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# High Processing Tolerances of Immunomodulatory Proteins in Enoki and Reishi Mushrooms

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This study investigated the processing tolerances of two mushroom proteins with immunomodulatory activities, including FVE from Enoki (*Flammulia velutipes*) and LZ8 from Reishi (*Ganoderma lucidum*) mushrooms, under food processing treatments such as heating, sterilization, frozen storage, extraction in acid/alkaline conditions, and dehydration. Results showed that the capability of these two proteins to induce IFN-$\gamma$ secretion by murine splenocytes remained after 100 °C heating for 30 min, 121 °C autoclaving for 15 min, and −80 °C freezing. The retained activities of both proteins on cell proliferation and IFN-$\gamma$ production did not decrease at 0.6 M hydrochloric acid (at pH 2) but strikingly dropped at 5 M sodium hydrate (at pH 13). After vacuum dehydration, FVE and LZ8 retained most of their activities on cell proliferation; nevertheless, the IFN-$\gamma$ secretion decreased to about half of the initial values. These findings suggest that these two mushroom proteins have a good thermal/freezing resistance, acid tolerance, and dehydration stability and are candidates for processing in food and nutraceutical utilization.

**KEYWORDS: Mushroom immunomodulatory protein; *Flammulia velutipes*; FVE; *Ganoderma lucidum*; LZ8; processing tolerance; stability**

## INTRODUCTION

Edible mushrooms are well-known as being a valuable part of the diet since they are a good source of proteins, vitamins, essential minerals (such as potassium, selenium, copper, and phosphorus), and are low in calories, fat, and sodium (*1*−*4*). Not only being a nutritious food source, some mushrooms also show potential in providing medicinal functions such as antitumor, immunomodulating, and antiviral activities (*5, 6*) and hence are good candidates for healthy food. Since many mushrooms are cooked or processed prior to consumption and because some food compartments are heat sensitive and unstable, it is important to understand their remaining activities after food processing and then to find the proper way to maintain their functions.

Enoki mushrooms (*Flammulia velutipes*) and Reishi mushrooms (*Ganoderma lucidum*) are popular mushrooms for dietary purposes. Enoki mushrooms, a familiar species in North America, Europe, and Asia, are rich in proteins and dietary fiber (20 and 29.3% on a dry basis, respectively) and low in fat and calories (*7*). In addition, Reishi mushrooms, which contain high levels of potassium, copper, and chromium, are known to be a good dietary supplement and a good source of minerals (*8*). For 30 years, both these two mushrooms have been reported to have immunoregulatory and antitumor activities. In some recent studies, the proteins in Enoki and Reishi mushrooms such as FVE and LZ8, respectively, were considered to have a relation to these strong immunomodulatory and antitumoral activities (*9, 10*). Therefore, these two mushroom proteins can be used for studying the relationship between food processing destruction and the retention of mushroom functions.

FVE, a protein with a molecular mass of 12.7 kDa, has been reported to stimulate human peripheral blood lymphocyte activation (*9*), induce the production of IFN-$\gamma$ through a p38 MAPK pathway (*11*), and inhibit food-allergic reactions in vivo (*12*). Furthermore, LZ8, a protein with molecular masses of 14 and 17 kDa, possesses in vitro biochemical and immunomodulatory capacities. It is a mitogen to murine splenocyte and human PMBC (*10, 13*), mediated by activating macrophages and T lymphocytes to induce the secretion of TNF-α, IL-2, and IFN-$\gamma$ (*14*). LZ8 also modulated adhesion molecules on immunocompetent cells (*15*) and prevented insulitis in nonobese diabetic mice in vivo (*16*).

Although Enoki and Reishi mushrooms were able to modulate immunity and antitumor activity, it is not clear as to whether their functions can be influenced by different processing conditions. The present study investigated the effects of different processing conditions on the immunomodulatory activities of mushroom proteins. The FVE and LZ8 proteins were used as biomarkers, and their immune-stimulating activities toward

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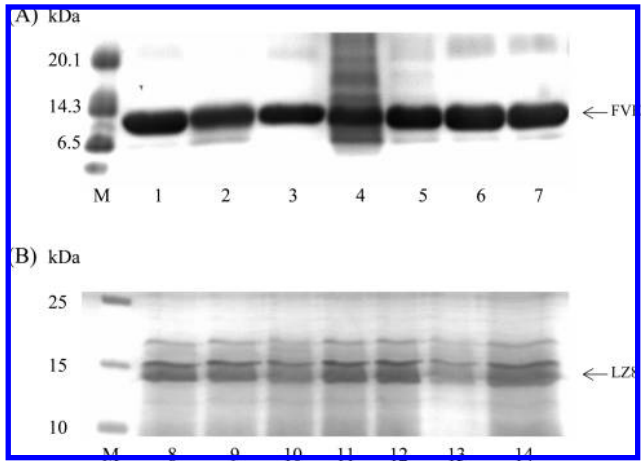


