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Publisher: Taylor & Francis

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Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/cfai20>

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Version of record first published: 01 Nov 2011.

To cite this article: Xiaoyu Liu, Yongkang Luo & Zheng Li (2012): Effects of pH, temperature, enzyme-to-substrate ratio and reaction time on the antigenicity of casein hydrolysates prepared by papain, Food and Agricultural Immunology, 23:1, 69-82

To link to this article: <http://dx.doi.org/10.1080/09540105.2011.604770>

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Effects of pH, temperature, enzyme-to-substrate ratio and reaction time on the antigenicity of casein hydrolysates prepared by papain

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(Received 29 March 2011; final version received 28 June 2011)

The effects of pH, temperature, enzyme-to-substrate ratio and reaction time on the antigenicity of casein hydrolysates were investigated. Response surface methodology (RSM) was employed to optimise the reaction conditions. Enzymatic hydrolysis with papain could reduce the antigenicity of α -casein and β -casein effectively and the reduction of antigenicity could be controlled by regulation of the reaction conditions. The model for optimal reaction conditions of a lower antigenicity of α -casein and β -casein was established. Under the range of conditions studied, enzyme-to-substrate ratio had the most significant effects on the antigenicity of α -casein and β -casein. The anti- α -casein IgG binding inhibition and anti- β -casein binding inhibition were both significantly negatively related with the degree of hydrolysis (DH).

Keywords: cow's milk allergy; α -casein; β -casein; hydrolysis; papain; ELISA; antigen; enzyme

1. Introduction

Food allergy has become a major public health concern around the world. Cow's milk is thought to be one of the most common food allergies (Fritsche, 2003). A number of studies has presented that cow's milk protein allergy (CMPA) has an incidence of 2–6% for infants or young children (Hill & Hosking, 1996, 1997; Hosking, Heine, & Hill, 2000), causing serious consequences for the infants' health. Therefore, it is very necessary to reduce or eliminate the milk allergens.

All milk proteins appear to be potential allergens, even those present in only trace amounts. However, the main allergens in cow's milk protein seem to be casein, α -LA and β -LG (Wal, 1998). Up to now, great attentions have been paid on how to reduce the allergenicity of whey protein, technological approaches involve heat processing (Bu, Luo, Zheng, & Zheng, 2009; Kleber & Hinrichs, 2007), enzymatic hydrolysis (Wróblewska, Jedrychowski, Hajós, & Szabó, 2008; Zheng, Shen, Bu, & Luo, 2008), fermentation (Bu, Luo, Zhang, & Chen, 2010), Maillard reaction (Bu, Lu, Zheng, & Luo, 2009; Bu, Luo, Lu, & Zhang, 2010) and high pressure (Kleber, Maier, & Hinrichs, 2007). Combined application of high pressure and hydrolysis is also used (Chicón, López-Fandiño, Alonso, & Belloque, 2008). Enzymatic hydrolysis with selected proteases is by far the most efficient process. Several investigators have evaluated the immunogenicity and allergenicity of enzymatically hydrolysed casein.

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Wróblewska, Jedrychowski, Szabo, & Hajos (2005) carried out experiments that commercial sodium caseinate isolate (SCI) was hydrolysed with Alcalase, pronase and papain in a two-step process (Alcalase–papain, pronase–papain and pronase–Alcalase) to determine the changes in the immunoreactivity of α -casein, β -casein and κ -casein, finding that the two-step process was an effective method in the reduction of immunoreactivity of casein, however, allergenic epitopes were still present in all peptide fractions. Hussein, Gelencse'r, Polga'r, & Hajo's (2000) found that enzymatic peptide modification with methionine enrichment seems to be an efficient method for the reduction of the potential allergenic character and for the improvement of the nutritive value of buffalo and cow milk caseins. Wróblewska, Jedrychowski, & Farjan (2007) used Alcalase, pepsin and lactozyme in a multi-step hydrolysis (Alcalase followed by pepsin and next lactozyme) to determine the allergenicity of a low molecular fraction of whey protein and sodium caseinate hydrolysates and found it did not decrease the allergenicity of both whey protein and sodium caseinate hydrolysates.

However, fewer studies were reported to predictive the effects of various hydrolysis parameters on the antigenicity of casein hydrolysates. Therefore, the aim of this study was to evaluate the effects of pH, temperature, enzyme-to-substrate ratio and reaction time on the antigenicity of casein hydrolysates prepared by papain, optimising the reaction conditions using the response surface methodology (RSM).

