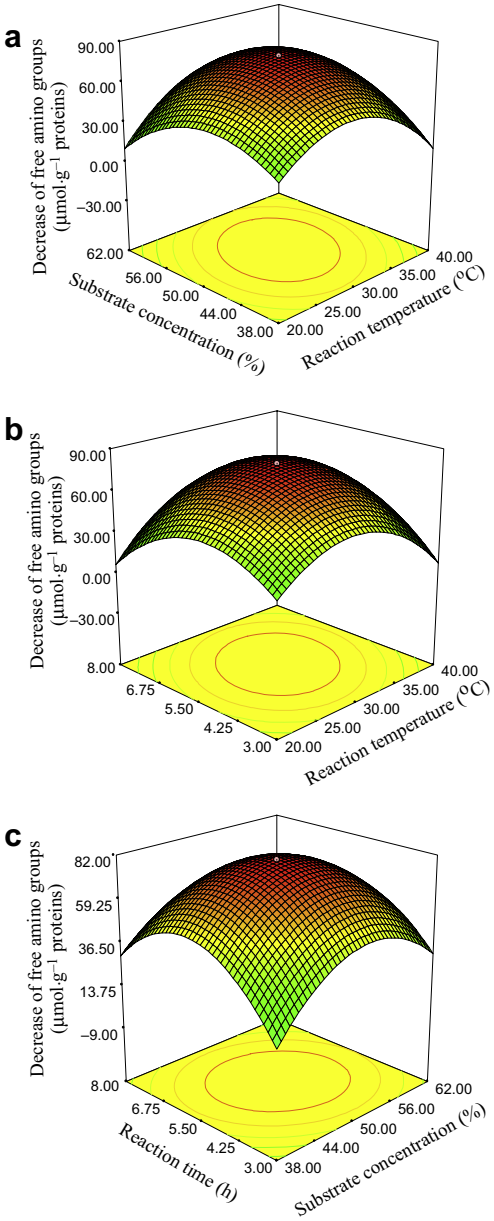

Figure 3. Response surface plots for the decrease of free amino groups in casein plasteins (Y) as a function of reaction temperature (X_1), substrate concentration (X_2) and reaction time (X_3). (a) response surface of substrate concentration and reaction temperature at $X_3 = 0$; (b) response surface of reaction time and temperature at $X_2 = 0$; and (c) response surface of reaction time and substrate concentration at $X_1 = 0$.

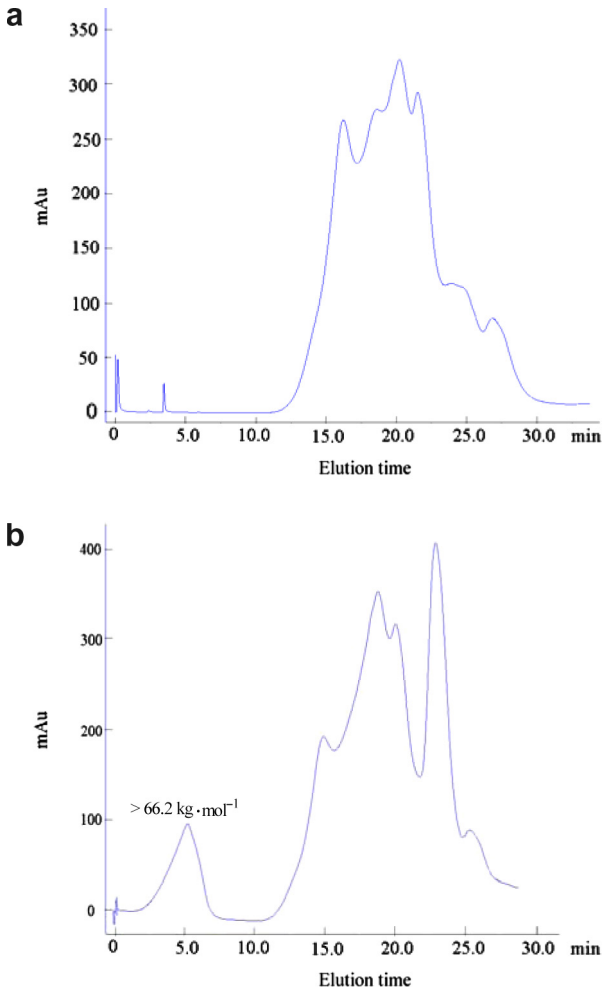

Figure 4. Chromatographic profiles of casein hydrolysates (a) or a casein plastein prepared with a decrease of free amino groups of 43.5 μmol·g^{-1} proteins (b) analyzed by size exclusion chromatography with Superdex-75 column. The analysis was performed at a flow rate of 0.9 mL·min^{-1}with 0.05 mol·L^{-1} sodium acetate-0.1 mol·L^{-1} NaCl buffer (pH 7.3) and monitored at 280 nm.