**Figure 1.** Electrophoresis analysis of FVE and LZ8 and their resistance against different treatments. The polyacrylamide gels were stained with Coomassie brilliant blue R-250 (**A** and **B**). FVE (**A**) and LZ8 (**B**) were pretreated with various processes including vehicle (untreated, lanes 1 and 8), freezing (−80 °C, lanes 2 and 9), boiling (100 °C, lanes 3 and 10), autoclaving (121 °C, lanes 4 and 11), 0.6 M hydrochloric acid (at pH 2) treatment (lanes 5 and 12), 5 M sodium hydrate (at pH 13) treatment (lanes 6 and 13), and dehydration (lanes 7 and 14). Lane M is the molecular mass marker.

mouse splenocytes were evaluated further. The tolerance of these mushroom proteins to heating, freezing, acid/alkali conditions, and dehydration treatments is discussed.

## MATERIALS AND METHODS

**Mice.** Female BALB/c and C57BL/6J mice were obtained from the animal center of National Taiwan University, and C57BL/10ScN (TLR4 deficiency mutant) mice were purchased from the Jackson Laboratory. Mice were 8−12 weeks old and were maintained in our SPF facilities. All animal studies were performed according to the institutional and National Institutes of Health guidelines for animal use and care.

**Reagents.** DEAE and CM-52 cellulose were purchase from Whatman (Maidstone, Kent, U.K.). Mono S HR 5/5 columns were from Amersham (Uppsala, Sweden), and dialysis membrane tubing (spectra/Por 1, WMCO 6−8 kDa) was from Spectrum (Rancho Dominguez, CA). Concanavalin A (ConA) from *Canavalia ensiformis* (jack bean) was obtained from Sigma Chemical (St. Louis, MO), and the BrdU Kit was from Roche (Penzberg, Germany).

**Purification of FVE and LZ8 Proteins.** FVE was purified from the fruiting body of *F. velutipes* as described by Ko et al. (*9*), and LZ8 was purified from the fruiting body of *G. lucidum* using the method of Kino et al. (*10*). The fruiting bodies were homogenized with ice-cold 5% (v/v) acetic acid in the presence of 0.1% (v/v) 2-mercaptoethanol, and soluble proteins in the supernatant were precipitated by the addition of ammonium sulfate to 95% saturation. The precipitate was collected and then dialyzed against 100 mM Tris-HCl buffer, pH 8.0 for 72 h with four changes of dialysis solution. The dialysate was fractionated on a DEAE-cellulose column (2.5 cm × 20 cm) that was equilibrated with 100 mM Tris-HCl buffer, pH 8.0. Subsequently, the column was washed with the equilibration buffer and eluted with 0−0.5 M NaCl in 0.01 M sodium acetate, pH 5.2. The main active fractions were further purified by a fast protein liquid chromatography (FPLC) system with a mono S HR 5/5 column, which was previously equilibrated with 0.01 M sodium acetate, pH 5.2. The active fractions containing FVE and LZ8 were collected for further experiment.

**Endotoxin Concentration Estimation (LAL Test).** The chromogenic kinetic *Limulus* amebocyte lysate (LAL) test kit (Charles River Laboratories, MA) was used to detect the endotoxin level within FVE and LZ8 (*17*). The *E. coli* endotoxin standard was dissolved in 5 mL of LAL reagent water to obtain an 80 EU/mL stock. FVE was diluted with the LAL reagent water to 0.5, 2, and 10 μg/mL, and LZ8 was diluted to 0.5, 2.5, and 10 μg/mL. Each of the diluted standard and

