

# Influence of Maillard reaction conditions on the antigenicity of bovine $\alpha$ -lactalbumin using response surface methodology

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## Abstract

**BACKGROUND:** Maillard reaction can modify functional properties of proteins. Bovine  $\alpha$ -lactalbumin ( $\alpha$ -LA) is often supplemented to the new generation of infant formulae, but it is considered to be a main allergen. However, there is little information on the effect of Maillard reaction on  $\alpha$ -LA antigenicity. The objective of this study was to investigate the influence of Maillard reaction on the antigenicity of  $\alpha$ -LA in conjugates of whey protein isolate (WPI) with glucose under different conditions of protein/sugar weight ratio (0.17–7.83), temperature (40–60 °C) and time (24–120 h) using response surface methodology.

**RESULTS:** Conjugation of WPI with glucose markedly reduced the antigenicity of  $\alpha$ -LA. This reduction in antigenicity could be controlled by regulating the three independent variables weight ratio, temperature and time. A model of optimal reaction conditions for lower antigenicity of  $\alpha$ -LA was established. According to the model, the minimum antigenicity of  $\alpha$ -LA was achieved at 52.8 °C, 78 h and 5.96:1 WPI/glucose weight ratio. WPI/glucose weight ratio had the greatest effect on the antigenicity of  $\alpha$ -LA, while reaction temperature influenced  $\alpha$ -LA antigenicity to a lesser extent.

**CONCLUSION:** Well-controlled Maillard reaction between WPI and glucose is an efficient method to reduce  $\alpha$ -LA antigenicity.

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**Keywords:**  $\alpha$ -lactalbumin; whey protein isolate; glucose; Maillard reaction; antigenicity; response surface methodology

## INTRODUCTION

Cow milk and its products are main nutrients for humans, especially infants. However, cow milk is considered an important allergen in food allergies. In many epidemiological studies, cow milk protein allergy has been reported to be the most prevalent allergy in infants and young children, with an incidence of 2–6%.<sup>1–3</sup> All cow milk proteins may be potential allergens, but the main allergens have been found to be  $\alpha$ -lactalbumin ( $\alpha$ -LA),  $\beta$ -lactoglobulin ( $\beta$ -LG) and caseins.<sup>4,5</sup> Other proteins such as bovine serum albumin and even lactoferrin (present in trace amounts) have also been shown to induce milk allergy.<sup>6</sup>

$\alpha$ -LA is a monomeric globular protein comprising 123 amino acids with four disulfide bonds and a molecular mass of approximately 14 kDa. It is present in all mammalian milks.<sup>6</sup>  $\alpha$ -LA is the second most abundant whey protein in bovine milk and the dominant whey protein in human milk. There is 74% amino acid sequence homology between  $\alpha$ -LA molecules of bovine milk and human milk. In addition,  $\alpha$ -LA is a good source of the essential amino acids tryptophan and cysteine. Infant formulae derived from bovine milk are increasingly 'humanised' by the addition of bovine whey protein fractions enriched with  $\alpha$ -LA.<sup>7,8</sup> However, it has been proven that bovine  $\alpha$ -LA is an important allergen. Many immunoglobulin E (IgE)- and IgG-binding epitopes on bovine  $\alpha$ -LA have been identified in serum from allergic patients and in animal serum.<sup>9–11</sup>

Several processing approaches have been applied to alter the allergenic potential of cow milk proteins, including heat treatment,

enzymatic hydrolysis, fermentation and glycation.<sup>4,12–14</sup> Of these processes, conjugation with reducing sugars through Maillard reaction seems to be a promising and safe method for masking food protein allergenicity and also an effective means to improve functional properties of proteins. Glycation reaction has been proposed as a way to change soy allergen structures, mask allergenicity and enhance functional properties.<sup>15</sup> It has been found that the antigenicity of soy protein isolate is greatly reduced by glycation with both fructose and fructooligosaccharides.<sup>16</sup> Some studies have revealed that  $\beta$ -LG allergenicity is modified by conjugation with various saccharides. Morgan *et al.*<sup>17</sup> indicated that lactosylated  $\beta$ -LG produced by Maillard reaction during heating changed the epitopic area. Hattori *et al.*<sup>18</sup> showed that the conjugate of  $\beta$ -LG and carboxymethyl dextran improved emulsifying properties and reduced immunogenicity.  $\beta$ -LG conjugated with acidic oligosaccharides has been shown to enhance thermal stability and reduce immunogenicity.<sup>13</sup> Thus well-controlled Maillard reaction can be a good method for protein processing in the food industry.

