

# Effects of Low-pH Treatment on the Allergenicity Reduction of Black Turtle Bean (*Phaseolus vulgaris* L.) Lectin and Its Mechanism

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**ABSTRACT:** A high content of potentially allergenic lectin in *Phaseolus vulgaris* L. beans is of increasing health concerns; however, understanding of the protein allergenicity mechanism on the molecular basis is scarce. In the present study, low-pH treatments were applied to modify black turtle bean lectin allergen, and a sensitization procedure was performed using the BALB/c mice for the allergenicity evaluation, while the conformational changes were monitored by the spectral analyses and the details were explored by the molecular dynamics simulation. Much milder anaphylactic responses were observed in BALB/c mice experiments. At the molecular level, the protein was unfolded in low acidic environments because of protonation, and  $\alpha$ -helix was reduced with the exposure of trypsin cleavage sites, especially the improvement of protease accessibility for Lys121, 134, and 157 in the B cell epitope structural alterations. These results indicate that a low-pH treatment might be an efficient method to improve the safety of legume protein consumption.

**KEYWORDS:** allergenicity, low-pH treatment, *P. vulgaris* lectin, structural alteration, molecular dynamics simulation

## INTRODUCTION

Allergic events induced by the consumption of *P. vulgaris* L. beans are increasing in recent years due to the high content of lectins (2.4–5% of total protein), which were accounting for 36.59% of food allergy events in 2017 with respect to allergenic plants in China.<sup>1</sup> Lectins are carbohydrate-binding proteins or glycoproteins of nonimmune origin<sup>2,3</sup> and play a critical role in triggering several food associated anaphylactic responses, such as nausea, bloating, vomiting, diarrhea and abdominal swelling in both children and adults.<sup>4,5</sup> Because of the high prevalence of lectin toxicity and allergenicity, safety guidelines regarding kidney bean (*P. vulgaris* L.) consumption have been proposed by public health laboratory services (PHLS), Colindale, U.K.<sup>2</sup> In 2006, more than 1 000 people had acute gastrointestinal symptoms and about 100 people were hospitalized in Japan because of consumption of insufficiently cooked white kidney beans (*P. vulgaris* L.).<sup>4</sup> In South Africa, due to the immunogenic potential of lectins, cultivation or consumption of a variety of the *P. vulgaris* beans by importation has been prohibited.<sup>6</sup> Nonetheless, *P. vulgaris* beans, including black turtle bean, kidney bean, pea bean, white bean, and yellow bean, etc., are among the most important varieties of edible cultivated legumes in the world.<sup>1</sup> The beans are grown in tropical and subtropical countries of the Americas, Europe, Africa, and Asia and are widely used for direct human consumption in several dishes such as salads, casseroles, macaroni, and Rajma-chawal due to the high contents of proteins, minerals, and vitamins.<sup>1,2,5</sup> Therefore, in order to ensure the application security of *P. vulgaris* L. beans, there is an urgent need for the development of novel hypoallergenic bean protein foods recently.

Several processing technologies, including thermal processing<sup>7,8</sup> and nonthermal treatments, such as ultrasonic,<sup>9,10</sup> high pressure,<sup>11,12</sup> as well as irradiation,<sup>13,14</sup> have shown tremendous potential in terms of reducing or eliminating lectin allergenicity. However, these methods are significantly hampered by the additional cost and safety risks and might not be adopted in the near future.<sup>14</sup> pH adjustment has been one of the basic principles in protein isolation and recovery. Recent studies have suggested that extreme pH treatments may have a major potential in protein property modifications<sup>15,16</sup> and might be an efficient and economical technique for the reduction of lectin immunogenicity without severe energy exchange or chemical reaction. However, related conformational information on protein allergenicity changes are lacking.<sup>17,18</sup>

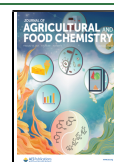
As a conventional *P. vulgaris* bean lectin, PHA (phytohemagglutinin) has been investigated in detail, and various potential biomedical applications, such as antimicrobial activity, insecticidal activity antitumor activity, etc., have been explored based on the lectin-carbohydrate specificity. Then more and more *P. vulgaris* bean lectins have already attracted great attention in recent studies as well as were purified and characterized from Zihua snap bean, white kidney bean, and many other south American bean varieties,

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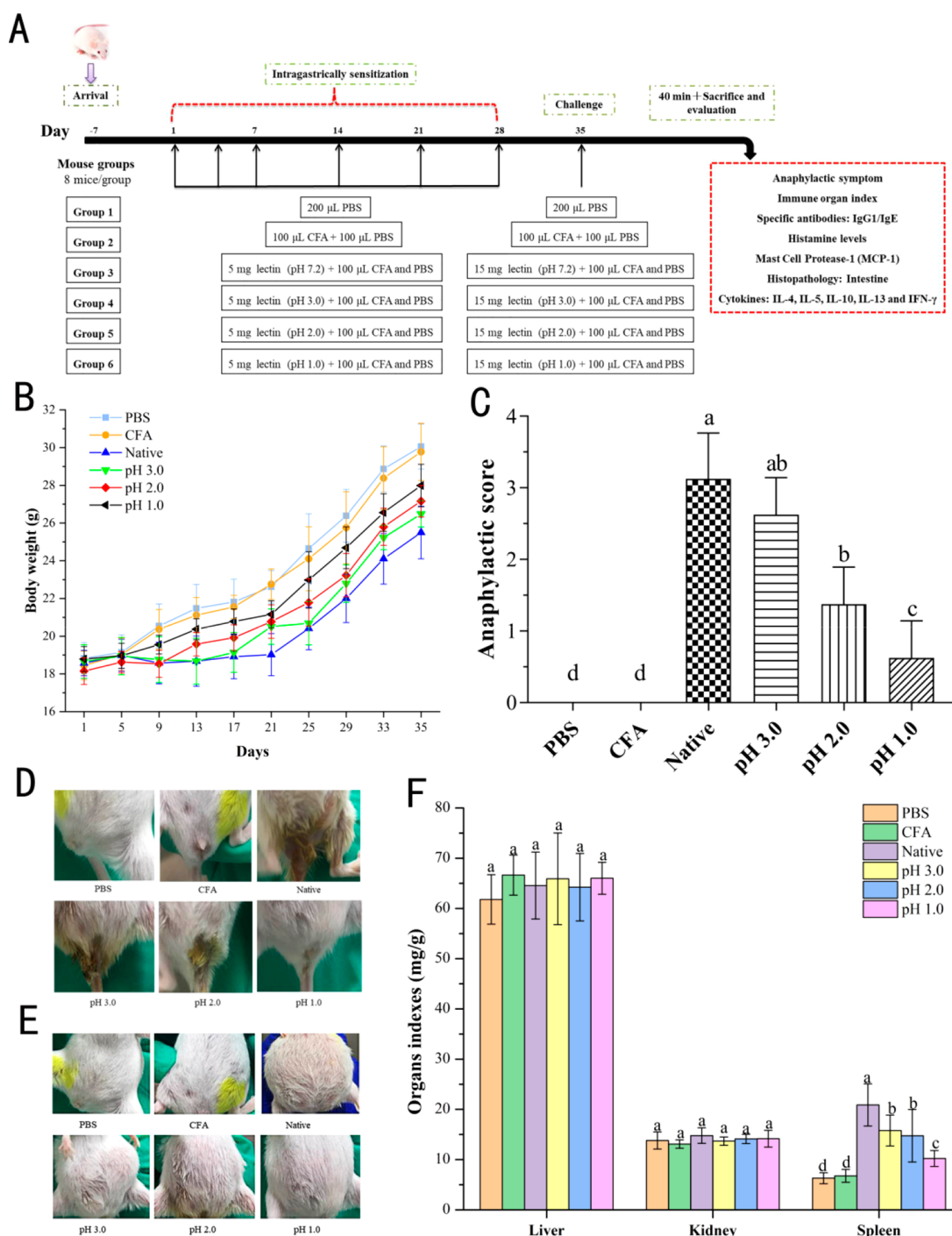