This interesting approach may also be applied to other protein hydrolysates, but the effect of plastein reaction needs to be confirmed.

Gómez-Ruiz et al. [10] had investigated the hydrolysis of different casein fractions with gastrointestinal enzymes and evaluated the antioxidant activity of hydrolysates by the ABTS^{+} assay. They found that the TEAC of α_s-casein fraction was 1.76 mg·mL^{-1} at 1 mg·mL^{-1}, while the TEAC of κ-casein fraction was 1.5 mg·mL^{-1}. Hernández-Ledesma et al. [12] reported that β-casein peptide (WSVPQPK) separated from human

Table IV. The scavenging activities of casein hydrolysates and six casein plasteins on DPPH radical and $ABTS^{\cdot+}$.

Samples	The decrease of free amino groups ($\mu mol \cdot g^{-1}$ proteins)	Scavenging activity on DPPH radical[e]		Scavenging activity on $ABTS^{\cdot+}$ [f]	
		TEAC ($mmol \cdot g^{-1}$ proteins)	IC_{50} ($\mu g \cdot mL^{-1}$)	TEAC ($\mu mol \cdot g^{-1}$ proteins)	IC_{50} ($\mu g \cdot mL^{-1}$)
Casein hydrolysates[g]	0	10.45 ± 0.64[b]	1854.46 ± 109.54	8.33 ± 1.14[d]	965.52 ± 56.77
Casein plasteins 1	7.07	76.50 ± 0.30[a]	253.29 ± 0.98	16.37 ± 1.97[a]	491.23 ± 24.58
Casein plasteins 2	10.87	73.41 ± 1.13[a]	263.95 ± 4.02	16.17 ± 2.29[c]	497.37 ± 52.74
Casein plasteins 3	18.43	77.47 ± 0.27[a]	250.14 ± 0.86	16.45 ± 1.21[a]	489.05 ± 7.70
Casein plasteins 4	78.95	86.58 ± 0.57[a]	223.81 ± 1.48	15.51 ± 0.12[b]	518.52 ± 1.60
Casein plasteins 5	80.50	84.73 ± 2.16[a]	228.72 ± 5.91	15.09 ± 0.62[b]	538.54 ± 9.01
Casein plasteins 6	81.01	90.31 ± 1.42[a]	214.57 ± 3.36	15.23 ± 1.09[b]	528.30 ± 15.94

[a–d] Data in the same column with different superscript letters differ significantly ($P < 0.05$) by Duncan's multiple range tests.
[e] The final concentration of casein hydrolysates or six casein plasteins for the assay of scavenging activity on DPPH radical was 100 $\mu g \cdot mL^{-1}$.
[f] The final concentration of casein hydrolysates or six casein plasteins for the assay of scavenging activity on $ABTS^{\cdot+}$ was 100 $\mu g \cdot mL^{-1}$.
[g] The content of free amino groups was 981.7 $\mu mol \cdot g^{-1}$ proteins and degree of hydrolysis was 9.6%.

milk hydrolysates by pepsin and pancreatin had TEAC 1.297 μmol Trolox equivs μmol^{-1} peptide by the $ABTS^{\cdot+}$ assay. Also, the study by Suetsuna et al. [30] had revealed that a peptide separated from casein hydrolysates had strong free radical scavenging activity on DPPH radical, and its IC_{50} was 98 $\mu mol \cdot L^{-1}$ (equal to 2.45 $mg \cdot mL^{-1}$). Nakajima et al. [18] had studied the hydrolysis of the muscle of four fish species with three enzymes (pepsin, pancreatin or thermolysin) and found that fish muscle hydrolysates by pancreatin possessed the highest DPPH radical scavenging activity than other hydrolysates, with IC_{50} ranging from 4.88 to 9.91 $mg \cdot mL^{-1}$. Compared to these results, it could be seen that casein plasteins prepared in our study had a better antioxidant activity, characterized by the lower value of IC_{50} (< 0.27 $mg \cdot mL^{-1}$ for DPPH radical or 0.54 $mg \cdot mL^{-1}$ for $ABTS^{\cdot+}$).