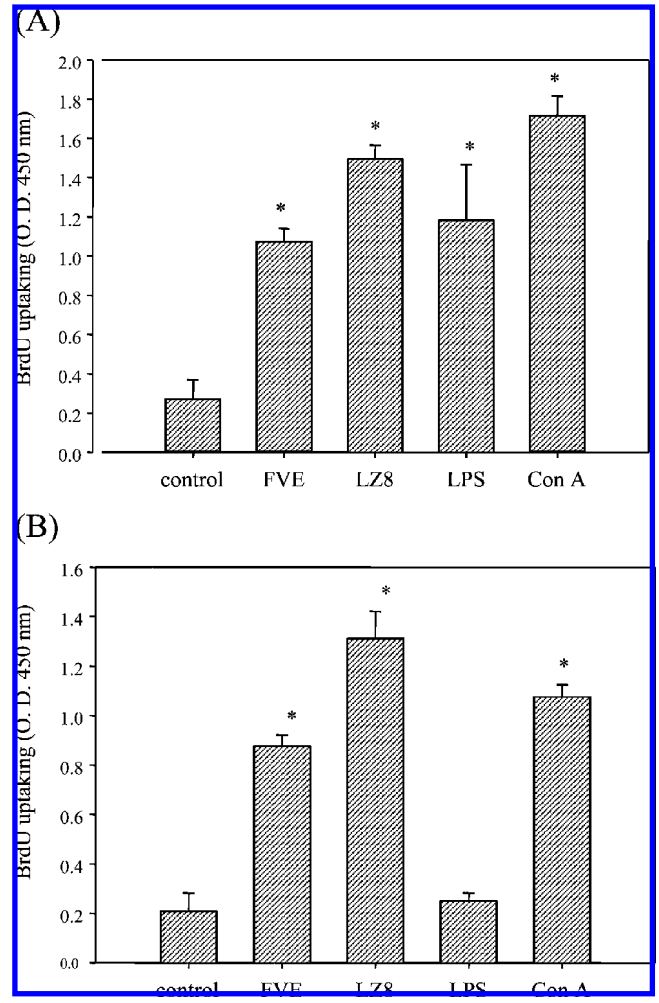


**Figure 2.** Effects of FVE and LZ8 on BrdU uptake by the C57BL/ScN ($TLR4^{-/-}$) splenocytes. FVE (2 μg/mL), LZ8 (2.5 μg/mL), LPS (10 μg/mL), and Con A (10 μg/mL) were cultured with splenocytes from C57BL/6J (**A**) and C57BL/ScN (**B**) for 3 days. Splenocytes cultured without stimuli were used as a control. Results are expressed as means ± SE by three independent experiments ($n = 3$). A $P$ value of <0.05 is considered to be significantly different from the control (*$P < 0.05$).

samples was mixed with the LAL reagent in a depyrogenate tube and incubated at 37 °C for 1 h. The level of endotoxin contamination was determined by the gelation of the mixture.

**Physical and Chemical Treatments of FVE and LZ8 Proteins.** To evaluate the stabilities of FVE and LZ8 proteins along with pharmaceutical and food processing, FVE and LZ8 were treated by different processing, and their activities were measured by cell experiments. To investigate their thermostability and freezing resistance, FVE and LZ8 samples were parafilm-sealed in Eppendorf tubes and autoclaved at 121 °C for 15 min, heated in boiling water at 100 °C for 30 min, or frozen at −80 °C overnight. To assess the effects of acid and alkali conditions on the activities of FVE and LZ8, the protein samples were dialyzed against 0.6 M hydrochloric acid (at pH 1−2) or 5 M sodium hydrate (at pH 12−13). After 2 h, the pH values of the dialysates were measured by pH indicator paper (pH 0−14, Whatman International, Ltd., Maidstone, Germany) to confirm their equilibration. The pH value was recovered by dialyzing against 0.05 M phosphate-buffered saline (PBS, at pH 7.2) for 24 h.

**Cell Proliferation Assay.** Splenocytes from BALB/c mice were cultured in the DMEM medium supplemented with 4 mM L-glutamine, 1.4 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mM sodium pyruvate (Gibco/BRL Life Technologies, Eggenstein, Germany), and 10% (v/v) FBS (Hyclone, Logan, UT). The cells were seeded into the wells of a 96-well plate ($5 \times 10^5$ cells/well) in the presence of FVE or LZ8 proteins treated by different processes. After 48 h of incubation at 37