**Figure 1.** Sensitization protocol and clinical signs of the BALB/c mice. (A) Diagrammatic representation of the sensitization protocol. (B) The body weight was measured on days 1, 5, 9, 13, 17, 21, 25, 29, 33, and 35. (C) Anaphylaxis symptoms scored after 40 min following challenge. (D,E) Diarrhea symptoms and abdominal swelling symptoms in the BALB/c mice sensitized and challenged with native or low-pH induced black turtle bean (*P. vulgaris* L.) lectin. (F) Organ (liver and kidney and spleen) indexes of the BALB/c mice administrated with native or low-pH induced black turtle bean (*P. vulgaris* L.) lectin. ( $n = 8$ , data presented as mean  $\pm$  SD, different letters above the bars represent significant differences among various treatment groups in anaphylactic scores (part C) and in organ indexes (part F), ANOVA with a Duncan's means comparison test were applied using SPSS version 11.5 (SPSS Inc. Chicago, IL) with a significance level of 0.05).

respectively.<sup>19–21</sup> Lectin from black turtle bean (*P. vulgaris* L.) with high hemeagglutinating activity and allergenicity has been purified in our laboratory, and its sequence was submitted to NCBI with accession no. AHB17899.1.<sup>22–24</sup> Lectin is a tetrameric protein, and the molecular weight of each subunit is about 31 kDa consisting of 275 amino acid residues, which is highly homologous with PHA-E.<sup>23,25</sup> Besides, four B-cell

epitopes of B1 (55–66, NVNDNGEPTLSS), B2 (116–125, VGSEPKDKGG), B3 (131–141, NNYKYDSNAHT), and B4 (149–160, LYNVHWDPKPRH) with high IgE binding capacities have been confirmed.<sup>26</sup> Furthermore, considering the widespread application of the pH-shift process in industrial protein production, the effects of low pH treatment on the allergenicity of *P. vulgaris* lectin have been *in vitro* explored in

our previous study, and the tertiary structure of lectin was found to be unfolded with concomitant reduction of IgE binding capacity.<sup>27</sup> However, the potential *in vivo* relationship between the lectin protein structure and immunogenicity for the legume food consumption is still scarce.

The present study evaluated the allergenicity of low-pH treated lectin by the BALB/c mouse model (Figure 1A), and the secondary structural changes of the lectin allergen were characterized by using circular dichroism (CD) and Fourier transform-infrared (FT-IR) experiments. Furthermore, molecule dynamics (MD) simulation via root-mean-square deviation (RMSD) and root-mean-square fluctuations (RMSF) were used to investigate the relationship between lectin conformational alterations and clinical anaphylactic reactions to gain the first deep insights into molecular structure versus function to guide legume protein production and consumption.

## MATERIALS AND METHODS

**Lectin Samples Preparation.** Lectin from black turtle beans (*P. vulgaris* L.) of purity >94% was prepared as per our previous study.<sup>22,24</sup> Then, lectin samples were dissolved in 10 mM specific pH buffer (pH 1.0, 2.0, 3.0 and 7.2, respectively) to a concentration of 2.0 mg/mL at room temperature ( $25 \pm 2$  °C) for 8 h. All the corresponding buffers were KCl–HCl (pH 1.0), Gly–HCl (pH 2.0), Na<sub>2</sub>HPO<sub>4</sub>–citric acid (pH 3.0), and Na<sub>2</sub>HPO<sub>4</sub>–NaH<sub>2</sub>PO<sub>4</sub> (PBS, pH 7.2), respectively, and were filtered through a 0.22 mm membrane prior to be used. The treated lectin solutions were freeze-dried and stored in a –80 °C freezer until use. The sample dispersed in PBS buffer (10 mM, pH 7.2) was selected as the native control.

**Sensitization and Challenge Protocols.** As outlined in Figure 1A, a total of 48 female BALB/c mice purchased from the Experimental Animal Center of Anhui Medical University (Hefei, Anhui, China) were randomly divided into six groups ( $n = 8/\text{group}$ ) and were maintained under standard animal housing conditions. The animal experiments were approved by the Biomedical Ethics Committee of Hefei University of Technology (No. 2019-03-020) and were strictly carried out in accordance with the protocol for the Care and Use of Laboratory Animals, National Institutes of Health. All the mice were intragastrically sensitized on days 1, 4, 7, 14, 21, and 28 after a 1-week acclimatization period and orally challenged on day 35. More specifically, the first group (PBS group) was sensitized and challenged with 200  $\mu\text{L}$  of PBS alone (pH 7.2, 10 mM), and the second group (CFA group) was sensitized and challenged with 100  $\mu\text{L}$  of Complete Freund's adjuvant (CFA) (Sigma-Aldrich Corporation, St. Louis, MO) mixing with 100  $\mu\text{L}$  of PBS (pH 7.2, 10 mM), which was also the control group. Groups 3–6 (i.e., native, pH 3.0, pH 2.0, and pH 1.0 treated lectin groups) were sensitized with 200  $\mu\text{L}$  emulsified lectin solution (5  $\mu\text{g}$  of the treated lectin antigens were emulsified with 100  $\mu\text{L}$  of CFA and 100  $\mu\text{L}$  of PBS) and challenged with 200  $\mu\text{L}$  of emulsified lectin solution containing 15 mg of lectin.<sup>28</sup> All BALB/c mice in the allergic group were orally challenged with 15 mg of lectin or pH treated lectin, followed by monitoring the clinical signs of allergy for approximately 40 min by three independent observers according to a validated anaphylactic scoring system,<sup>29</sup> and then the mice were euthanized by CO<sub>2</sub> inhalation.

The BALB/c mice were administered with different treatments for 5 weeks (experimental weeks), and body weights were monitored at days 1, 5, 9, 13, 17, 21, 25, 29, 33, and 35. The organ indexes, including liver, kidney, and spleen indexes, were calculated by the equation: organ index = organ weight (mg)/body weight (g).

**Pathological Examination of Intestine.** In order to assess pathologic alterations, histological analyses were carried out by direct microscopic examination with a Pannoramic 250/MIDI microscope (3DHISTECH Ltd., Budapest, Hungary). The duodenum samples were fixed in 10% formalin in PBS and embedded in paraffin, and then 5  $\mu\text{m}$  sections were cut and were stained with hematoxylin and eosin (H&E).

**Assessment of Lectin-Specific Immunoglobulins.** Whole blood from each BALB/c mouse was collected and centrifuged at 3000 rpm for 15 min at 4 °C to collect sera and kept at –80 °C until analysis. Lectin allergen specific IgE and IgG<sub>1</sub> levels were assayed by enzyme-linked immunosorbent assay (ELISA) as described earlier.<sup>30</sup> Briefly, 96-well microtiter plates were coated either with native or treated lectin (5  $\mu\text{g}/\text{well}$ ). The sera in each BALB/c mouse were diluted to 1:50 for IgE and 1:2000 for IgG<sub>1</sub>. HRP-conjugated goat antimouse IgE and IgG<sub>1</sub> antibodies (diluted to 1:5000) were applied. The optical density (OD value) was measured at 450 nm within 15 min by a microplate reader (Tecan Infinite 200Pro, Tecan Group Ltd., Mannedorf, Switzerland).