As indicated in Figure 2, an excessive or insufficient hydrolysis of casein would impair the radical scavenging activity of casein hydrolysates, which is contradictory to the results obtained for casein plasteins that had improved radical scavenging activities as shown in Table IV. This conflict might be explained by the possible peptide modifications brought about by the plastein reaction in casein plasteins. Therefore, further study is needed to reveal the actual changes in the peptide composition and peptide distribution in casein plasteins after a plastein reaction at the molecular level, especially those peptides formed by condensation or further hydrolysis.

4. CONCLUSIONS

Casein was hydrolyzed at selected conditions by papain for 2 h to prepare casein hydrolysates with a DH of 9.6%. Casein hydrolysates were then subjected to papain-catalyzed plastein reaction to prepare casein plasteins with higher radical scavenging activity. The optimal conditions of plastein reaction were studied by RSM with the decrease of free amino groups of casein plasteins as response, and the obtained condition parameters were as follows: the addition level of papain fixed at 500 $U \cdot g^{-1}$ proteins, reaction temperature 30 °C, the concentration of casein hydrolysates 50% and reaction time 5.6 h. With these conditions, the predicted decrease of free amino groups of casein plasteins was 80.01 $\mu mol \cdot g^{-1}$ proteins, very close to the actual result of 80.06 $\mu mol \cdot g^{-1}$ proteins. The results from size exclusion chromatography confirmed that plasteins with molecular weight large than 66.2 $kg \cdot mol^{-1}$ were generated in the casein plasteins prepared. The results showed that casein plasteins had a better radical scavenging activity than that of casein hydrolysates, which was characterized by their lower IC_{50} value. The scavenging activity of the casein plasteins prepared with different reaction extents on DPPH radical had a seven- or ninefold increase compared to that of casein hydrolysates, while the scavenging activity of the casein plasteins on $ABTS^{\cdot+}$ doubled. Plastein reaction might have served as an effective approach to prepare antioxidant peptides with higher activity from proteins.

Acknowledgements: The authors gratefully acknowledge Mr. Axel Rau for his kindly and valuable help in paper revision, and the financial support from the National High Technology Research and Development Program ("863" Program) of China (No. 2006AA10Z324) and National Natural Science Foundation of China (No. 30972132).

REFERENCES

[1] Adler-Nissen J., Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid, J. Agric. Food Chem. 27 (1979) 1256–1261.

[2] Andrews A.T., Alichanidis E., The plastein reaction revisited: evidence for a purely aggregation reaction mechanism, Food Chem. 35 (1990) 243–261.

[3] Bougatef A., Hajji M., Balti R., Lassoued I., Triki-Ellouz Y., Nasri M., Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases, Food Chem. 114 (2009) 1198–1205.

[4] Chang C.Y., Wu K.C., Chiang S.H., Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates, Food Chem. 100 (2007) 1537–1543.

[5] Chiu S.C.K., Kitts D.D., Antioxidant characterization of caseinphosphopeptides from bovine milk, ACS Symposium Series 871 (2004) 279–289.

[6] Chu K.T., Ng T.B., First report of a glutamine-rich antifungal peptide with immunomodulatory and antiproliferative activities from family Amaryllidaceae, Biochem. Biophy. Res. Comm. 325 (2004) 167–173.

[7] Church F.C., Swaisgood H.E., Porter D.H., Catignani G.L., Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins, J. Dairy Sci. 66 (1983) 1219–1227.

[8] Combes D., Lozano P., α-Chymotrypsin in plastein synthesis. Influence of water activity, Annals of the New York Academy of Sciences 672 (Enzyme Engineering XI), New York, USA, 1993.

[9] Fiat A.M., Migliore-Samour D., Jolles P., Drouet L., Sollier C.B.D., Caen J., Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities, J. Dairy Sci. 76 (1993) 301–310.

[10] Gómez-Ruiz J.A., López-Expósito I., Pihlanto A., Ramos M., Recio I., Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC–MS/MS, Eur. Food Res. Technol. 227 (2008) 1061–1067.

[11] Hernández M.J.M., Domingo E.B., Camañas R.M.V., Alvarez-Coque M.C.G., Evaluation of the proteolysis degree with the *o*-phthalaldehyde/N-acetyl-L-cysteine reagent, Fresenius' J. Anal. Chem. 338 (1999) 62–65.