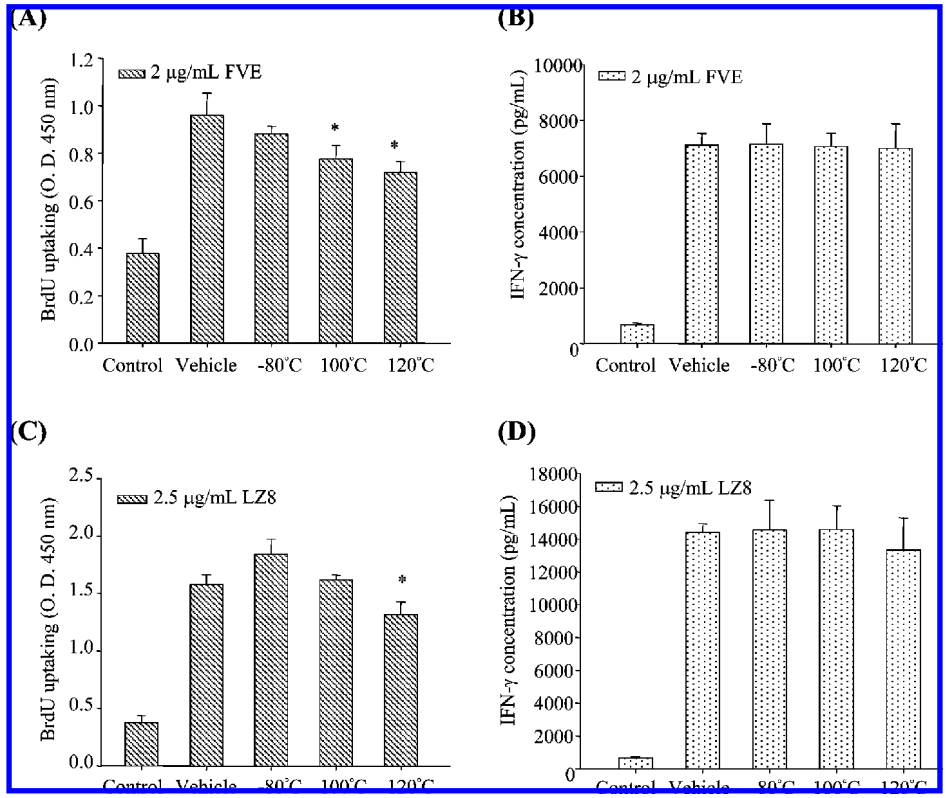


**Figure 3.** Temperature tolerances of FVE and LZ8 in their BrdU uptake and IFN-$\gamma$ production by BALB/c splenocytes. FVE (2 $\mu$g/mL) (**A** and **B**) and LZ8 (2.5 $\mu$g/mL) (**C** and **D**) were pretreated with different temperatures (−80, 100, and 121 °C) and then incubated with BALB/c splenocytes for 3 days. Variations in activity were compared to FVE and LZ8 without any thermal treatments (vehicle). Splenocytes cultured without stimuli were used as the control. Results are expressed as means ± SE by three independent experiments ($n = 3$). A $P$ value of <0.05 is considered to be significantly different from the vehicle (*$P < 0.05$).

**Table 1.** Remaining Activities of FVE and LZ8 Proteins on BrdU Proliferation and Secretion of IFN-$\gamma$ by BALB/c Splenocytes after Various Treatments[a]

| | | residual activity (%)[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | temp (°C) | | | acid/alkali | | |
| stimulus | production | −80 | 100 | 121 | 2 | 13 | dehydration |
| FVE | BrdU | 94.21 ± 3.36 | 86.38 ± 5.92 | 82.14 ± 4.99 | 100.05 ± 5.89 | 72.68 ± 9.2 | 86.42 ± 6.37 |
| | IFN-$\gamma$ | 102.38 ± 10.17 | 95.07 ± 6.85 | 89.5 ± 12.3 | 96.95 ± 3.87 | 66.97 ± 2.28 | 54.38 ± 6.15 |
| LZ8 | BrdU | 113.01 ± 8.34 | 101.91 ± 2.56 | 86.64 ± 6.79 | 99.07 ± 8.63 | 54.11 ± 4.78 | 74.54 ± 2.07 |
| | IFN-$\gamma$ | 101.44 ± 12.58 | 101.79 ± 9.97 | 87.03 ± 13.6 | 107.54 ± 4.14 | 63.06 ± 1.86 | 39.97 ± 10.44 |

[a] Results are expressed as means ± SE; $n = 3$. [b] Residual activities of stimuli were presented as relative activity as compared to the vehicle (medium only).

°C and 5% $CO_2$ conditions, the splenocytes were added to 10 $\mu$M 5-bromo-2′-deoxyuridine (BrdU). After another 24 h, the amount of BrdU that was uptaken by lymphocytes was quantified by ELISA using a match paired anti-BrdU antibody (HRP) specific to BrdU in newly synthesized, cellular DNA. The absorbance was measured at 450 nm on a BioRad-3550 microplate reader (Hercules, CA).

**Determination of IFN-$\gamma$ Production.** Splenocytes from BALB/c mice were cultured in a 96-well plate ($5 \times 10^5$ cells/well) in the presence of various treatments of FVE or LZ8 for 72 h. The levels of IFN-$\gamma$ in the supernatants were measured by using OptEIA mouse IFN-$\gamma$ ELISA set (BD PharMingen, San Diego, CA).

**Data Analysis.** Data were presented as the mean ± SD of three separate experiments performed in triplicate. Statistical analysis was made by means of one-way ANOVA with a Duncan multiple comparisons test. Differences were considered as statistically significant when the $P$ value was below 0.05.

## RESULTS

**Electrophoresis Evaluation on Mushroom Protein Stability.** The purity and identity of FVE and LZ8 proteins that were extracted from the mushroom fruiting bodies and isolated with FPLC anion-exchange chromatography were analyzed using electrophoresis. The SDS/polyacrylamide gel showed that FVE displayed a single band with an apparent molecular mass of 12 kDa (**Figure 1A**, lane 1), and LZ8 yielded an apparent molecular mass of 14 and 17 kDa (**Figure 1B**, lane 8). These observations confirmed that FVE and LZ8 proteins prepared in the current study showed comparable biochemical characteristics to those as described in the literature (*9, 10*).

The mushroom protein samples were then treated with various operations mimicking food processing conditions such as autoclaving at 121 °C for 15 min, heating at 100 °C for 30 min, freezing at −80 °C overnight, acidification at 0.6 M hydrochloric acid (pH 2), alkalization at 5 M sodium hydrate (pH 13), and vacuum dehydration. The integrity and activities of these proteins after the treatments were analyzed by SDS/polyacrylamide gel electrophoresis. As shown in **Figure 1A**,**B**, significant bands for FVE and LZ8 were found, showing that there was little degradation through the foregoing processes. The results demonstrated the tolerance and resistance of FVE and LZ8 against these processing conditions.

