Effects of Maillard reaction conditions on the functional properties of WPI chitosan oligosaccharide conjugates

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Revised: 28 September 2011 / Accepted: 29 January 2013 / Published online: 6 February 2013
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Abstract Whey proteins are widely used as food material, but their functional properties are affected by processing conditions, in which Maillard reaction commonly occurs. The objective of this study was to investigate the effects of reaction conditions (saccharide rate (*SR*), setting time and setting temperature) on the functional properties of conjugates of whey protein isolates (WPI) - chitosan oligosaccharide (COS), which were produced by Maillard reaction. Response surface methodology (RSM) was used in this study, models for solubility, heat stability (*HS*), emulsifying activity (*EA*) and emulsifying stability (*ES*) of WPI-COS were established. Results indicated that the solubility of WPI-COS was 98.8 % at 29.8 % *SR*, 17.4 h, 46.7 °C; the heat stability of WPI-COS was 92.95 % at 27.2 % *SR*, 18.1 h, 48.7 °C; the *EA* of WPI-COS was 1.87 at 25.3 % *SR*, 20.0 h, 46.1 °C; the *ES* of WPI-COS was 13.28 min at 32.5 % *SR*, 12.0 h, 35.0 °C and setting temperature was the major factor affecting *EA* and *ES* while *SR* was the major factor affecting solubility and heat stability. Conditions were optimized for the preparation of WPI-COS to obtain improved functional properties.

Keywords Whey protein isolate · Chitosan oligosaccharide · Solubility · Heat stability · Emulsifying property

Introduction

Whey proteins are well known for high nutritional value. They contain β -lactoglobulin (β -LG) and α -lactalbumin

(α -LA) accounting for 70 % of total whey protein. Whey proteins are widely used in food products due to their good functional properties, e.g. solubility and emulsifying properties. But these functional properties are usually limited by processing duration and temperature, which hampers the wide usage of whey proteins in diverse food systems and processing methods. Therefore, it is necessary to expand the usage of whey proteins in foods safely and easily, and the technique of protein modification is common to obtain desired protein properties.

Many efforts have been dedicated to protein modifications by chemical, enzymatic and/or physical methods (Defaye and Ledward 1995; Galazka et al. 1995; Ouattara et al. 2002; Ven et al. 2002; Zheng et al. 2008), which have been proved to be effective in improving functional properties of proteins. Preparing protein-saccharide conjugates by means of Maillard reaction is widely used among those methods. The Maillard reaction is well known as an amino acid-reducing sugar reaction, in which different kinds of products responsible for food color and flavor are generated. This type of reaction often occurs during food processing (O'Brien and Morrissey 1989; Ames 1998; Chuyen 1998). Different kinds of proteins have been modified in this method. Glucose, glucose 6-phosphate and dextran were separately conjugated to improve the emulsifying properties of ovalbumin (Kato et al. 1988, 1990, 1995). Also, 6-phosphate and galactomannan were used to make better applications of both egg protein and whey proteins (Aoki et al. 1994). Matsudomi et al. (1994) found that galactomannan improved emulsifying properties of protamine. Paraman et al. (2007) discovered that the product of glycosylation of rice endosperm protein had better solubility and emulsifying properties than those of enzymic hydrolysis and original proteins.

In terms of whey proteins, they were modified using different saccharides. By conjugating different saccharides (glucosamine, glucosamineoctase, carboxymethyl dextran, chitosan, etc) under varied circumstances, *EA* and stability

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of β -LG were obviously improved by forming β -LG-saccharide (Waniska and Kinsella 1988; Bertrand-Harb et al. 1990; Hattori et al. 1994, 2000; Akhtar and Dickinson 2003). Chevalier et al. (2001) revealed how β -LG had been changed by analyzing modifications of amino acid of β -LG, which was conjugated with glucose. Furthermore, Kobayashi et al. (2001) proved that conjugation of carboxymethyl dextran was an effective method to reduce immunogenicity of β -LG. Most studies focus on β -LG, the dominating protein in whey proteins. However, WPI is usually used as a whole in food industry instead of β -LG because of its lower price and easier separation technique, which makes studies on whole WPI more important and applicable.