2. Materials and methods

2.1 Materials

The antigen proteins used for sensitisation studies and enzyme-linked immunosorbent assay (ELISA) were α -casein (C-6780; purity > 90%) and β -casein (C-6905; purity > 95%) purchased from Sigma Chemical Company (St. Louis, MO, USA). Commercial casein obtained from Beijing Bio-technology Company (Beijing, China) was used as hydrolysis reaction substrate. Papain used for the hydrolysis of casein was purchased from FangShan Enzyme Preparation Factory (Beijing, China).

2.2 The preparation of rabbit antiserum

Rabbits for the sensitisation studies were 4–5 months old at the start of the study and weight was about 2.0 kg. Rabbits were sensitised with bovine α -casein (Sigma, C-6780) or β -casein (Sigma, C-6905) as shown in Table 1. Seven days after the fourth sensitisation, blood samples were obtained from the rabbit hearts. Blood samples were incubated for 1 h at room temperature and left overnight at 4°C, then centrifuged for 10 min at 3000 $\times$ g to obtain sera. The sera were stored at –80°C until the following analyses on the antigenicity of α -casein and β -casein by indirect competitive ELISA.

2.3 Proteolytic activity of protease

Procedures refer to folin phenol reagent method (Lowry, Rosebrough, Farr, & Randall, 1951).

Table 1. Immunisation procedure of New Zealand rabbits.

Sequence	Intervals(days)	Antigen sensitisation	Dose (mg protein/ kg weight)	Treatment
First	0	Antigen/Freud complete adjuvant	0.5	Crural intramuscular
Second	14	Antigen/Freud incomplete adjuvant	0.5	Dorsal subcutaneous
Third	10	Antigen	1.0	Otic plexus
Fourth	10	Antigen	1.0	Otic plexus

The proteolytic activity of protease was determined by folin phenol reagent method. The protease activity of papain used in the experiment is about 98253 units g⁻¹ protein.

2.4 Casein hydrolysis

Coded and uncoded settings of the independent variables for casein hydrolysis according to central composite rotatable design are presented in Table 2.

Casein was hydrolysed with papain under the conditions which were shown in Table 3. Prior to hydrolysis, casein was dissolved in 0.067 mol/L phosphate buffer, stirring for 15 min at the pre-treatment temperature of 50°C. During hydrolysis, pH was maintained by the addition of 1 M NaOH and 1 M HCl. The hydrolysis process was terminated by heating the solution at 100°C for 10 min and then cooling immediately. The DH was determined according to the trinitrobenzene sulfonic acid (TNBS) method (Nissen, 1979).

2.5 Estimation of antigenicity of casein hydrolysates by indirect competitive enzyme-linked immunosorbent assay (ELISA)

Specific procedures of indirect competitive ELISA refer to the method used earlier (Zheng et al., 2008).

The residual antigenicity of casein hydrolysates was estimated by indirect competitive ELISA. Microtitre plates with 96 wells (flat-bottomed; Costar, Corning Inc., Corning, NY, USA) were coated with α -casein (or β -casein) which

Table 2. Coded and uncoded settings of the independent variables for casein hydrolysis according to central composite rotatable design.

Coded level	Independent variables			
	pH	Temperature(°C)	E/S(%)	Reaction time(min)
2	6.80	60.0	3.0	140
1	6.40	55.0	2.5	115
0	6.00	50.0	2.0	90
-1	5.60	45.0	1.5	65
-2	5.20	40.0	1.0	40

was diluted in 50 mM carbonate buffer (pH 9.6) at $4\ \mu\text{g mL}^{-1}$ (at $2\ \mu\text{g mL}^{-1}$ for β -casein) and incubated overnight at 4°C . In test tubes, 1 mg/mL solutions of various casein hydrolysates were incubated overnight at 4°C with an equivalent volume of rabbit anti- α -casein or anti- β -casein antiserum diluted in 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 1% BSA and 0.1% Tween 20 (PBS–BSA–Tween 20) (1:240,000 for anti- α -casein antiserum and 1:120,000 for anti- β -casein antiserum). The plates were washed four times the next day with 10 mM PBS containing 0.05% Tween 20 (PBS–T). After washing the plates, all wells were filled with 100 μL per well of PBS–BSA–Tween 20 in order to block residual free binding sites, and incubated for 1 h at 37°C . The plates were washed and then 100 μL per well of reactive mixtures of casein hydrolysates and polyclonal rabbit antibodies (IgG) were added and incubated for 1 h at 37°C . Meanwhile, the addition of 100 μL of individual anti- α -casein or anti- β -casein serum was taken for the noncompetitive model. After washing the plates, the wells were filled with 100 μL per well of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (diluted 1:10,000) in PBS–BSA–Tween 20. After incubation for 1 h at 37°C , the plates were washed again and then 100 μL per well of 3,3', 5,5'-tetramethylenbenzidine (TMB, Amresco) substrate solutions were immediately added. The plates were incubated at 37°C for 10 min. Finally, 50 μL per well of 2 mol/L H_2SO_4 were added to stop the reaction. Absorptions were read spectrophotometrically at dual wavelengths of 450 nm and 630 nm by Multiskan MK3 ELISA plate reader (Thermo Labsystems, Franklin, MA, USA).

