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Potential allergenicity response to structural modification of irradiated bovine α -lactalbumin

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Bovine α -lactalbumin (α -La) is a major food allergen found in milk and is characterized by high conformational stability because of its four disulfide bridges and being calcium bound. This study aimed to describe the influence of gamma irradiation on the structure and potential allergenicity of α -La. The prepared α -La was irradiated at doses of 1–10 kGy. The changes in structure were characterized through SDS-PAGE, circular dichroism spectroscopy, ultraviolet absorption spectroscopy, and fluorescence spectroscopy. The potential allergenicity of the irradiated α -La was evaluated *in vitro* through IgG/IgE inhibition ELISA and the human basophil KU812 degranulation assay. The results showed that the secondary and tertiary structures of α -La significantly changed and caused extensive protein denaturation and aggregation. IgG and IgE binding properties remarkably decreased, and the degranulation capacity of basophils weakened. The results suggested that structural damage of α -La induced by irradiation significantly reduces the potential allergenicity of α -La.

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1. Introduction

Cow milk and dairy products have been widely introduced in the human diet because of their excellent nutritional and functional properties. Unfortunately, cow milk protein allergy (CMPA) is a type of food allergy that can cause serious allergic reactions in infants or young children.¹ Population-based studies in developed countries have revealed that the incidence of CMPA ranges from 2% to 7.5% and from approximately 2% to 3% in the first year of life.^{2,3} The majority of children outgrow CMPA by the age of 3–4 years, but 15% of infants retain their allergic reaction to this food throughout their lifetime.^{4,5} Thus far, the complete avoidance of cow milk proteins in diet has been considered as the most effective approach to prevent CMPA. However, the presence of milk-derived components in manufactured foods impedes the application of this approach; the elimination of cow milk proteins may cause nutritional imbalance and may thus affect the growth of infants and children. Therefore, improved hypoallergenic dairy products need to be developed.

Gamma irradiation is considered as a successful physical technique that could reduce the allergenicity of allergens and preserve food with minimum changes in sensory and nutritional properties.⁶ The effects of irradiation on the allergenicity of certain food allergens have been reported in several studies.^{6,7} For instance, Seo *et al.* (2007) indicated that the irradiation of ovalbumin (OVA) suppresses humoral and cellular immune responses specific to OVA.⁸ The irradiation of shrimp allergen extracts at 10 kGy significantly reduces the IgE binding, as indicated by the immunoblotting and inhibition ELISA of the pooled sera from 15 shrimp allergy patients.⁹ Oh *et al.* (2009) investigated the allergenicity of peanut allergen and discovered that irradiation downregulates the Th2 lymphocyte activity in peanut-sensitized mice.¹⁰ The influence on allergenicity is mainly attributed to changes in protein secondary and tertiary structures as a result of cross-linking, aggregation, fragmentation, and amino acid modification.¹¹ Nevertheless, regarding milk proteins, systematic studies related to the effect of structural changes on the allergenicity after irradiation treatment have not yet been conducted. As such, irradiation should be further investigated before it can be applied to reduce the allergenicity of milk proteins.

Alpha-lactalbumin (α -La), as well as major milk proteins, exhibits an allergenic potential.¹² Approximately 30%–35% of individuals allergic to milk possess specific IgE against α -La. IgE binding epitopes play a key role in the cross-linking high-affinity IgE receptor Fc ϵ RI to activate effector cells, such as basophils or mast cells.^{13,14} The residual allergenicity of processed proteins has been widely investigated through *in vitro*

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IgE-binding capacity testing; the decrease in IgE binding capacity is not correlated well with a low amount of basophils responding to degranulation following the allergen-induced cross-linking of IgE-bound Fc ϵ RI.^{15,16} This finding has been confirmed in certain nuts and legumes; for instance, thermal and pressure treatments can decrease the IgE-binding properties of protein extracts from peanuts and reduce the ability to activate basophils. A conflicting result has been observed in peanut flour, that is, hydrolysis reduces the IgE-binding capacity but does not decrease the IgE cross-linking capacity in basophil activation testing.^{17,18} Therefore, IgE-binding capacity is insufficient to assess the allergenicity of processed foods containing allergens. The effect on biological activities, such as degranulation capacity, should also be considered.

This study aimed to determine the influence of gamma irradiation on the structure and allergenicity of bovine α -La. Considering the safety of irradiation for human consumption,⁶ we irradiated bovine α -La at doses of 1–10 kGy. We then examined the structural changes in the irradiated α -La through circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy, and fluorescence spectroscopy. We also evaluated the potential allergenicity of the irradiated α -La by determining the IgG/IgE binding abilities and by performing the basophil degranulation assay *in vitro*.

2. Material and methods

2.1. Materials

1-Anilinonaphthalene-8-sulfonate (ANS), Freund's complete adjuvant, Freund's incomplete adjuvant, *o*-phenylenediamine (OPD), horseradish peroxidase (HRP)-labeled anti-rabbit IgG, HRP-labeled streptavidin, and biotinylated-labeled anti-human IgE were purchased from Sigma-Aldrich Chemical Co. (St Louis, USA). Flow analysis reagents were supplied by eBioscience (San Diego, CA, USA). Solvents and other reagents were of analytical grade. Solutions were prepared with Milli-Q water.

