Effect of fermentation times and extracting solvents on the in vitro immune potentials of the soluble extracts of mucor-fermented Mao-tofu

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Received: 27 April 2016 / Revised: 20 October 2016 / Accepted: 2 February 2017 / Published online: 29 May 2017
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Abstract Mucor-fermented Mao-tofu at 3–7 days was extracted using water (pH 4.5 and pH 6.5) and ethanol solutions (40 and 80%, v/v). At protein concentration of 40 µg/mL, all the extracts showed immune activities via activating macrophages, splenocytes, and natural killer cells, which enhances the secretion of interleukin (IL)-2, interferon- γ , tumor necrosis factor- α , IL-1 β , IL-6, inducible nitric oxide synthase, and lysozyme, but inhibits the secretion of IL-4. All the extracts exhibited higher activities than that of soybean protein, demonstrating that Mucor-mediated fermentation enhanced the immune potentials of Mao-tofu. Fermentation time of 6 days ensured the extracts the highest activities, whereas lower activities were detected. The highest and lowest activities were detected on using a solution of water (pH 6.5) and 40% ethanol, respectively. The extract obtained using water (pH 6.5) contained more Arg and Cys with immune significance, which partially contributed to its high activities. Both fermentation times and extracting solvents were thus proved to affect the immune activities of soluble Mao-tofu extracts.

Keywords Mao-tofu · *Mucor* · Fermentation · Soluble extract · Immune activity

Introduction

Immune function is important to prevent and control infection and neoplasia in the body. Nutritional interventions that involve optimized intake of nutrients and consumption of promising functional foods can exert health-promoting effects on immune system. Lymphoid organs are influenced directly by environment, particularly by daily diets [1]. Dietary components and relatively excessive food intake affect lymphoid organs and immune cells, and thus can modulate the activation and functions of these organs and cells [2]. Immune system thus can be modulated by daily diets; for example, consumption of traditionally fermented legumes is suggested to protect the body from chronic diseases [3]. Therefore, production and consumption of these fermented legumes have increased.

In comparison with other legumes, soybean is rich in protein and has been consumed for thousands of years in Oriental countries. Soybean contains several bioactive phytochemicals, including flavonoids, saponins, phytosterols, and sphingolipids [4], and possesses high immune potentials [5]. China practices a long-time tradition of soybean processing such as the well-known tofu production and soybean fermentation. Microbial fermentation as a valuable bioprocess of food materials can generate water-soluble extracts with healthcare functions [6]. Fermented soybean products (e.g., sufu and stinky tofu) that exhibit good nutrition and healthcare functions therefore have been paid considerable attention [7]. In general, bioactivities of these fermented soybean products are controlled by fermentation conditions, including the applied microorganisms and fermentation times. However, healthcare benefits (particularly, immune potentials) of another fermented soybean product called Mao-tofu, which is a soft cheese-

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type tofu produced by the short-time action of *Mucor* sp. [8], is still less studied.

Fermented soybean products have stronger anti-oxidative properties than non-fermented ones [9] and possess considerably stronger anti-cancer and anti-metastatic activities than soybean itself [10]. Furthermore, fermented soybean products have other desired physiological functions such as anti-hypertension [11], anti-diabetes [12], anti-inflammation [13], and immune functions [14]. Mao-tofu extracts obtained using water of different pH values and water–ethanol solutions as extracting solvents have been assessed for their in vitro anti-oxidation and anti-hypertension [15, 16], which are mainly contributed by the degraded products of soybean protein (i.e., peptides). Mao-tofu has thus revealed desired healthcare functions in terms of anti-oxidation and anti-hypertension. Proteins from different resources are important sources to generate immunomodulatory peptides [17]. Immune potentials of Mao-tofu extracts should be assessed to provide further scientific information about healthcare functions of Mao-tofu.

In this study, Mao-tofu was fermented by *Mucor* for 3–7 days and then extracted using different solvents to obtain respective soluble extracts. The four extracting solvents used were water (pH 4.5 and pH 6.5) and two ethanol solutions (comprising 40 and 80% ethanol, v/v). The immune activities of these extracts were assessed using three immune cells, namely macrophages, natural killer (NK) cells, and splenocytes. The extracts were also assayed to determine their effects on the production of eight immune molecules, namely interleukin-2 (IL-2), IL-4 and interferon- γ (IFN- γ) secreted by splenocytes, as well as IL-6, IL-1 β , tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), and lysozyme (LZM) secreted by macrophages. The aim of this study was to verify the immune potentials of Mao-tofu and to assess the effects of fermentation times and extracting solvents on immune activities of the resultant soluble Mao-tofu extracts.

