# Properties of Casein Hydrolysate as Affected by Plastein Reaction in Ethanol-Water Medium

*Yang ZHANG[1] and Xin-Huai ZHAO[1,2]*

*[1]Key Laboratory of Dairy Science of Ministry of Education, Northeast Agricultural University, Harbin, P.R. China; [2]Department of Food Science, Northeast Agricultural University, Harbin, P.R. China*

**Abstract**

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Casein hydrolysate with *in vitro* ACE-inhibitory activity of 44.4% at 0.3 mg/ml was generated from casein by Alcalase and modified by the Alcalase-catalysed plastein reaction in an ethanol-water medium. Eight treated hydrolysates were prepared using the reaction time of 1–8 h under ethanol or substrate concentration, Alcalase addition and reaction temperature of 56.8% (v/v) or 56.8% (w/v), 8.4 kU/g peptides and 37.5°C, respectively. Most of the treated hydrolysates showed enhanced ACE-inhibition compared to casein hydrolysate, and a reaction time of 4 h brought about the highest ACE-inhibition. All treated hydrolysates had lower zinc- or calcium-chelation but slightly higher iron(II)-chelation than casein hydrolysate, and a reaction time of 4 or 2 h could grant the treated hydrolysates the highest zinc- or calcium-chelation. Kinetic evaluation indicated that casein hydrolysate and two treated hydrolysates were competitive inhibitors to ACE. ACE-inhibition of these evaluated hydrolysates originated from themselves but was uncorrelated with their zinc-chelation, while their $CaCO_3$ precipitation inhibition was clearly correlated with their measured calcium-chelation ($P < 0.05$).

**Keywords:** angiotensin converting enzyme; casein; hydrolysate; metal chelation; plastein reaction

Functional properties of food proteins can be modified by specific treatments, such as enzymatic modification. Enzymatic proteolysis has been widely used for food proteins to obtain protein hydrolysates with helpful bioactivities (Haque & Chand 2008; Ren *et al.* 2010; Li & Zhao 2011), or to improve their functional properties (Surówka & Żmudziński 2004; Stangierski *et al.* 2012). The plastein reaction is a protease catalysed reaction that results in the formation of new peptides (Yamashita *et al.* 1976), and was used in the past to modify food proteins for the improvement of functional properties (Kolakowski *et al.* 1997) or nutritive values (Yamashita *et al.* 1979). In recent years, the plastein reaction was also used as an interesting way to enhance *in vitro* ACE-inhibition (Zhao & Li 2009; Zhao & Zhao 2013) and antioxidation (Zhao *et al.* 2010) of protein hydrolysates.

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) converts inactive decapeptide angiotensin I by cleaving a dipeptide from C-terminal into octapeptide angiotensin II, which has a tendency to increase the blood pressure (Li *et al.* 2004). Food protein-derived peptides are suggested as potential alternatives to ACE inhibitor drugs (Ni *et al.* 2012), and now are well-known as so-called ACE-inhibitory peptides. Some studies evaluated ACE-inhibition of protein hydrolysates (Li & Zhao 2011), characterised the primary structure of ACE-inhibitory peptides (Jang & Lee 2005), and investigated the relationship between the peptide structure and potential activities (Alemán *et al.*

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2011). Casein, a major protein fraction of milk, is considered as one of the good sources of ACE-inhibitory peptides (Fitzgerald *et al.* 2004). Protein hydrolysates also have other beneficial properties, such as chelation on nutritious metal ions. It is important for the protein hydrolysates or peptides to prevent the formation of metal precipitates of lower bioavailability. Strong chelating activity of food proteins or peptides towards calcium (Bao *et al.* 2007), iron (Kim *et al.* 2007a), and zinc (Wang *et al.* 2011) could lead to enhanced bioavailability (Chaud *et al.* 2002).