2.2. Gamma irradiation treatment

Bovine α -La was purified in our laboratory in accordance with previously described methods.¹⁹ The purity and identity of the prepared α -La were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS; purity >96%).

α -La was prepared in phosphate buffered saline (0.05 M, pH 6.8) at a concentration of 1 mg mL⁻¹, and was irradiated at different doses (1, 3, 5, 7, and 10 kGy) in a cobalt-60 gamma irradiator (Jiangxi Academy of Agricultural Sciences, Nanchang, China) at a rate of 0.32 kGy h⁻¹.

2.3. Electrophoresis

SDS-PAGE was performed in accordance with Laemmli²⁰ to detect the total protein patterns of irradiated α -La. Protein samples (8 μ g) were resolved on 12% separating gel and 4% stacking gel with a constant current of 6–12 mA per gel. Gels

were stained with Coomassie Blue and destained with a solution containing 5% methanol and 7.5% acetic acid.

Low-molecular-weight (MW) peptides were analyzed through tricine-SDS-PAGE²¹ by using a discontinuous system containing 4%, 10%, and 16.5% acrylamide. The gels were immersed in a fixative solution containing ethanol, acetic acid, and water (4 : 1 : 5 v/v/v) for 30 min and then stained with Coomassie Blue and destained with 7.5% acetic acid.

2.4. CD spectroscopy

CD measurements were performed using a spectropolarimeter (JASCO J810, Tokyo, Japan). Far-UV CD spectra (190–250 nm) were obtained at a protein concentration of 0.2 mg mL⁻¹ in phosphate-buffered saline (PBS) with pH 6.8 at 25 °C. The optical path length was 0.1 cm, with a scan speed of 60 nm min⁻¹ and a bandwidth of 1.0 nm. The average of the results of each sample was determined and then expressed as the mean residue ellipticity $[\theta]$, defined as $[\theta] = \theta_{\text{obs}}/(10Cl_n)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm), and n is the number of amino acid residues. The secondary structural contents were calculated using the JASCO secondary structure software (JASCO, Japan).

2.5. UV absorption spectroscopy

UV absorption was evaluated using a UV spectrophotometer (TU-1901, Purkinje General, Beijing). The samples were homogeneously mixed at 0.1 mg mL⁻¹ in PBS (pH 6.8) and then scanned from 230 nm to 350 nm at 25 °C.

2.6. Fluorescence spectroscopy

The fluorescence emission spectrum of the irradiated samples was obtained at a concentration of 0.1 mg mL⁻¹ in PBS (pH 6.8) at 25 °C by using a spectrophotofluorometer (F-4500, Hitachi, Japan) by using a quartz cuvette with a 1.0 cm path length. For intrinsic fluorescence measurement, the excitation wavelength was 325 nm. The emission spectrum was recorded at 280–450 nm, with 2 nm band pass filters in both excitation and emission.

The surface hydrophobicity of the irradiated α -La was determined using ANS fluorescent probes by using the F-4500 spectrophotofluorometer (Hitachi Limited, Japan). The samples were transferred to a quartz cuvette and then mixed with 80 μ M bis-ANS. The fluorescence emission spectrum was obtained from 400 nm to 650 nm, with an excitation at 350 nm.

2.7. Preparation of anti- α -La antibodies

Two rabbits were purchased from the Institute of Occupational Medicine of Jiangxi (Nanchang, China; permission number "SCXK (Gan) 2014-0005"), which were used to produce polyclonal antibodies against α -La. All experimental procedures used in the present study were approved by the Animal Care Review Committee, Nanchang University. The rabbits were subcutaneously immunized with 1 mg of α -La by using an equal volume of Freund's complete adjuvant. Subsequent injections

Table 1 Clinical data of milk-allergic patients

Patient no.	Sex	Age	Milk-related clinical symptoms	α -La S-IgE ($\text{kU}_\text{A} \text{L}^{-1}$)
1	Male	1 year	AS	4.14
2	Male	5 year	AB	0.45
3	Female	5 year	C	12
4	Male	1 year	AS	2.42
5	Male	4 year	NK	0.75
6	Male	1–2 years	L	1.09
7	Male	NK	D	25.3
8	Male	4–5 years	R	3.18

AS, asthma; AB, asthenatoid bronchopneumonia; C, cough variant asthma; NK, not known; L, leukocytosis; D, damp obstruction; R, rhinoconjunctivitis.

using the same immunogen concentration at the same volume of Freund's incomplete adjuvant were re-stimulated 6 times at 2-week intervals. When the titer reached a stable level by indirect ELISA, the rabbits were finally bled and serum separated by centrifugation at 4000g for 10 min at 4 °C. All sera were stored at –80 °C until use.

Moreover, all sera samples from patients allergic to α -La were generously provided by the First Affiliated Hospital of Guangxi Medical University (Guangxi, China), and their availability was approved by the internal ethical committee of the hospital. All experiments were performed in compliance with the relevant laws and institutional guidelines in China, and written informed consent was obtained from each individual before conducting the test. Anti- α -La specific IgE levels in milk allergic human sera are reported in Table 1, and they varied from 0.45 to 25.3 $\text{kU}_\text{A} \text{L}^{-1}$. The same volume of mixed serum pool was used for the subsequent ELISA and mediator release assay.