It has been reported that glycation of  $\alpha$ -LA with glucose and lactose can lead to higher solubility and better emulsifying

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properties compared with nonglycated  $\alpha$ -LA.<sup>19,20</sup> However, only a few studies have focused on changes in the antigenicity of  $\alpha$ -LA during Maillard reaction. Moreover, the influence of Maillard reaction conditions such as temperature, time and protein/sugar weight ratio on the antigenicity of proteins has not been studied systematically. In order to establish a predictive model of the effects of reaction conditions of glycation on  $\alpha$ -LA antigenicity, response surface methodology (RSM) was employed in the present study to optimise reaction conditions, since RSM has been proven to be a valuable tool for simultaneous optimisation of several process parameters in biological and chemical science.<sup>21,22</sup>

Whey proteins are widely used in food products as a kind of functional additive owing to their high nutritional value and excellent functional properties. In this study, whey protein isolate (WPI) conjugates with glucose were investigated. Glucose is a common alimentary sugar in the food industry and is easily absorbed and utilised by the body. Therefore the purpose of the study was to investigate the effects of different dry-heating conditions of Maillard reaction (WPI/glucose weight ratio, temperature and time) on the antigenicity of  $\alpha$ -LA and to use RSM to select optimal conditions for lower antigenicity of  $\alpha$ -LA.

## MATERIALS AND METHODS

### Materials

The antigen used for sensitisation studies and enzyme-linked immunosorbent assay (ELISA) was  $\alpha$ -LA (L5385, purity >90%) purchased from Sigma Chemical Company (St Louis, MO, USA).

WPI was obtained as a powder from Murray Goulburn Co-operative Co. Ltd (Melbourne, Australia). It contained 852 g total protein, 61 g moisture, 33 g ash and 3 g carbohydrate kg<sup>-1</sup>. Glucose was purchased from Beijing Chemical Reagents Company (Beijing, China). All other reagents used were of analytical grade.

### Preparation of rabbit antiserum

For the sensitisation studies, four young male New Zealand white rabbits from the Experimental Animal Center, China Agricultural University (Beijing, China) were used. Antibodies were obtained from the rabbits by applying the immunisation protocol shown in Table 1. The titres of antibodies were controlled by the indirect ELISA method by taking blood samples from the rabbit marginal veins 2 days before the subsequent scheduled immunisation. Ten days after the fourth sensitisation, blood samples were collected from the rabbit hearts.<sup>23</sup>

The blood samples were incubated at room temperature for 1 h and left overnight at 4 °C, then centrifuged at 3000  $\times$  g for 20 min to obtain serum samples. The serum samples were stored

at –20 °C until subsequent analysis of the antigenicity of  $\alpha$ -LA by indirect competitive ELISA.

### Preparation of WPI/glucose conjugates

WPI/glucose (WPI/G) conjugates were prepared by Maillard reaction according to a previous study.<sup>24</sup> WPI and glucose in various weight ratios based on the experimental design were dissolved in distilled water and lyophilised. The WPI/G powders were kept at different temperatures in desiccators equilibrated to 79% relative humidity using saturated solutions of KBr for various incubation times according to the experimental design, then the powders containing glycation products were stored at –20 °C until analysis. Control samples were obtained by incubation of lyophilised WPI alone for 120 h under the same dry-heating conditions. WPI without dry-heating treatment was also used as a control.

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of conjugates

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the WPI/G conjugates was performed on 120 g L<sup>-1</sup> acrylamide plates according to Laemmli.<sup>25</sup> The resolving gel was prepared in 1.5 mol L<sup>-1</sup> Tris-HCl buffer (pH 6.8) and the stacking gel comprised 40 g L<sup>-1</sup> acrylamide diluted in 0.5 mol L<sup>-1</sup> Tris-HCl buffer (pH 8.8). All samples, each containing 8  $\mu$ g of protein, were prepared in denaturing buffer with  $\beta$ -mercaptoethanol and heated at 100 °C for 3 min before electrophoresis.

The gels were run in Tris-glycine buffer (pH 8.3) and the electrophoresis was carried out at a constant voltage of 80 V for 1 h in the stacking gel, then at 100 V for 1.5 h in the resolving gel or until the tracking dye reached the bottom of the gel. After electrophoresis, proteins in the gels were stained with Coomassie Brilliant Blue R-250.