The plastein reaction shows an impact on ACE-inhibition of casein hydrolysate; however, whether it also has an influence on metal chelation has not been well-studied so far. In the present study, a casein hydrolysate was prepared from casein by Alcalase and then modified by the Alcalase-catalysed plastein reaction in an ethanol-water medium. Casein hydrolysate and some treated hydrolysates of different reaction extents were evaluated for their chelating activities towards zinc, calcium and iron(II), and inhibitory activities towards ACE or $CaCO_3$ precipitation *in vitro*, to reflect the impact of the plastein reaction on metal chelation as well as ACE or $CaCO_3$ precipitation inhibition. A potential relationship between the evaluated zinc-chelation and ACE-inhibition, or calcium-chelation and $CaCO_3$ precipitation inhibition of these treated hydrolysates, was revealed by correlation analysis. ACE-inhibition kinetics of some hydrolysates was assayed the using Michaelis-Menten equation. The aim was to provide more information about the impact of plastein reaction on these properties of casein hydrolysate.

## MATERIAL AND METHODS

***Material***. Caseinate, hippuryl-histidyl-leucine (HHL), rabbit lung acetone powder (as ACE source), and ferrozine were from Sigma-Aldrich Co. (St. Louis, USA). *o*-Cresolphthalein complexone (o-CPC) was from Aladdin-reagent Co. Ltd. (Shanghai, China). Alcalase (118 kU/ml) was from Novozyme China (Tianjin, China). All other chemicals and reagents used were of analytical grade. Highly purified water was prepared by Milli-Q PLUS (Millipore Corporation, Billerica, USA) and used to prepare all buffers and solutions.

***Preparation and modification of casein hydrolysate***. Casein hydrolysate was prepared by the procedure of Zhao and Li (2009), and then modified by the Alcalase-catalysed plastein reaction in an ethanol-water medium for 1–8 h at the selected conditions, which were obtained from a previous study using response surface methodology with central composite design (CCD). In the CCD, reaction time was fixed at 6 h, and a decrease of free amino groups of the treated hydrolysates was used as the responses. Ethanol or substrate concentration, Alcalase addition and reaction temperature were investigated in five levels, among which zero levels were set at 40.0% (v/v) or 40.0% (w/v), 5.0 kU/g peptides and 30.0°C, with step levels of 10.0% (v/v) or 10.0% (w/v), 2.0 kU/g peptides and 10.0°C, respectively. After reaction, eight treated hydrolysates prepared thereof were heated at 95°C for 15 min to inactivate Alcalase, lyophilized, ground into fine powder (particle size less than 75 μm), and stored at –20°C for future study.

***Assaying of protease activity and content of free amino groups***. Protease activity was assayed according to the method of Sarath *et al.* (2001). Protein content of all samples was measured by the Kjeldahl method (IDF 1993). Content of free amino groups of all samples was measured by *o*-pthaldialdehyde (OPA) assay (Church *et al.* 1983) with a UV-2401 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and used to calculate the degree of hydrolysis (DH) of hydrolysates described by Adler-Nissen (1979).

***Evaluation of ACE-inhibitory activity and inhibition kinetics***. ACE-inhibitory activity was measured by a spectrophotometric method used by Zhao and Zhao (2013). Lineweaver-Burk plots were applied to measure Michaelis-Menten equation and inhibition kinetics (Barbana & Boye 2011). Inhibition constants ($K_i$) were determined from the Lineweaver-Burk plots, and calculated by the equation (Sun *et al.* 2011) as below:

$$K_i = \frac{[S]}{(K'_m/K_m) - 1}$$

where:

[S] – concentration of peptides added (mg/ml)

$K_m$, $K'_m$ – Michaelis-Menten constant in the absence or presence of an inhibitor at fixed concentration [I]

***Assaying of chelating activity towards zinc, iron, and calcium***. Zinc-chelating activity was measured according to the method of Wang *et al.* (2012). Iron(II)-chelating activity was assayed

by a reported method (Li *et al.* 2011), and EDTA at a final concentration of 10 μmol/l was used as positive control. Calcium-chelating activity was assayed by the method of Bao *et al.* (2007). The amount of calcium in the supernatants was determined by the method of Lorentz (1982) with some modifications. Standard calcium or sample solution of 100 μl was mixed with 1 ml chromogenic agent (0.1 mmol/l o-CPC, 8 mmol/l 8-hydroxyquinoline, 5 mol/l urea, 43 mmol/l acetic acid, and 25% ethanol) and 10 ml water. After incubation at room temperature for 20 min, the absorbance was measured at 570 nm in the spectrophotometer, and used to calculate calcium content using the obtained standard curve.