Materials and methods

Materials and chemicals

Commercial tofu was purchased from a local market of Harbin City (Heilongjiang Province, China). Soybean protein isolate (SPI) containing 90.1% protein on dry basis was prepared using defatted soybean flour purchased from Harbin High-tech Soybean Foods Co. Ltd. (Harbin, China), as previously described [18]. Trypan blue and neutral red both were purchased from Amresco Inc. (Los Angeles, CA, USA). Fetal bovine serum (FBS) was purchased from

Gibco Co. (Grand Island, NY, USA). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Dojindo Laboratories, Kyushu, Japan). Concanavalin A (Con A) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse iNOS and LZM enzyme-linked immunosorbent assay (ELISA) kits were purchased from Beijing Equation Biotechnology Science and Technology Co. Ltd. (Beijing, China). Cytokine kits were acquired from Wuhan Boster Biological Engineering Co. Ltd. (Wuhan, Hubei Province, China). All chemicals used were of analytical grade. The water used was generated from the Milli-Q PLUS (Millipore Corporation, New York, NY, USA).

Mucor, animals, and cells

A *Mucor* strain was isolated from commercial Mao-tofu produced in Xiuning County (Anhui Province, China), as per the study [19]. Spore suspension of *Mucor* used in Mao-tofu fermentation was also prepared, as previously described [20].

BALB/c mice (female, 6–8 weeks old) were provided by Beijing Vital River Experimental Animal Technical Co., Ltd. (Beijing, China) and maintained in an animal facility for 1 week prior to the experiment. All animal procedures were performed in accordance with the Ethical Guidelines of the Animal Care and Use Committee of Northeast Agricultural University (Harbin, China).

YAC-1 cells used in the assay of NK cell activity were provided by the Chinese Academy of Sciences (Shanghai, China).

Preparation and extraction of Mao-tofu

The tofu (in 6 cm $\times$ 3 cm $\times$ 2 cm pieces) was autoclaved at 121 °C for 30 min to inactivate the polluted microorganisms and enzymes, as previously described [16], slightly dried after the sterilization, coated with the spore suspension on the surface, and placed in an incubator at 20 °C to conduct *Mucor*-fermentation.

The Mao-tofu samples fermented for 3–7 days were selected randomly, and heated at 95 °C for 5 min to stop the action of *Mucor* or enzymes. The Mao-tofu samples (30 g) were subsequently mixed with one of the prior selected extracting solvents (100 mL) and stirred by a high-speed homogenizer (Type DS-1, Shanghai Jingke Ltd., Shanghai, China) for 1 min. After centrifugation at 4000 $\times$ g for 20 min, the supernatants (i.e., soluble Mao-tofu extracts) were collected, concentrated via solvent evaporation, and freeze-dried at an initial temperature of –24 °C and under a fixed pressure of 10 Pa for 12 h to obtain extract powders, which were stored at –20 °C. These powders and SPI (as the protein control) were dissolved in water and used at

fixed protein concentration of 40 µg/mL in these activity evaluations.

Chemical assays of Mao-tofu extracts

Protein contents were assayed using the Kjeldahl method together with two conversion factors 5.71 (the extracts) and 6.25 (SPI) [21].

The extracts and SPI samples (about 200 mg protein) were hydrolyzed in vacuum-sealed glass tubes by 6 mol/L HCl at 110 °C for 24 h. Both Cys and Met were oxidized by performic acid prior to hydrolysis and detected as cysteic acid and methionine sulfone, respectively. Pre-column derivatization used the ninhydrin reaction system. Seventeen amino acids (except Trp) were detected in an automated amino acid analyzer (L-8800, Hitachi Co. Ltd., Tokyo, Japan) using the recommended protocols and amino acid standards.