2.8. Inhibition ELISA analysis

The IgG- and IgE-binding ability of α -La were analyzed by inhibition ELISA with the rabbit polyclonal antibodies and sera from patients allergic to α -La.

Then, 100 μL of 0.5 $\mu\text{g mL}^{-1}$ non-irradiated α -La in 0.05 M carbonate buffer (pH 9.6) was coated into each well of a 96-well microtiter plate overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween 20. Each well was blocked for 1 h at 37 °C with 3% gelatin in PBS. Equal volumes of antisera samples (1:30 000 diluted rabbit sera to evaluate the antigenicity and 1:20 diluted human sera to analyze the IgE-binding ability) and irradiated α -La (inhibitors) were mixed and incubated for 1 h at 37 °C. The blocked plates were washed as previously described; afterward, 100 μL of each inhibited antisera sample mixture was added to the plate and incubated for 1 h at 37 °C. The plate was washed, and 100 μL of the diluted 1:5000 antisera (HRP-labeled anti-rabbit IgG/biotinylated-labeled anti-human IgE) was added to the wells and subsequently incubated at 37 °C for 1 h. The plate was washed again, and 100 μL of substrate solution (0.4 mg mL^{-1} OPD in a citrate buffer with 0.04% H_2O_2) was added to the

wells. Another step was performed before a chromogenic reaction was induced to determine the IgE-binding ability of α -La. In this step, avidin was added to react with biotin to expand the chromogenic reaction. The reaction was stopped by adding 50 μL per well of 2 M sulfuric acid. Absorbance was determined at 490 nm by using a microplate reader (Model 1860, Bio-Rad, USA). The inhibition rate was calculated using the following equation: % inhibition = $1 - B/B_0 \times 100$, where B and B_0 are the absorbance values of the well with and without the inhibitor, respectively. IC_{50} is the analyte concentration that causes a 50% inhibition of antibody binding.

2.9. Mediator release assay from human basophil leukemia (KU812) cells

Human basophilic KU812 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). They were maintained in IMDM (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Gibco, Life, Australia), 2 mM L-glutamine, 25 mM HEPES buffer, 100 units per mL of penicillin G, and 100 mg mL^{-1} of streptomycin and cultured at 37 °C under a humidified atmosphere with 5% CO_2 . The cells were subcultured every 3–4 days. First, the cell surface Fc ϵ RI expression was identified in this study. KU812 cells (1.0×10^6 cells per mL) were pre-incubated with specific allergic- α -La human serum (v/v = 5:1) for 24 h. Cell surface staining of anti-human Fc ϵ RI α chain antibody CRA-1 (0.5 μg per test, eBioscience; clone AER-37) was conducted for 45 min in the dark at 4 °C. The cells were then washed twice with a flow cytometry staining buffer and then subjected to flow cytometry. Data were analyzed with the BD FACSDiva software (BD Biosciences, CA, USA).

Second, intracellular Ca^{2+} concentration was evaluated in a KU812 cell model. KU812 cells were seeded in a 48-well round-bottom microtiter plate and incubated with human serum for 24 h at 37 °C and 5% CO_2 . After 24 h, the irradiated α -La (10 μg) was added to the wells and then continuously incubated for 4 h under the same atmospheric conditions. The cells were then stained with the calcium fluorescent dye Fluo-3 AM (0.5 μL per test, Beyotime, Jiangsu, China) for 45 min in the dark at 37 °C and then washed twice with flow cytometry staining buffer. The dyed cells were examined, and the data were analyzed as described in previous studies.

Finally, histamine release was further analyzed through the basophil degranulation assay. KU812 cells (1.0×10^6 cells per mL) were incubated with human serum for 24 h and then stimulated with 10 μg of α -La at different doses of irradiation (0, 1, 3, 5, 7, and 10 kGy) for 4 h at 37 °C. After centrifugation was performed at 120g for 5 min, the supernatant was collected, and histamine content was measured using a commercial Human His ELISA kit (Fusheng Industry Co., Shanghai, China).

2.10. Statistical analysis

Three samples from each irradiation dose were analyzed, and all the assays from every independent treatment were carried out in triplicate. The results are expressed as mean values and

standard deviation (SD). Appropriate data were analyzed to determine statistical significance through ANOVA (SPSS for Windows 2003, Microsoft Corp., version 13.0, Chicago, IL, USA) and Fisher's least significant difference (LSD, $P < 0.05$) test.

3. Results

3.1. Molecular weight analysis

The molecular weight profile of the irradiated α -La is characterized as shown in Fig. 1A and B. Comparison of the SDS-PAGE profile of α -La in its native (without irradiation) and irradiated forms revealed major changes at different irradiation doses (Fig. 1A). An irradiation dose of 1 kGy did not exhibit evident changes in the intensity of the protein image, whereas a weak-intensity protein image appeared at 3 kGy. The samples irradiated at a dose above 5 kGy showed a marked reduction in the intensity of the protein image. The intensity of the cross-linked components (molecular weight more than 130 kDa, Fig. 1A, arrows) increased.