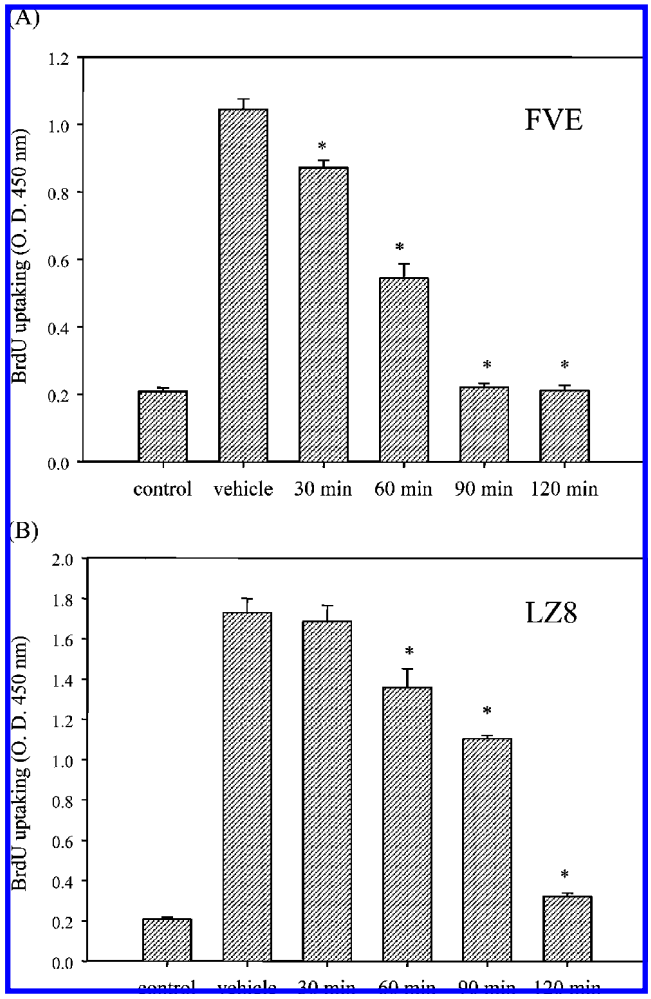


**Figure 4.** Time manner after thermal treatment of FVE and LZ8 in BrdU uptaking by BALB/c splenocytes. Two $\mu$g/mL FVE (**A**) and 2.5 $\mu$g/mL LZ8 (**B**) were heated at 100 °C for different times including 30, 60, 90, and 120 min and then incubated with BALB/c splenocytes for 3 days. Variations in activity were compared to FVE and LZ8 without any thermal treatments (vehicle). Splenocytes cultured without stimuli were used as the control. Results are expressed as means ± SE by three independent experiments ($n = 3$). A $P$ value of <0.05 is considered to be significantly different from the vehicle (*$P < 0.05$).

**Endotoxin Detection.** To exclude the possibility that FVE and LZ8 were contaminated by endotoxin, we detected the endotoxin concentrations of FVE and LZ8 using the LAL test. The endotoxin-LAL mixtures were found to gelate while endotoxin levels were greater than 0.015 EU/mL. However, none of the FVE and LZ8 samples (0.5−10 $\mu$g/mL) caused the gelation, suggesting that any possible endotoxin contamination in these two samples was below 0.015 EU/mL. On the other hand, we used splenocytes from the C57BL/10ScN mouse, which was a TLR4 deficiency mutant and was known not to express TLR4 and not to respond to endotoxin LPS induction (*18*), to evaluate as to whether FVE and LZ8 gave a null response on these endotoxin-specific cells or not. As shown in **Figure 2**, FVE and LZ8 could stimulate cell proliferation of the splenocytes obtained from both the C57BL/6J wild type (**Figure 2A**) and the C57BL/10ScN (**Figure 2B**) mouse, while endotoxin LPS could not induce cell proliferation of the C57BL/10ScN cells. These results clearly demonstrated that FVE- and LZ8-induced splenocyte activation did not result from endotoxin LPS, and those results ruled out the possibility of endotoxin contamination within the FVE and LZ8 samples.

**Thermal and Freezing Resistances.** The effects of heat processing on the activities of FVE or LZ8 proteins (0.5−40 $\mu$g/mL) were determined by the change of cell proliferation and IFN-$\gamma$ production in BALB/c splenocytes using the BrdU assay and ELISA technique, respectively. The concentrations of FVE or LZ8 at 2 and 2.5 $\mu$g/mL, respectively, resulting in the highest stimulatory effect were used as vehicle treatments in the following experiments.