### Indirect competitive ELISA

The antigenicity of  $\alpha$ -LA in dry-heated WPI and WPI/G conjugates was estimated by indirect competitive ELISA.<sup>23</sup> Microtitre plates with 96 flat-bottomed wells (Costar, Corning, New York, USA) were coated with 100  $\mu$ L per well of antigen (2  $\mu$ g mL<sup>-1</sup>  $\alpha$ -LA, as determined earlier by indirect ELISA) diluted in 50 mmol L<sup>-1</sup> carbonate buffer (pH 9.8) and incubated overnight at 4 °C. WPI solution and standard antigen ( $\alpha$ -LA) were separately incubated overnight at 4 °C in test tubes with an equivalent volume of rabbit anti- $\alpha$ -LA serum (1 : 128 000) diluted in 10 mmol L<sup>-1</sup> phosphate-buffered saline (PBS, pH 7.4) containing 10 g L<sup>-1</sup> bovine serum albumin (BSA) and 1 g L<sup>-1</sup> Tween 20 (PBS-BSA-T). Next day the plates were washed four times with 10 mmol L<sup>-1</sup> PBS (pH 7.4) containing 0.5 g L<sup>-1</sup> Tween 20 (PBS-T). This washing procedure was repeated after each step of ELISA. After washing, the wells were filled with 100  $\mu$ L per well of PBS-BSA-T to block residual

**Table 1.** Immunisation procedure of New Zealand white rabbits

| Sequence | Interval (weeks) | Antigen sensitisation             | Dose (mg protein kg <sup>-1</sup> weight) | Treatment            |
|----------|------------------|-----------------------------------|-------------------------------------------|----------------------|
| First    | 0                | Antigen/Freud complete adjuvant   | 1                                         | Crural intramuscular |
| Second   | 2                | Antigen/Freud incomplete adjuvant | 1                                         | Dorsal subcutaneous  |
| Third    | 4                | Antigen                           | 1                                         | Dorsal subcutaneous  |
| Fourth   | 6                | Antigen                           | 1                                         | Otic plexus          |

free binding sites and incubated for 1 h at 37 °C. After further washing, 100 µL per well of reactive mixtures of samples or standard antigen and polyclonal rabbit antibodies (IgG) were added and incubated for 1 h at 37 °C. After washing again, the wells were filled with 100 µL per well of horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma A6154, 1 : 10 000) in PBS-BSA-T. After incubation for 1 h at 37 °C and washing, 100 µL of 3,3',5,5'-tetramethylenbenzidine substrate solution (Amresco, Ohio, USA) was immediately added to each well and incubated for 15 min at 37 °C. Finally 50 µL of 2 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction. Absorptions were determined at dual wavelengths of 450 and 630 nm using a Multiskan MK3 ELISA plate reader (Thermo Labsystems, Franklin, MA, USA).

The antigenicity was calculated from a standard curve of α-LA, and a linear logarithmic correlation was observed in the range 0.05–200 µg mL<sup>-1</sup>. All analyses were carried out in triplicate and average values were converted to concentration equivalents (µg mL<sup>-1</sup>).

The inhibition rate of α-LA antigenicity by Maillard reaction was calculated as follows:

$$\text{inhibition rate (\%)} = [(B_0 - B)/B_0] \times 100 \quad (1)$$

where *B* is the antigenicity of α-LA in WPI/G conjugates and *B*<sub>0</sub> is the antigenicity of α-LA in WPI.

### Experimental design

In the experimental design, WPI/G weight ratio (*X*<sub>1</sub>), temperature (*X*<sub>2</sub>) and time (*X*<sub>3</sub>) were chosen as independent variables (*k* = 3). The dependent *Y* variable was used to evaluate the antigenicity of α-LA in the conjugates of WPI with glucose. The independent variables were optimised using a central composite rotatable design (CCRD) containing five levels for each independent variable, coded as -1.682, -1, 0, 1 and 1.682. The correspondence between these coded values and the actual values of independent variables is given in Table 2. The complete central composite design consisted of 2<sup>3</sup> experiments for a full factorial design plus 2 × 3 star experiments and nine centre experiments, resulting in a total of 23 experiments (Table 3). The centre experiments were chosen to verify any change in the estimation procedure as a measure of precision. Each glycation experiment was run in duplicate.