***Evaluation of inhibition towards calcium precipitation.*** Inhibition towards $CaCO_3$ precipitation was evaluated by the method of Inoue *et al.* (2001) with some modifications. $CaCO_3$ precipitation was assayed by recording the turbidity of a solution containing $NaHCO_3$ solution (0.22 mol/l) of 1 ml and sample solution (10 mg/ml) of 0.4 ml, after $CaCl_2$ solution (0.22 mol/l) of 1 ml was added to the mixed solution. Turbidity changes for the resultant solution were measured for 5 min at 570 nm by the spectrophotometer, and used to reflect the formation of $CaCO_3$ precipitation.

***Statistical analysis.*** All experiments or analyses were performed in triplicate. The data were expressed as means ± standard deviations. Differences between the means of multiple groups were evaluated by one-way analysis of variance (ANOVA) with Duncan's multiple comparison tests. Bivariate correlations procedure was used to calculate Pearson's correlation coefficient ($R^2$). SPSS 16.0 software (SPSS Inc., Chicago, USA) was used to analyse the data.

## RESULTS AND DISCUSSION

### Plastein reaction of casein hydrolysate and its impact on ACE-inhibition

Casein hydrolysate prepared in the present study had a DH of 11.6% and *in vitro* ACE-inhibitory activity of 44.4% at 0.3 mg/ml. For its plastein reaction in an ethanol-water medium, the suitable ethanol or substrate concentration, Alcalase addition and reaction temperature selected from the CCD were 56.8% (v/v) or 56.8% (w/v), 8.4 kU/g peptides and 37.5°C, respectively. Under these conditions, eight treated hydrolysates with different reaction extents were prepared using reaction times of 1–8 h, respectively. A decrease of free amino groups and ACE-inhibitory activities of these treated hydrolysates are listed in Table 1. The decrease of free amino groups of the treated hydrolysates showed an increasing trend as the reaction time prolonged from 1 h to 8 h, implying that peptide condensation occurred during the plastein reaction. The treated hydrolysates exhibited higher ACE-inhibitory activities than casein hydrolysate (46.5–62.5% vs. 44.4%) as the reaction time increased from 1 to 4 h; after then, they showed decreased activities (35.6–56.4% vs. 62.5%) as the reaction time increased from 5 h to 8 hours. A reaction time of 4 h conferred the highest activity on the treated hydrolysate (TCH4). The treated hydrolysate (TCH8) by a reaction time of 8 h showed an activity of 35.6%. This value was the lowest one among all treated hydrolysates, even lower than that of casein hydrolysate. This fact means that only the plastein reaction to a lower extent had a beneficial impact on the ACE-inhibition of casein hydrolysate.

ACE-inhibition of the peptides is correlated with their primary peptide structure, as the peptides will bind to the active site of ACE (Li *et al.* 2004). It seems that amino acids (esp. hydrophobic amino acids) at each of the three C-terminal positions are very important to the ACE-inhibition of the peptides (López-Fandiño *et al.* 2006). During the plastein reaction, some new peptides are formed as a result of condensation or transpeptidation (Yamashita *et al.* 1976). The treated hydrolysates (e.g. TCH1-4) thus would contain some new peptides generated from the plastein reaction with different amino acid sequences from their parent peptides, and thus would have higher inhibition towards ACE. ACE-inhibitory peptides usually contain 2–12 amino acid residues (Li *et al.* 2004). When the plastein reaction was carried out for a too long time, the peptides in the treated hydrolysate were too much condensed (i.e. the generated peptides had a longer peptide chain). Undesirably, this would give rise to the blocked active sites of the ACE-inhibitory peptides, and thus lower activity of the treated hydrolysate (e.g. TCH8). A similar result was found out in the previous study (Zhao & Zhao 2013), in which the too much long plastein reaction of casein hydrolysate in a propanol-water medium resulted in the lower ACE-inhibition of the obtained products. The plastein reaction is a