**Determination of Serum Histamine Levels and Mouse Mast Cell Protease-1.** Histamine and mouse mast cell protease-1 (mMCPT-1) concentrations in all BALB/c mice sera were determined by ELISA assay using a commercially available ELISA kit (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

**Spleen Cell Culture and Quantitation of Cytokines.** Murine splenocyte culture was carried out as reported previously.<sup>31</sup> In brief, individual spleen from each BALB/c mouse was collected aseptically and single-cell suspensions were prepared. Cells ( $2 \times 10^6/\text{well}$ ) were cultured at 37 °C in a flat-bottom 96-well plate without any stimulation or in the presence of native or treated lectin (2 mg/mL) for 24 h in a 5% CO<sub>2</sub> incubator. Supernatants were collected and stored at –80 °C until analysis. IL-4, IL-5, IL-13, and IFN- $\gamma$  were determined using commercially available ELISA Kits (YuanYe Biotechnology Co., Ltd., Shanghai, China).

**CD Analysis.** Lectin sample was dissolved in PBS (10 mM, pH 7.2) for a concentration of 0.2 mg/mL, and the CD spectra were collected using a Chirascan qCD circular dichroism spectrometer (Applied Photophysics Ltd., Tokyo, U.K.) at 25 °C. The spectra data between 190 and 260 nm were recorded at a 90 nm/min scanning speed in a 1 mm path length quartz cuvette under nitrogen atmosphere, and each sample was scanned 3 times to obtain an averaged value.

**FT-IR Spectra.** The lyophilized lectin samples (5 mg) were mixed with KBr (100 mg) and ground into a fine powder in an agate mortar incubated with infrared light and then pressed into a pellet. FT-IR spectra data were obtained in the wavenumber range from 400 to 4000  $\text{cm}^{-1}$  during 32 scans with 0.09  $\text{cm}^{-1}$  resolution using a Nicolet 6700 FT-IR spectrometer (Thermo Nicolet Corporation). Data were analyzed by Omnic 8.2 software (Thermo Fisher Scientific Inc., Madison, WI) and Peakfit 4.12 (Systat Software, San Jose, CA).

**MD Simulations.** All MD simulations were performed using Discovery Studio 2019 software (San Diego, CA, USA), and the CHARMM force field was applied. Solvation was carried out by the explicit periodic boundary model, and then the system was energy minimized in 50 000 steps using the steepest descent algorithm, and 100 000 steps equilibrations were performed under 298.15 K in the NVT ensemble. Finally, a total of four MD productions were performed in NPT conditions at 298.15 K for each complex at a 12 ns time scale with a 2.5 ps time step. The knowledge extracted from the trajectory files was used to understand the behavior of protein in the explicit solution environment with different pH conditions. Secondary structure, RMSD, and RMSF were analyzed.

**Statistical Analyses.** All experiments were reported as mean  $\pm$  SD with a minimum of triplicates. Statistical analyses of variance (ANOVA) and Duncan's means comparison test were performed by using SPSS software (version 11.5, SPSS Inc. Chicago, IL) with a significance level of  $P < 0.05$ .

## RESULTS

**Determination of Body Weight during the Animal Sensitization.** We consistently monitored the body weight changes of intragastrically sensitized BALB/c mice and found that the weight of each group remained stable after 1 week adaption, but various weight gain trends were observed during the experiment days from the first to the 35<sup>th</sup> days (Figure 1B). The highest body weights of  $30.05 \pm 1.19$  and  $29.77 \pm 1.52$  g

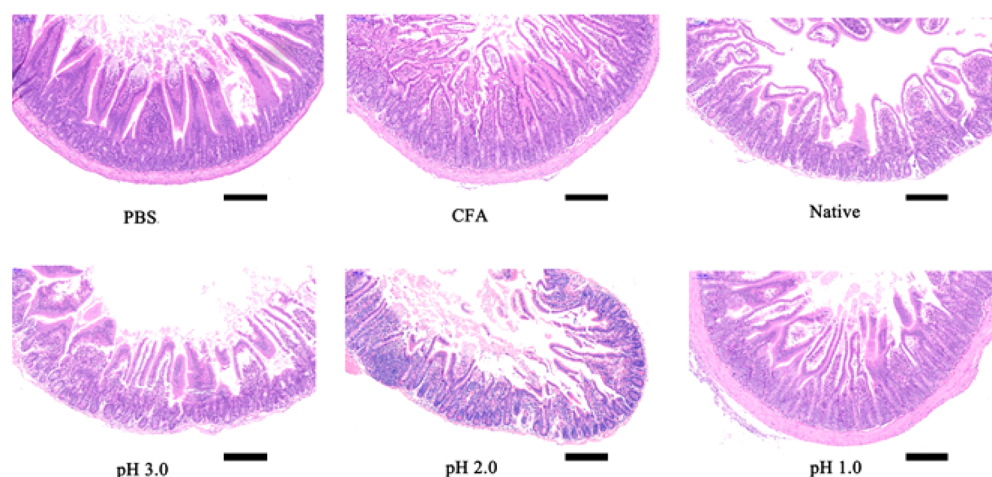


**Figure 2.** Histopathology alterations of the intestine of the BALB/c mice administrated with native or treated black turtle bean (*P. vulgaris* L.) lectin (bar = 100  $\mu$ m). Intestine tissues were collected 40 min after the challenge and fixed in neutral-buffered formaldehyde. 5  $\mu$ m sections were stained with hematoxylin and eosin, and then the sections were examined histologically at  $\times 100$  magnification using a light microscope.