### Statistical analysis

Experimental data were statistically analysed using SAS Version 8.2 (SAS Institute Inc., Cary, NC, USA). Response surface analysis was

based on multiple regressions taking into account main, quadratic and interactive effects according to the following equation:

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

where *b*<sub>0</sub>, *b*<sub>*i*</sub>, *b*<sub>*ii*</sub> and *b*<sub>*ij*</sub> are constant and regression coefficients of the model (*b*<sub>0</sub> for constant, *b*<sub>*i*</sub> for linear terms, *b*<sub>*ii*</sub> for quadratic terms and *b*<sub>*ij*</sub> for interactive terms) and *x*<sub>*i*</sub> is the coded value of the independent variable corresponding to the uncoded value *X*<sub>*i*</sub>. As three independent variables were chosen, ten *b* coefficients were estimated. The significance of the *b* coefficients for each dependent variable was assessed by Student's *t* test. The level of significance was defined at *P* < 0.05. The goodness of fit of the model was evaluated by analysis of variance (ANOVA). Three-dimensional response surface plots were drawn using the fitted fully quadratic polynomial equations obtained by holding one of the independent variables at a constant value and varying the levels of the other two variables within the experimental range.

## RESULTS AND DISCUSSION

### SDS-PAGE analysis of conjugates

The SDS-PAGE patterns of native WPI, dry-heated WPI and WPI/G conjugates are presented in Figs 1–3. It can be observed from Fig. 1 that dry-heated WPI showed a similar band pattern to native WPI. The faint bands of high molecular mass for dry-heated WPI, especially at 60 °C, indicated protein polymerisation. There were almost no changes in mobility of WPI/G mixtures without dry-heating treatment compared with native WPI (Fig. 3).

When the protein and saccharide reacted, a covalent bond was formed; the ε-amino residue of the protein and the reducing end of the saccharide were expected to attach themselves to each other.<sup>26</sup> As seen in Figs 2 and 3, the mobility of major whey proteins (α-LA, β-LG, β-LG dimer and BSA) decreased in glycated WPI compared with native WPI, while the molecular mass of these proteins increased. The results proved that some glucose bound to whey proteins during the reaction. With increasing temperature and reaction time (Fig. 2), whey protein components decreased gradually and the formation of high-molecular-mass conjugates increased. It can be seen in lane 4 of Fig. 2 that a small amount of protein was retained in the stacking gel, indicating the formation of polymers between WPI and glucose and an increase in glycation degree. In addition, the formation of numerous polymers could reflect structural modification, and the antigenicity of polymers might be altered compared with the native form.<sup>16</sup>

### Assessment of model of α-LA antigenicity for three independent variables

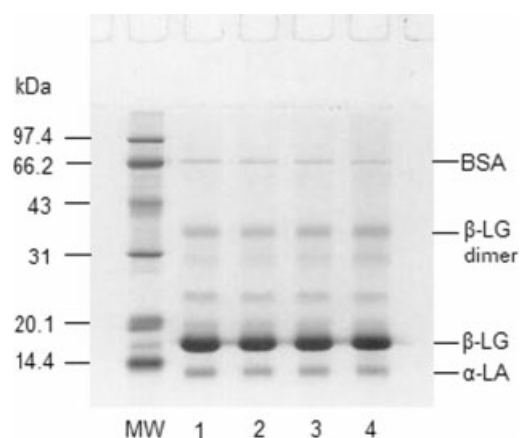
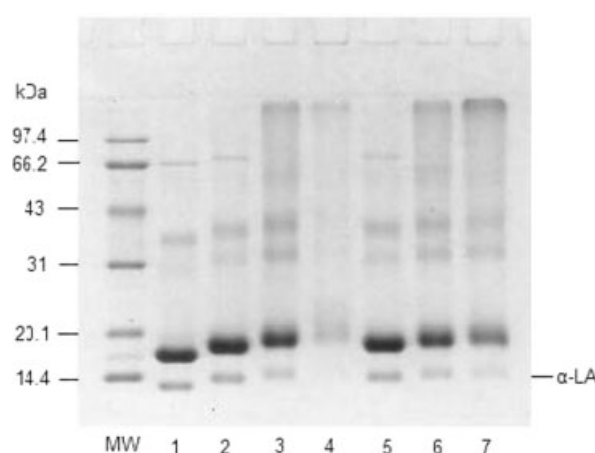
Indirect competitive ELISA indicated that the antibody prepared against α-LA was specific to α-LA, and no cross-reactivity was observed with other cow milk proteins. The antigenicity of α-LA in WPI and WPI/G conjugates was determined by indirect competitive ELISA. The experimental design and results are presented in Table 3. Variations in the antigenicity of α-LA under different reaction conditions were observed. From regression analysis on 23 experiments, the regression coefficients and overall performance of the initial regression analysis performed with all regression variables (Table 4, 'full model') revealed that several terms were not significant (*P* > 0.05). In order to fit the full second-order model, non-significant terms were eliminated step by step from the

