Two horseradish peroxidase-based modifications result in two milk protein products with ordered secondary structure and enhanced in vitro antigenicity

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\begin{abstract}
In this study, two horseradish peroxidase (HRP)-based modifications were used to treat whey protein isolate (WPI) and skim milk powder (SKMP) at protein concentration of 50 g/L, pH 7.0, and 37°C, via dityrosine formation in protein molecules. When HRP, glucose oxidase, and glucose were used to treat WPI and SKMP, suitable usages of HRP, glucose oxidase, and glucose, and reaction time were selected from single factor trials based on relative dityrosine contents of the modified products. After that, the selected HRP plus the calculated H\textsubscript{2}O\textsubscript{2} were used to treat WPI and SKMP. Electrophoretic analysis demonstrated that the modified products contained some cross-linked proteins with greater molecular weights. Other analyses showed that the modified products had more ordered secondary structure, and higher in vitro α-lactalbumin and β-lactoglobulin antigenicity than the respective substrates. HRP–glucose oxidase–glucose system was more potent than HRP–H\textsubscript{2}O\textsubscript{2} system to enhance in vitro antigenicity of WPI and SKMP.
\end{abstract}

\begin{keyword}
Whey protein isolate; skim milk powder; cross-linking; horseradish peroxidase; secondary structure; in vitro antigenicity
\end{keyword}

\begin{palabras clave}
Aislado proteínico de lactosuero; leche desnatada en polvo; reticulación; peroxidasa de rábano picante; estructura secundaria; antigenicidad in vitro
\end{palabras clave}

Introduction

Food allergies affect the health up to 6% of young children and 3–4% of adults (Wang & Sampson, 2011). Cow’s milk is widely used in the production of infant foods; however, also, it is one of the most common food allergies in childhood. Cow’s milk allergy is clinically an abnormal immunological reaction to cow milk proteins due to IgE-mediated reactions induced by one or more milk proteins (El-Agamy, 2007), such as the well-known two components (α-lactalbumin and β-lactoglobulin) of whey proteins. Whey proteins are protein ingredients widely used in food products, especially in the manufacture of infant milk powder. It is well known that food processing can alter the allergenic properties of proteins via hiding, destroying, or disclosing allergenic epitopes through conformational changes. Modification of whey proteins by physical, chemical, and enzymatic approaches is a hot spot, as functional and allergenic properties of whey proteins can be changed after the treatments. High-pressure treatment results in change of the epitopic regions of whey proteins and hydrolysates (Kleber, Maier, & Hinrichs, 2007; Peñas, Snel, Floris, Préstamo, & Gomez, 2006). Enzymatic hydrolysis by Alcalase and acid proteinases can reduce the antigenicity of α-lactalbumin and β-lactoglobulin (Lakhman et al., 2011; Zheng, Shen, Bu, & Luo, 2008). Lactic acid fermentation also is suggested to have effect on protein allergenicity (El-Ghaish et al., 2011). Conjugating whey protein isolate (WPI) with maltose through the Maillard reaction approach can effectively reduce the antigenicity of α-lactalbumin and β-lactoglobulin (Li, Luo, & Feng, 2011; Taheri-Kafani et al., 2009). Transglutaminase (TGase, EC 2.3.2.13) is well known to induce protein cross-linking (Gaspar & de Góes-Favoni, 2015) and can conjugate amido-containing saccharides (e.g., glucosamine and oligochitosan) into...
proteins (Jiang & Zhao, 2011). Glucosamine or oligochitosan glycation and cross-linking of whey proteins by TGase have been found resulting in lower in vitro antigenicity (Gaspar & de Góes-Favoni, 2015; Villas-Boas, Fernandes, Zolliner, & Netto, 2012; Zhang, Liu, Xu, & Zhao, 2016).