Chitosan oligosaccharides are usually obtained from partial hydrolysis of chitosan, which is derived from fully or partially deacetylated chitin (Shahidi et al. 1999). With low polymerization degree, COS tend to have better solubility than chitin. Moreover, the bindings of COS to negatively charged surfaces are strong as COS have positive charges, which are different from other polysaccharides, and this should results in most of the functionalities (Kim and Rajapakse 2005). Because of their well-known physiological and chemical properties, chitosan oligosaccharides are promising in pharmacy and functional food industry (Hirano et al. 1991; Jeon et al. 2001). A large quantity of shellfish and shrimps are produced every year, leaving by-products to be disposed of. Also, there are different sources of chitin with large amounts, as it is abundant in crustaceans, insects and fungi. Chitosan and its derivants have been widely used due to good functionality and economical viability. Chitosan oligosaccharide was then selected as a saccharide supplier in this study.

RSM was employed to discover optimized conditions for preparing WPI-COS conjugates with better water solubility, heat stability and emulsifying properties. So far, most of such researches have been conducted under various conditions and related to effects of one or more individual parameters. Hence it is difficult to compare results from such reported data to predict the differing functional behavior of any given system. RSM was applied instead to generate proper models within the experimental design of this study to understand the trend of multiple factors affecting functional properties of WPI-COS systematically and make reasonable predictions of their effects. The result may be useful in expanding the applications of whey proteins.

Materials and methods

Materials

WPI was obtained from Murray Goulburn Co-operative Co. LTD (Melbourne, Australia), the total protein content was 85.17 ± 0.81 % (measured in Kjeldahl method); the β -LG

content was 59.34 ± 0.4 %, α -LA content was 11.6 ± 0.2 % [measured by RP-HPLC, using a SPD-M10AVP (Shimadzu Corp., Tokyo, Japan) with a Kromasil C8 column].

Chitosan oligosaccharide was obtained from Haidebei Marine Bioengineering Co., Ltd., Jinan, China. The average molecular weight of the chitosan oligosaccharide was less than 10 kDa, the degree of deacetylation was 85.7 % and the insoluble content was less than 0.2 %.

Preparation of WPI-COS

The preparation method was set referring to the report of Kato (2002). WPI and chitosan oligosaccharide were mixed at different saccharide rates (percentage of saccharide content in saccharide-protein mixture) following experimental design, dissolved in distilled water (10 %, w/v) and lyophilized (FD-1PF, Detianyou Tech. Co., Ltd., Beijing, China). Different lyophilized products were put in the desiccator where the relative humidity was controlled at 79 % by saturated potassium bromide solution. The desiccator containing the lyophilized products was kept under the experimental conditions of setting times and temperatures, which were determined by preliminary experiments and shown in Table 1.

Determination of the solubility of WPI-COS

The determination of solubility was following the method of Jiménez-castaño et al. (2005) with some modification. The solubility of the conjugate was measured by dissolving the powder in distilled water to make a protein concentration of 1 mg/ml. After 30 min of stirring at room temperature, the samples were centrifuged (TGL-16A, Pingfan Co., Ltd., Changsha, China) for 15 min at 4 °C and $3,500 \times g$. Protein concentrations of the supernatants were determined by measuring the absorbances at 280 nm (UV-2600A, Unico Co., Ltd., Shanghai, China) and using a standard curve for WPI. Before all the solutions were measured, they were diluted with 8 M urea (1:1) in order that non-soluble solids interferences were prevented. All measurements were done in

Table 1 Coded and uncoded levels of the independent variables for the solubility, heat stability, emulsifying activity and emulsifying stability of WPI-COS

Coded levels	Independent variables		
	Saccharide Rate (%)	Time (h)	Temperature (°C)
+1.682 (+ α)	40.0	36.0	55
+1	33.9	29.5	51
0	25.0	20.0	45
−1	16.1	10.5	39
−1.682 (− α)	10.0	4.0	35

duplicate. The solubility was calculated by the following equation:

$$\text{Solubility}(\%) = C_1/C_0 \times 100\%$$

where C_1 is the protein concentration of the supernatant after centrifugation, and C_0 is the protein concentration of the original solution before centrifugation.