Moreover, to detect whether low-molecular-weight products were generated after irradiation, the samples were analyzed through tricine-SDS-PAGE. Fig. 1B indicates that irradiation could decrease the intensity of the native α -La (14.2 kDa). In addition to the formation of aggregates, no other lower-molecular-weight bands were observed in tricine-SDS-PAGE.

3.2. Secondary structure analysis

The far-UV CD spectra of α -La were used to define the extent of secondary structural changes induced by irradiation at various doses (Fig. 2). The spectrum of native α -La exhibited a strong positive peak at 192 nm and prominent double negative peaks at 208 and 216 nm. Fig. 2A shows a progressive decrease in molar ellipticity at different doses, and major changes were observed in the 190–200 nm region. When α -La was irradiated at 1 kGy, the intensity of the positive molar

ellipticity decreased, and the positive molar ellipticity disappeared at 3 kGy. In all of the samples irradiated at doses of 1, 3, 5, 7, and 10 kGy, the negative molar ellipticity exhibited a prominent decline at 205–220 nm. In addition, the secondary structure of native α -La was composed of 40% α -helix, 34% random coil, 20% β -turn, and 6% β -sheet (Fig. 2B). The β -sheet exhibited an increasing trend when the doses were increased; by contrast, the random coil did not display drastic changes. The β -turn increased at 1–5 kGy and declined at 7–10 kGy. The content of the α -helix revealed an opposite variation pattern compared with that of β -turn. A transition from α -helix to β -sheet occurred when the doses were increased.

3.3. Tertiary structure analysis

The alteration in the tertiary structure of α -La as a function of irradiation can be characterized on the basis of UV absorption spectra and fluorescence spectra (Fig. 3). The UV absorbance of irradiated α -La was observed at a wavelength of 230–350 nm (Fig. 3A). The non-irradiated α -La exhibited a maximum absorbance at 285 nm. Compared with the untreated sample, the irradiated α -La showed an increase in UV absorbance and exhibited a great blue shift in the location of the maximum absorption peak.

The fluorescence emission intensity of the irradiated α -La decreased as the irradiation dose increased (Fig. 3B). However, the ANS-fluorescence intensities of the irradiated α -La increased upon the irradiation dose increase (Fig. 3C). In contrast to that of the untreated sample, the ANS-fluorescence intensities of the treated samples increased 22.02- and 61.98-fold at doses of 1 and 3 kGy, respectively. When the samples were treated at 5, 7, and 10 kGy, the ANS-fluorescence intensities showed a profile similar to that of the 3 kGy-treated samples. Moreover, a slight blue shift of the location of the ANS-fluorescence peak was observed between the irradiation-treated samples and the non-irradiated ones.

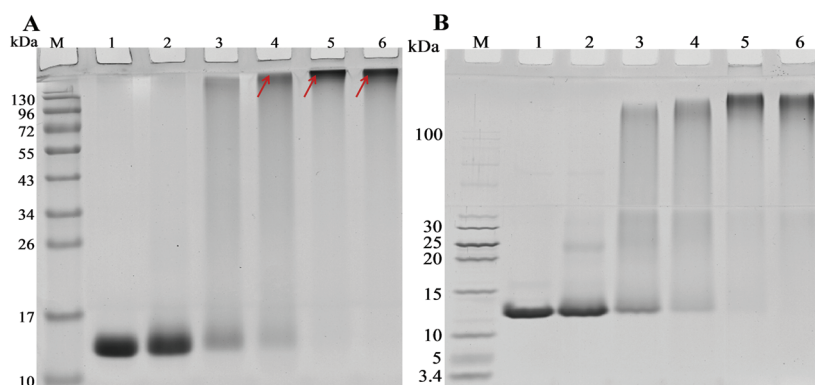


Fig. 1 Effect of gamma irradiation on the molecular weight behavior of the prepared α -La. (A) SDS-PAGE profile of the irradiated α -La at different doses. 8 μ g of protein was loaded into each well of 4% stacking gel and 12% separating gel. Lane M: prestained marker. Lane 1: the native α -La. Lanes 2–6: the irradiated α -La at doses 1, 3, 5, 7, and 10 kGy, respectively. (B) Tricine-SDS-PAGE profile of the irradiated α -La at different doses. 8 μ g of protein was loaded into each well of the discontinuous system containing 4, 10, and 16.5% acrylamide. Lane M: prestained marker. Lane 1: the native α -La. Lanes 2–6: the irradiated α -La at doses 1, 3, 5, 7, and 10 kGy, respectively.