Table 1. ACE-inhibitory and zinc-chelating activities of casein hydrolysate and eight treated hydrolysates

| Samples | Reaction time (h) | Decrease of free amino groups (μmol/g peptides) | ACE-inhibitory activity (%) | Zinc-chelating activity (mg/g peptides) |
|---|---|---|---|---|
| CH | 0 | 0 | $44.4 \pm 0.3^{b}$ | $4.22 \pm 0.04^{D}$ |
| TCH1 | 1 | $156.2 \pm 2.2^{A}$ | $46.5 \pm 3.2^{b}$ | $1.97 \pm 0.35^{A}$ |
| TCH2 | 2 | $223.7 \pm 2.6^{B}$ | $55.4 \pm 2.2^{c}$ | nd |
| TCH3 | 3 | $251.4 \pm 7.7^{C}$ | $53.9 \pm 3.6^{c}$ | $3.28 \pm 0.54^{B}$ |
| TCH4 | 4 | $275.7 \pm 1.3^{D}$ | $62.5 \pm 0.3^{d}$ | $3.86 \pm 0.31^{CD}$ |
| TCH5 | 5 | $295.9 \pm 2.3^{E}$ | $56.4 \pm 1.5^{c}$ | $3.62 \pm 0.18^{BC}$ |
| TCH6 | 6 | $300.9 \pm 2.1^{E}$ | $52.1 \pm 3.0^{c}$ | $3.50 \pm 0.25^{BC}$ |
| TCH7 | 7 | $302.1 \pm 1.3^{E}$ | nd | nd |
| TCH8 | 8 | $340.4 \pm 8.2^{F}$ | $35.6 \pm 4.8^{a}$ | $3.37 \pm 0.13^{B}$ |

CH – casein hydrolysate; TCH – plastein reaction treated hydrolysates; ACE – inhibitory activity was determined at a peptide concentration of 0.3 mg/ml; decrease of free amino groups was calculated by subtracting the content of free amino groups of TCH from that of CH; values with different letters as superscripts in the same column indicate that one-way ANOVA of the mean values is different significantly ($P < 0.05$); nd – not detected

kinetically driven reversal of the protein hydrolysis (Yamashita *et al.* 1976). This means that water will have a significant influence on the extent of plastein reaction. Our previous studies revealed that the plastein reaction of casein hydrolysate in water and propanol-water medium for 6 h or 8 h would result in the decrease of free amino groups of the treated products about 180 and 350–400 mmol/g peptides, respectively (Zhao & Li 2009; Zhao & Zhao 2013). It indicated that the incorporation of propanol into water was responsible for the greater decrease of free amino groups of the treated products. Propanol added at 58.5% (v/v) decreased the water concentration (i.e. water activity) of the reaction system, shifted the reaction equilibrium towards condensation, and finally brought about a greater decrease of free amino groups. In the present study, ethanol as part of the reaction solvent was added into the reaction system at a level of 56.8% (v/v), thus causing a similar impact on the plastein reaction as propanol did, leading to the decrease of free amino groups of TCH6–8 about 300–340 mmol/g peptides (Table 1).

### Chelating activity of treated casein hydrolysates towards three metal ions

Chelating activities of casein hydrolysate and some treated hydrolysates towards three metal ions, zinc, calcium and iron (II), are listed in Tables 1 and 2.