Determination of the heat stability of WPI-COS

The determination of heat stability was conducted referring to the method of Chevalier et al. (2001) and Jiménez-castaño et al. (2005). The heat stability of conjugates was measured by making solutions of 1 mg/mL WPI concentration samples. The samples were heated for 15 min at 90 °C, then cooled to room temperature by ice-water and centrifuged for 15 min at 4 °C and $3,500 \times g$. The protein contents of the supernatants were determined as described above. All measurements were done in duplicate. The heat stability was calculated by the following equation:

$$HS(\text{heat stability})(\%) = C_2/C_0 \times 100\%$$

where C_2 is the protein concentration of the supernatant after centrifugation, and C_0 is the protein concentration of the original solution before centrifugation.

Determination of the emulsifying properties of WPI-COS

The emulsifying property of WPI-COS was measured as per the method described by Pearce and Kinsella (1978) with slight modifications. Samples were dissolved in 0.1 M phosphate buffer (pH7.4), and the protein concentrations were adjusted to 0.1 % (w/v). Each 3 ml sample solution was homogenized at 12,000 rpm at 20 °C for 1 min with 1 mL peanut oil in order to form emulsion. From the bottom of each test tube, 100 μ L of emulsion was taken out at different times and diluted with 4 mL of 0.1 % SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm. All measurements were done in duplicate. *EA* was expressed by the following equation:

$$\text{EAI (Emulsifying Activity Index)} = 2T/\Phi C$$

where T (turbidity)= $2.3A/L$ (A : the absorbance at 500 nm, L : the light path(10^{-2} m)), Φ (oil phase volume)=0.25, C =the concentration of sample(10^3 gm^{-3}). Values were the means of results of triplicate samples.

And the *ES* was expressed by the following equation:

$$\text{ES} = A_0 \times t/(A_0 - A_t)$$

where t is the interval of two determinations of *EAI* of the same sample, A_0 is the absorbance at time 0, and A_t is the absorbance at time t .

Experimental design and statistic analysis

Equation (1) was fitted to the experimental data, which is shown in Table 2. RSM was applied to study the effect of *SR*, setting time and setting temperature on the solubility, heat stability and emulsifying properties of WPI-COS. The experimental design was based on a three-factor orthogonal composite rotatable design with a total of 23 experimental runs. Based on preliminary experiments and references, three parameters, including *SR* (X_1), setting time (X_2) and setting temperature (X_3), were identified as key factors responsible for the properties observed in this study. The coded values of the independent variables ($-1.68, -1, 0, 1, 1.68$) were calculated according to the design. Correspondence between these coded values and actual values is given in Table 1. The complete design consisted of 23 experimental points that included 9 replications of the center point. The four dependent Y variables to evaluate the conjugate functional properties were solubility (Y_1), heat stability (Y_2), *EA* (Y_3) and *ES* (Y_4). The results were statistically analyzed using SAS 8.2 (SAS Institute Inc., Cary, NC, USA) by response surface regression to fit the following second-order polynomial to all dependent Y variables:

The level of significance was defined at $P < 0.05$. The 3-dimensional response surface plots were generated by SAS 8.2 for illustrating the main effects of the independent variables on the conjugates' functional properties.

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 b_{ij} X_i X_j \quad (1)$$

Results and discussion

Assessment on model of functional properties for Maillard reaction conditions

Four equations were obtained and tested for adequacy and the qualities were evaluated based on Eq. (1). The main results of the multiple regressions were developed for each of the dependent variables, with the corresponding coefficients of determination. The models developed indicated how these variables should be related. The regression analysis showed that the experimental results were well described by the model within the experimental field ($P < 0.01$). Table 3 indicated that the model established for *HS*, *EA* and *ES* were all valid and significant with the P values all lower than 0.05, amongst which the P values for *EA* and *ES* were both lower than 0.01, indicating these two models were highly significant.