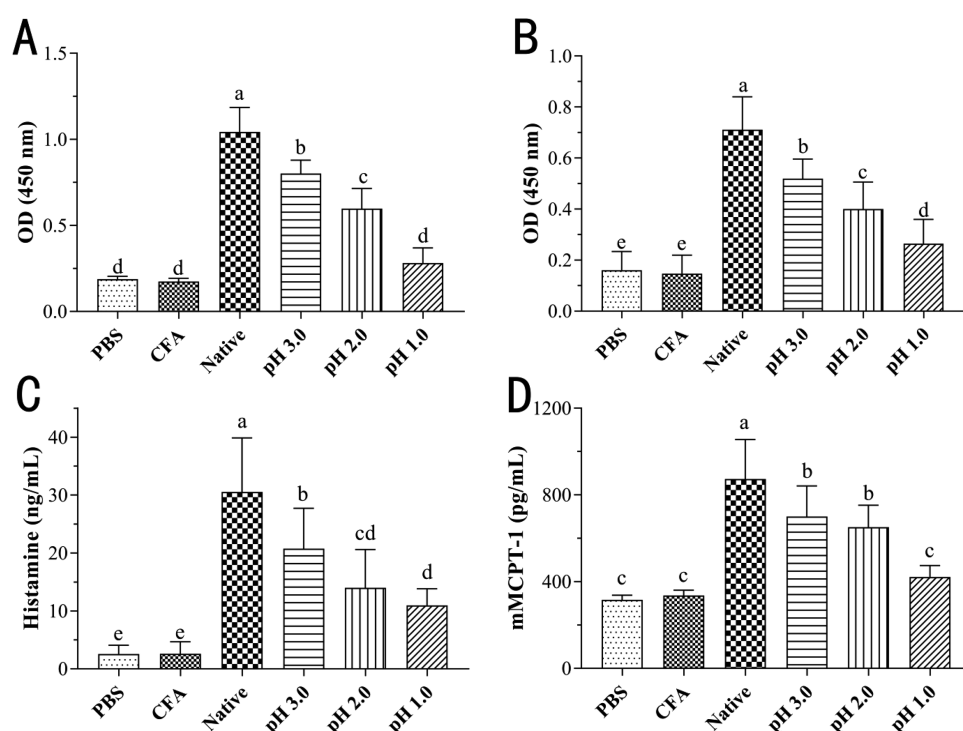


**Figure 3.** Anaphylaxis in the BALB/c mice sensitized and challenged with native or treated black turtle bean (*P. vulgaris* L.) lectin. (A–D) Lectin-specific IgE and IgG<sub>1</sub> levels and concentration of histamine and mMCPT-1 in 40 min after orally challenged the BALB/c mice serum, receptively. Lectin-specific IgE and IgG<sub>1</sub> activities were measured by ELISA with HRP-conjugated goat antimouse IgE and IgG<sub>1</sub> antibodies. The results were represented as optical density (OD). The concentration of histamine and mMCPT-1 in each BALB/c mice was determined by a commercial ELISA kit according to the manufacturer's instructions ( $n = 5$ , data presented as mean  $\pm$  SD, different letters above the bars represented significant differences, ANOVA with Duncan's means comparison test were applied using SPSS version 11.5 (SPSS Inc. Chicago, IL) with a significance level of 0.05).

were obtained in the PBS group (control group) and CFA group (sham-sensitized group) at the end of the sensitization protocol (35<sup>th</sup> day), respectively; and the body weights of the groups administrated with the low pH treated lectin were significantly higher than that of the group administered native lectin ( $25.50 \pm 1.39$  g) with the order of pH 1.0 group > pH 2.0 group > pH 3.0 group > native group.

**Assessment of Anaphylactic Scores.** The BALB/c mice were challenged orally at the 35<sup>th</sup> day after initial sensitization, and the anaphylactic scores are shown in Figure 1C. There was

no evident anaphylactic reaction or difference between the PBS and CFA groups. By contrast, the highest anaphylactic score was obtained in the native group sensitized by untreated lectin as evidenced by severe anaphylactic reactions such as severe diarrhea, abdominal swelling, shivering, and no activity after stimulation. This was followed sequentially by anaphylactic scores of the pH 3.0, pH 2.0, and pH 1.0 groups, while only subtle anaphylactic symptoms were observed in the BALB/c mice from the pH 1.0 group (Figure 1C,D).

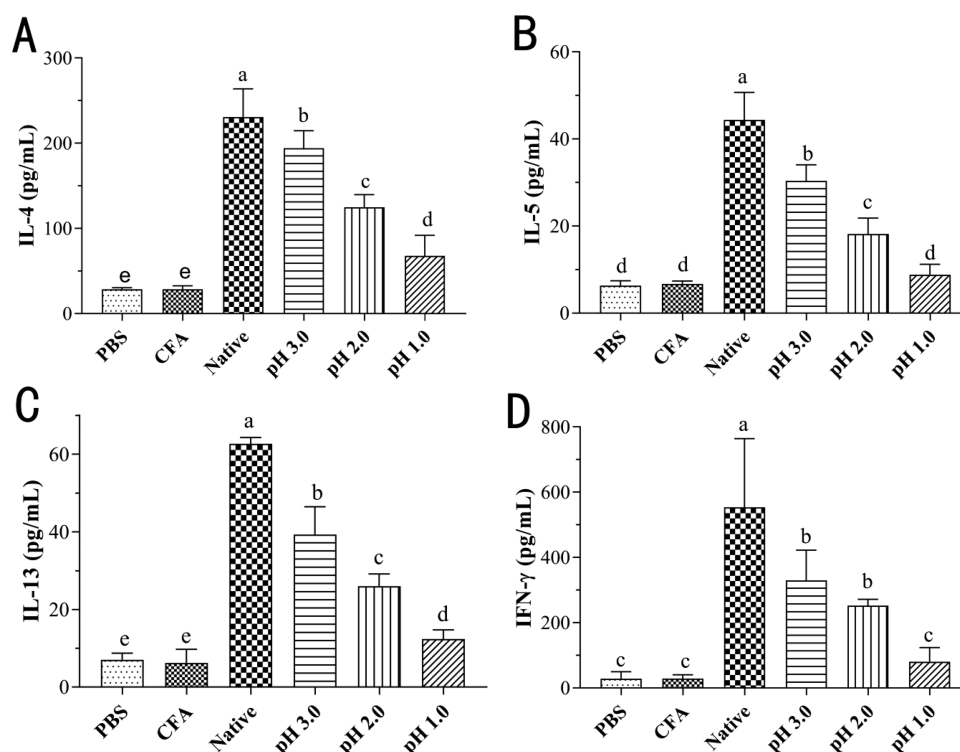


**Figure 4.** Cytokines producing of the BALB/c mice splenocyte stimulated by native or treated lectin. (A–D) Levels of cell culture supernatants for IL-4, IL-5, IL-13, and IFN-γ were determined by a commercial ELISA kit ( $n = 5$ , data presented as mean  $\pm$  SD, different letters above the bars represented significant differences, ANOVA with Duncan's means comparison test were applied using SPSS version 11.5 (SPSS Inc. Chicago, IL) with a significance level of 0.05).

**Determination of Organ Indexes.** The organ indexes of the kidney, liver and spleen were calculated and are shown in Figure 1F. Compared with the PBS and CFA groups, no significant differences ( $P > 0.05$ ) of both the liver and kidney indexes were observed among the mice groups fed with native lectin and low pH treated lectins. Nevertheless, significantly higher ( $P < 0.05$ ) spleen indexes were obtained in the BALB/c mice from lectin sensitization groups, while spleen indexes significantly ( $P < 0.05$ ) decreased with decreasing the pH for treated lectin, e.g., the spleen indexes of the pH 1.0 group decreased to 46.56% of that of the native lectin group.

**Histopathological Examination of Intestinal Segments.** As shown in Figure 2, the villi structure of the small intestine appeared tidy and intact in PBS and CFA groups without obvious pathological changes, whereas typical lectin-induced disruptions with small intestine villi atrophy, brush border membranes detachment, and even partial necrosis of intestinal cells, were all observed in the native lectin-sensitized group. Although slight swelling and intestinal cell disorder were also found in the low pH treated lectin groups, the damages of the small intestine were much milder compared with the native lectin group, and the order was similar to spleen index as follows: native group > pH 3.0 group > pH 2.0 group > pH 1.0 group, i.e., ranging from severe to mild. Particularly, compared with the PBS and CFA groups, the villi structures of BALB/c mice small intestine in pH 1.0 group were tidy and intact without obvious inflammation.

**Levels of Specific IgE and IgG<sub>1</sub>.** The immune response was further characterized by measuring serum-specific IgE and IgG<sub>1</sub> of challenged BALB/c mice (Figure 3A,B). Negligible amounts of lectin-specific IgE and IgG<sub>1</sub> were detected in the serum of BALB/c mice in the PBS and CFA groups, while the

native group mice showed the highest IgE and IgG<sub>1</sub> levels. However, significantly lower levels ( $P < 0.05$ ) of both serum IgE and IgG<sub>1</sub> were observed in low-pH induced lectin-administrated mice in decreasing order of native group > pH 3.0 group > pH 2.0 group > pH 1.0 group.

