Plastein reaction enhanced bile-acid binding capacity of soybean protein hydrolysates and whey protein hydrolysates

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Abstract Plastein reaction is a modification reaction that can improve the functional properties of protein hydrolysate. The product of the reaction is a thixotropic aggregation of peptides. This study investigated the formation condition of soybean-whey plastein and bile acid binding capacity of plastein. Soy protein and whey protein were hydrolyzed by pepsin. The mixture (1:1, w/w) of two hydrolysates was modified by pepsin again. After the reaction, the decrease in free amino groups and the turbidity of the modified hydrolysate were measured to obtain appropriate reaction condition. Results showed that the concentration of hydrolysates 40% (w/v), enzyme ratio of 2.0 KU/g protein, pH 5.0, 37 °C, reaction time of 3.0 h respectively, were showed maximum changes in protein hydrolysates. Tricine SDS-PAGE analysis under denaturing conditions revealed that whey protein was more sensitive to pepsin and yielded different polypeptides (PPs) of molecular weight ranged from 3.5–17 kDa. However, a high molecular weight PP was completely hydrolyzed while PPs of 14.2–26 kDa were partially digested after pepsin treatment. Native page analysis further revealed the presence of a high-molecular weight PP in crude and purified plastein product. The bile acid binding capacity was improved by the plastein reaction. The amount of binding sodium deoxycholate, sodium taurocholate, and sodium cholate were 0.75, 2.0 and 1.87 $\mu\text{mol}/100\text{ mg}$ respectively.

Keywords Soybean protein hydrolysate · Whey protein hydrolysate · Pepsin · Plastein reaction · Bile acid binding capacity

Introduction

Plastein reaction was formed as protein-like from concentrated protein hydrolysates or a mixture of oligopeptide in the presence of active proteases under a given condition (Gong et al. 2015). It was thought to be an aggregation of peptides, which occurred not only in the sole protein hydrolysate, but also in mixed peptides from various proteins. It was known that peptides exhibited bioactivities due to their inherent amino acid sequence and composition (Udenigwe and Aluko 2012). However, recently plastein has been produced and assessed for a number of enhanced biological activities related to human health promotion. Plastein reaction was mainly a 3-step process. The first step was protein hydrolysis by proteases to produce protein hydrolysates or a low mixture of small peptides. The second step was a concentration stage, and the third was aggregation again after modification by proteases. Pepsin was stable in the gastric environment. We hoped that the soybean-whey plastein could exist stably in the stomach, so pepsin was chosen as a catalyst for hydrolysis and aggregation in this work.

Cardiovascular disease (CVD) is a key cause of high morbidity and mortality in the world, and the rise of CVD is strongly associated with the increased value of blood lipid. The total cholesterol level in blood is controlled not only by diet, but also associated with the level of bile acid. Bile acid is the metabolite of cholesterol in liver and then eliminated, which is the major route of discharging bile acid from the body. Some agents bind bile acid in the intestines and disturb the intestinal loop, leading to an accelerated reduction of cholesterol (Rui et al. 2010; Costarelli et al. 2002). Protein hydrolysate had a capacity to bind bile acids that contributed to the conversion of cholesterol to bile acids (Howard and Udenigwe 2013).

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This capacity could be enhanced by plastein reaction. The plastein from chicken meat hydrolysates demonstrated that the peptides aggregates enhanced the capacity to bind bile acid more significantly than the parent hydrolysates (Udenigwe et al. 2015). A follow-up study from the same group demonstrated that isolated casein plastein had a higher binding capacity than the crude casein plastein (Mohan and Udenigwe 2015).

In some instances, high amounts of hydrophobic amino acid residues of peptides were thought to be crucial for bile acid binding capacity. In addition, hydrophobic force also played a key role in plastein reaction. Thus, in this work, the plastein was produced by hydrolysis of soybean protein and whey protein to improve bile acid binding capacity of hydrolysates and studied the formation condition of soybean-whey plastein.

Materials and methods

Preparation of hydrolysate

Soybean protein (protein content: 87.9%, Shandong Jiahua Co. Ltd., China) and whey protein (protein content: 80.8%, Mielel, Germany) were dispersed in a phosphate buffer solution (0.05 mol/L, pH 2.0) to a concentration of 4% (w/v) respectively, then subjected to heat treatment at 80 °C for 15 min, cooled to room temperature. The protein solution was then treated with pepsin (V900497, Sigma-Aldrich/Vetec, USA) at E/S ratio of 1.0 KU/g protein, incubated at 37 °C for 6 h. The reaction was terminated by heating the protein solution in boiling water for 15 min and then the sample was adjusted to pH 4.5 by an addition of 0.1 mol/L NaOH solution, centrifuged (5804 R, Eppendorf, Germany) at 10000 r/min for 15 min to separate the soluble hydrolysates from the non-soluble substances. Soluble hydrolysates were lyophilized to obtain soybean protein hydrolysate (SH) and whey protein hydrolysate (WH) and then stored at − 20 °C until analyzed.