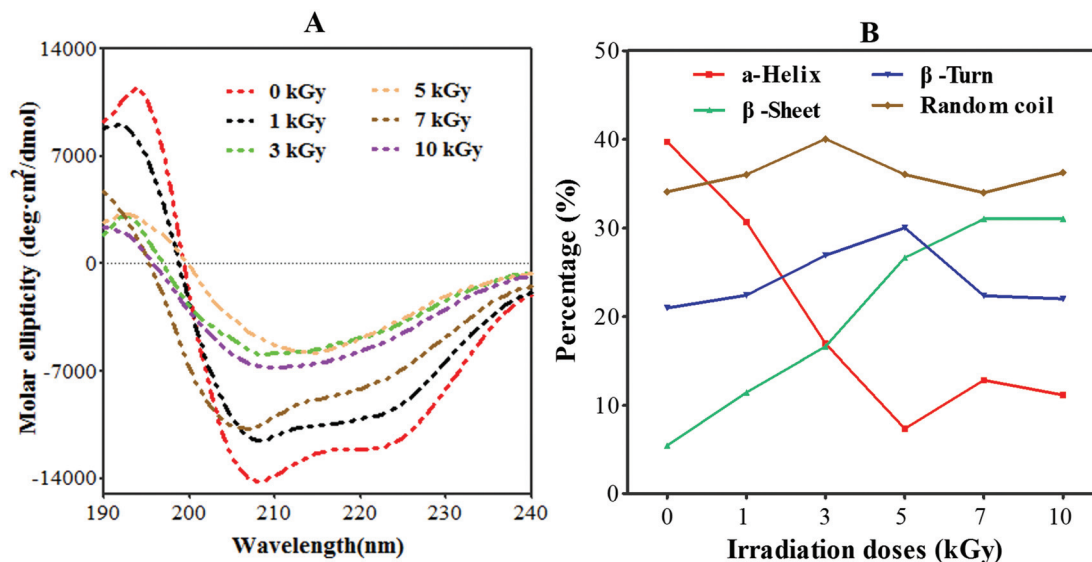


Fig. 2 Far-UV CD spectra of the prepared α -La irradiated at different doses (0, 1, 3, 5, 7, and 10 kGy). (A) CD spectra were recorded in the Far-UV range (190–250 nm). All of the measurements were done at 25 °C, and the sample concentration was 0.2 mg mL⁻¹. (B) The content of protein secondary structure of the irradiated α -La.

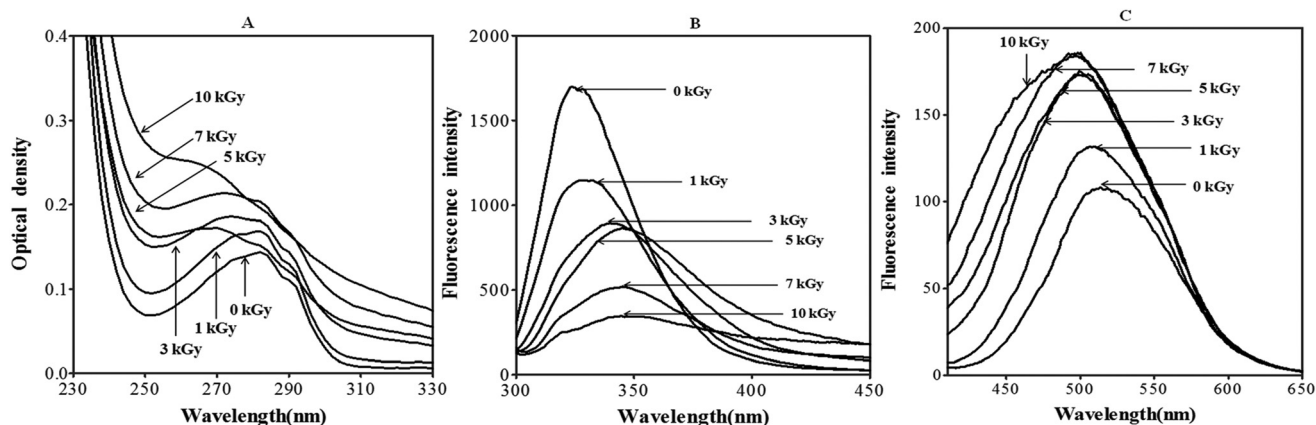


Fig. 3 UV absorption and fluorescence of the α -La irradiated at different doses (0, 1, 3, 5, 7, and 10 kGy). (A) UV absorption spectra of the irradiated α -La at a wavelength of 230–350 nm. (B) Intrinsic fluorescence of the irradiated α -La at various doses. The excitation wavelength was 325 nm, while the emission spectrum was recorded in the range of 280–450 nm. (C) ANS fluorescence of the irradiated α -La at various doses. Protein samples (0.1 mg mL⁻¹) and ANS solution (8 mM) was mixed and measured (excitation, 350 nm, and emission, 400–650 nm).

3.4. IgG- and IgE-binding capacity

Inhibition ELISA was conducted to assess the IgG- and IgE-binding ability of the irradiated α -La with anti- α -La rabbit anti-sera and the sera from patients allergic to bovine α -La, respectively (Fig. 4). As shown in Fig. 4A, the trend of the IC₅₀ inhibitory curve toward a higher inhibitor concentration changes, indicating that irradiation processing induces a decrease in the antigenicity of α -La. The IC₅₀ values for the 1 kGy-treated sample (0.27 μ g mL⁻¹) were equivalent to non-irradiated α -La (0.25 μ g mL⁻¹). At doses of 3 (0.78 μ g mL⁻¹), 5 (1.23 μ g mL⁻¹), 7 (3.18 μ g mL⁻¹), and 10 (4.74 μ g mL⁻¹) kGy, IC₅₀ of the irradiated α -La were 3.12-, 4.92-, 12.72-, and 18.96-fold higher than that of the native α -La.