**Table 2.** Coded and uncoded settings of independent variables for dry-heating according to central composite rotatable design

| Coded level | Independent variables |                  |          |
|-------------|-----------------------|------------------|----------|
|             | Weight ratio          | Temperature (°C) | Time (h) |
| 1.682       | 7.83                  | 60               | 120      |
| 1           | 6.28                  | 56               | 100.5    |
| 0           | 4                     | 50               | 72       |
| -1          | 1.72                  | 44               | 43.5     |
| -1.682      | 0.17                  | 40               | 24       |

**Table 3.** Full factorial central composite design matrix for antigenicity of  $\alpha$ -lactalbumin ( $\alpha$ -LA) in whey protein isolate/glucose conjugates

| Assay | Independent variables <sup>a</sup> |                                    |             | Dependent variable: $\alpha$ -LA antigenicity ( $\mu\text{g mL}^{-1}$ ) <sup>b</sup> | Inhibition rate (%) |
|-------|------------------------------------|------------------------------------|-------------|--------------------------------------------------------------------------------------|---------------------|
|       | Weight ratio                       | Temperature ( $^{\circ}\text{C}$ ) | Time (h)    |                                                                                      |                     |
| 1     | 1.72 (−1)                          | 44 (−1)                            | 43.5 (−1)   | 31.65 $\pm$ 2.07                                                                     | 31.27               |
| 2     | 1.72 (−1)                          | 44 (−1)                            | 100.5 (1)   | 15.67 $\pm$ 1.12                                                                     | 65.97               |
| 3     | 1.72 (−1)                          | 56 (1)                             | 43.5 (−1)   | 29.71 $\pm$ 2.26                                                                     | 35.48               |
| 4     | 1.72 (−1)                          | 56 (1)                             | 100.5 (1)   | 7.65 $\pm$ 0.60                                                                      | 83.39               |
| 5     | 6.28 (1)                           | 44 (−1)                            | 43.5 (−1)   | 15.98 $\pm$ 0.62                                                                     | 65.30               |
| 6     | 6.28 (1)                           | 44 (−1)                            | 100.5 (1)   | 11.20 $\pm$ 0.32                                                                     | 75.68               |
| 7     | 6.28 (1)                           | 56 (1)                             | 43.5 (−1)   | 4.16 $\pm$ 0.23                                                                      | 90.97               |
| 8     | 6.28 (1)                           | 56 (1)                             | 100.5 (1)   | 1.72 $\pm$ 0.05                                                                      | 96.26               |
| 9     | 0.17 (−1.682)                      | 50 (0)                             | 72 (0)      | 30.39 $\pm$ 0.91                                                                     | 34.00               |
| 10    | 7.83 (1.682)                       | 50 (0)                             | 72 (0)      | 0.26 $\pm$ 0.04                                                                      | 99.44               |
| 11    | 4 (0)                              | 40 (−1.682)                        | 72 (0)      | 28.21 $\pm$ 1.14                                                                     | 38.74               |
| 12    | 4 (0)                              | 60 (1.682)                         | 72 (0)      | 12.37 $\pm$ 1.35                                                                     | 73.14               |
| 13    | 4 (0)                              | 50 (0)                             | 24 (−1.682) | 26.70 $\pm$ 0.62                                                                     | 42.02               |
| 14    | 4 (0)                              | 50 (0)                             | 120 (1.682) | 10.25 $\pm$ 0.51                                                                     | 77.74               |
| 15    | 4 (0)                              | 50 (0)                             | 72 (0)      | 9.84 $\pm$ 0.64                                                                      | 78.63               |
| 16    | 4 (0)                              | 50 (0)                             | 72 (0)      | 9.23 $\pm$ 0.86                                                                      | 79.96               |
| 17    | 4 (0)                              | 50 (0)                             | 72 (0)      | 11.15 $\pm$ 0.57                                                                     | 75.79               |
| 18    | 4 (0)                              | 50 (0)                             | 72 (0)      | 8.33 $\pm$ 0.14                                                                      | 81.91               |
| 19    | 4 (0)                              | 50 (0)                             | 72 (0)      | 10.50 $\pm$ 0.43                                                                     | 77.20               |
| 20    | 4 (0)                              | 50 (0)                             | 72 (0)      | 12.36 $\pm$ 0.38                                                                     | 73.16               |
| 21    | 4 (0)                              | 50 (0)                             | 72 (0)      | 8.54 $\pm$ 0.59                                                                      | 81.45               |
| 22    | 4 (0)                              | 50 (0)                             | 72 (0)      | 10.01 $\pm$ 0.44                                                                     | 78.26               |
| 23    | 4 (0)                              | 50 (0)                             | 72 (0)      | 10.67 $\pm$ 0.31                                                                     | 76.83               |