The residual antigenicity of casein hydrolysates was calculated as follows:

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

where B is the absorbance measured in the presence of casein hydrolysate and B_0 is the absorbance measured in the absence of casein hydrolysate. Low inhibition rates indicate low residual antigenicity of casein hydrolysate for α -casein and β -casein.

2.6 Experimental design

In the experimental design, pH (X_1), temperature (X_2), enzyme-to-substrate ratio (X_3) and reaction time (X_4) were chosen as independent variables. The two dependent Y (Y_1 and Y_2) variables were to evaluate the residual antigenicity of casein hydrolysates. Y_1 reflects the residual antigenicity of α -casein of casein hydrolysates and Y_2 reflects the residual antigenicity of β -casein of casein hydrolysates. Central composite rotatable design (CCRD) was used to optimise independent variables, which contained five levels (Table 2) for each independent variable, coded as -2 , -1 , 0 , $+1$ and $+2$. Table 2 shows the correspondence among coded values and actual values of independent variables. Table 3 illustrates the complete central composite design, which consisted of 2^4 experiments for a full factorial design plus 2×4 star experiments and 12 centre experiments, resulting in 36 experiments.

Table 3. Full factorial central composite design matrix for anti- α -casein and anti- β -casein IgG binding inhibition.

Assay	Independent variables ^a				Dependent variables		
	pH	T(°C)	E:S(%)	t(min)	Y ₁ : Anti- α -casein IgG binding inhibition (%)	Y ₂ : Anti- β -casein IgG binding inhibition (%)	DH(%)
1	5.6(−1)	45(−1)	1.5(−1)	65(−1)	15.69	30.48	9.34
2	5.6(−1)	45(−1)	1.5(−1)	115(1)	14.14	27.23	11.58
3	5.6(−1)	45(−1)	2.5(1)	65(−1)	10.60	21.38	12.07
4	5.6(−1)	45(−1)	2.5(1)	115(1)	9.41	19.27	16.31
5	5.6(−1)	55(1)	1.5(−1)	65(−1)	13.76	26.96	13.73
6	5.6(−1)	55(1)	1.5(−1)	115(1)	12.86	25.34	9.98
7	5.6(−1)	55(1)	2.5(1)	65(−1)	9.75	19.33	15.60
8	5.6(−1)	55(1)	2.5(1)	115(1)	11.89	17.77	13.84
9	6.4(1)	45(−1)	1.5(−1)	65(−1)	19.84	38.71	8.71
10	6.4(1)	45(−1)	1.5(−1)	115(1)	15.86	31.14	10.28
11	6.4(1)	45(−1)	2.5(1)	65(−1)	12.24	24.39	11.51
12	6.4(1)	45(−1)	2.5(1)	115(1)	11.33	20.67	11.57
13	6.4(1)	55(1)	1.5(−1)	65(−1)	18.44	37.07	9.01
14	6.4(1)	55(1)	1.5(−1)	115(1)	17.11	33.11	7.68
15	6.4(1)	55(1)	2.5(1)	65(−1)	14.05	27.94	8.37
16	6.4(1)	55(1)	2.5(1)	115(1)	15.15	29.74	8.71
17	5.2(−2)	50(0)	2(0)	90(0)	13.02	25.64	9.08
18	6.8(2)	50(0)	2(0)	90(0)	18.76	36.99	7.63
19	6(0)	40(−2)	2(0)	90(0)	15.90	32.12	8.92
20	6(0)	60(2)	2(0)	90(0)	14.02	27.75	11.20
21	6(0)	50(0)	1(−2)	90(0)	23.53	39.17	9.17
22	6(0)	50(0)	3(2)	90(0)	12.52	25.04	11.01
23	6(0)	50(0)	2(0)	40(−2)	16.96	34.55	9.59
24	6(0)	50(0)	2(0)	140(2)	14.02	27.78	14.57
25	6(0)	50(0)	2(0)	90(0)	9.64	23.57	11.41