**Histamine and Mice mMCPT-1 Release.** The concentrations of serum histamine and mMCPT-1 of the challenged BALB/c mice were measured by ELISA. As shown in Figure 3C,D, compared with the BALB/c mice challenged with native lectin, significantly lower ( $P < 0.05$ ) histamine and mMCPT-1 releases were found in the BALB/c mice challenged with low-pH induced lectin. In particular, histamine and mMCPT-1 concentrations significantly decreased ( $P < 0.05$ ) with decreasing of the pH group for lectin treated, and the histamine and mMCPT-1 concentrations from the pH 1.0 group were close to the values obtained from PBS and CFA groups, which were reduced by 64.03% and 51.77%, respectively, compared to the native group.

**Cytokines Production in Splenic Cells.** To further evaluate the immunoreactivity of low-pH induced lectin, cytokines production of IL-4, IL-5, IL-13, and IFN-γ were analyzed in the BALB/c mice splenocyte (Figure 4A–D). The cell cultures prepared from PBS and CFA groups showed negligible cytokine release. Significantly higher IL-4, IL-5, IL-13, and IFN-γ secretions were found in native lectin-administered BALB/c mice than the PBS and CFA groups. In contrast, the group administered low-pH induced lectin showed remarkable decreases ( $P < 0.05$ ) in IL-4, IL-5, IL-13, and IFN-γ levels, whereas the IL-4, IL-5, IL-13, and IFN-γ levels of pH 1.0 group were reduced to 29.46%, 19.87%, 19.80%, and 14.57%, respectively, of those in the native group.

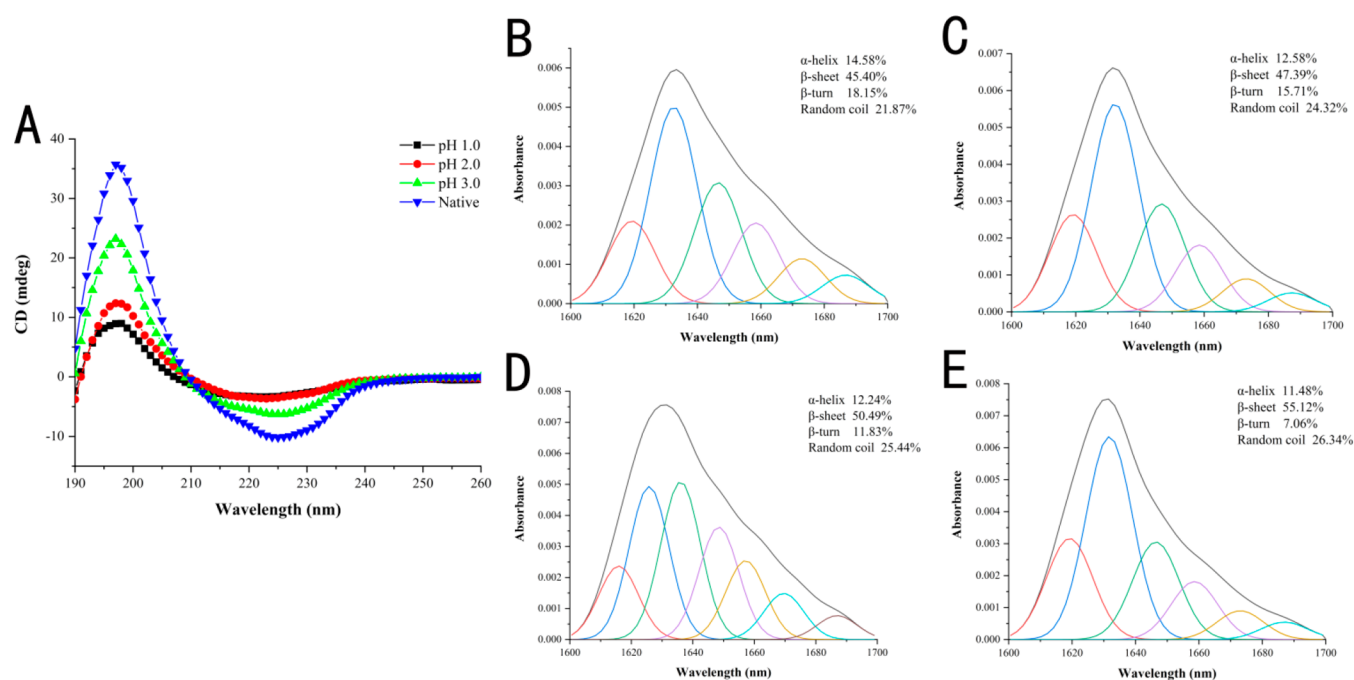


**Figure 5.** The analysis of the secondary structure of native or treated black turtle bean (*P. vulgaris*L.) lectin. (A) Changes in CD spectra of native or treated lectin. Self-deconvoluted FT-IR spectrum and their curve fitting results of amide I (1600–1700  $\text{cm}^{-1}$ ) of native (B) or low-pH induced (C) pH 3.0, (D) pH 2.0, and (E) pH 1.0) black turtle bean (*P. vulgaris* L.) lectin.

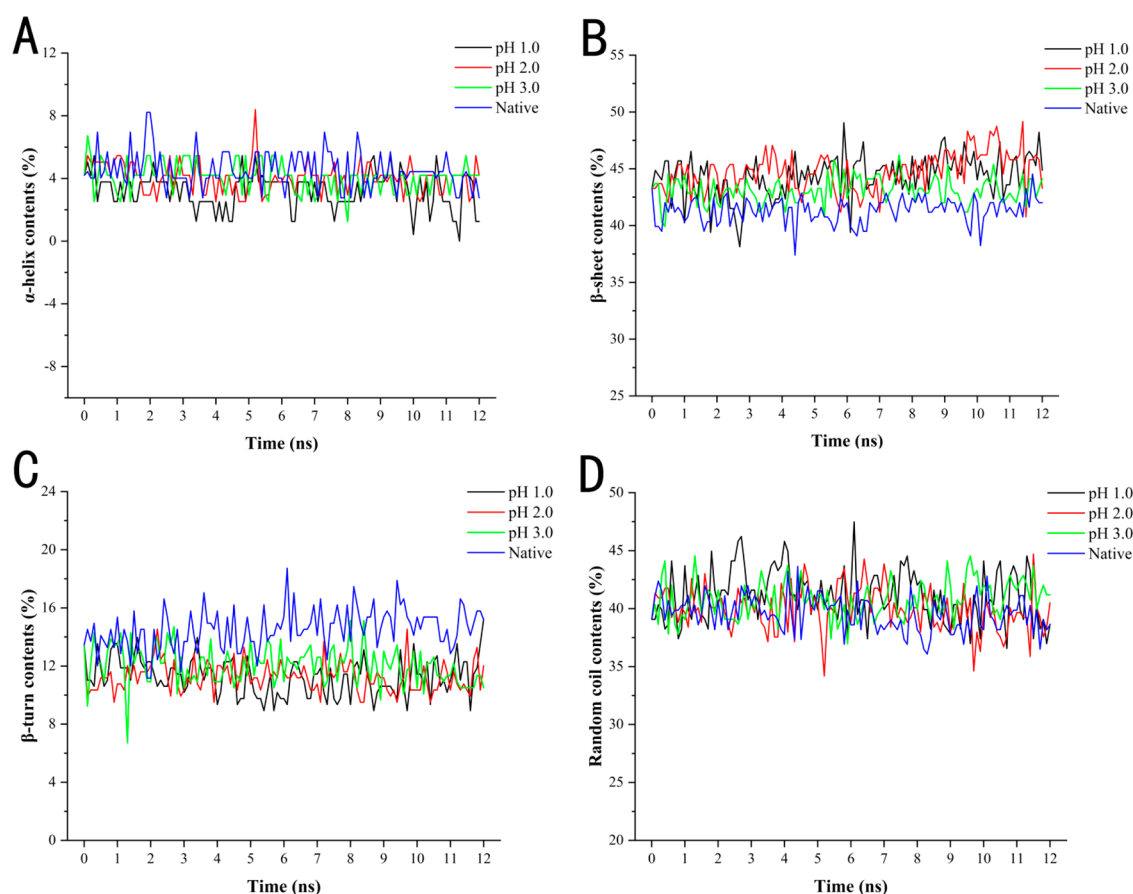


**Figure 6.** Secondary structure changes of black turtle bean (*P. vulgaris*L.) lectin in different pH environments during 12 ns MD simulations: (A)  $\alpha$ -helix, (B)  $\beta$ -sheet, (C)  $\beta$ -turn, and (D) random coil.