Some studies have utilized horseradish peroxidase (HRP, EC 1.11.1.7) to modify proteins (Chang & Zhao, 2012; Dhayal, Sforza, Wierenga, & Gruppen, 2015; Saricay, Wierenga, & de Vries, 2013), as HRP can induce the cross-linking of protein molecules through the radicalization of tyrosine residues and subsequent formation of dityrosine (Heijnis, Wierenga, Janssen, van Berkel, & Gruppen, 2010). HRP requires one molecule of H$_2$O$_2$ for each catalytic cycle. This enables a mixture containing HRP, glucose oxidase (EC 1.1.3.4), and glucose capable of cross-linking caseinate and soy protein (Chang & Zhao, 2012; Ciaurriz, Bravo, & Hamad-Schifferli, 2014), as glucose oxidase can catalyze glucose oxidation to generate H$_2$O$_2$. However, if cross-linking of milk proteins by this mixture also has impact on antigenic properties of milk proteins is still unknown in the present time.

In this study, two HRP-based modifications using respective HRP-glucose oxidase-glucose and HRP-H$_2$O$_2$ systems were applied to treat two milk protein products, WPI and skim milk powder (SKMP). The modified products were assessed for their secondary structural features and in vitro α-lactalbumin and β-lactoglobulin antigenicity. The aim of this study was to reveal potential effects of the two HRP-based modifications on antigenicity of milk proteins.

Materials and methods

Materials and chemicals

The used WPI and SKMP with respective protein contents of 87.95% and 34.35% were purchased from Brewster Dairy (Brewster, OH, USA). Glucose oxidase (type X-S, from Aspergillus niger) with declared activity of 130 kU/g, α-lactalbumin, and β-lactoglobulin were obtained from Sigma (Saint Louis, MO, USA), whilst HRP with declared activity of 20 kU/g was purchased from Aladdin Biological Technology Co. Ltd. (Shanghai, China). All enzymes were kept at −20°C before using. Goat anti-rabbit antibody and tetra-methylbenzidine were provided by Shanghai Immune Biotech Co. Ltd. (Shanghai, China). Other chemicals used were of analytical grade, while water used for the whole study was prepared by Milli-Q PLUS (Millipore Corporation, New York, NY, USA).

Two HRP-based modifications of two milk protein products

When using the HRP-glucose oxidase-glucose system, the reaction was carried out at protein concentration of 50 g/L, pH 7.0, and 37°C. The suitable levels of other reaction factors (HRP, glucose oxidase, and glucose levels, and reaction time) were selected from single factor trials briefly. For the modification of WPI, the studied levels of glucose, glucose oxidase, HRP, and reaction time were 0–0.03 mmol/g, 0–5 U/g, 0–500 U/g protein, and 1–5 h, respectively. For the modification of SKMP, the studied levels of glucose, glucose oxidase, and HRP, and reaction time were 0–0.03 mmol/g, 0–10 U/g, 0–500 U/g protein, and 1–5 h, respectively. After the reaction, the modified products were heated at 85°C for 10 min to inactivate the enzymes, diluted by a phosphate buffer solution (0.2 mol/L, pH 7.4) into protein concentration of 0.5 g/L, and detected for their relative dityrosine contents (which were then used to select suitable levels of the four factors). With these selected and fixed reaction conditions, WPI and SKMP were bulk treated, assigned as MWPI-1 and MSKMP-1, respectively, freeze-dried, and stored at 4°C before future analysis.

When using the HRP-H$_2$O$_2$ system, WPI and SKMP were bulk treated as above without glucose oxidase and glucose addition but with the addition of the calculated H$_2$O$_2$. The modified products were treated as above, assigned as MWPI-2 and MSKMP-2, respectively, freeze-dried, and stored at 4°C before future analysis.

Indirect enzyme-linked immunosorbent assay

An indirect enzyme-linked immunosorbent assay (ELISA) was used to estimate in vitro antigenicity of these modified products as per reference (Zheng et al., 2008). Each sample was dissolved and diluted into a protein concentration of 1 mg/L and then detected using a microplate reader (Bio Rad Laboratories, Hercules, CA, USA). In vitro antigenicity of the analyzed sample was evaluated by detecting the amounts (µg/mL) of α-lactalbumin and β-lactoglobulin, which could bind with the anti-α-lactalbumin (or anti-β-lactoglobulin) antiserum on the ELISA plates. Two standard curves generated from pure α-lactalbumin and β-lactoglobulin, respectively, were used in the calculation.