Table 2 Response of dependent variables to the functional properties of the conjugates

n	Independent variables			Dependent variables			
	Rate (%)	Time (h)	Temperature(°C)	Solubility (%,Y1)	Heat Stability (%,Y2)	Emulsifying Activity(Y3)	Emulsifying Stability (min,Y4)
1	16.1	10.5	39	66.0	84.4	1.306	12.00
2	16.1	10.5	51	92.5	87	1.301	10.58
3	16.1	29.5	39	96.6	83.3	1.260	11.95
4	16.1	29.5	51	78.5	87.1	1.141	11.05
5	33.9	10.5	39	84.9	87.6	1.139	13.51
6	33.9	10.5	51	85.4	87.4	1.293	10.76
7	33.9	29.5	39	80.5	86.4	1.171	12.16
8	33.9	29.5	51	85.9	86.8	1.148	10.89
9	10.0	20.0	45	65.6	70.4	1.317	10.32
10	40.0	20.0	45	95.4	85.3	1.544	11.17
11	25.0	4.0	45	80.2	83.5	1.436	10.89
12	25.0	36.0	45	66.9	71.4	1.686	11.12
13	25.0	20.0	35	68.5	83.3	1.293	12.14
14	25.0	20.0	55	80.3	89.2	1.796	10.63
15	25.0	20.0	45	97.3	92.1	1.857	12.42
16	25.0	20.0	45	97.5	92.1	1.848	12.45
17	25.0	20.0	45	97.2	91.9	1.865	12.40
18	25.0	20.0	45	97.0	92.3	1.879	12.38
19	25.0	20.0	45	96.9	92.0	1.859	12.44
20	25.0	20.0	45	97.8	92.4	1.851	12.41
21	25.0	20.0	45	97.7	92.5	1.849	12.42
22	25.0	20.0	45	97.5	92.0	1.848	12.43
23	25.0	20.0	45	97.9	92.9	1.859	12.46

Interactive effects of saccharide rate, setting time and setting temperature on the functional properties of WPI-COS

The interactive effect of saccharide rate and setting time on the solubility of WPI-COS is shown in the response surface plot (Fig. 1a) with the setting temperature variable being 45 °C. Both saccharide rate and setting time had positive effects on the solubility of WPI-COS, meaning that an increase in saccharide rate or setting time could increase the solubility. For setting time, the quadratic effect was highly significant, indicating that an optimal value of setting time exists within the experimental field. From Fig. 1a, it is shown that the solubility increases markedly when the reaction time was extended from 5 h to 20 h, and then started to decrease after 20 h. An improved solubility was discovered where saccharide rate was around 32.5 %.

The effect of saccharide rate and setting time on the heat stability of WPI-COS was much similar to that on solubility, which is shown in Fig. 1b, however, the effect of setting time on the WPI-COS heat stability was opposite to that on the solubility. For saccharide rate, the quadratic effect was

highly significant ($P<0.01$), meaning that the peak value of heat stability existed during saccharide rate increase at a given time. Highest heat stability was observed where saccharide rate increased to 27 % and then decreased slightly.

The interactive effect on the emulsifying activity is presented in Fig. 1c. Table 3 shows that the quadratic effect is highly significant ($P<0.01$) for saccharide rate, indicating that within the change of saccharide rate existed an optimized emulsifying activity. For setting time, an improved emulsifying activity also existed due to the highest quadratic effect. From Fig. 1c, it is shown when saccharide rate was increased to about 25 %, the emulsifying activity of WPI-COS increased to the highest point within a given time range, and then went down with further increase of saccharide rate.

The interactive effect on the emulsifying stability is described in Fig. 1d. Both saccharide rate and setting time had positive effects on emulsifying stability. Emulsifying stability increased either with the increase of *SR* or the increase of setting time, moreover, the optimal point located in the area where both *SR* and setting time were high. For *SR* and

Table 3 Regression coefficients and analysis of the second-order polynomials for four response variables of the conjugates

Coefficients	Response variables			
	Solubility(Y1)	Heat stability(Y2)	Emulsifying activity(Y3)	Emulsifying stability(Y4)
b_0	97.23	92.13	1.86	12.41
Linear				
b_1	3.90	2.30	0.009	0.23 ^a
b_2	−0.71	−1.70	0.008	−0.03
b_3	2.50	1.21	0.062	−0.65 ^b
Quadratic				
b_{11}	−4.13	−3.76 ^b	−0.21 ^b	−0.46 ^b
b_{22}	−6.59 ^b	−3.90 ^b	−0.16 ^b	−0.37 ^b
b_{33}	−6.29 ^a	−0.79	−0.17 ^b	−0.23 ^a
Interactions				
b_{12}	−2.56	−0.10	0.01	−0.21
b_{13}	−0.31	−0.78	0.03	−0.21
b_{23}	−4.96	0.225	−0.04	0.25
R^2	0.70	0.74	0.80	0.88
F	3.36	4.09	5.95	10.27
Probability of F	0.0238	0.0110	0.0021	0.0001

^a Significant at the 0.05 level^b Significant at the 0.01 level

setting time, the quadratic effects were highly significant, showing that the *ES* achieved highest within the experimental area of *SR* and time.