The IgE binding to the irradiated α -La was evaluated by inhibition ELISA assays with sera from patients allergic to bovine α -La to assess the influence of irradiation treatment on the potential allergenicity of α -La (Fig. 4B). Interestingly, the change in trend of the IgE inhibitory curve was highly similar to that of the IgG inhibitory curve. IC₅₀ of the irradiated samples exhibited a tendency toward the direction of the high-concentration extension. The calculated IC₅₀ of the native α -La was the lowest (3.22 μ g mL⁻¹), and IC₅₀ gradually increased when the irradiation doses were increased to 1 (4.12 μ g mL⁻¹, 1.28-fold), 3 (13.55 μ g mL⁻¹, 4.21-fold), 5 (15.00 μ g mL⁻¹, 4.66-fold), 7 (48.41 μ g mL⁻¹, 15.03-fold), and 10 (59.65 μ g mL⁻¹, 18.52-fold) kGy, indicating a decrease in IgE binding capacity.

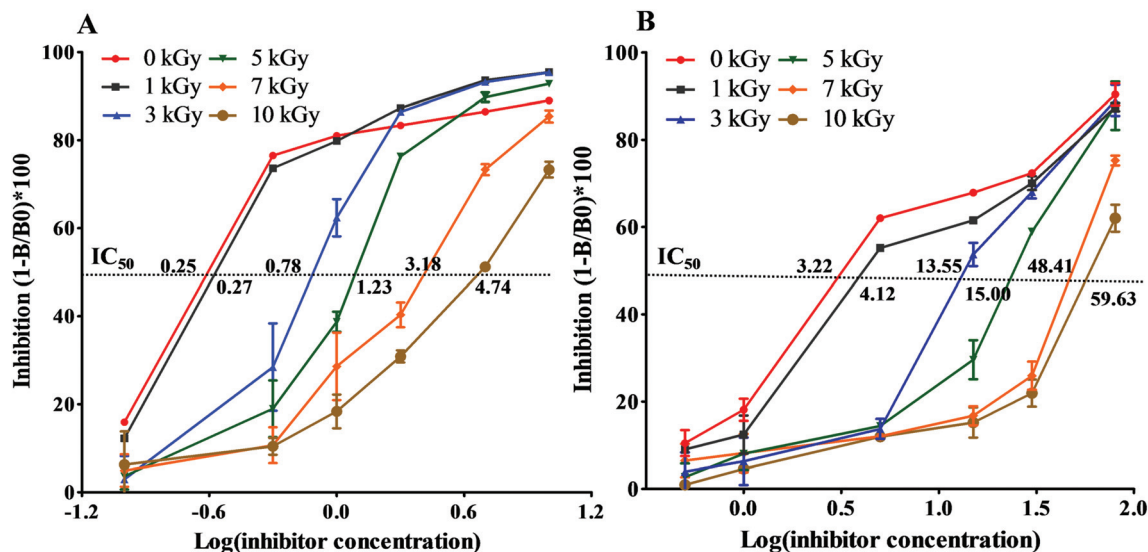


Fig. 4 IgG/IgE-binding ability of native and irradiated α -La was defined by inhibition ELISA. IC_{50} : the concentration of inhibitors that causes a 50% inhibition of antibody binding ($\mu\text{g mL}^{-1}$). (A) Changes in IgG-binding ability of α -La. Anti- α -La rabbit pooled sera (60 μL per well) were incubated separately with 0.1, 0.5, 1, 2, 5, and 10 $\mu\text{g mL}^{-1}$ (60 μL per well) of the corresponding irradiated α -La as inhibitors. (B) Changes in IgE-binding ability of α -La. Anti- α -La patients' pooled sera (60 μL per well) were incubated separately with 0.5, 1, 5, 15, 30, and 80 $\mu\text{g mL}^{-1}$ (60 μL per well) of the corresponding irradiated α -La as inhibitors.

3.5. Effect of irradiation on the mediator-releasing capacity

The biological mediator-releasing activity of the irradiated α -La was determined in intracellular Ca^{2+} expression and histamine release by using human basophilic KU812 cells. After the KU812 cells were sensitized with IgE from patients allergic to α -La, Fc ϵ RI, which is a high-affinity IgE receptor considered as

a key molecule in inducing basophils degranulation,²² was significantly expressed (30%) on the cell surface. The cells were then challenged by native and irradiated α -La; as a result, the release of allergic mediators is dose-dependent.

Intracellular Ca^{2+} plays an essential role in the induction of Fc ϵ RI-mediated basophil or mast cell degranulation.²³ A calcium-specific fluorescent probe Fluo-3 AM was used to