Plastein reaction of soy protein and whey protein

Soybean protein hydrolysate (SH) and whey protein hydrolysate (WH) were mixed at equal weight, and were diluted in the phosphate buffer solution (pH 2.0) to obtain protein solution with different concentrations. The pepsin was added in the protein solution, and then the protein solution was incubated at different temperatures for plastein reaction. After termination of the plastein reaction by heating the solution in boiling water for 15 min, the crude plastein (PC) product was obtained. A portion of PC was lyophilized and the other portion was washed with water to precipitate aggregates by centrifugation at 10000 r/min,

and then lyophilized to obtain the isolated plastein (PI) and stored at − 20 °C for further analysis.

Free amino groups determination

The varying amount of free amino groups during plastein was analyzed using O-phthalaldehyde (OPA). OPA of 40 mg was dissolved in 1 ml of methyl alcohol and was mixed with 25 mL of sodium borate buffer (0.1 mol/L) containing 0.5 g of sodium dodecyl sulfate (SDS), then added 25 µmol of mercaptoethanol. The volume of the solution was adjusted to 50 mL to obtain the OPA reagent. The OPA reagent (1 mL) was mixed with 0.05 mL of sample, incubated at 35 °C and the absorbance of the solution was measured by a Spectrophotometer (Multiskan G1510, Thermo Fisher Scientific, USA) at 340 nm after 2 min. Leucine (0–0.5 mg/mL) was used as the standard, and the free amino group was calculated by µmol of NH₂/g of protein.

Turbidity measurement

Soy-Whey plastein was the precipitate formed by aggregating of two hydrolysates, which could make the turbidity of hydrolysates solution higher. Therefore, the turbidity of hydrolysate was an important and convenient index to monitor the amount of plastein during reaction. The turbidity of the modified product solution was monitored with a method from Chibuike et al. (2014) during plastein reaction. The solution was diluted with the equal volume water, and absorbance at 460 nm was measured. The initial absorbance was deducted.

Tricine-SDS-PAGE and Native-PAGE

The gel of Tricine-SDS-PAGE and Native-PAGE were prepared according to the procedure described by Schagger et al. (1987, 1991). The vertical electrophoresis unit (AE8135, ATTO, Japan) was used in conjunction with the power supply. In Tricine-SDS-PAGE, separating gel (15%), spacer gel (10%) and stacking gel (4%) were composed for the separation of low molecular weight protein. In Native-PAGE, just separating gel (15%) and stacking gel (5%) were used to form the separation system. Each sample with protein content of 5 µg was loaded into the gel prepared previously. All electrophoretic separation was carried out starting at 30 V. The running condition was set at 100 V, when samples had completely entered into the spacer gel. The protein bands were fixed in a solution containing 50% methanol and 10% acetic acid for 30 min, before they were stained with 0.025% Coomassie Brilliant Blue G-250, and 10% acetic for 1 h. The gel was destained in 10% acetic acid with several changes of destaining

solution for 1 h. Destained gels have been scanned using electrophoresis gel imaging system (Gel Doc^{TM} XR+, Bio-Rad, USA).

Determination of the bile acid binding capacity

The capacity of the bile acid binding of soy protein hydrolysate (SH), whey protein hydrolysate (WH), the crude plastein (PC) and isolated plastein (PI) were measured.

Samples of 30 mg were digested in 1 mL of 0.1 mol/L sodium phosphate buffer (pH 6.3), mixed with 1 mL of bile acid (2 mg/mL) and incubated under agitation for 2 h at 37 °C, then centrifuged at 10000 r/min. Free bile acid in supernatant was determined by the Total Bile Acid Assay Kit (Shanghai Xitang Co. Ltd., China). Ten milligram of cholestyramine powder was used as positive control. Bile acid binding capacity was reported as μmol of bile acid bond by 100 mg of sample.

Statistical analysis

All experiments were performed in triplicate. The obtained data was reported as mean values. Multivariate regression statistical analysis was conducted using Origin 8.5. The significance of the difference between the mean values was determined with F-test ($p < 0.05$) using IBM SPSS version 20.0.