Thermal treatment was frequently used for food sterilization. To investigate the thermal stability of FVE and LZ8 proteins, these two mushroom proteins were autoclaved at 121 °C for 15 min and boiled at 100 °C for 30 min. An evaluation of the splenocyte-activating ability showed that autoclaving could keep the proliferation of the FVE- and LZ8-treated mouse splenocytes to an extent higher than half of their initial activities (**Figure 3**). As compared to the vehicle, the IFN-$\gamma$ secretion activities of FVE and LZ8 were reduced to 89.5 and 87.03%, respectively (**Table 1**). The boiling treatment resulted in a higher residual activity of FVE and LZ8 (86.38 and 101.91%, respectively, as compared to the vehicles) (**Table 1**). Furthermore, we investigated the decay of the activity of FVE and LZ8 after 100 °C thermal treatment for various times. As shown in **Figure 4**, results indicated that both the two proteins lost their stimulatory activities time dependently. FVE and LZ8 kept half of their activities in 60 and 90 min of 100 °C treatment, respectively. These results suggested that only mild damage to these mushroom proteins could occur in food sterilization and regular thermal processing. However, a long time of high temperature treatment, such as heating exceeding 90 min at 100 °C, could destroy most activities of FVE and LZ8.

Freezing treatment was important for food preservation, but repeated cycles of freezing and thawing could lead to the destruction of some food components. The resistances of FVE and LZ8 to freezing treatments were observed, while 94.21 and 113.01%, respectively, of cell proliferation remained (**Table 1**). An induction of IFN-$\gamma$ secretion by the cells also was observed (**Figure 3B**,**D**). These results demonstrated that these mushroom proteins had superior thermal and freezing stabilities to keep most of their immunomodulatory activities toward murine splenocytes.

**Acid and Alkali Resistances.** Acidification was usually involved in the production of sugaring foods, and alkalization was applied in the extraction of chitin compounds from mushrooms and some other foods. To evaluate the tolerance of mushroom proteins toward different acid and alkali conditions, the protein samples were dialyzed against 0.6 M hydrochloric acid (at pH 1−2) or 5 M sodium hydrate (at pH 12−13) and then concentrated to a working concentration for the following cell experiments. The remaining splenocyte-stimulating activities of acid-/alkali-treated FVE and LZ8 are shown in **Figure 5**. It was observed that there were no significant ($P > 0.05$) changes in the stabilities of FVE and LZ8 in 0.6 M hydrochloric acid (at pH 2) as compared to the vehicles, revealing that these two proteins could resist acidification. In 5 M sodium hydrate (at pH 13), both cell proliferation and IFN-$\gamma$ production of FVE and LZ8 were significantly ($P < 0.05$) reduced (**Figure 5**) as compared to the vehicle. The cell proliferations of the FVE- and LZ8-treated mouse splenocytes decreased to 72.68 and 54.11%, respectively, and the secretions of IFN-$\gamma$ were diminished to 66.95 and 63.06%, respectively (**Table 1**). The results demonstrated that these two mushroom proteins could keep more