Both casein hydrolysate and the treated hydrolysates were able to bind zinc. Zinc-chelating activities of the treated hydrolysates were 1.97 to 3.86 mg/g peptides, markedly lower ($P < 0.05$) than that of casein hydrolysate (4.22 mg/g peptides) (Table 1). The plastein reaction extent showed an impact on the binding reaction between the treated hydrolysate and zinc. As the plastein reaction time increased from 1 h to 4 h, zinc-chelating activities of the treated hydrolysates were enhanced; after then, a decreasing trend of zinc-chelating activities

Table 2. Calcium- and iron(II)-chelating activities of EDTA, casein hydrolysate, and six treated hydrolysates

| Samples | Calcium-chelating activity (mg/g peptides) | Iron(II)-chelating activity (%) |
|---|---|---|
| EDTA | nd | $57.7 \pm 0.3^{d}$ |
| CH | $10.48 \pm 0.41^{D}$ | $52.6 \pm 0.4^{a}$ |
| TCH1 | $7.81 \pm 0.21A^{B}$ | $53.3 \pm 0.4^{a}$ |
| TCH2 | $8.60 \pm 0.18^{C}$ | $54.8 \pm 0.3b^{c}$ |
| TCH3 | $8.23 \pm 0.24^{BC}$ | $55.5 \pm 0.6^{c}$ |
| TCH4 | $8.04 \pm 0.15^{B}$ | $54.6 \pm 0.5^{bc}$ |
| TCH6 | $7.88 \pm 0.31^{AB}$ | $54.3 \pm 0.4^{b}$ |
| TCH8 | $7.51 \pm 0.05^{A}$ | $53.2 \pm 0.1^{a}$ |

CH – casein hydrolysate; TCH – plastein reaction treated hydrolysates; final peptide concentration of CH or TCH used in the analysis was 100 mg/ml, while that of EDTA was 10 mmol/l; values with different letters as superscripts in the same column indicate that one-way ANOVA of the mean values is different significantly ($P < 0.05$); nd – not detected

was observed. This change profile of zinc-chelation of the treated hydrolysates was similar to that of ACE-inhibition. The applied plastein reaction accounted for this irregular change in zinc-chelating activity, as it led to the treated hydrolysates with less free amino groups (i.e. lower DHs). This result was consistent with the result reported by Wang *et al.* (2011), who found that enzymatic hydrolysis led to the lower zinc-binding activity of the obtained yak casein hydrolysates (more free amino groups) than native yak casein (less free amino groups). Another support was found in a recent study in which the zinc-binding capacity of a zinc-binding peptide from oyster protein hydrolysate was found to be impacted by *in vitro* simulated digestion of three proteases (Chen *et al.* 2013). The unfavourable impact of proteolysis (or plastein reaction) on the zinc-binding capacity of a peptide (or protein hydrolysates) resulted from the damage to the original spatial structure of the zinc-binding peptides, which could form stable complexes with metal ions (Harding *et al.* 2010).

The correlation analysis showed that ACE-inhibitory activities of casein hydrolysate and the treated hydrolysates were poorly correlated with their corresponding zinc-chelating activities ($R^2$ = 0.195, $P$ > 0.05). This fact implied that the detected ACE-inhibition originated directly from these samples themselves, and did not depend on their zinc-chelation. ACE is a zinc metalloenzyme and zinc at active sites is essential (Bünning & Riordan 1985). Binding of inhibitors to the zinc in ACE can decrease ACE activity (Haque & Chand 2008). It has been indicated that competitive binding of the peptides to the active sites of ACE can prevent substrate binding and thus result in ACE-inhibition (Cheung *et al.* 1980; Rao *et al.* 2012). This conclusion gave a support to the present result, i.e. the binding of these hydrolysate to ACE (but not to the zinc in ACE) led to ACE-inhibition. Many researchers have found that some protein hydrolysates or peptides were competitive inhibitors to ACE. Some examples will be given in the forthcoming section of this paper, providing more supports to the present result.