Assay of spleen lymphocyte proliferation

Spleens were treated, as previously described [22], to obtain a single-cell suspension (cell viability >98%). The splenocytes at final density of 1×10^5 cells/mL were seeded onto a 96-well plate (50 µL/well), and then incubated with or without the extract solutions (40 µL/well) in a total volume of 100 µL of RPMI-1640 medium supplemented with 10% FBS. Con A (10 µL) was used at 5 µg/mL as the positive control. The blank control wells received only the RPMI-1640 medium, whereas the positive control wells received both Con A and RPMI-1640 medium. The SPI solution was used as the protein control. After incubation at 37 °C under 5% CO₂ atmosphere and 95% humidity for 48 h, CCK-8 (10 µL) was added. The cells were incubated for another 4 h. The absorbencies were measured at 450 nm by a microplate reader (Bio Rad Laboratories, Hercules, CA, USA). Lymphocyte proliferation expressed as stimulation index (SI) was calculated accordingly [23].

Assay of macrophage phagocytosis

Peritoneal macrophages were prepared as previously described [22] with a cell viability of greater than 98%. Macrophages at 1×10^5 cells/mL in the RPMI-1640 medium containing 10% FBS were seeded onto a 96-well plate (100 µL/well), and incubated at 37 °C for 4 h. The non-adherent cells were discarded, whereas the adherent cells were treated with fresh RPMI-1640 medium containing 10% FBS (100 µL/well). Sterilized water, SPI, and extract solutions (100 µL/well) were subsequently added into the wells. The cells were incubated again at 37 °C for another 24 h. The supernatants were discarded, followed

by addition of 100 µL of 1% neutral red solution into the wells. After further incubation for 30 min, the cells were washed thrice with 1 mol/L phosphate buffer (pH 7.4) and incubated with cell lysis buffer (1 mol/L acetic acid:ethanol = 1:1, v/v) at 100 µL/well for another 2 h. The absorbances were measured at 540 nm by the microplate reader to reflect macrophage phagocytosis.

Assay of NK cell activity

In brief, 50 µL of splenocytes (1×10^5 cells/mL) and YAC-1 cells (1×10^4 cells/mL) were plated onto a 96-well plate. Sterilized water, SPI, and the extract solutions were separately added into the test wells (50 µL/well). The plate was incubated for 20 h at 37 °C, followed by addition of CCK-8 (15 µL). After another co-culture for 4 h, the absorbances were measured at 450 nm by the microplate reader. The absorbances were also recorded for the target and effector cell controls. NK cell activity was calculated as previously described [24].

Assays of IL-2, IL-4, IFN-γ, IL-1β, IL-6, TNF-α, iNOS, and LZM

Splenocytes or macrophages (1×10^5 /mL, 1 mL) were seeded onto 24-well plates. Sterilized water, SPI, and extract solutions (1 mL) were separately added into each well. The cells were cultured at 37 °C for 48 h, and then centrifuged at 1500×g for 20 min. The collected cellular supernatants were assayed for IL-2, IL-4, IFN-γ, IL-1β, IL-6, TNF-α, iNOS, and LZM levels using the ELISA kits and manufacturers' protocols.

Statistical analysis

All reported data were collected from three independent experiments and expressed as mean values or mean values ± standard deviations. Differences between the mean values of multiple groups were analyzed by one-way ANOVA with Duncan's multiple range tests using the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results and discussion

Immune potentials of the extracts to activate immune cells

In the Mucor-mediated fermentation, mycelial growth was observed on the surface of Mao-tofu fermented for 2 days, and white mycelia covered the surface of Mao-tofu at fermentation time of 5 days (Fig. 1). Mao-tofu fermented

Fig. 1 Mycelia on the surface of the Mucor-fermented Mao-tofu at day 5

for 3–7 days was thus used to prepare soluble Mao-tofu extracts. Using the four extracting solvents, 20 soluble Mao-tofu extracts were obtained. The results in Fig. 2 report the detected immune activities of these extracts to three immune cells in terms of lymphocyte proliferation, macrophage phagocytosis, and NK cell activity. These data reflected the impacts of fermentation times and extracting solvents on the immune potentials of these extracts. All extracts could promote lymphocyte proliferation (Fig. 2(A)), as they increased SI values to 1.176–1.452 (higher than control SI value 1.151). Most of the 20 extracts (particularly those from Mao-tofu with longer fermentation times) showed higher SI values than that of the control protein SPI (SI value 1.234). This indicated that