<sup>a</sup> Values in parentheses are coded levels of independent variables.<sup>b</sup> Values are mean  $\pm$  standard deviation of three experiments.**Figure 1.** SDS-PAGE analysis of whey protein isolate (WPI) dry-heated at different temperatures for 120 h: MW, molecular weight markers; lane 1, native WPI; lane 2, WPI dry-heated at 40  $^{\circ}\text{C}$ ; lane 3, WPI dry-heated at 50  $^{\circ}\text{C}$ ; lane 4, WPI dry-heated at 60  $^{\circ}\text{C}$ .**Figure 2.** SDS-PAGE analysis of dry-heated whey protein isolate (WPI)/glucose (G) conjugates (weight ratio 4:1): MW, molecular weight markers; lane 1, native WPI; lane 2, WPI/G dry-heated at 40  $^{\circ}\text{C}$  for 72 h; lane 3, WPI/G dry-heated at 50  $^{\circ}\text{C}$  for 72 h; lane 4, WPI/G dry-heated at 60  $^{\circ}\text{C}$  for 72 h; lane 5, WPI/G dry-heated at 50  $^{\circ}\text{C}$  for 24 h; lane 6, WPI/G dry-heated at 50  $^{\circ}\text{C}$  for 72 h; lane 7, WPI/G dry-heated at 50  $^{\circ}\text{C}$  for 120 h.

regression model, starting with quadratic and interactive terms, ensuring that the determination coefficient ( $R^2$ ) of the model did not change significantly (backward elimination). This procedure resulted in a second-order model with seven regression terms (Table 4, 'fitted model'). ANOVA indicated that the  $P$  value of the 'fitted model' was 0.0001. In addition, the adjusted  $R^2$  of the 'fitted model' was 0.904. The very low  $P$  value and high adjusted  $R^2$  value indicated that the second-order model was highly significant and

adequate to represent the actual relationship between responses and independent variables.

#### Effect of dry-heating conditions on antigenicity of $\alpha$ -LA

Regression coefficients and response surfaces were used to study the effects of various independent variables on the antigenicity of

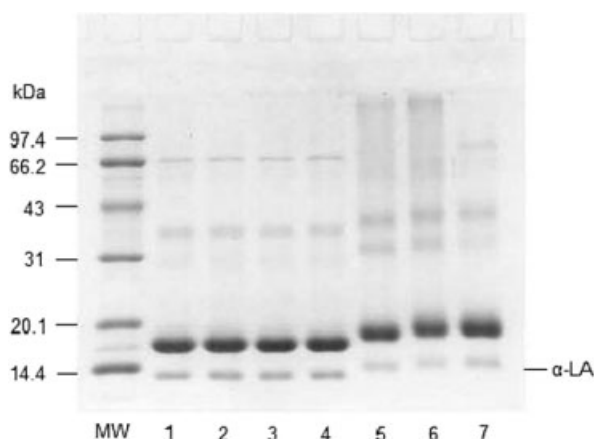


**Figure 3.** SDS-PAGE analysis of whey protein isolate (WPI)/glucose (G) mixtures without dry-heating treatment (lanes 2–4) and dry-heated at 50 °C for 72 h (lanes 5–7): MW, molecular weight markers; lane 1, native WPI; lanes 2 and 5, WPI/G weight ratio 7.83 : 1; lanes 3 and 6, WPI/G weight ratio 4 : 1; lanes 4 and 7, WPI/G weight ratio 0.17 : 1.

**Table 4.** Regression coefficients (*b*) of linear regression model for prediction of antigenicity of  $\alpha$ -lactalbumin

|                             | Full model |          | Fitted model |          |
|-----------------------------|------------|----------|--------------|----------|
|                             | <i>b</i>   | <i>P</i> | <i>b</i>     | <i>P</i> |
| Intercept                   | 10.15      |          | 10.80        |          |
| <i>Linear</i>               |            |          |              |          |
| Weight ratio                | −7.49      | 0.000    | −7.49        | 0.000    |
| Temperature                 | −4.24      | 0.000    | −4.24        | 0.000    |
| Time                        | −5.34      | 0.000    | −5.34        | 0.000    |
| <i>Quadratic</i>            |            |          |              |          |
| (Weight ratio) <sup>2</sup> | 1.07       | 0.134    | –            | –        |
| (Temperature) <sup>2</sup>  | 2.83       | 0.001    | 2.82         | 0.001    |
| (Time) <sup>2</sup>         | 2.18       | 0.006    | 2.18         | 0.008    |
| <i>Interactions</i>         |            |          |              |          |
| Weight ratio × temperature  | −1.42      | 0.157    | –            | –        |
| Weight ratio × time         | 3.85       | 0.001    | 3.85         | 0.001    |
| Temperature × time          | −0.47      | 0.629    | –            | –        |
| <i>Other statistics</i>     |            |          |              |          |
| <i>R</i> <sup>2</sup>       | 0.915      |          | 0.904        |          |
| <i>F</i>                    | 27.32      | 0.0001   | 35.58        | 0.0001   |