[12] Hernández-Ledesma B., Quirós A., Amigo L., Recio I., Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin, Int. Dairy J. 17 (2007) 42–49.

[13] IDF, Milk. Determination of the nitrogen (Kjeldahl method) and calculation of the crude protein content, IDF Standard 20B, International Dairy Federation, Brussels, Belgium, 1993.

[14] Kim G.N., Jang H.D., Kim C.I., Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase, Food Chem. 53 (2007) 1359–1365.

[15] Kitts D.D., Antioxidant properties of casein phosphopeptides, Trends Food Sci. Technol. 16 (2005) 549–556.

[16] López-Fandiño R., Otte J., van Camp J., Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity, Int. Dairy J. 16 (2006) 1277–1293.

[17] Mao X.Y., Wu S.J., Fan J.B., Xu S.P., Wang J., Gu J.N., Ren F.Z., Scavenging activity of enzymatic hydrolysate of yak casein on DPPH radical, Modern Food Sci. Technol. 24 (2007) 624–626, 634.

[18] Nakajima K., Yoshie-Stark Y., Ogushi M., Comparison of ACE inhibitory and DPPH radical scavenging activities of fish muscle hydrolysates, Food Chem. 114 (2009) 844–851.

[19] Nsimba R.Y., Kikuzaki H., Konishi Y., Antioxidant activity of various extracts and fractions of *Chenopodium quinoa* and *Amaranthus* spp. seeds, Food Chem. 106 (2008) 760–766.

[20] Ono S., Kasai D., Sugano T., Ohba K., Takahashi K., Production of water soluble antioxidative plastein from squid hepatopancreas, J. Oleo Sci. 53 (2004) 267–273.

[21] Peng X.Y., Xiong Y.L., Kong B.H., Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance, Food Chem. 113 (2009) 196–201.

[22] Pihlanto L.A., Antioxidative peptides derived from milk proteins, Int. Dairy J. 16 (2006) 1306–1314.

[23] Pihlanto L.A., Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides, Trends Food Sci. Technol. 11 (2001) 347–356.

[24] Re R., Pellegrini N., Proteggente A., Yang M., Rice-Evans C., Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Rad. Bio. Med. 26 (1999) 1231–1237.

[25] Rival S.G., Boeriu C.G., Wichers H.J., Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to

lipoxygenase inhibition, J. Agric. Food Chem. 49 (2001) 295–302.

[26] Rival S.G., Fornaroli S., Boeriu C.G., Wichers H.J., Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties, J. Agric. Food Chem. 49 (2001) 287–294.

[27] Rossini K., Noreña C.P.Z., Cladera-Olivera F., Brandelli A., Casein peptides with inhibitory activity on lipid oxidation in beef homogenates and mechanically deboned poultry meat, LWT-Food Sci. Technol. 42 (2009) 862–867.

[28] Sakanaka S., Tachibana Y., Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates, Food Chem. 95 (2006) 243–249.

[29] Sarath G., De La Motte R.S., Wagner F.W., Protease assay methods, in: Beynon R.J., Bond J.S. (Eds.), Proteolytic Enzymes, A Practical Approach, IRL Press, Oxford, UK, 1989, pp. 25–55.

[30] Suetsuna K., Ukeda H., Ochi H., Isolation and characterization of free radical scavenging activity peptides derived from casein, J. Nutr. Biochem. 11 (2000) 28–131.

[31] Sukan G., Andrews A.T., Application of the plastein reaction to caseins and to skim milk powder I. Protein hydrolysis and plastein formation, J. Dairy Res. 49 (1982) 265–278.

[32] Sukan G., Andrews A.T., Application of the plastein reaction to caseins and to skim milk powder II. Chemical and physical properties of the plasteins, J. Dairy Res. 49 (1982) 279–293.

[33] Williams R.J., Brownsell V.L., Andrews A.T., Application of the plastein reaction to mycoprotein: I. Plastein synthesis, Food Chem. 72 (2001) 329–335.

[34] Yamashita M., Arai S., Fujimaki M., Plastein reaction for food protein improvement, J. Agric. Food Chem. 24 (1976) 1100–1104.

[35] Zhao X.H., Li Y.Y., An approach to improve ACE inhibitory activity of casein hydrolysates with plastein reaction catalyzed by Alcalase, Eur. Food Res. Technol. 229 (2009) 795–805.