The interactive effect of *SR* and setting temperature on the solubility of WPI-COS is displayed in Fig. 2a with the

setting time variable being 20 h. Quadratic effect of temperature was significant ($P < 0.05$). From Fig. 2a, it is observed that the effect of *SR* and setting temperature on the solubility of WPI-COS is similar to the interactive effect of *SR* and setting time on this particular property. *SR* and setting

Fig. 1 Interactive effects of *SR* and setting time on functional properties of WPI-COS at a setting temperature of 45 °C. (a) shows the effect on the solubility of WPI-COS; (b) shows the effect on the heat stability of WPI-COS; (c) shows the effect on the emulsifying activity of WPI-COS; (d) shows the effect on the emulsifying stability of WPI-COS

Fig. 2 Interactive effects of SR and setting temperature on functional properties of WPI-COS at a setting time of 20 h. (a) shows the effect on the solubility of WPI-COS; (b) shows the effect on the heat stability of WPI-COS; (c) shows the effect on the emulsifying activity of WPI-COS; (d) shows the effect on the emulsifying stability of WPI-COS

temperature closing to low points resulted in extremely low *HS*, and the solubility at high setting temperature point was higher than that at low setting temperature point where *SR* was defined as a constant value within the experimental field.

In Fig. 2b, the interactive effect on heat stability is presented. For setting time, the optimal existed due to its highly significant quadratic effect ($P < 0.01$). The *HS* of WPI-COS changed more dramatically with the change of *SR* than with the change of setting temperature. Given a constant value of *SR*, *HS* increases along with the increase of setting temperature; moreover, *HS* at high temperatures were better than those at low temperatures. The optimum *HS* existed around the *SR* of 25 %.

The effect of *SR* and setting temperature on the *EA* of WPI-COS is displayed in Fig. 2c. The interactive effect was much similar to the *SR*-setting time effect on *EA*, the optimum value of *EA* exists within the experimental field. For each given *SR*, *EA* increased with the increase of setting temperature to about 45 °C then went down. Also, high setting temperature resulted in better *EA*.

Figure 2d shows the interactive effect on *ES*, indicating that the *ES* is better at higher *SR* and lower setting temperature when the setting time variable was defined as a constant value. At low *SR* within the experimental field, the *ES* did not change much with the increase of setting temperature. However, an increase in setting temperature resulted in a sharp decrease in *ES* at high *SR*.

Figure 3a shows how the solubility of WPI-COS changes with the interactive effect of setting time and setting temperature

with *SR* being 25 % as a constant value. Similarly, low setting temperature and short setting time condition resulted in an extremely low solubility. An increase in setting time or setting temperature while the other was set to a constant value resulted in a remarkable increase in solubility. The peak solubility arrived at maximum at high setting temperature and medium setting time within the experimental field.

The interactive effect on the heat stability of WPI-COS is presented in Fig. 3b. For each given setting time, an increase in setting temperature caused an increase in *HS*, and longer setting time sharpened the increase degree. At shorter setting time, a prolonged setting time resulted in an increase in *HS*, while at longer setting time, a decreased *HS* appeared with a prolonged setting time.

Figure 3c presents the interactive effect of setting time and setting temperature on *EA*, the shape of which is similar to the plot reflecting how the solubility was affected by the interactive effect of *SR* and setting temperature. The quadratic effect of setting temperature is highly significant, indicating that the optimal *EA* existed within the experimental field. *EA* at low setting temperature point was lower than that at high setting temperature point. When temperature was around 46 °C, *EA* achieved peak value.

As is displayed in Fig. 3d, high setting temperature or prolonged setting time does not improve *ES*, which was in accordance with the analysis for Fig. 2d. The optimum *ES* existed in the area where setting temperature was low and setting time was medium within the experiment.