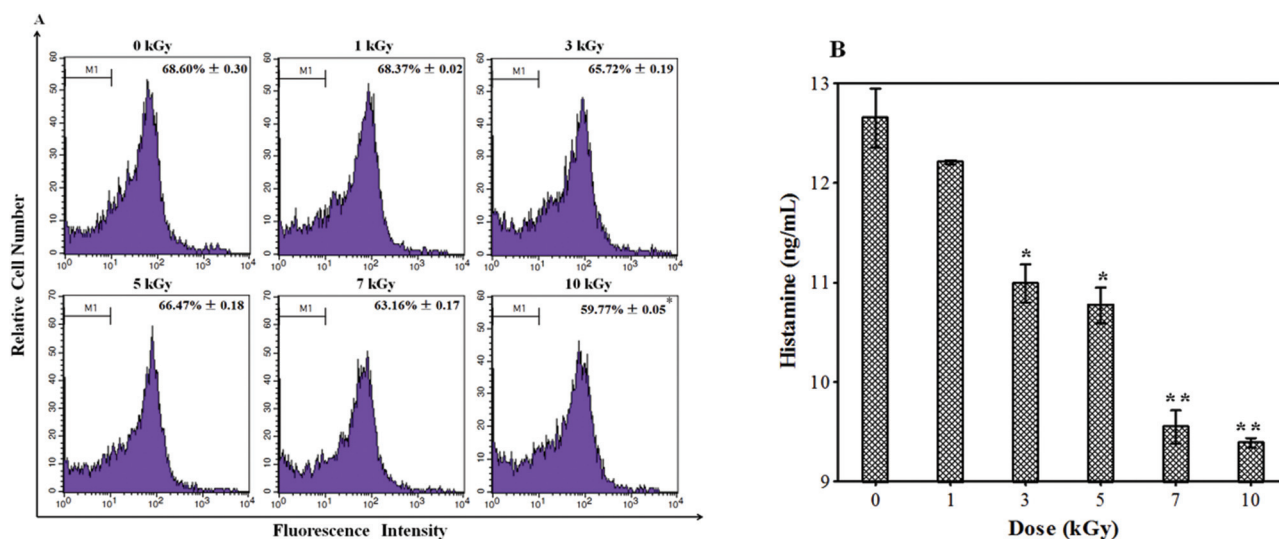


Fig. 5 Intracellular Ca^{2+} level and histamine release from KU812 cells stimulated with native and irradiated α -La. (A) Flow cytometry identifies the concentration of intracellular Ca^{2+} of the native and irradiated α -La. Human serum sensitized KU812 cells, followed stimulating by irradiated samples for 4 h. Afterwards, it was loaded with Ca^{2+} indicator Fluo-3 AM, followed by detecting the positive fluorescent expression. Representative data from one out of three independent experiments. * $P < 0.05$ indicates significant difference as compared to that of the control (0 kGy). (B) Inhibitory effect of histamine release from KU812 cells stimulated with irradiated α -La. Each data value is expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ indicates significant difference as compared to that of the control (0 kGy).

investigate the effect of the irradiated samples on intracellular Ca^{2+} concentration, and the expression value of intracellular Ca^{2+} was evaluated through flow cytometry (Fig. 5A). When the cells were stimulated with the irradiated α -La at 1 kGy, the expression of Ca^{2+} was almost similar to that of the control sample (non-irradiated α -La). Regarding the irradiated α -La at 3, 5, 7, and 10 kGy, the intracellular Ca^{2+} expression decreased, and the suppression effects were significantly reduced by 12.9% at 10 kGy ($P < 0.05$).

Histamine release is a characteristic and critical function of basophils. Fig. 5B shows the histamine release from the KU812 cells treated with the native and irradiated α -La upon stimulation with specific sera from patients allergic to α -La. In the control group (non-irradiated α -La treated cells), the histamine release was 12.65 ng mL^{-1} . After α -La was irradiated at varying doses of 1, 3, 5, 7, and 10 kGy, the histamine release for the cells decreased to 12.21, 11.00 ($P < 0.05$), 10.77 ($P < 0.05$), 9.55 ($P < 0.01$), and 9.38 ($P < 0.01$) ng mL^{-1} , respectively. The decrease in the histamine release was positively correlated with the reduction in the intracellular Ca^{2+} expression.

4. Discussion

Gamma irradiation, a non-thermal method of food processing, has been extensively investigated to modify the structure and functional properties of foods and food components.^{24,25} To date, the application of irradiation in dairy products is not only limited to prolong shelf-life and enhance food safety without a detrimental effect on the sensorial and nutritional quality when applying the appropriate dose (below 10 kGy), but also widely used in other application such as proteins together with other milk components in building or stabilizing gels, foams, emulsions and fibrous structures.^{6,26} Depending on the adsorbed irradiation dose, various effects can be achieved resulting in protein aggregation, carbohydrate degradation, vitamin loss, and fat oxidation.²⁶ In particular, a high dose of irradiation can promote lipid oxidation, leading to undesirable nutritional and sensorial properties.²⁴ Therefore, to provide further insight into the effect of different irradiation doses on changes in the structure and potential allergenicity of the treated α -La, 5 different safe irradiation doses were chosen in this study.

The molecular weight of the irradiated α -La was modified during irradiation. Electrophoresis analysis confirmed that irradiation caused the formation of high-molecular-weight aggregates without the appearance of any fragmented polypeptides. Under irradiation, the damage to proteins is manifested as either fragmentation or aggregation of molecules.^{27,28} Proteins in the dry state are damaged directly by interactions with ionizing radiation, which causes protein fragmentation. However, for proteins in solution, the direct effects are negligible, whereas the indirect effects are predominant. The indirect effects on proteins mainly involved free radicals ($\text{H}^\bullet$, $\text{OH}^\bullet$, $\text{HO}_2^\bullet$) generated because of water radiolysis, which reacts efficiently with protein molecules and causes cross-linking of

molecules, leading to protein aggregation.⁷ In this study, the bovine α -La samples were in the moist state, thus confirming that high-molecular-weight aggregation occurs as an indirect effect of irradiation. This phenomenon is not unique, Lee *et al.* also observed similar results in other milk proteins, such as α -casein (ACA) and β -lactoglobulin (BLG).²⁹