**CD and FT-IR Analyses.** The far UV region (190–260 nm) of the CD spectrum was employed to give a quantitative

analysis of the secondary structure changes. As shown in Figure 5A, low-pH induced lectin showed evident decreases in

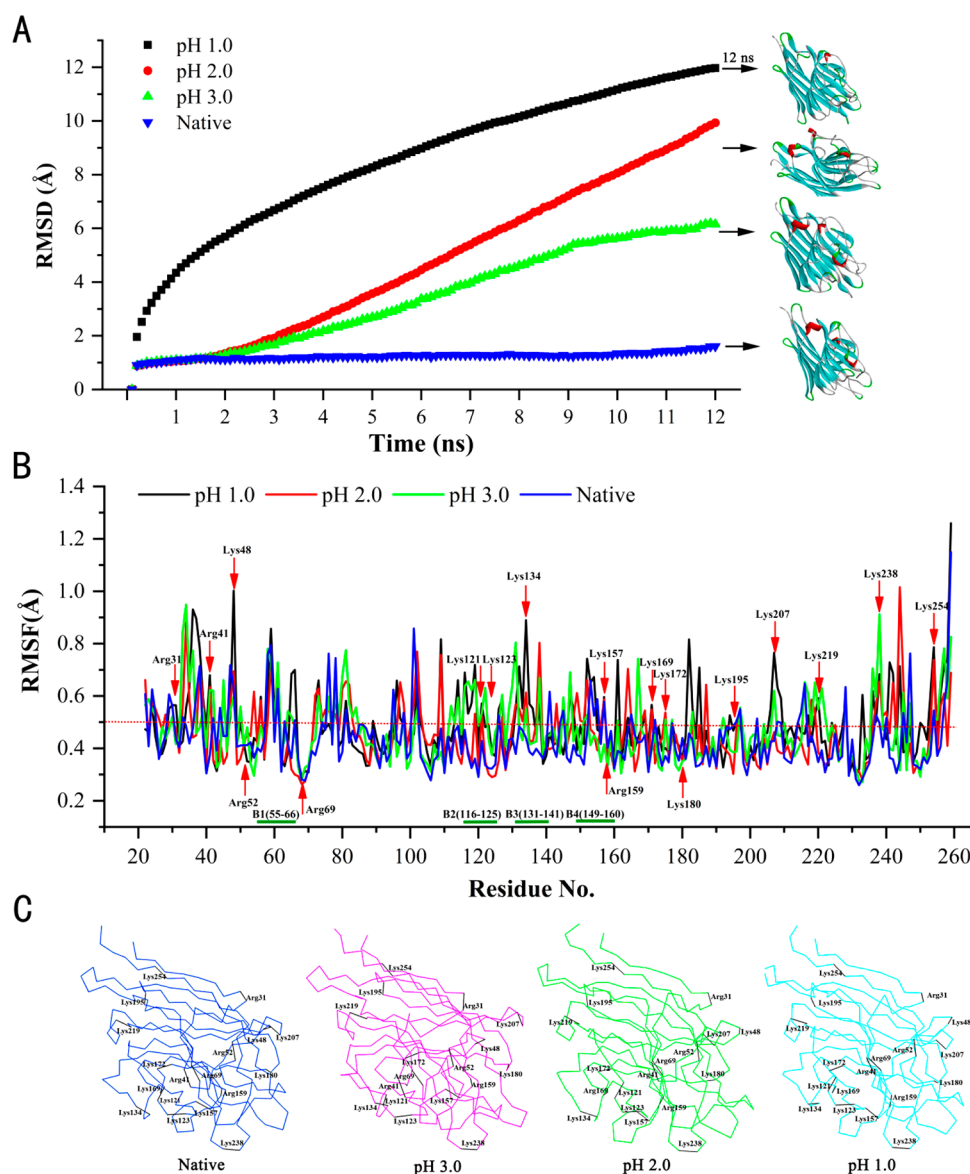


**Figure 7.** RMSD, RMSF and trypsinolysis analyses of black turtle bean (*P. vulgaris* L.) lectin in different pH environments during 12 ns MD simulation. (A) Plot describing the variations in the black turtle bean (*P. vulgaris* L.) lectin backbone RMSD values in different pH environments on the basis of 12 ns MD simulations. RMSD values were calculated by comparing the structure of simulated protein during simulation with the native lectin reference. (B) RMSF curves showing the variations in the backbone residues of the black turtle bean (*P. vulgaris* L.) lectin with respect to the crystal structure at different pH environments during 12 ns MD simulations. Arrows represent the trypsinolysis of the RMSF. Green lines represent the sequences of epitopes. (C) Trypsinolysis variations of the lectin monomers in different pH environments at the end of 12 ns MD simulations.

negative ellipticity at 208 and 222 nm with respect to the native group, and an evident blue shift of the peak wavelength near 222 nm was obtained.

FT-IR spectra of low-pH induced lectin in the region of 1700–1600  $\text{cm}^{-1}$  (amide I band) are shown in Figure 5B–E. The peak position of the amide I band of native lectin was shifted from 1649 to 1654.2  $\text{cm}^{-1}$  with decreasing treatment in pH values, suggesting changes of the lectin secondary structure. The curve-fitted spectra of the infrared amide I band were employed to calculate the secondary structure contents of treated lectin. Thus, compared to the native lectin, the contents of the  $\alpha$ -helix and  $\beta$ -turn decreased from 14.58% to 11.48% and from 18.15% to 7.06%, respectively, while the  $\beta$ -sheet content increased from 45.40% to 55.12%, and the

increase of random coil content was from 21.87% to 26.34% with the decreasing of treatment in pH values (Figure 5D,E).

**MD Simulations Analysis.** MD simulations were applied to analyze the lectin monomer under periodic boundary conditions using the CHARMM force field. As shown in Figure 6, native lectin was calculated to consist of 4.20%  $\alpha$ -helix, 43.27%  $\beta$ -sheet, 13.45%  $\beta$ -turn, and 39.07% random coil. The secondary structures of the native lectin remained steady within 12 ns of the MD simulation, while low pH induced lectin secondary structures showed significant changes. In general, the  $\alpha$ -helix and  $\beta$ -turn contents of the lectin displayed an evident increase trend under low pH environments compared to native lectin, whereas the content of the  $\beta$ -sheet increased significantly with decreasing pH, which was in good agreement with the spectral results.