the Mucor-mediated fermentation resulted in the extracts with higher lymphocyte proliferation compared to soybean protein itself. Figure 2(A) also shows that (1) the extracts using ethanol solvents (particularly 40% ethanol solution) resulted in lower lymphocyte proliferation than those using water with two pH values; (2) the extracts using pH 6.5 water had higher lymphocyte proliferation than those using pH 4.5 water; and (3) a fermentation time of 6 days usually conferred the extracts with the highest lymphocyte proliferation; however, further fermentation (e.g., 7 days) clearly led to decreased lymphocyte proliferation. Consistent results could also be found when macrophage phagocytosis and NK cell activity were also used as assaying indices (Fig. 2(B), (C)), because (1) all extracts showed increased macrophage phagocytosis and NK cell activity; (2) most extracts showed enhanced macrophage phagocytosis and NK cell activity than in SPI; and (3) fermentation time of 6 days and pH 6.5 water ensured the resultant extract with the highest activities. A conclusion that is firstly arrived here is that the Mucor-mediated fermentation conferred Mao-tofu with enhanced immune potentials than of SPI to activate the three immune cells and that both fermentation times and extracting solvents could influence the immune potentials of the resultant extracts.

Fermentation of tofu with mushroom mycelia has been shown to improve its physiological functions [25]. This calls an investigation to assess the possible effect of the Mucor-mediated fermentation on the immune potentials of Mao-tofu using soluble extracts as target samples. The spleen is a major immune organ and contains both T- and B-lymphocytes. Splenocyte proliferation is related to immunity improvement of B-lymphocytes and especially T-lymphocytes. The present results indicated that these assayed extracts directly caused mitogen effects on splenocytes by stimulating lymphocyte proliferation. The

Fig. 2 Splenocyte proliferation (A), macrophage phagocytosis (B), and natural killer (NK) cell activity (C) of Mao-tofu extracts. Different lowercase letters above the data in same line indicate significant differences of mean values ($p < 0.05$). Lymphocyte

proliferation (stimulation index, SI), phagocytosis macrophages, and NK cell activity for the control were 1.151, 0.093, and 39.7, whereas those for soybean protein isolate were 1.234, 0.152, and 56.6, respectively

study by Kong et al. [26] reached a conclusion similar to the present study as hydrolyzed and fermented soybean protein demonstrate higher immune activity in term of murine splenic lymphocyte proliferation. Macrophages possess numerous immune functions and can be activated by immune enhancers such as polysaccharides and components of fermented foods [27, 28]. This also supports that these assayed extracts could promote macrophage phagocytosis. NK cells are a group of specialized lymphocytes that can spontaneously kill pathogen-infected cells and tumor cells [29]. These assayed extracts were also observed to be able to enhance NK cell activity. This result was similar to the finding reported in another research [26]. These assayed extracts (or Mao-tofu itself) were thus evidenced to have immune potentials via activating the three immune cells. Moreover, the *Mucor*-mediated fermentation resulted in the degradation of soybean protein and generation of peptides with different sequences and sizes [15, 16]. These assayed extracts thus contained hydrolyzed soybean proteins (i.e., peptides) and therefore would possess higher immune potentials than that of the non-hydrolyzed soybean protein (i.e., SPI) [26]. These generated peptides were more soluble in water; consequentially, the extracts using water showed higher activities than those using ethanol solutions. Using pH 6.5 water could extract more peptides from Mao-tofu than using pH 4.5 water, as the latter solvent possessed an iso-electric pH value similar to that of soybean protein. It is reasonable that the extracts using pH 6.5 water had the highest activity compared to other extracting solvents. The different properties of these extracting solvents thus accounted for the observed influences of extracting solvents on immune activities of these soluble Mao-tofu extracts. However, long-time fermentation of Mao-tofu can result in excessive protein degradation and low inhibition on angiotensin I-converting enzyme [16]. The present study reached a similar conclusion. Fermentation time of 7 days in this study also led to further degradation of these immune peptides, which was partially accounted for the reduced immune potentials of the respective extracts.