The strong loss in secondary structures of irradiated α -La was obtained by using CD spectra. The patterns of α -helix, β -sheet, β -turn, and random coil content of α -La were almost irregular, suggesting alteration in the ordered structure of the polypeptide chain and resulting in total structural damage. These changes in α -La secondary structure strongly supported the argument that free radicals, generated in solution during irradiation, disrupt the ordered polypeptide chain and lead to structural dispersion. Many researchers have also described similar results in concanavalin A and the soybean trypsin inhibitor.^{30,31}

In addition, irradiation resulted in important changes in the α -La tertiary structure as demonstrated by UV and fluorescence spectroscopy. The UV spectra of the native α -La reflected the maximum absorption peak at around 280 nm, indicating that the UV absorption spectrum of α -La was mainly dependent on tyrosine (Tyr) and tryptophan (Trp) residue side-chain groups. Moreover, an increase in the absorption intensity was observed as the dose increased. These results were mainly dependent on protein structure unfolding, suggesting that the Trp and Tyr residues were gradually exposed on the surface of the protein molecule. In the case of intrinsic fluorescence spectra, a decrease in fluorescence intensity with a significant red shift occurred. This observation indicated that increase in irradiation doses leads to fluorescence quenching, thereby changing the polarity of Trp and Tyr in the micro-environment, which could change the primary structure of α -La.^{31,32} An additional support for structural changes induced by irradiation was defined by ANS fluorescence. The increase in the intensity of ANS fluorescence indicated that the protein was unfolded, exposing more hydrophobic regions after irradiation, thereby increasing the hydrophobicity of the irradiated α -La. Hydrophobic surfaces in proteins are more easily attacked by hydroxyl radicals, and this change in protein hydrophobicity is considered as the prevailing factor for structural collapse.³³ Moreover, more hydrophobic surfaces indicated the complete unfolding of proteins, leading to peptides that strongly bind to the bis-ANS and exhibit a tendency toward aggregation.^{30,34,35} Collectively, these studies clearly demonstrated that changes in the tertiary structure caused by irradiation led to protein unfolding and subsequently, to aggregation.

Irradiation-induced alteration of the protein structure could affect potential allergenicity. In this study, a significant decrease in the overall IgG- and IgE-binding ability of the irradiated α -La was demonstrated. With the increase in irradiation dose, the structure gradually unfolded and exposed more hydrophobic groups. Given that the interior buried linear and conformational epitopes of the native molecule are mainly hydrophobic amino acid residues, more IgG and IgE epitopes

were exposed on the molecule surface as the dose was increased.³⁶ The exposed epitopes were destroyed severely by irradiation, resulting in a decrease in IgG- and IgE-binding ability of the irradiated α -La. Previous studies have confirmed that irradiation treatment could destroy linear and conformational epitopes by denaturation, aggregation, fragmentation, and amino acid modification of proteins.^{6,37} In addition, similar results were indicated in other studies on irradiated food proteins. For instance, Byun *et al.* found that the IgE binding abilities of irradiated proteins (BLG, OVA and shrimp tropomyosin) were all reduced depending upon the dose (0–10 kGy).³⁸ In another study, Oh *et al.* reported that 10 kGy was an effective dose to reduce the allergenicity of peanut extract and downregulated the Th2 lymphocyte activity in the peanut-sensitized mice.¹⁰ Combined with the finding by Oh *et al.*, Luo positively concluded that irradiation can reduce the antigenicity and allergenicity of the peanut allergen Ara h 6, particularly at a dose of 10 kGy; the reduction of allergenicity primarily relies on the irradiation-induced alteration of the protein structure.³⁹

Several studies that have corroborated on the ability to bind IgE do not necessarily indicate that the protein can effectively cross-link IgE/Fc ϵ RI complexes to trigger effector cell degranulation and subsequent histamine release.^{40,41} In addition to the markedly reduced IgE binding ability of α -La, decreased histamine-releasing capacity was also observed in the irradiated α -La. These results indicate that irradiation treatment could diminish the allergic effector activity. The Fc ϵ RI-elicited intracellular Ca²⁺ was significantly reduced when the cell was stimulated with irradiated α -La at varying doses, which positively correlated with histamine release in the allergic reaction system. Although intracellular Ca²⁺ is considered one of the most important and ubiquitous mechanisms of basophil degranulation, the specific role of α -La in the regulation of the Fc ϵ RI-mediated Ca²⁺ signaling pathway requires further clarification.

In conclusion, this study provided an important insight into the relationship between protein structure and potential allergenicity of irradiated α -La. In contrast to native α -La, irradiation was able to damage the structural integrity in proteins, and produce a decrease on the IgG/IgE binding properties and the IgE cross-linking capacity of α -La. However, these results were acquired only from *in vitro* experiments. Further investigations, such as animal or clinical studies, are still needed to confirm if the irradiation-treated α -La can significantly reduce the allergic symptoms related to consumption of a regular α -La counterpart. If confirmed, gamma irradiation might have potential as a food processing technology to reduce the allergenic properties of milk allergens.

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