Table 3 (Continued)

Assay	Independent variables ^a				Dependent variables		
	pH	T(°C)	E:S(%)	t(min)	Y ₁ : Anti- α -casein IgG binding inhibition (%)	Y ₂ : Anti- β -casein IgG binding inhibition (%)	DH(%)
26	6(0)	50(0)	2(0)	90(0)	10.67	27.26	9.66
27	6(0)	50(0)	2(0)	90(0)	9.54	24.82	9.93
28	6(0)	50(0)	2(0)	90(0)	11.53	23.48	10.01
29	6(0)	50(0)	2(0)	90(0)	8.68	23.52	9.96
30	6(0)	50(0)	2(0)	90(0)	9.30	22.54	10.44
31	6(0)	50(0)	2(0)	90(0)	10.63	23.41	10.61
32	6(0)	50(0)	2(0)	90(0)	9.64	23.40	11.72
33	6(0)	50(0)	2(0)	90(0)	9.50	22.12	9.74
34	6(0)	50(0)	2(0)	90(0)	9.20	23.05	10.23
35	6(0)	50(0)	2(0)	90(0)	10.59	22.56	9.63
36	6(0)	50(0)	2(0)	90(0)	9.67	23.41	9.82

^aValues in parentheses are the coded levels of independent variables

2.7 Statistical analysis

SAS 8.2 (SAS Institute Inc., Cary, NC, USA) was applied to analyse the experimental data. Response surface analysis took into account the main, the quadratic and the interaction effects, according to the following equation:

$$Y = b_0 + \sum_{i=1}^4 b_i x_i + \sum_{i=1}^4 b_{ii} x_i^2 + \sum_{i < j=2}^4 b_{ij} x_i x_j \quad (2)$$

where b_0 is constants of the model. b_i , b_{ii} and b_{ij} are regression coefficients, respectively, for linear terms, quadratic terms and interaction terms of the model. x_i is the independent variable in the coded value. Student's- t test was employed to assess the significance of the b -coefficients for each dependent variable. The level of significance was defined at $P < 0.05$. The ANOVA is applied to evaluate the significance and the fitness of the model, as well as the effects of individual terms and their interactions on the responses. The three-dimensional response surface plots were drawn using the fit quadratic polynomial equations by SAS, holding two of the independent variables at a constant value and varying the other two variables within the experimental range.

3. Results and discussion

3.1 Assessment on models of antigenicity of casein hydrolysates for four independent variables

Table 3 illustrates the experimental design and results. The results for the regression analysis are shown in Table 4. The significance and the fitness of the model were assessed by ANOVA, in addition, the effects of individual terms and their interactions on the responses were evaluated by ANOVA. Table 4 ('full model') revealed that several terms were not significant ($P > 0.05$). The nonsignificant terms were eliminated to fit the full second-order model. For α -casein, the linear effects of temperature and reaction time are not significant. However, the quadratic effects of temperature and reaction time are significant. So the linear terms should be retained in the model. In addition, for β -casein, the linear effect of temperature is not significant, but the interaction effects between temperature and pH is significant. So the linear terms of temperature should be retained in the model. This procedure resulted in the second-order model for α -casein with eight regression terms and that for β -casein with eight regression terms, respectively (Table 4, 'fitted model'). Table 4 showed that P -values of the 'fitted model' for α -casein and for β -casein were 0.0001, respectively. The adjusted R^2 of the 'fitted model' for α -casein is 0.861 and that for β -casein is 0.816. The small P -values and high adjusted R^2 values demonstrate that the model could give a good description of the relationship between responses and independent variables.

3.2 Inhibition of anti- α -casein IgG binding to α -casein by casein hydrolysates

The effects of four independent variables on the anti- α -casein IgG binding inhibition could be observed by regression coefficients (Table 4) and response surfaces (Figure 1). It is presented that both linear and quadratic effects of pH on

Table 4. Regression coefficients for the regression model for prediction of anti- α -casein and anti- β -casein IgG binding inhibition.