As shown in Table 2, the treated hydrolysates had lower calcium-chelating activities than casein hydrolysate (7.51–8.60 vs. 10.48 mg/g peptides). A reaction time of 2 h conferred the highest calcium-chelation on the treated hydrolysate. Both casein hydrolysate and the treated hydrolysates showed lower iron(II)-chelating activities than the positive control EDTA (Table 2). It was unexpected that plastein reaction and reaction extent exhibited a slightly helpful influence on iron(II)-chelating activities of the treated hydrolysate, as the assayed values were enhanced from 52.6% to 53.2–55.5% (Table 2).

Food proteins or peptides with good metal chelation can improve mineral bioavailability and provide the body with sufficient minerals. Transition metal ions, such as copper and iron, can induce the generation of reactive oxygen species (ROS), which are adverse to foods (Chung *et al.* 2002). Chelating activity of food proteins or peptides towards copper and iron will decrease ROS formation, and is beneficial to food storage. Some researches showed that the enzymatic hydrolysis of soybean (Bao *et al.* 2007) and casein (Wang *et al.* 2011) was beneficial to mineral absorption. Enzymatic deamidation and hydrolysis of casein also resulted in enhanced calcium- and iron(II)-chelating activities (Li & Zhao 2011). Bao *et al.* (2007) found that the hydrolysate fractions with average molecular weight of 22.5 kDa had much higher calcium-binding ability than those with the weight of 8.76 kDa, and molecular weight, number of amino acid residues and functional groups contained in the peptide chain governed the calcium-binding ability of soybean protein hydrolysates. Whey protein hydrolysates digested by nine proteases *in vitro* had better iron-binding than whey protein concentrates, among which was Alcalase-generated hydrolysate possessing the highest iron-binding (Kim *et al.* 2007b). This study showed that the differences in the type of amino acids, net charge and peptide length had an impact on the metal-binding of the hydrolysates, and gave a support to the present result, i.e. the treated hydrolysates had different peptide compositions, thus being different in calcium- or iron(II)-chelating activities. Unfortunately, whether the plastein reaction had an impact on the metal chelation of other protein hydrolysates was not investigated in these studies. More study is needed to reveal the potential impact of the plastein reaction on metal chelation of other protein hydrolysates.

### ACE-inhibition kinetics of the treated casein hydrolysates

The Lineweaver-Burk plots of ACE with the evaluated samples at different substrate (HHL)