With respect to the reference of native lectin molecule, RMSD was applied to predict the arithmetic values of the deviations in the atoms, and significant effects of low pH environments on the RMSD values of the lectin allergen were observed during the 12 ns trajectories (Figure 7A). Specially, the RMSD value of native lectin was calculated to be 1.61 Å, while the lectin incubated in the pH range of 1.0–3.0 showed a significant increase with RMSD values of 4.54 Å at pH 3.0, 8.32 Å at pH 2.0, and 10.36 Å at pH 1.0.

RMSF values of native and low-pH induced lectin monomers were plotted to determine higher residual fluctuation regions of protein (Figure 7B). The magnitude of the residual fluctuation was represented by the height of the peaks in the RMSF analysis. An evident change of fluctuation upon the pH values was observed, and the fluctuation increased with decreasing pH values, where relatively higher RMSF values were obtained in the pH 1.0 treated lectin. Trypsin cleavage sites and epitopes in the lectins were marked with a red arrow and green line in Figure 7B, respectively. The RMSF values of almost all cleavage sites by trypsin were higher than 0.45 Å in low acidic conditions. In fact, if the cut RMSF value was set at 0.50 Å, the positive predictive values to trypsinolysis for the susceptibility of target cut sites in lectin allergen are 38.89%, 50.00%, and 61.11%, respectively, in low pH conditions (pH 3.0, pH 2.0, and pH 1.0), while resistance of native lectin to proteolytic action was 77.78%. Moreover, higher RMSF values were observed in all epitope regions, especially for cleavage sites to trypsin (Lys121, 134 and 157) located in four B-cell epitopes of B2 (116–125, VGSEPKDKGG), B3 (131–141, NNYKYDSNAHT), and B4 (149–160, LYNVHWDPKPRH).

## DISCUSSION

Food allergies mostly occur through allergen-specific IgE-mediated type I anaphylaxis responses and can elicit powerful T helper cell type 2 (Th2) responses including systemic, gastrointestinal, and mucosal immune systems symptoms.<sup>5,32,33</sup> In our present study, BALB/c mice were sensitized with native and low-pH treated lectins, respectively. During the experimental period or at the end of the sensitization protocol, higher body weight, lower spleen indexes, and anaphylactic scores were observed in the treated lectin groups compared with native lectin administrated BALB/c mice. Less weight loss of mice was also reported in an earlier study by feeding with irradiated wheat germ agglutinin (WGA).<sup>34</sup> Many previous studies also confirmed that dietary lectins could interfere with nutrient digestion and absorption, and lectins at high intakes could seriously threaten the growth and health of consuming animals.<sup>5,34,35</sup> As shown in Figure 1B–D, the BALB/c mice body weights followed a decreasing order of PBS and CFA groups > pH 1.0 group > pH 2.0 group > pH 3.0 group > native group, while the anaphylactic scores and spleen organ indexes displayed the opposite trend. In particular, no signs or very little diarrhea and abdominal swelling were observed in the pH 1.0 group, indicating that the lectin after the incubation in the pH 1.0 environment might have lower immunoreactivity to the BALB/c mice.

The gastrointestinal hypersensitivity was further measured by intestinal histopathological examination. The BALB/c mice administrated with native lectin showed severe intestinal villi atrophy as well as shedding and necrosis of intestinal cells (Figure 2). Similar intestinal pathology features have been frequently observed during food allergic reactions.<sup>5,30,34,36</sup>

Previous studies indicated that lectin molecules could be resistant to breakdown in the gastrointestinal tract by proteolytic enzymes, thus they would bind to the surface epithelium of the digestive tract and potentially elicit antinutritional, impaired digestion, feeble growth, mild allergic, or other subclinical effects in humans and animals.<sup>2,5,35</sup> Additionally, attenuated small intestine damages were clearly observed in the low-pH treated lectin-fed mice through histopathological examination, and the pH 1.0 group presented similar tissue changes with that of PBS and CFA groups.

It is believed that anaphylaxis in BALB/c mice might be triggered by multiple mechanisms, and that serum-specific IgE antibodies would play a crucial role in mediating type I allergic reactions.<sup>33,37</sup> Our earlier *in vitro* research by indirect ELISA clearly indicated that IgE binding capacity significantly decreased for lectins after lower pH treatments.<sup>27</sup> However, measuring IgE levels by ELISA may not reflect the accuracy of the allergic symptoms.<sup>38</sup> Accordingly, the *in vivo* serum-specific IgE levels of BALB/c mice were detected after oral challenge. Our results indicated that lectin-specific IgE levels were significantly ( $P < 0.05$ ) lower when the mice were injected with low-pH treated lectin, and no differences ( $P > 0.05$ ) in IgE contents were observed among pH 1.0, PBS and CFA groups, compared with the native group (Figure 3A). In addition, lectin allergen-specific production of the IgG<sub>1</sub> level in the sera was also significantly decreased in the low pH-induced lectin-fed BALB/c mice than in the native group (Figure 3B) with the order of native group > pH 3.0 group > pH 2.0 group > pH 1.0 group > PBS and CFA groups. Previous studies have confirmed that systemic anaphylaxis in mice could also be mediated largely through IgG<sub>1</sub>, and the serum IgE and IgG<sub>1</sub> levels were positively correlated in the T helper cell type 2 (Th2) immune response.<sup>33,39</sup> Then both the reduction of serum IgE and IgG<sub>1</sub> levels in the present study suggested the decrease in immunogenicity of the low-pH induced lectins, especially for the pH 1.0 treated sample. Furthermore, significantly lower levels of histamine and mMCPT-1 were subsequently confirmed in the low-pH treated lectin-administrated mice (pH 3.0, 2.0, and 1.0 groups) with respect to the native lectin group ( $P < 0.05$ ), and the order was similar to the antibody levels (Figure 3C,D). Since the histamine and mMCPT-1 were released from the activated mast cells or basophils after secondary exposure of allergens,<sup>2,32</sup> the reductions of anaphylactic mediators provided further evidence for the alleviation of anaphylactic symptoms.

Moreover, the IL-4, IL-5, IL-13, and IFN- $\gamma$  levels were detected to evaluate the allergenicity of low pH treated lectin by using BALB/c mice splenocytes after *in vitro* stimulation. According to the previous studies, a variety of IL-4, IL-5, and IL-13 cytokines would be secreted by a subset of T helper lymphocytes (Th2 cells) and play important roles in mediating Th2 allergic reactions by including B-cell proliferation and differentiation as well as IgE and IgG<sub>1</sub> synthesis.<sup>25,32</sup> The Th1 immune response could be indicated by the IFN- $\gamma$  level which might result from the increasing production of IgG<sub>2a</sub> antibodies and Th1-type cytokines.<sup>30,40</sup> Compared with the groups administrated with low pH-treated lectin, a higher secretion of Th2-type cytokines was obtained in the splenocytes from the native group, which would trigger and aggravate the inflammatory responses (Figure 4A–D).<sup>32,41</sup> The higher anaphylactic scores and more serious intestinal damage that occurred in the native group could also be explained by potential features of higher cytokines levels. Previous studies

demonstrated that several plant-derived lectins, including Con A, *P. vulgaris* erythroagglutinin (PHA-E), *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), and *Sambucus nigra* agglutinin (SNA) could effectively trigger the release of IL-4, and high IL-4 concentration would enhance secretions of IL-13 and histamine.<sup>42</sup> Similar to the low pH treated lectin, gamma radiated lectin has also been found to lose the inducing ability for the secretions of IL-4, IL-5, and IL-13, which might be related to the protein conformational changes.<sup>34,43,44</sup> Besides, an up-regulation of Th1-type cytokine IFN- $\gamma$  in splenocytes was found in the native group compared with the groups administered with low pH treated lectins, PBS, and CFA. In order to keep the balance of the Th1/Th2 ratio to counteract harmful inflammation,<sup>30,40</sup> IFN- $\gamma$  might be released in a high level in the native lectin group and would also enhance the release of the IgE level and various other mediators that might be responsible for allergic reactions.<sup>41</sup> After low-pH treatment for the lectin, significantly decreased levels of IL-4, IL-5, IL-13, and IFN- $\gamma$  were observed in splenocytes stimulated with pH treated samples (Figure 3), which was consistent with our earlier *in vitro* research by indirect ELISA and simulated gastrointestinal digestion,<sup>27</sup> suggesting that low-pH treatment might reduce the immunogenicity potential of lectin. Despite the explanations being unclear yet, the improvement of *in vitro* digestibility of lectin was presumed to be related to the structural modifications of the protein induced by the acidic environment and would contributed to the loss of allergenicity.<sup>18,27</sup>