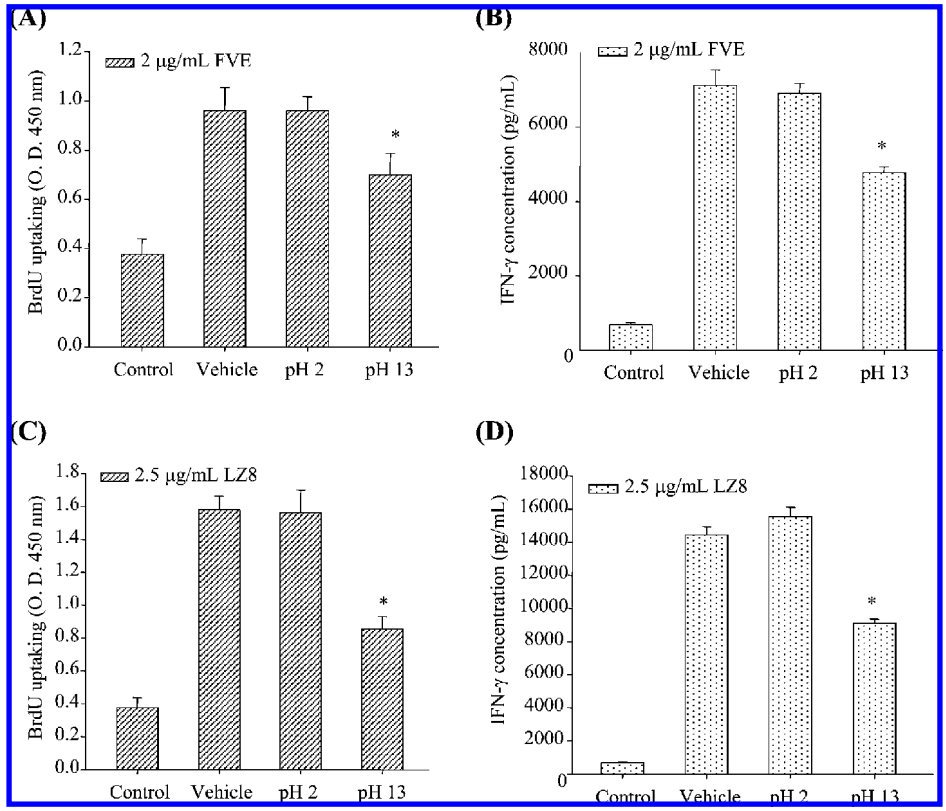


**Figure 5.** Acid/alkali resistances of FVE and LZ8 in their BrdU uptake and IFN-$\gamma$ production by BALB/c splenocytes. FVE (2 $\mu$g/mL) (**A** and **B**) and LZ8 (2.5 $\mu$g/mL) (**C** and **D**) were pretreated with 0.6 M hydrochloric acid (at pH 2) or 5 M sodium hydrate (at pH 13) reagents and then incubated with BALB/c splenocytes for 3 days. Variations in activity were compared to FVE and LZ8 without any acid/alkali treatments (vehicle). Splenocytes cultured without stimuli were used as the control. Results are expressed as means $\pm$ SE by three independent experiments ($n = 3$). A $P$ value of <0.05 is considered to be significantly different from the vehicle (*$P < 0.05$).

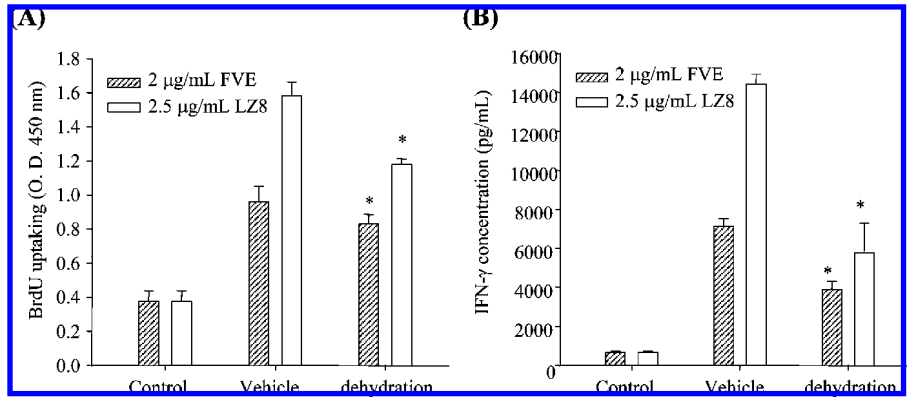


**Figure 6.** Effects of drying treatment on FVE and LZ8 to induce BALB/c splenocytes uptaking BrdU and secreting IFN-$\gamma$. FVE (2 $\mu$g/mL) (**A** and **B**) and LZ8 (2.5 $\mu$g/mL) (**C** and **D**) were vaccuum-dried and then incubated with BALB/c splenocytes for 3 days. Variations in activity were compared to FVE and LZ8 without any drying treatments (vehicle). Splenocytes cultured without stimuli were used as the control. Results are expressed as means $\pm$ SE by three independent experiments ($n = 3$). A $P$ value of <0.05 is considered to be significantly different from the vehicle (*$P < 0.05$).

than half of their activities upon alkali decomposition. It was suggested that both FVE and LZ8 mushroom immunomodulatory proteins were stable in acidic processing but quite liable for damage in alkaline conditions.

**Dehydration Resistance.** Most mushrooms were sun-dried or mechanically dehydrated for storage. The residual activities of the mushroom proteins were evaluated after dehydration treatment. After vacuum dehydration at room temperature, the cell proliferation abilities of mouse splenocytes activated by FVE and LZ8 were reduced to 86.42 and 74.54%, respectively, as compared to the vehicles, and their abilities to induce IFN-$\gamma$ production also were decreased significantly to 54.38 and 39.97%, respectively (**Table 1**). As indicated in **Figure 6**, it was suggested that FVE and LZ8 retained most of their activities to excite cell proliferation after the dehydration process but only had some capability left to stimulate IFN-$\gamma$ secretion. The residual activities of FVE and LZ8 treated by dehydration shifted to be more sensitive at IFN-$\gamma$ secretion than at cell proliferation.