Results and discussion

Influence of hydrolysate concentration on turbidity and decrease of free amino groups

Plastein reaction is an aggregation of peptides at a higher concentration of hydrolysate with precipitation forming during the reaction. There are several ways that can reflect the plastein aggregation from peptide, such as measuring the amount of precipitation separated through centrifuge with water or trichloroacetic acid (TCA). The turbidity of substrate solution was changed during the reaction. It was also a good index for detecting the aggregation quickly. The decrease in free amino groups of the substrate was used in the researches of Zhao and Li (2009). However, the approaches mentioned above were significantly different and had different meanings for studying plastein aggregation.

During the plastein reaction, the hydrolysate not only produced larger molecular weight plastein than that which could be precipitated by TCA, but also could still be hydrolyzed. The free amino acid released by hydrolysis could not be detected by TCA. Therefore, two approaches

in the study of plastein reaction processes were different in mechanism and results. In order to study the effect of condition for plastein reaction, two ways were selected to monitor plastein aggregation together, and tried to explain the discipline of the reaction.

The effect of hydrolysate concentration (SH/WH) was 1/1 on turbidity and the amount of free amino groups during the reaction as shown in Fig. 1a, b. As shown in Fig. 1a, b, the tendency of both curves climbed up to a peak at a concentration of 40% (w/v), and then declined with the hydrolysate concentration increasing. Thus, the results implied that two kinds of hydrolysates aggregated during the reaction.

Plastein reaction is a multiple reaction, including continuous hydrolysis and aggregation of peptides. Based on results listed in Fig. 1a, as the substrate concentration below 25%, turbidity of substrate solution increased slightly, but the decreased amount of free amino groups was minus. This paradoxical situation was probably a consequence from the hydrolysis of the substrate being stronger than the aggregation of peptides at substrate concentration below 25%. Thus, decrease of free amino was minus although plastein was still formed in the

Fig. 1 Influence of substrate concentration on plastein modification and at E/S ratio of 2.0 KU/g protein, pH 5.0, 37 °C for 3 h. Different small letters represent significantly different mean values with F-test ($p < 0.05$). Based on results in Fig. 1, we considered concentration 25% was a boundary that aggregation of peptide was stronger than hydrolyzation, and the aggregation was best at concentration 40%

substrate solution. This suggested that plastein reaction could be a kind of dynamic equilibrium. In this study, we considered concentration 25% was a boundary at which aggregation of peptide began to be stronger than hydrolysis, and the aggregation was best at concentration 40%.

Influence of pH on turbidity and decrease of free amino groups

The pH of the reaction medium was a major factor that influenced plastein formation. The turbidity and decrease of free amino firstly increased then fell off within the pH range 2.0–8.0 as Fig. 2a, b. There were a few plastein products and a decrease of free amino below zero when $\text{pH} < 4.0$, which meant that the degree of hydrolysis was stronger than the degree of aggregation in the reaction. Pepsin was an acidic protease, and the reaction condition with a lower pH for enzymatic activity was necessary for protein hydrolysis. Similarly, the pH over 7.0 resulted in poorer formation, and the amount of free amino was relatively stable. This was possibly due to the ability of pepsin could not be activated in surroundings with weak alkali.

Fig. 2 Influence of pH on plastein modification and at E/S ratio of 2.0 KU/g protein, substrate concentration 40%, 37 °C for 3 h. Different small letters represent significantly different mean values with F-test ($p < 0.05$). Based on results in Fig. 2, the decrease of free amino and turbidity of solution on the verge of pH 5.0 was significantly higher than other pH

Near neutral pH has been proven to promote plastein formation, but the optimal pH could be affected by protease mode. In this experiment, the decrease of free amino and turbidity of solution at pH 5.0 was significantly higher than other pH ($p < 0.05$).

Influence of time and temperature on plastein modification

The combined effects of incubation time and temperature on plastein formation were illustrated in Fig. 3. Different temperatures contributed to the discrepancies of turbidity and the decrease of free amino groups. As can be observed from Fig. 3a, there was a rapid initial phase of turbidity which then leveled off into a plateau region. The decrease of free amino groups during the reaction was found (Fig. 3b) that the initial decrease of free amino groups increased and then fell down at a different temperature.