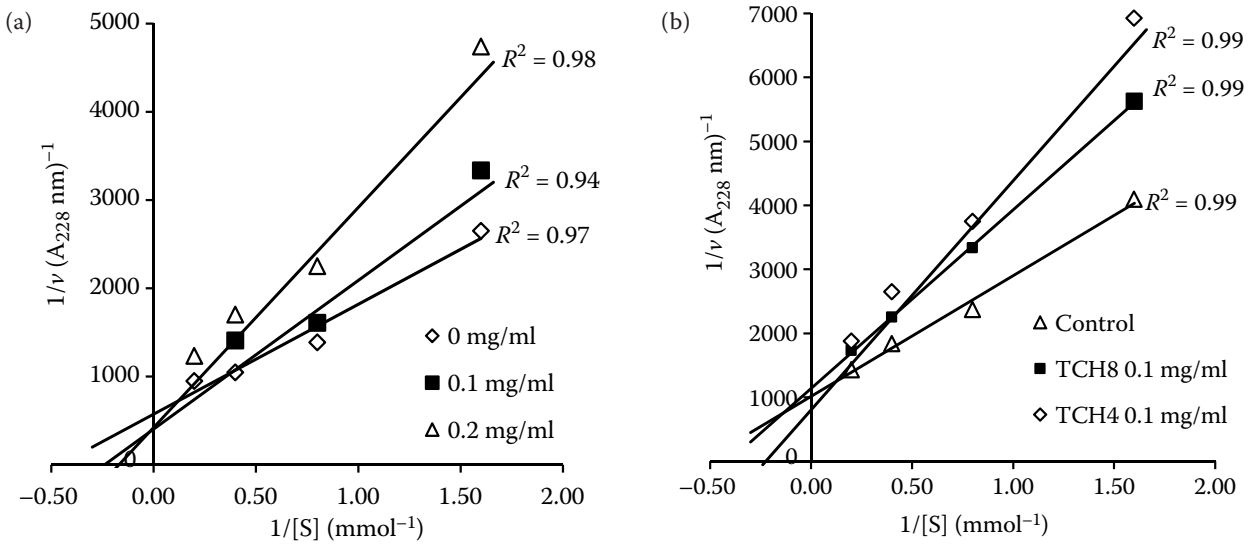


Figure 1. Lineweaver-Burk plots of the inhibition of ACE by (a) casein hydrolysate and (b) two treated casein hydrolysates (TCH4 and TCH8) (water was used as control)

[S] – substrate concentration; 1/v – enzymatic reaction velocity

concentrations are shown in Figure 1. The line intersection on the 1/v axis indicates that casein hydrolysate and the treated hydrolysates were competitive inhibitors to ACE, i.e. the active sites of ACE were bound competitively by casein hydrolysate and the treated hydrolysates, showing a classic competitive inhibition pattern. Some studies indicated that ACE-inhibitory peptides from food proteins were competitive inhibitors to ACE, such as freshwater clam hydrolysate (Sun *et al.* 2011), separated peptides from fermented oyster sauce (Je *et al.* 2005) and chymotryptic hydrolysate of α-kafirin (Kamath *et al.* 2007). The present result was consistent with these reported results. On the contrary, two studies also stated that two peptides, IFL from tofuyo (fermented soybean) and VECYGPNRPQF from algae protein hydrolysates, appeared to be uncompetitive inhibitors to ACE (Kuba *et al.* 2003; Sheih *et al.* 2009). All these mentioned results pointed out one fact, i.e. the inhibitory peptides can bind into ACE sites but may result in different effects.

The calculated $K_i$ values for casein hydrolysate, TCH4 and TCH8 were 0.231, 0.137 and 0.313 mg/ml, respectively, i.e. TCH4 and TCH8 were the strongest and the weakest inhibitor to ACE. This result was supported by their different ACE-inhibitory activities (Table 1). A purified peptide containing thirteen amino acid residues had a $K_i$ value of 21.6 μmol/l (Qian *et al.* 2007). Based on the molecular weight of the peptide, this value equals to 0.033 mg/ml. A hydrolysate (LPH2) prepared from hen egg white lysozyme by simulated gastrointestinal digestion was a competitive inhibitor to ACE, and had a $K_i$ value of 0.0134 mg/ml (Rao *et al.* 2012). It is thus concluded that TCH4 as a peptide mixture had weaker ACE-inhibition than these mentioned peptides and hydrolysate.

## Calcium carbonate precipitation inhibition of the treated casein hydrolysates

The inhibition of casein hydrolysate and the treated hydrolysates towards $CaCO_3$ precipitation is shown in Table 3. It was observed that as the reaction time prolonged from 1 min to 5 min, more $CaCO_3$ precipitation was formed in the reaction system, reflected by the enhanced turbidity. This result was similar to the result reported by Inoue *et al.* (2001). Casein hydrolysate showed the more powerful ability to inhibit $CaCO_3$ precipitation formation than the treated hydrolysates. Among the six treated hydrolysates evaluated, TCH2 caused the strongest inhibition towards $CaCO_3$ precipitation. If the plastein reaction time longer than 2 h was applied for the treated hydrolysate, lower $CaCO_3$ precipitation inhibition was observed. This fact implied that there existed a possible relationship between calcium-chelating activity and $CaCO_3$ precipitation inhibition. The analysis of results revealed that $CaCO_3$ precipitation inhibition of