$\alpha$ -LA (Table 4, Fig. 4). From the analysis of regression coefficients, the linear effect of WPI/G weight ratio on the antigenicity of  $\alpha$ -LA was highly significant ( $P < 0.01$ ). Both linear and quadratic effects of temperature and time were highly significant ( $P < 0.01$ ). The interactive effect between WPI/G weight ratio and time was also highly significant ( $P < 0.01$ ). However, the interactive effects between WPI/G weight ratio and temperature and between temperature and time were not significant in comparison with the linear and quadratic effects. Among the three independent variables, the effect of WPI/G weight ratio was the most important, which was reflected by the highest absolute regression coefficient.

The effect of WPI/G weight ratio and temperature at a fixed time of 72 h is shown by the response surface plot in Fig. 4(a). WPI/G weight ratio had a negative effect on the antigenicity of  $\alpha$ -LA, so an increase in WPI/G weight ratio resulted in a decrease in antigenicity. The quadratic effect of temperature was highly significant, which

means that there is an optimal value of temperature. From Fig. 4(a) it appears that the antigenicity decreased initially with an increase in temperature and then increased as the temperature rose further. It was observed that the minimum value of antigenicity was attained at 50–55 °C. Therefore a relatively high temperature is advantageous to reduce the antigenicity of  $\alpha$ -LA during Maillard reaction of WPI with glucose. In addition, the result indicates that the decrease in antigenicity may be related to the quantity of sugar. The present study suggests that a higher WPI/G weight ratio can induce a lower antigenicity of  $\alpha$ -LA. However, Wróblewska and Jedrychowski<sup>27</sup> found that the larger the amount of polyethylene glycol (PEG), the lower was the immunoreactivity of  $\alpha$ -LA and  $\beta$ -LG in conjugates of cow milk whey protein with PEG. This discrepancy may be explained by the different nature of the conjugates used in the two studies, which might modulate the immune response of proteins.

The effect of WPI/G weight ratio and time at a fixed temperature of 50 °C is shown by the response surface plot in Fig. 4(b). A negative effect of WPI/G weight ratio on the antigenicity of  $\alpha$ -LA was observed. An optimal value of reaction time exists, as demonstrated by its highly significant quadratic effect. From Fig. 4(b) it appears that the antigenicity was minimal when the reaction time was about 70–80 h. It can be concluded from these results that a longer reaction time did not always bring about a lower antigenicity, suggesting that Maillard reaction products at different stages may contain different structural characteristics inducing antigenicity changes. Thus it is important to control the time of Maillard reaction in order to obtain a lower antigenicity.

The effect of temperature and time at a fixed WPI/G weight ratio of 4 : 1 is shown by the response surface plot in Fig. 4(c). The minimum antigenicity was observed at a temperature of 50–55 °C and a reaction time of 70–80 h. In this study it was also found that the antigenicity of  $\alpha$ -LA in native WPI and WPI dry-heated at 40, 50 and 60 °C for 120 h was 46.05, 46.53, 47.16 and 48.28  $\mu\text{g mL}^{-1}$  respectively. These results proved that there was a slight increase in  $\alpha$ -LA antigenicity in dry-heated WPI compared with native WPI. However, the present study showed that the addition of glucose to WPI during dry-heating induced a decrease in  $\alpha$ -LA antigenicity, which may suggest that glucose could shield the epitopes on  $\alpha$ -LA by Maillard reaction. Furthermore, it has been demonstrated in this study that there was a considerable reduction in the antigenicity of  $\alpha$ -LA by controlling reaction temperature and time.

The three independent variables WPI/G weight ratio, temperature and time showed different effects on the antigenicity of  $\alpha$ -LA. The optimal values of the three independent variables yielding minimum antigenicity were a WPI/G weight ratio of 5.96 : 1, a temperature of 52.8 °C and a time of 78 h. Under these reaction conditions the antigenicity of  $\alpha$ -LA was determined as 2.20  $\mu\text{g mL}^{-1}$ , indicating that the antigenicity of  $\alpha$ -LA could be reduced by 95.22% by Maillard reaction compared with native WPI. The antigenicity of  $\alpha$ -LA determined using rabbit IgG serum decreased markedly in this research, but it is uncertain whether the allergenicity of  $\alpha$ -LA can be reduced in humans, because the rabbit IgG model may be not broadly indicative of human allergic reaction. Therefore the allergenicity of  $\alpha$ -LA during Maillard reaction will be investigated using human milk-allergic IgE serum in future work.