	Full model				Fitted model			
	Anti- α -casein IgG binding inhibition (%)		Anti- β -casein IgG binding inhibition (%)		Anti- α -casein IgG binding inhibition (%)		Anti- β -casein IgG binding inhibition (%)	
	<i>b</i> - coefficient	P	<i>b</i> - coefficient	P	<i>b</i> - coefficient	P	<i>b</i> - coefficient	P
Intercept	9.883		23.595		9.883		24.183	
Linear								
pH	1.558	0.0001	3.238	0.0001	1.558	0.0001	3.238	0.0001
Temperature	0.005	0.984	-0.198	0.716	0.006	0.985	-0.198	0.725
(E:S)	-2.304	0.0001	-4.075	0.0001	-2.304	0.0001	-4.075	0.0001
Time	-0.520	0.080	-1.480	0.012	-0.521	0.103	-1.480	0.013
Quadratic								
pH ²	1.134	0.0001	1.226	0.015	1.134	0.0002	1.226	0.017
Temperature ²	0.901	0.001	0.881	0.072	0.901	0.002		
(E:S) ²	1.668	0.0001	1.424	0.006	1.668	0.0001	1.424	0.006
Time ²	1.034	0.0004	1.189	0.018	1.034	0.0006	1.189	0.020
Interactions								
pH $\times$ temperature	0.441	0.216	1.369	0.0496			1.369	0.055
pH $\times$ (E:S)	-0.230	0.514	-0.314	0.637				
pH $\times$ Time	-0.226	0.520	-0.307	0.645				
Temperature $\times$ (E:S)	0.664	0.069	0.884	0.193				
Temperature $\times$ Time	0.540	0.134	0.707	0.294				
(E:S) $\times$ Time	0.556	0.123	0.676	0.316				
Other statistics								
<i>R</i> ²	0.909		0.867		0.861		0.816	
<i>F</i>	15.05	0.0001	9.789	0.0001	20.921	0.0001	14.979	0.0001

the anti- α -casein IgG binding inhibition were highly significant ($P < 0.01$). The linear effect of temperature was not significant ($P > 0.05$), however, the quadratic effects of temperature were highly significant ($P < 0.01$). For enzyme-to-substrate ratio, both linear and quadratic effects were highly significant ($P < 0.01$). The linear effect of reaction time was not significant ($P > 0.05$), however, the quadratic effects of reaction time were highly significant ($P < 0.01$). The interactive effects among independent variables did not appear to be significant ($P > 0.05$). Enzyme-to-substrate ratio was with the highest absolute regression coefficient, indicating that enzyme-to-substrate ratio was the most significant among four independent variables.

Figure 1a presents the effects of pH (X_1) and temperature (X_2), where the enzyme-to-substrate ratio and reaction time are set on constant values of 2% (w/w) and 90 min, respectively. From Figure 1a, the anti- α -casein IgG binding inhibition decreases with the increase of pH up to 5.5–6.0, then the anti- α -casein IgG binding inhibition increases again. It indicates that when the pH is 5.5–6.0, the minimal anti- α -casein IgG binding inhibition exists. Lieske & Konrad (1996) hydrolysed α -LA and β -LG by papain and found enzymic availability was best for α -LA when pH is 3.5