Table 3. Turbidity changes of the solutions containing $NaHCO_3$ and casein hydrolysate or treated hydrolysates after addition of $CaCl_2$ solution

| Samples | Absorbencies at 570 nm | | | | |
|---|---|---|---|---|---|
| | 1 min | 2 min | 3 min | 4 min | 5 min |
| CH | 0.092 ± 0.005$^{A}$ | 0.220 ± 0.005$^{a}$ | 0.299 ± 0.003$^{A}$ | 0.346 ± 0.003$^{a}$ | 0.379 ± 0.004$^{A}$ |
| TCH1 | 0.097 ± 0.006$^{A}$ | 0.243 ± 0.008$^{b}$ | 0.325 ± 0.007$^{C}$ | 0.379 ± 0.010$^{c}$ | 0.413 ± 0.002$^{C}$ |
| TCH2 | 0.090 ± 0.002$^{A}$ | 0.227 ± 0.004$^{a}$ | 0.311 ± 0.004$^{B}$ | 0.364 ± 0.005$^{b}$ | 0.401 ± 0.006$^{B}$ |
| TCH3 | 0.097 ± 0.007$^{A}$ | 0.248 ± 0.003$^{b}$ | 0.333 ± 0.006$^{C}$ | 0.385 ± 0.005$^{c}$ | 0.422 ± 0.003$^{C}$ |
| TCH4 | 0.111 ± 0.007$^{B}$ | 0.263 ± 0.006$^{c}$ | 0.351 ± 0.003$^{D}$ | 0.404 ± 0.005$^{d}$ | 0.440 ± 0.003$^{D}$ |
| TCH6 | 0.130 ± 0.006$^{C}$ | 0.293 ± 0.003$^{d}$ | 0.381 ± 0.003$^{E}$ | 0.431 ± 0.004$^{e}$ | 0.454 ± 0.010$^{E}$ |
| TCH8 | 0.188 ± 0.005$^{D}$ | 0.361 ± 0.008$^{e}$ | 0.447 ± 0.009$^{F}$ | 0.500 ± 0.004$^{f}$ | 0.529 ± 0.005$^{F}$ |

CH – casein hydrolysate; TCH – plastein reaction treated hydrolysates; final peptide concentration of CH or TCH used in the analysis was 10 mg/ml; values with different capital or lowercase letters as superscripts in the same column indicate that one-way ANOVA of the mean values is different significantly ($P < 0.05$)

casein hydrolysate and the treated hydrolysates was well-correlated with their calcium-chelating activities ($R^2 = 0.703$, $P < 0.05$), suggesting that it was calcium chelation of the hydrolysates that suppressed the formation of $CaCO_3$ precipitation.

Casein phosphopeptide (CPP) showed inhibition towards $Ca_3(PO_4)_2$ and $CaCO_3$ precipitation (Sato *et al.* 1991). The present results showed that all evaluated samples could inhibit $CaCO_3$ precipitation as the CCP did. This was also reported by another research results, in which CPP showed inhibition of hydroxyapatite crystal formation (Holt *et al.* 1996), or a cysteine-rich Mdm2 peptide decreased calcite crystal growth by 22–58% in the concentration range tested (Inoue *et al.* 2004). Molecular size and acidic amino acid residues of the peptides are key factors to cause the precipitation inhibition (Jin *et al.* 2000). Casein hydrolysate and the treated hydrolysates were different in their peptide composition, thus they were different in their $CaCO_3$ precipitation inhibition.

## CONCLUSION

The carried out plastein reaction showed an impact on some properties of a casein hydrolysate prepared by Alcalase, but kept the inhibition pattern of these treated hydrolysates towards ACE as competitive inhibitors. Compared to casein hydrolysate, the treated hydrolysates had higher *in vitro* ACE-inhibitory activities, lower zinc- or calcium-chelating and slightly higher iron(II)-chelating activities. The ACE-inhibition originated from the treated hydrolysates themselves but not their zinc-chelation. The inhibition of $CaCO_3$ precipitation of the treated hydrolysates was lower than that of casein hydrolysate, but well-correlated ($P < 0.05$) with their assayed calcium chelation.

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*Corresponding author:*

Prof. Xin-Huai Zhao, Northeast Agricultural University, Key Laboratory of Dairy Science, Ministry of Education, Harbin 150030, P.R. China; E-mail: zhaoxh@mail.neau.edu.cn or xhzhao63@sina.com.cn