In reaction medium, there was a rapid enhancement of plastein formation before 1.0 h. However, the tendency of

Fig. 3 Combined effects of temperature and time on plastein modification at E/S ratio of 2.0 KU/g protein, substrate 40%, pH 5.0. The significance of the differences between the mean values was determined with F-test ($p < 0.05$). In Fig. 3, it has been shown that the balance of plastein reaction, aggregation or hydrolysis, affected by temperature. The higher temperature contributed to the faster aggregation. In contrast, a slow aggregation occurred when the temperature was lower. The decrease of free amino groups, in our measurement was 119.67 μmol/g protein at 37 °C for 3.0 h

turbidity and free amino groups was different at various temperatures. The turbidity and the decrease of free amino groups still increased slowly at 37 and 22 °C after 1.0 h, which was different from the situation at 52 °C. That turbidity was unchanged but the decrease of free amino fell off. On the basis of these results, we tentatively enhanced that a higher temperature, such as 52 °C or higher, which could promote a rapid formation of a portion of substrate before 1.0 h, but also contribute to a still hydrolysis of another portion after 1.0 h, which probably resulted from the rapid formation, that made the concentration of substrate too low (< 25%) to promote plastein formation.

It has been found that the balance of plastein reaction, aggregation or hydrolysis, was affected by temperature. The higher temperature contributed to the faster aggregation. In contrast, a slow aggregation occurred when the temperature was lower. The decrease of free amino groups in our measurement was 119.67 $\mu\text{mol/g}$ protein at 37 °C for 3.0 h.

Influence of time and enzyme ratio on plastein modification

The impact of time and enzyme ratio on plastein formation was shown in Fig. 4. As enzyme ratio was 3.5 KU/g protein, the decrease of free amino increased until 1.0 h and then decreased below zero after 3.0 h. At enzyme ratio at 0.5 KU/g protein, the decrease of free amino was minus first and then increased after 1.0 h. A similar phenomenon had been seen that the speed of plastein aggregation was also affected by enzyme ratio, and an excessive enzyme ratio could contribute to the hydrolysis of substrate. Thus, in Fig. 4b the amount of plastein was not the highest at a higher enzyme ratio. But the maximum point was 118.38 $\mu\text{mol/g}$ protein at 2.0 KU/g protein for 3.0 h.

It was known that active enzyme was responsible for plastein formation. But the enzyme also contributed to the hydrolysis of substrate. The enzyme played a major role during the reaction speed of plastein aggregation.

Tricine-SDS-PAGE and Native-PAGE

The results of Tricine-SDS-PAGE in the presence of SDS were shown in Fig. 5a, and the results of Native-PAGE electrophoresis are shown in Fig. 5b. In Tricine-SDS-PAGE, different peptides were produced from two proteins by pepsin. And the molecular weight of two hydrolysates was distributed in the range of 1.0–4.0 kDa (Lane 3 and Lane 4). Compared with hydrolysis, a slight discrepancy appeared in 14.2 kDa of the isolated plastein (Lane 6), but there was no peptide with a higher molecular weight formed in the plastein. Thus, there was no clearly defined

Fig. 4 Combined effects of enzyme ratio and time on plastein modification at substrate 40%, pH 5.0, 37 °C. The significance of the differences between the mean values was determined with F-test ($p < 0.05$). In Fig. 4, a parallel discipline has been showed that the speed of plastein aggregation also affected by enzyme ratio, and an excessive enzyme ratio could contribute to hydrolysis of substrate. The amount of plastein was not highest at higher enzyme ratio. And the maximum point of the decrease of free amino was 118.38 $\mu\text{mol/g}$ protein at 2.0 KU/g protein for 3.0 h

covalent bond product obtained during the plastein reaction. There was a completely different situation observed in the profiles in Native-PAGE profiles as shown in Fig. 5b. Some new bands were presented in the crude plastein (Fig. 5b, Lane 5) and isolated plastein (Fig. 5b, Lane 6), suggesting that there was protein with higher molecular weight formed during plastein reaction.

SDS-PAGE is an electrophoresis in presence of SDS and β -mercaptoethanol, which are strong denaturant known for disrupting physical protein associations. Hydrophobic force and hydrogen bond are key forces for keeping the secondary and tertiary associations of protein, and these forces can be disrupted by some denaturants. The Native-PAGE has no denaturant SDS and β -mercaptoethanol, and so the plastein formation might not be affected in native electrophoresis. Sukan and Andrews (1982) studied the molecular weight changes of caseins plastein reaction in SDS-PAGE, and also obtained the similar results to our