The present results showed that the degree of WPI glycation increased gradually with increasing temperature and reaction time (Fig. 2). However, the antigenicity of  $\alpha$ -LA was reduced initially, followed by an increase with increasing temperature and reaction time (Fig. 4). This suggests that there is no direct

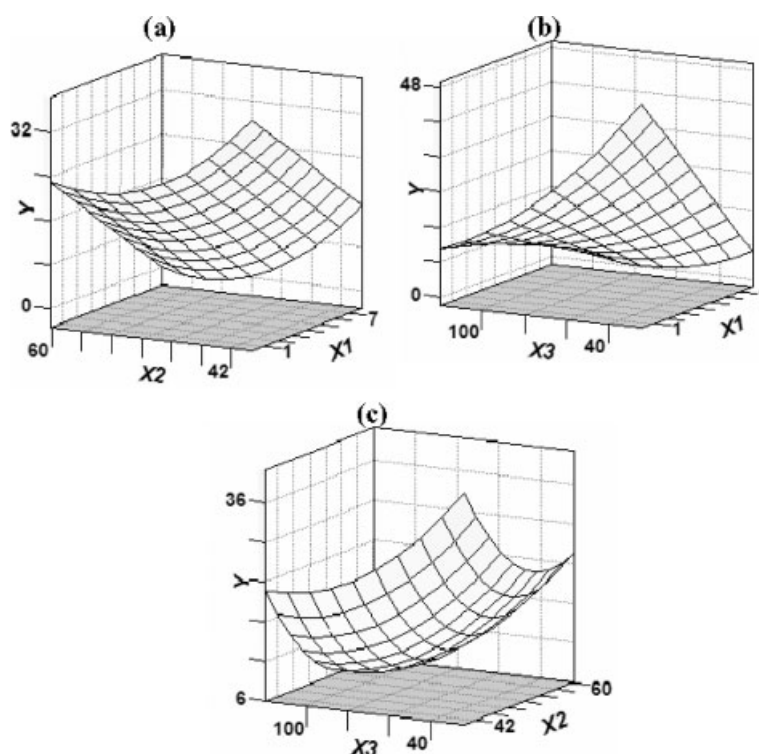


**Figure 4.** Response surfaces of antigenicity of  $\alpha$ -lactalbumin ( $Y$ ) using  $b$  coefficients from fitted model (Table 4): (a) effect of whey protein isolate/glucose (WPI/G) weight ratio ( $X_1$ ) and temperature ( $X_2$ ) on antigenicity at fixed time of 72 h; (b) effect of WPI/G weight ratio ( $X_1$ ) and time ( $X_3$ ) on antigenicity at fixed temperature of 50 °C; (c) effect of temperature ( $X_2$ ) and time ( $X_3$ ) on antigenicity at fixed WPI/G weight ratio of 4 : 1.

relationship between antigenicity and glycation degree. The changes in  $\alpha$ -LA antigenicity depend largely on the Maillard reaction conditions. In addition, the antigenicity of  $\alpha$ -LA might be related to the conformational changes caused by Maillard reaction. It was found that one of the mechanisms responsible for the reduced immunogenicity of  $\beta$ -LG-acidic oligosaccharides was the shielding of the epitopes due to the conjugation.<sup>13</sup> Therefore further investigations are needed to elucidate the structural characteristics of conjugates of WPI and glucose resulting in the decrease in antigenicity of  $\alpha$ -LA.

## CONCLUSIONS

The conjugation with glucose reduced the antigenicity of  $\alpha$ -LA effectively in this study. Under the range of conditions studied, the WPI/G weight ratio had the greatest effect on  $\alpha$ -LA antigenicity, while the temperature of Maillard reaction influenced the antigenicity of  $\alpha$ -LA to a lesser extent. RSM was an effective method to optimise several independent variables of the glycation reaction simultaneously, resulting in a WPI/G conjugate with minimum antigenicity of  $\alpha$ -LA. Although the antigenicity of  $\alpha$ -LA (a main allergen in whey proteins) was not completely eliminated, it is concluded that the glycation of whey protein with glucose under optimal reaction conditions (WPI/G weight ratio, temperature and time) is a promising way of achieving lower  $\alpha$ -LA antigenicity.

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