## DISCUSSION

A large proportion of mushrooms is consumed in the form of industrially processed products. Processing techniques to preserve and even to enhance the nutritional quality of foods

might comprise thermal sanitation, freeze storage, pH value dialysation, dehydration, and other methods, which could bring detrimental effects to mushroom components. To investigate the impact of processing on the stability and function of bioactive mushroom proteins is crucial work in the industry. We therefore evaluated the immune-stimulating activity of FVE and LZ8 after some treatments, mimicking different food processing conditions in this study. We first ruled out the possibility of the existence of endotoxin in our samples by using the LAL assay and the TLR4 deficiency mice model (**Figure 2**). Treatments on foods including blanching, heating, sterilization, and frozen storage might cause protein denaturation and result in the failure of its bioactivity. The SDS-PAGE results demonstrated that autoclaving (121 °C, 15 min), boiling (100 °C, 30 min), and freezing treatments (−80 °C, 24 h) did not degrade the conformations and properties of FVE and LZ8. After the freezing and autoclaved treatments, both FVE and LZ8 maintained their capabilities to activate mouse splenocytes in the cytokine secretion and cell proliferation (82−100% against the vehicles). Nevertheless, long time thermal treatments might decrease part of their immunostimulatory activities. In time manner experiments, FVE kept half of its activity after heating treatment at 100 °C for 60 min, and LZ8 retained most of its activity after 90 min of heating. These results suggest that FVE and LZ8 have a great tolerance to frozen storage and could maintain most of their immunostimulatory activities during food sterilization and regular cooking processes. However, a long time of thermal treatment such as heating for 90 min or above could destroy portions of their activity.

On the other hand, some common practices in mushroom processing such as salting, sugaring, fermentation, and other preservative methods might introduce mushrooms to high acid or alkali conditions. These treatments could easily interfere with the ionic force of the proteins' structure and then decrease the stability of the proteins or induce intermolecular hydrolysis and decomposition. In this study, FVE and LZ8 retained almost 100% activity in 0.6 M hydrochloric acid (pH 2, 2 h) as compared to the vehicle, showing a great acid resistance. However, FVE and LZ8 in 5 M sodium hydrate (pH 13, 2 h) lose part of their functions and activities (declining to 72 and 52%, respectively, as compared to the vehicle). These results demonstrate that it is favorable to process Enoki and Reishi mushrooms in acid conditions (e.g., sugaring) rather than in alkali conditions (e.g., salting). After vacuum dehydration, a reduction in the activity of FVE and LZ8 (86 and 74%, respectively, as compared to the vehicle) was observed, which suggested that FVE and LZ8 could be somewhat sensitive to the drying and hydration cycles. It was proposed that alkali and drying conditions could break some hydrogen bonds or eliminate hydrophobic forces in the protein structure, resulting in some conformational changes of proteins as well as a decrease in the interaction between cell surface and proteins (i.e., FVE or LZ8). Taken together, FVE and LZ8 displayed a great thermal/freezing and acidic stability as well as good alkali and dehydration resistances. Therefore, it is inferred that Enoki and Reishi mushrooms could retain their immune-activating activities in most food processing conditions.

The unique tolerance of FVE and LZ8 to various processing conditions could result from their lectin-like structures. An X-ray diffraction study reported that FVE is a lectin having an N-terminal helix structure that was associated with the noncovalently linked homodimer and the immunomodulatory activity of FVE (*19, 20*). LZ8 was structurally similar to FVE with a 61.4% identity in amino acid sequence and also had a high similarity in activity, suggesting that LZ8 was a lectin resembling the structure of FVE (*9*). Lectins, widely found in beans, vegetables, fruits, and mushrooms, are carbohydrate-binding proteins specifically recognizing restricted monosaccharides or oligosaccharides (*21, 22*). Some lectins exhibited hemagglutinating and immunoregulatory activities through binding glycolipids and glycoproteins on the cell surface (*23, 24*). Some recent thermodynamic studies revealed that the folding processes of lectins, such as legume lectins, were reversible and cooperative, thus forming a conformationally compact structure with six to eight $\beta$-sheets and a number of loops to interconnect the sheets to maintain a high thermostability and pH tolerance (*25, 26*). In some other studies, Concanavalin A (a lectin from *C. ensiformis*), pea lectin, legume lectin, and certain mushroom lectins were found to be thermally stable and retained their hemagglutinating activities in the presence of acid/alkali solutions (*27, 28*) . From our previous findings, *Agaricus bisporus* lectin (ABL) and *Auricularia polytricha* lectin (APP) were found to retain an overall 70−100% of their activities in activating RAW 264.7 macrophages based on the same treatments as in this study (*29*). Taken together, the compact and stable structures of FVE and LZ8 also contributed to their resistances against various processing challenges and retained their immune-stimulating activities.

It was previously reported that FVE could resist the digestion of pepsin (*9*) and that the oral administration of FVE could enhance the in vivo activation of immunity without any adverse effect, serving the purpose of immunoprophylaxis for food allergy and other allergy diseases (*12*). LZ8 could prevent insulitis in nonobese diabetic mice without harmful effects via intraperitoneal administration (*16*). On the basis of our findings, FVE and LZ8 had superior thermal and acid stabilities and a moderate resistance to alkali and dehydration treatments. The potential of Enoki and Reishi mushrooms to be a choice of fresh vegetables and processed foods was revealed to offer immunoregulatory proteins to strengthen adaptive immunity. This study demonstrated the thermal/freezing stability, acid/alkali resistance, and dehydration tolerance of immunomodulatory proteins in Enoki and Reishi mushrooms after various food processing conditions.

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