Other assays

Relative fluorescence intensity of each diluted sample was measured at a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) with a slit of 5 nm, together with respective excitation and emission wavelengths of 320 and 410 nm. The detected value was used to indicate the relative dityrosine content of the analyzed sample.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed as per the method (Laemmli, 1970). The samples were diluted to a protein concentration of 1 g/L by the sample buffer containing Tris–HCl (0.0625 mol/L, pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. Sample solutions of 15 µL were loaded into each well. Protein markers with molecular weights of 14.4–97.4 kDa were used in analysis.

Far-ultra violet circular dichroism (CD) spectra (190–260 nm) of the analysis samples were obtained by a Jasco J-815 CD spectrometer (Jasco Corporation, Tokyo, Japan) at 25°C, using light path length of 0.1 cm. The analyzed samples were prepared at protein content of 0.1 g/L with another phosphate buffer (0.1 mmol/L, pH 7.0). Mean residue ellipticity (θ) was thus obtained and expressed as deg cm$^2$/dmol.

Statistical analysis

All data were reported as means ± standard deviations from at least three independent trials. The differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan’s multiple range tests. SPSS16.0 software (SPSS Inc., Chicago, IL, USA)
and MS Excel 2007 (Microsoft Corporation, Redmond, WA, USA) were used to analyze and report the data.

**Results and discussion**

*Modification of WPI and SKMP by two HRP-based approaches*

In this study, two HRP-based approaches were used to treat WPI and SKMP, respectively, as they could mediate the same protein reaction. When HRP–glucose oxidase–glucose system was used, HRP, glucose oxidase, and glucose levels, and reaction time were first studied for WPI and SKMP. Relative dityrosine contents of the modified products were measured and then used for condition selection. Based on the obtained results shown in Figure 1, the four conditions for WPI and SKMP modifications were thus selected. For the modification of WPI, the optimal levels of glucose, glucose oxidase, HRP for protein substrates of 1 g were 0.01 mmol, 2 U, and 200 U, respectively, whilst suitable reaction time was 2 h. For the modification of SKMP, the optimal levels of glucose, glucose oxidase, HRP for protein substrate of 1 g were 0.015 mmol, 2 U, 200 U, respectively, but a longer reaction time of 4 h was needed. With these conditions, MWPI-1 and MSKMP-1 thus prepared were detected to have relative dityrosine contents of (920.1 ± 32.2) and (2477.0 ± 27.6), respectively. If HRP and H$_2$O$_2$ were used to induce protein modification, WPI was treated with the calculated H$_2$O$_2$ of 0.01 mmol/g protein, HRP of 200 U/g protein, and a reaction time of 2 h, whilst SKMP was treated with the calculated H$_2$O$_2$ of 0.015 mmol/g protein, HRP of 200 U/g protein, and a reaction time of 4 h. MWPI-2 and MSKMP-2 thus prepared were detected to have relative dityrosine contents of (527.5 ± 16.3) and (1570.2 ± 23.1), respectively. SDS–PAGE analysis results (Figure 2) demonstrate that MWPI-1, MWPI-2, MSKMP-1, and MSKMP-2 all contained some cross-linked protein products, in comparison with respective WPI and SKMP. As seen from Figure 2(a), lanes 1 (MWPI-1) and 2 (MWPI-2) were observed to have deeper color than lane 3 (WPI) at the positions with molecular weights larger than 66.2 kDa. A similar phenomenon was also observed in Figure 2(b) reporting analysis results of SKMP, MSKMP-1, and MSKMP-2. These results proved that the two HRP-based approaches were also able to induce the cross-linking of WPI and SKMP.

HRP has been used by some researchers to cross-link caseinate (Chang & Zhao, 2012; Matheis & Whitaker, 1984) and soybean protein (Jiang & Zhao, 2014; Stuchell & Krochta, 1994). However, due to substrate differences, the used cross-linking conditions usually have some

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Figure 1. Impacts of glucose, glucose oxidase, horseradish peroxidase addition levels (A–C), and reaction times (D) on the cross-linking of whey protein isolate (WPI) and skim milk powder (SKMP). Different capital and lowercase letters above the respective columns indicate that one-way ANOVA of the mean values is different significantly ($P < 0.05$).