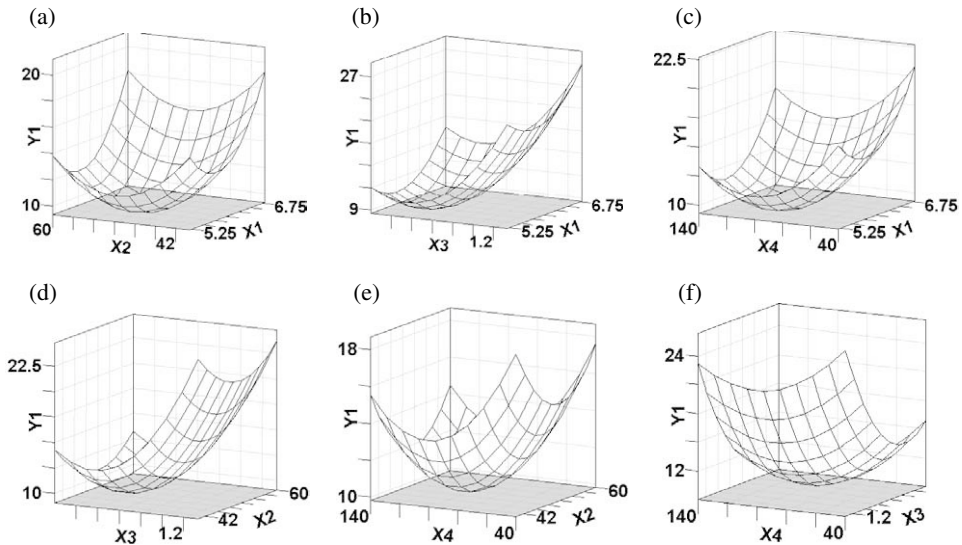


Figure 1. Response surfaces of the anti- α -casein IgG binding inhibition (Y_1) using the b -coefficients from the fitted model (Table 4). (a) The effect of pH (X_1) and temperature (X_2) on the inhibition at E/S = 2% (w/w), reaction time 90min; (b) the effect of pH (X_1) and E:S (X_3) on the inhibition at a temperature of 50°C, reaction time 90min; (c) the effect of pH (X_1) and reaction time (X_4) on the inhibition at temperature of 50°C, E/S = 2% (w/w); (d) the effect of temperature (X_2) and E:S (X_3) on the inhibition at a pH = 6, reaction time 90min; (e) the effect of temperature (X_2) and reaction time (X_4) on the inhibition at a pH = 6, E/S = 2% (w/w); (f) the effect of E:S (X_3) and reaction time (X_4) on the inhibition at pH = 6, temperature of 50°C.

and enzymic availability was best for β -LG when pH > 7.5. This suggests that papain may have different effects on different substrates; and for different purposes, favourite conditions are different even using the same enzyme. The anti- α -casein IgG binding inhibition decreases with the increase of temperature up to 48–54°C, then the anti- α -casein IgG binding inhibition increases again, which suggests that when the temperature is 48–54°C, the minimal anti- α -casein IgG binding inhibition exists. Temperature that exceeds 55°C was not effective for the reduction of antigenicity of α -casein. However, Zheng (2009) found that temperature had no effect on the antigenicity of β -LG but had a negative effect on the anti- α -LA IgG binding inhibition when hydrolysing whey protein by papain. This may be that papain has different effects on different substrates.

The effect of pH (X_1) and enzyme-to-substrate ratio (X_3) is shown in the response surface plot (Figure 1b) where the temperature and reaction time are set on constant values of 50°C and 90 min, respectively. From Figure 1b, the inhibition decreases initially with the increase of enzyme-to-substrate ratio and then the inhibition increases again. The minimal inhibition exists when enzyme-to-substrate ratio is about 2.5%. It may be that with the enzyme-to-substrate ratio increasing to 2.5%, some allergenic epitopes were destroyed, resulting in the reduction of the allergenicity. But if the enzyme-to-substrate ratio was too high exceeding 2.5%, the hydrolysis may be too active, some allergenic epitopes that hidden inside may expose outside, causing the increase of the allergenicity again.

It is illustrated in Figure 1c the effect of pH (X_1) and reaction time (X_4), where the temperature and enzyme-to-substrate ratio are set on constant values of 50°C and 2%, respectively. It shows that when reaction time is 80–100 min and pH is 5.5–6.0, the minimal inhibition exists. If the hydrolysis last for a longer time, some hidden allergenic epitopes may be outside, causing the allergenicity increasing again. On the other hand, that prolonging the reaction time may cause some bitter peptides occur, it is not good for taste. It is better to control the reaction time around 80–100 min.

Figure 1d, Figure 1e, Figure 1f show, respectively, the effect of temperature (X_2) and E:S (X_3), temperature (X_2) and reaction time (X_4), E:S (X_3) and reaction time (X_4) on the anti- α -casein IgG binding inhibition. It can be comprehensively concluded from the three figures that the minimal inhibition of anti- α -casein IgG binding to α -casein exists when temperature is 48–54°C, enzyme-to-substrate ratio (E:S) is 2.5% and reaction time is 80–100 min.

The effects of the four independent variables (pH, temperature, enzyme-to-substrate ratio and reaction time) are different on the anti- α -casein IgG binding inhibition. Finally, the optimum values of the four independent variables to reach minimum levels of inhibition for α -casein were as follows: pH = 5.60, T = 50°C, E/S = 2.5% (w/w), reaction time = 90 min. Under the above hydrolysis conditions, the inhibition for α -casein was 9.05% and that for β -casein was 19.25%.

3.3 Inhibition of anti- β -casein IgG binding to β -casein by casein hydrolysates

As we can see in Table 4 and Figure 2, the linear effects of pH on the anti- β -casein IgG binding inhibition are highly significant ($P < 0.01$), the quadratic effects of pH were significant ($P < 0.05$). Neither the linear effect nor the quadratic effects of temperature is significant ($P > 0.05$). For enzyme-to-substrate ratio, both linear and quadratic effects are highly significant ($P < 0.01$). And both linear and quadratic effects of reaction time are significant ($P < 0.05$). The interactive effects between pH and temperature appear to be significant ($P < 0.05$). The effect of enzyme-to-substrate ratio is most significant among four independent variables for inhibition of anti- β -casein IgG binding to β -casein.