Herein, in order to explore the key structural modifications responsible for the reductions of immunogenicity, CD, FT-IR spectra, and MD simulation were further applied. Two weak negative bands at around 208 and 222 nm were found in CD spectra (Figure 5A), which were characteristics of the  $\alpha$ -helix of protein, and a negative peak at around 218 nm is attributed to  $\beta$ -sheet contents.<sup>35,46</sup> The blue shift of the peak wavelength (from 222 to 218 nm) suggested that low-pH treatments might induce a decrease in  $\alpha$ -helix content of lectin accompanied by a simultaneous increase in the  $\beta$ -sheet structure.<sup>17</sup> The structural changes of lectin via low-pH induced treatments were further determined by FT-IR (Figure 5B–E). The infrared amide I band (1600–1700  $\text{cm}^{-1}$ ) is highly sensitive to alterations in the secondary structure of proteins, which has been widely utilized in analyzing the protein secondary structural changes, with 1610–1638  $\text{cm}^{-1}$  belonging to the  $\beta$ -sheet, 1638–1648  $\text{cm}^{-1}$  attributed to the random coil, 1649–1660  $\text{cm}^{-1}$  belonging to the  $\alpha$ -helix, and 1660–1692  $\text{cm}^{-1}$  attributed to the  $\beta$ -turn structures.<sup>47</sup> The curve-fitted spectra of the infrared amide I band clearly suggested that low-pH treatments could cause a decrease in the  $\alpha$ -helix content, and the value was reduced to 11.48% in lectin treated at pH 1.0, while the  $\beta$ -turn content was decreased to 7.06%, which was accompanied by simultaneous increases in the  $\beta$ -sheet and random coil contents. Combining *in vivo* experimental results, these structural changes might lead to the unfolding of lectin allergen, and then conformational or linear epitopes might be easier to be destroyed and degraded, resulting in decreases of antigenicity and allergenic response.<sup>14,18</sup>

To further study the structure of lectin molecules, MD simulations were performed in different pH environments for 12 ns (Figure 6). The low-pH treated protein MD simulation results also revealed a similar decrease in  $\alpha$ -helix and  $\beta$ -turn structures and a simultaneous decrease in the  $\beta$ -sheet and random coil contents. The hydrogen bond between the amino

terminal hydrogen (NH–) and carbonyl terminal oxygen (–C=O) plays a crucial role in the formation and stabilization of the helical structure.<sup>48,49</sup> As the pH of the medium was far away from the isoelectric point of lectin (around pH 4.5), the amino acid groups (–OH, –COOH, and –NH<sub>2</sub>) on protein molecules would be protonated, and intramolecular electrostatic repulsions would drive the protein molecules to a partially unfolded state, causing the alteration of secondary structures.<sup>50,51</sup> Similarly, an earlier research demonstrated that the hydrogen bonds could be destroyed by proton transfer between the solution and the peptide at low pH environments by using reactive molecular dynamics study, and consequently, a significant amount of helix would unwind and display a pH-dependent transition from a helix to a coil.<sup>52</sup> In our MD simulation results, a similar pH dependency in helical structure loss and helix to coil transition were also confirmed, such as the helix regions of Glu-Thr-Asn (AA 34–36) and Gly-Gly-Leu (AA 124–126) changed to coil structures. Based on our previous analysis, reductions of the helical structures with an increase of the  $\beta$ -sheet contents might be negatively well correlated with the severity of anaphylaxis responses, which would mask and destroy lectin epitope regions and cause a decline of the IgE binding capacity.<sup>27</sup> A similar decrease in  $\alpha$ -helix and antigenicity, accompanied by increasing  $\beta$ -sheet contents under low acid conditions, was also observed in low pH treated tropomyosin from the short-neck clam (*Ruditapes philippinarum*).<sup>18</sup> The helical structure might play a critical role in protein antigenicity, and previous research demonstrated that the content of  $\alpha$ -helices of the peanut allergen decreased with the reduction in antigenicity after combination of high-pressure and heat treatments,<sup>53,54</sup> indicating that epitopes might be probably destroyed due to the loss of  $\alpha$ -helical structure.

RMSD were further calculated for monitoring distance deviations during the entire simulation time. The lectin backbone RMSD attained a significantly higher value under low pH conditions (Figure 7A), as the final RMSD value was about 1.61 Å at neutral conditions (pH 7.2), but it increased to around 6.15 Å at pH 3.0, and 9.93 Å at pH 2.0, and 11.97 Å at pH 1.0, which further confirmed the unfolding of the helical secondary structures in low acidic conditions.<sup>55</sup> Although similar research studies are scarce, the increase of RMSD values was also observed in collagen-like peptide, human prion protein, and calcium-calmodulin dependent protein kinase IV (CAMKIV) in low acidic conditions during the MD situation<sup>56–58</sup> and indicated partially unfolded states of proteins. Besides, in our previous research, the lectin intact structure gradually becoming loose could be obtained by the dynamic light scattering value displayed a significant fluctuation in low acidic environments with respect to the native state during the MD simulation (Figure 7B), which was mainly because of the transitions among the amino acid residues, thus causing partial secondary structural adjustments, especially for the loss of the helical secondary structures. Furthermore, high allergenicity was well related with the digestion resistibility of allergens.<sup>2,26</sup> All trypsin-sensitive target sites for lectin allergen were identified in this study (Figure 7B), and our data showed that under low pH conditions, the lectin trypsin cleavage sites showed higher RMSF values, and more trypsin cleavage sites were exposed (Figure 7B,C), thereby rendering the lectin allergen would be more susceptible to proteolytic attack. Furthermore, it is worth noting that the trypsin cleavage sites (Lys 48, 134, and 157) in

the B-cell epitopes of B2, B3, and B4 were exposed under low pH conditions, which would improve the possibility of epitope hydrolysis and abolish the IgE binding, which directly resulted in the immunogenicity decrease of the protein.<sup>32</sup>

Taken together, we first provided an efficient method to reduce the potential allergenicity of lectin protein and elucidated its mechanisms. Through the use of low-pH incubation, the treated lectin allergen displayed a weaker anaphylaxis response as well as significantly reducing the releases of IgE, IgG<sub>1</sub>, histamine, mMCPT-1, and cytokines in BALB/c mice. CD, FT-IR, and MD simulation suggested a hypoallergenic response was well correlated with the decreasing of  $\alpha$ -helix and exposure of more cleavage sites for trypsin, especially for Lys121, 134, and 157, due to the unfolding of the protein in extremely low acidic environments. Thus, low-pH treatments have good potential to enhance the safety of legume protein consumption.

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### Notes

The authors declare no competing financial interest.

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