Figura 1. Impactos de los niveles de adición (A–C) de glucosa, glucosa oxidasa, peroxidasa de rábano picante, además de los tiempos de reacción (D) en la reticulación de aislado proteínico de lactosuero (WPI) y leche desnatada en polvo (SKMP). Las diferentes letras mayúscula y minúscula sobre las columnas respectivas indican que ANOVA de único sentido de los valores promedio es significativamente distinto ($P < 0.05$).
differences. For example, glucose oxidase of 6 U/g, glucose of 0.05 mol/g, HRP of 200 U/g protein, and reaction time of 2 h have been used in caseinate cross-linking (Chang & Zhao, 2012), whilst glucose oxidase of 4 U/g, glucose of 0.01 mol/g, HRP of 200 U/g protein, and reaction time of 3 h are required for the cross-linking of soybean protein (Jiang & Zhao, 2014). This study used some similar conditions to treat WPI and SKMP as the two mentioned studies, such as addition levels of glucose oxidase and HRP, and reaction time. At the same time, electrophoretic analysis is usually conducted to confirm the resultant protein cross-linking (Djoullah, Krechiche, Husson, & Saurel, 2016; Ercili-Cura et al., 2012; Giosafatto et al., 2012). This study also used SDS-PAGE analysis to confirm the cross-linking of the four modified products and shared a consistent conclusion to the mentioned three studies.

Secondary structural features of the modified products

If the conducted modifications had impacts on secondary structure of WPI and SKMP was briefly investigated through the CD analysis. Secondary structural features of WPI, SKMP, and their modified products are shown in Figure 3. The obtained CD results demonstrated that the four modified products (MWPI-1, MWPI-2, MSKMP-1, and MSKMP-2) all had much greater absorption at 208, 222 (α-helix structure), and 215 (β-folding structure) nm. These results proved that the carried out two modifications totally resulted in the four modified products with more ordered secondary structure than the respective substrates.

Enzymatic cross-linking of proteins is found to result in changes in secondary structure. Cross-linking of bovine gelatin by HRP–glucose oxidase–glucose system totally brings about a more ordered secondary structure (Han & Zhao,

Figure 2. Electrophoretic profiles of whey protein isolate (WPI), skim milk powder (SKMP), and their modified products. The lanes 1–3 in (a) represent HRP–glucose oxidase–glucose modified WPI (MWPI-1), HRP–H₂O₂ modified WPI (MWPI-2), and WPI; whilst the lanes 1–3 in (b) represent HRP–glucose oxidase–glucose modified SKMP (MSKMP-1), HRP–H₂O₂ modified SKMP (MSKMP-2), and SKMP, respectively. The lanes M and H represent protein markers with molecular weights of 14.4–97.4 kDa and horseradish peroxidase, respectively.

Figure 2. Perfiles electroforéticos de aislado proteínico de lactosuero (WPI), leche desnatada en polvo (SKMP) y sus productos modificados. Las líneas 1–3 en (a) representan WPI modificado HRP-glucosa oxidasa-glucosa (MWPI-1), WPI modificado HRP–H₂O₂ (MWPI-2) y WPI; mientras que las líneas 1–3 en (b) representan SKMP modificado HRP-glucosa oxidasa-glucosa (MSKMP-1), SKMP modificado HRP–H₂O₂ (MSKMP-2) y SKMP, respectivamente. Las líneas M y H representan los indicadores proteínicos con peso molecular entre 14,4–97,4 kDa y peroxidasa de rábano picante, respectivamente.

Figure 3. CD spectra of whey protein isolate (WPI), skim milk powder (SKMP), and their modified products. MWPI-1 and MSKMP-1 represent HRP–glucose oxidase–glucose modified WPI and SKMP, whilst MWPI-2 and MSKMP-2 represent HRP–H₂O₂ modified WPI and SKMP, respectively.