The effect of pH (X_1) and temperature (X_2) on anti- β -casein IgG binding inhibition (Y_2) is presented in the response surface plot (Figure 2a), where the enzyme-to-substrate ratio and reaction time are set on constant values of 2% (w/w) and 90 min, respectively. From Figure 2a, the anti- β -casein IgG binding inhibition decreases with the increase of pH, indicating that pH has negative effects on anti- β -casein IgG binding inhibition. Zheng (2009) studied the antigenicity of WPC[AQ2] hydrolysates prepared by papain, the results suggested when pH is around 5.5, the antigenicity of α -LA is minimal. So it may be that papain could perform well in acid environment. Temperature had negative effects on the anti- β -casein IgG binding inhibition. With the temperature increases, the anti- β -casein IgG binding inhibition decreases.

Figure 2b shows the effect of pH (X_1) and enzyme-to-substrate ratio (X_3) on the anti- β -casein IgG binding inhibition (Y_2), where the temperature and reaction time are set on constant values of 50°C and 90 min, respectively. When the

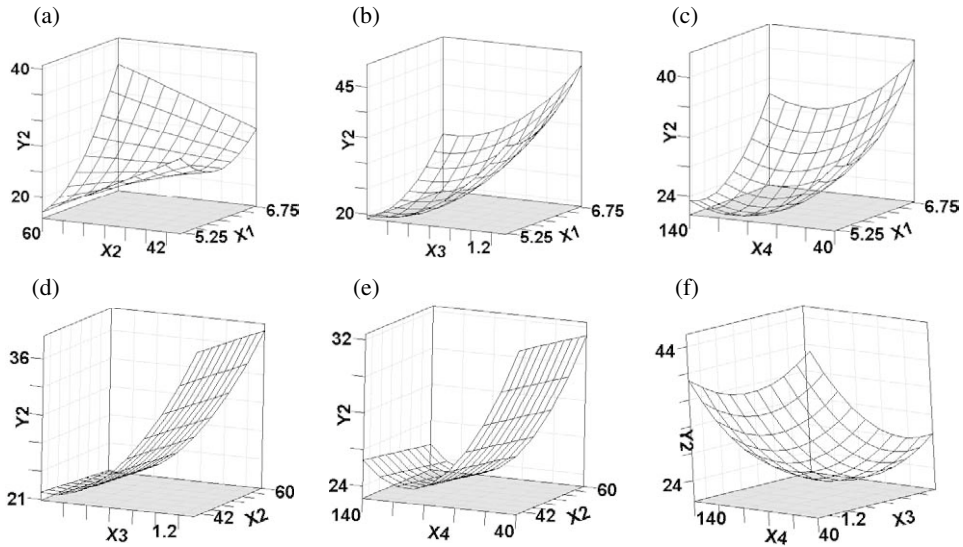


Figure 2. Response surfaces of the anti- β -casein IgG binding inhibition (Y_2) using the b -coefficients from the fitted model (Table 4). (a) The effect of pH (X_1) and temperature (X_2) on the inhibition at $E/S = 2\%$ (w/w), reaction time 90min; (b) the effect of pH (X_1) and E:S (X_3) on the inhibition at a temperature of 50°C , reaction time 90min; (c) the effect of pH (X_1) and reaction time (X_4) on the inhibition at a temperature of 50°C , $E/S = 2\%$ (w/w); (d) the effect of temperature (X_2) and E:S (X_3) on the inhibition at a pH = 6, reaction time 90min; (e) the effect of temperature (X_2) and reaction time (X_4) on the inhibition at a pH = 6, $E/S = 2\%$ (w/w); (f) the effect of E:S (X_3) and reaction time (X_4) on the inhibition at pH = 6, temperature of 50°C .

enzyme-to-substrate ratio is increased from 1% to 2.4%, the inhibition decreases rapidly and when the enzyme-to-substrate ratio exceeds 2.5%, the inhibition starts to change slightly. Therefore, relatively high enzyme-to-substrate ratio is advantageous to reduce the inhibition for β -casein, but ratio exceeds 2.5% has no significant impact on the inhibition.

The effect of pH (X_1) and reaction time (X_4) on anti- β -casein IgG binding inhibition (Y_2) is observed in Figure 2c, where the temperature and enzyme-to-substrate ratio are set on constant values of 50°C and 2%, respectively. It shows that the inhibition is low when reaction time is 80–100 min along with low pH.