Fig. 5 **a** Tricine SDS-PAGE analysis under denaturing conditions revealed that whey protein was more sensitive to pepsin and yielded different polypeptides (PPs) of molecular weight ranged from 3.5–17 kDa. However, a high molecular weight PP was completely hydrolyzed while PPs of 14.2–26 kDa were partially digested after pepsin treatment. Native page analysis further revealed the presence of a high-molecular weight PP in crude and purified plastein product. Tricine-SDS-Page analysis of hydrolysates and plastein. M, Marker; 1, Soybean protein; 2, Whey protein; 3, Soy protein hydrolysate; 4, Whey protein hydrolysate; 5, Plastein product crude; 6, Plastein isolated. The Tricine-SDS-Page electrophoresis were shown in **a**, a slight discrepancy appeared in of 14.2 kDa of the isolated plastein (Lane 6), but there was no peptide pattern of higher molecular weight formed in the plastein. Thus, no clearly defined covalent bond product was obtained during plastein reaction. **b** Native-PAGE analysis of hydrolysates and plastein. 1, Soybean protein; 2, Whey protein; 3, Soy protein hydrolysate; 4, Whey protein hydrolysate; 5, Plastein product crude; 6, Plastein isolated. In **b**, some new bands were presented in the crude plastein (Lane 5) and isolated plastein (Lane 6), suggesting that there was higher molecular weight formed through some kinds of force during plastein reaction

research. The results mentioned above might suggest that hydrophobic force and hydrogen bond were responsible for plastein formation.

Bile acid binding assay

Bile acid in the human body mainly existed in the form of bile salts, containing 30% sodium cholate and 20% sodium deoxycholate (Debruyne et al. 2001). Furthermore, sodium taurocholate is representative of bile salts that are more difficult to bind with other materials. In this assay, the bile acids mentioned above chosen by us were used for the study of ability of plastein. As shown in Fig. 6, soybean protein hydrolysate (SH) was found to exhibit a higher capacity to bind bile acid than whey protein hydrolysate (WH). The capacity to bind bile acid was advanced by plastein modification. Specially, the binding capacity of the crude plastein to sodium cholate was higher. It was 2.5-fold higher than SH. More notably, the isolated plastein (PI) had a higher binding capacity to three kinds of bile acid than the crude plastein (PC). This indicated that the precipitate formed during plastein was a crucial factor for enhancing the capacity of bile acid binding. Bile acid is a compound containing the structures of hydrophobicity and hydrophilicity. It binds hydrophobic amino acid of protein through hydrophobic force (Higaki et al. 2006). At the same time, the hydrophobicity of hydrolysate was changed during plastein reaction (Doucet et al. 2003), which could influence the capacity of binding bile acids.

Cholestyramine has been clinically used as a cholesterol-lowering agent. This agent is to bind bile acids, leading to accelerated conversion of cholesterol to bile acid. But

Fig. 6 Bile-acid binding ability of soybean protein hydrolysate, whey protein hydrolysate, hydrolysate and modified hydrolysate. SH Soy protein hydrolysate, WH Soy protein hydrolysate, PC the crude plastein, PI the isolated plastein. Bars with different small letters, small letters with * and capital letters represent significantly different adsorbing capacity of sodium deoxycholate, Sodium taurocholate and sodium cholate respectively ($p < 0.05$). Based on results in Fig. 6, the capacity of the hydrolysis was advanced by plastein modification. The binding capacity of the crude plastein for sodium cholate was 2.5-fold higher than SH. More notably, the isolated plastein had higher binding capacity of three kinds of bile-acid than the crude plastein (PC). This indicated that the precipitate formed during plastein was a crucial factor for enhancing capacity of bile-acid binding

the long-term use of cholestyramine can bring patients side effects. According to our data, the amount of binding bile acid to sodium deoxycholate, sodium taurocholate and sodium cholate were 0.75, 2.0, 1.87 $\mu\text{mol}/100\text{ mg}$ protein respectively, and was 19.3, 10.5, 15.5% for cholestyramine. Therefore, the plastein of soybean protein and whey protein could be seen as a functional protein product for lowering cholesterol.

Conclusion

The plastein was seen as a dynamic equilibrium reaction between aggregation and further hydrolysis. Our results confirmed that hydrolysis also appeared in unaggregated peptide. This could lead to an increase of free amino groups although the plastein has been formed. And a rapid aggregation promoted by higher temperature and amount of enzyme, but contributed to hydrolysis in parent hydrolysate. The condition for soybean-whey plastein reaction was hydrolysate concentration 40%, enzyme ratio 2.0 KU/g protein, pH 5.0, 37 °C, reaction time 3.0 h respectively.

The study demonstrated that the plastein formed from parent hydrolysate could be destroyed by protein denaturing agent. Moreover, this study demonstrated that the plastein reaction could promote the bile acid binding capacity of two hydrolysates, and evaluated amount of bile acid binding in vitro. It can be concluded that the isolated plastein has a better prospect as a functional food for managing hypercholesterolemia and promoting health.

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