Figure 3. Espectro CD de aislado proteínico de lactosuero (WPI), leche desnatada en polvo (SKMP) y sus productos modificados. MWPI-1 y MSKMP-1 representan WPI modificado HRP-glucosa oxidasa-glucosa y SKMP, mientras que MWPI-2 y MSKMP-2 representan WPI modificado HRP–H₂O₂ y SKMP, respectivamente.
The antigenicity of WPI, SKMP, and their modified products was assessed by ELISA technique. The obtained results are listed in Table 1. It is interesting to see that MWPI-1 and MWPI-2 (or MSKMP-1 and MSKMP-2) had higher antigenicity than WPI (or SKMP). For WPI, the two modifications resulted in about 150–310% increases in α-lactalbumin antigenicity, or about 250–310% increases in β-lactoglobulin antigenicity. For SKMP, the two modifications led to about 160–320% increases in α-lactalbumin antigenicity, or about 100–140% increases in β-lactoglobulin antigenicity. In addition, MWPI-1 (or MSKMP-1) showed higher antigenicity than MWPI-2 (or MSKMP-2) (P < 0.05). These results demonstrate that HRP–glucose oxidase–glucose system was more potent than HRP–H₂O₂ system to confer the modified products with enhanced in vitro antigenic properties.

Table 1. In vitro antigenicity of whey protein isolate (WPI) and skim milk powder (SKMP) as well as their modified products evaluated by an indirect ELISA.

| Samples   | α-Lactalbumin (μg/mL) | β-Lactoglobulin (μg/mL) |
|-----------|-----------------------|------------------------|
| WPI       | 3.99 ± 0.01a          | 5.91 ± 0.028a          |
| MWPI-1    | 16.43 ± 0.01a         | 24.35 ± 0.01d          |
| MWPI-2    | 9.97 ± 0.02b          | 20.36 ± 0.02c          |
| SKMP      | 5.04 ± 0.03a          | 1.70 ± 0.03a           |
| MSKMP-1   | 23.33 ± 0.01c         | 4.32 ± 0.01b           |
| MSKMP-2   | 13.31 ± 0.02b         | 3.58 ± 0.02c           |

Each sample was diluted into a protein concentration of 1 mg/L before assaying. Different lowercase letters after the data as the superscripts in the same column indicate that one-way ANOVA of the mean values is significantly different (P < 0.05).

Conclusion

With these fixed and studied reaction conditions, two HRP-based modifications using HRP–glucose oxidase–glucose and HRP–H₂O₂ systems, respectively, could induce cross-linking of WPI and SKMP via the dityrosine formation. The two modifications conferred the modified products with more ordered secondary structure and especially enhanced α-lactalbumin and β-lactoglobulin antigenicity. HRP–glucose oxidase–glucose system was more potent than HRP–H₂O₂ system to enhance in vitro antigenicity of WPI and SKMP. Special attention should thus be paid for the HRP-based modifications of other protein allergens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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in vitro antigenic properties of the modified products

In vitro antigenicity of WPI, SKMP, and their modified products was assessed by an ELISA technique. The obtained results are listed in Table 1. It is interesting to see that MWPI-1 and MWPI-2 (or MSKMP-1 and MSKMP-2) had higher antigenicity than WPI (or SKMP). For WPI, the two modifications resulted in about 150–310% increases in α-lactalbumin antigenicity, or about 250–310% increases in β-lactoglobulin antigenicity. For SKMP, the two modifications led to about 160–320% increases in α-lactalbumin antigenicity, or about 100–140% increases in β-lactoglobulin antigenicity. In addition, MWPI-1 (or MSKMP-1) showed higher antigenicity than MWPI-2 (or MSKMP-2) (P < 0.05). These results demonstrate that HRP–glucose oxidase–glucose system was more potent than HRP–H₂O₂ system to confer the modified products with enhanced in vitro antigenic properties.

Changing antigenicity property of food proteins is a result of conformational changes in the epitopes. Enzymatic modifications of milk proteins usually result in decreased in vitro antigenic properties, for example, cross-linked whey protein (Villas-Boas et al., 2012), glycated and cross-linked whey protein (Zhang et al., 2016). Other studies also utilized protein hydrolysis or protein hydrolysis followed by protein cross-linking to treat whey protein and found that the antigenicity of whey protein can be decreased by using acid proteinases, alcalase, or alcalase plus TGase (Lakshman et al., 2011; Villas-Boas, Benedé, de Lima Zollner, Netto, & Molina, 2015; Zheng et al., 2008). This study obtained a result inconsistent to these reported works. However, heating of β-lactoglobulin and wheat gluten at low pH were found to enhance antigenic property (Rahaman, Vasiljevic, & Ramchandran, 2015, 2016), which confirm that protein modification also
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