Figure 2d, Figure 2e, Figure 2f present, respectively, the effect of temperature (X_2) and E:S (X_3), temperature (X_2) and reaction time (X_4), E:S (X_3) and reaction time (X_4) on anti- β -casein IgG binding inhibition (Y_2). They suggest that temperature has negative effect on the inhibition; and when enzyme-to-substrate ratio reaches 2.5% and when reaction time is 80–100 min, the minimal inhibition exists.

The optimum values of the four independent variables to yield minimum levels of inhibition for β -casein are as follows: pH = 5.20, $T = 55^\circ\text{C}$, $E/S = 2.5\%$ (w/w), reaction time = 90min. Under the above hydrolysis conditions, the inhibition for α -casein was 11.81% and that for β -casein was 18.83%.

3.4 The relation between the inhibition and the degree of hydrolysis (DH)

Figure 3 and Table 5 present the relation between the inhibition and the degree of hydrolysis (DH). It can be observed from Figure 3 that the anti- α -casein IgG binding inhibition and DH are negatively related. An increase in DH results in a reduction in the inhibition for α -casein. The R^2 (Table 5) between anti- α -casein IgG binding inhibition and DH shows highly significant negative relation. For β -casein, the anti- β -casein IgG binding inhibition is also highly significant negative with DH. This may be that with the DH increasing, more antigen epitopes are destroyed, so that the antigenicity reduces. However, many findings indicate that enzyme specificity, rather than the DH seems to determine the residual antigenicity of milk protein. Ena, van Beresteijn, Robben, & Schmidt (1995) found that the reduction in antigenicity varied with the enzyme used, this may be due to the fact that even if the DH is very high, the short peptides might contain the antigen epitopes. Jost, Monti, & Pahud (1987) found that DH had no relationship with the antigenicity. Zheng et al. (2008) concluded that the anti- α -LA IgG binding inhibition was significantly negatively related with the DH, while the anti- β -LG binding inhibition was not related with the DH. So we can conclude that the hydrolysis is different for different enzymes because of the high specificities of the enzymes, also the hydrolysis is different even using the same enzyme because of different substrates.

4. Conclusion

Enzymatic hydrolysis with papain can reduce the antigenicity of α -casein and β -casein effectively, however, the antigenicity of casein was not completely eliminated.

Under the range of conditions studied, enzyme-to-substrate ratio had the most influential effect to reduce the allergenicity of casein; pH was found to influence inhibition of α -casein and β -casein to a less extent. RSM was adequate to optimise several independent variables of the hydrolysis process simultaneously, resulting in a casein hydrolysate with minimum antigenicity for α -casein and β -casein. The model

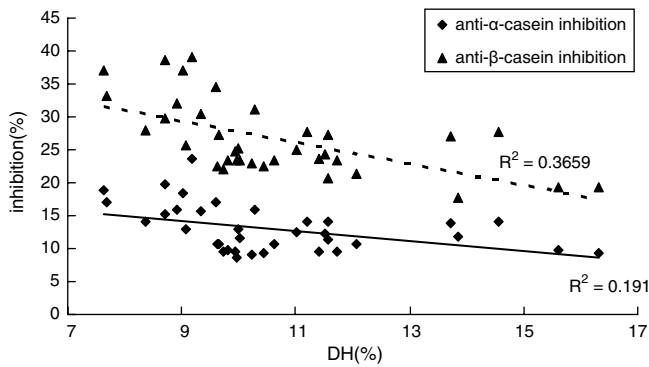


Figure 3. The relation curve between anti- α -casein (or anti- β -casein) IgG binding inhibition and the DH. Anti- α -casein inhibition: anti- α -casein IgG binding inhibition; anti- β -casein inhibition: anti- β -casein IgG binding inhibition; – linearity (anti- α -casein IgG binding inhibition);...linearity (anti- β -casein IgG binding inhibition).

Table 5. Statistics analysis of correlation between anti- α -casein (or anti- β -casein) IgG binding inhibition and the DH.

	R ²	R ^a	P-value
Anti- α -casein IgG binding inhibition	0.191	-0.437**	0.008 < 0.01
Anti- β -casein IgG binding inhibition	0.366	-0.605**	0.000 < 0.01

^aCorrelation coefficient.

**significant at 0.01.

was adequate to represent the actual relationship between responses and independent variables. The anti- α -casein IgG binding inhibition and the anti- β -casein IgG binding inhibition were both highly significant negative with DH.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (award numbers 30471224 and 30871817) and National Science and Technology Ministry of China (award numbers 2006BAD27B04 and 2006BAD04A06).

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