Fig. 3 Interactive effects of setting time and setting temperature on functional properties of WPI-COS at a SR of 25.0 %. (a) shows the effect on the solubility of WPI-COS; (b) shows the effect on the heat stability of WPI-COS; (c) shows the effect on the emulsifying activity of WPI-COS; (d) shows the effect on the emulsifying stability of WPI-COS

Saccharides have long been known as protective agents for protein solubility, preventing protein coagulations. Interaction between hydrophobic groups is strengthened with the attachment of saccharide molecules. The dry-heating process might have introduced chitosan oligosaccharide to whey proteins as hydrophobic group stabilizer. Shu et al. (1996) reported the improvement of heat stability of lysozyme modified by glycosylation, indicating that several kilodaltons of saccharide may be necessary for converting lysozyme into a stable form and to stabilize against heating. The result from the present study was much similar in the improvement of heat stability. However, Kato (2002) concluded that oligosaccharide was little effective in improving heat stability, based on the results from lysozyme-xylloglucan. It can be deduced that the structure of chitosan oligosaccharide contributed in stabilizing whey protein heat-resistant form.

With the variable of setting time increasing, emulsifying activity initially increases then decreases, with the separating time point of 20 h, which is partly in agreement with the study result of Miralles et al. (2007) that the emulsifying activity index (EAI) of β -LG-chitosan went up at first and then went down with the extension of setting time. There are also some differences in the conclusions above due to the differences in categories of proteins and saccharides which were used in this study, of which chitosan oligosaccharide had lower molecular weight and different amount of acetyls in comparison with chitosan and the use of WPI could also alter the results. According to the research results of Hattori (2002), increased saccharide content improved emulsifying properties of β -LG. The result showed some differences

because α -LA was involved as an important part and the reaction conditions were differed from the report.

The increase of emulsifying properties with the increase of saccharide rate within a limited range should be due to the relatively high molecular rate of the saccharide (Nagasawa et al. 1996). The increase in saccharide rate probably caused the increase in positive charge, which was an important aspect of improving *ES* (Kato 2002).

Similar results were also observed in the research of Hattori (2002), where the emulsifying ability of β -LG under unfavorable conditions was markedly improved by conjugation of carboxymethyl dextrans of different molecular weights, finding that the role of saccharides in improving *EA* were to modify the net charge of β -LG, resulting in the down-shift of the *pI*. According to the result of Hattori (2002), the more saccharide content was in the conjugate, the lower *pI* could be achieved, and the better improved *EA* was observed. However, the kind of saccharide used in the present study was different, causing different effect of net charge modification. The α -LA content also affected the conjugation of WPI and COS, altering the *pI* of the conjugates. Einhorn-Stoll et al. (2005) reported conjugation of WPI with pectin, where the emulsifying properties were markedly improved and the higher saccharide content proved to be advantageous to improving WPI emulsifying properties. In the present study, COS was used as the saccharide in conjugation, and the reaction conditions were much different, therefore, the result came to be some difference.

Higher setting temperature may be advantageous to keeping the WPI unfolded structure to maximize the degree of Maillard reaction (Einhorn-Stoll et al. 2005).

Conclusions

Modifications by attachment of chitosan oligosaccharide molecules through glycosylation improved solubility, heat stability and emulsifying properties of WPI, and models for these properties were established using RSM, which helped to understand the interactive effects of these factors. The models were significant to predict how the functional properties should be affected by the dry-heating conditions (*SR*, setting time and setting temperature). According to the models, the solubility of WPI-COS was 98.8 % at 29.8 % *SR*, 17.4 h, 46.7 °C; the heat stability of WPI-COS was 92.95 % at 27.2 % *SR*, 18.1 h, 48.7 °C; the *EA* of WPI-COS was 1.87 at 25.3 % *SR*, 20.0 h, 46.1 °C; the *ES* of WPI-COS was 13.28 min at 32.5 % *SR*, 12.0 h, 35.0 °C. Verification experiments are needed, especially on the emulsifying properties, and further researches on the mechanism aspect are needed. On the other hand, detailed investigation on solubility model will be performed due to its lower R-square value. Overall, the results of this study still make sense in the utilization of whey proteins.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (award nr 30871817 and 31171715) and National Science and Technology Ministry of China (award nr 2011BAD09B03) and Chinese Universities Scientific Fund.

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