Effects of the extracts on IL-2, IL-4, and IFN- γ secretion in splenocytes

The data shown in Fig. 3 report the measured levels of the three immune molecules (IL-2, IL-4, and IFN- γ) in the splenocytes. When these extracts were used, the respective levels of IL-2, IL-4, and IFN- γ were 32.9–55.3, 18.9–37.9, and 42.9–77.9 pg/mL. These extracts all displayed abilities to enhance IL-2 and IFN- γ secretion but inhibit IL-4 secretion, and thus showed immune activities via activating splenocytes. Fermentation times and extracting solvents both influenced the three activities of these extracts. The

extracts using Mao-tofu fermented for 6 days showed the highest activities, indicating that further fermentation (i.e., increased protein degradation) adversely affected the immune potentials of Mao-tofu. Using pH 6.5 water also ensured the extracts high activities to increase IL-2 and IFN- γ secretion as well as to reduce IL-4 secretion. In total, the pH 6.5 water was the most suitable extracting solvent to obtain soluble Mao-tofu extract with high immune potentials, followed by the pH 4.5 water, 80% ethanol, and 40% ethanol solutions. SPI enhanced IL-2 and IFN- γ secretion from 25.8 to 31.7 pg/mL (control) to 33.1 and 39.4 pg/mL and reduced IL-4 secretion from 39.8 (control) to 38.1 pg/mL, respectively, displaying its immune potentials to activate splenocytes. However, a comparison of these immune molecule levels also demonstrates that these extracts had higher activities than SPI. That is, the *Mucor*-mediated fermentation conferred Mao-tofu with enhanced immune potentials than soybean protein to activate splenocytes.

IL-2 is necessary for the growth and functions of T cells and is normally produced during an immune response. IFN- γ is critical for innate and adaptive immunity against viral and intracellular bacterial infections and also for tumor control. Splenocyte activation results in increased or decreased secretion of some cytokines. A protein from Jew's Ear mushroom (*Auricularia polytricha*) can activate splenocytes by promoting IFN- γ secretion [30]. The constituents from edible beans can enhance IL-2 and IFN- γ secretion in splenocytes [31]. The two results therefore provide a consistent support to the present study. IL-4 is critical for T cell survival and proliferation, whereas anaphylactic activities of IgG depend on IL-4 level [32]. Yang et al. [33] found that germinated soybean protein could reduce IL-4 production in cultured spleen cells in comparison with soybean protein. This finding shows consistency with the present assaying results about IL-4 secretion.

Effects of the extracts on IL-1 β , IL-6, TNF- α , iNOS, and LZM secretion in macrophages

The obtained results are listed in Table 1. On treating the macrophages with these extracts, the respective levels of IL-1 β , IL-6, TNF- α , iNOS, and LZM were 95.9–123.3, 37.1–66.1, 95.3–127.1, 113.6–139.3 pg/mL, and 27.5–44.2 ng/mL. However, when SPI was used, the respective levels of these five immune molecules were 81.9, 35.3, 92.2, 99.7 pg/mL, and 25.6 ng/mL. These extracts were clearly stronger than SPI to impact the secretion of these five immune molecules, demonstrating again that the *Mucor*-mediated fermentation conferred Mao-tofu with enhanced immune potentials to activate macrophages. Fermentation times and extracting solvents

Fig. 3 In vitro immune potentials of Mao-tofu extracts in terms of their effects on IL-2 (A), IL-4 (B), and IFN-γ (C) secretion. Different lowercase letters above the data in same line indicate significant differences of mean values ($p < 0.05$). Secretion levels of IL-2, IL-4,

and IFN-γ in the control were 25.8, 39.8, and 31.7 pg/mL, whereas those for soybean protein isolate were 33.1, 38.1, and 39.4 pg/mL, respectively

Table 1 In vitro secretion of L-1β, IL-6, TNF-α, iNOS, and LZM responsible to soybean protein isolate and soluble Mao-tofu extracts

Samples	Fermentation times (day)	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	iNOS (pg/mL)	LZM (ng/mL)
SPI	NA	81.9 ± 1.4 ^d	35.3 ± 2.7 ^d	92.2 ± 1.8 ^d	99.7 ± 7.1 ^d	25.6 ± 1.7 ^d
Extracts using pH 6.5 water	3	99.8 ± 1.4 ^c	47.5 ± 1.8 ^c	101.6 ± 3.6 ^c	117.9 ± 2.5 ^c	31.7 ± 1.8 ^c
	4	109.1 ± 1.5 ^b	49.7 ± 2.6 ^c	107.2 ± 1.3 ^c	122.7 ± 4.1 ^b	36.1 ± 1.2 ^b
	5	117.2 ± 1.8 ^a	57.9 ± 2.5 ^b	115.3 ± 2.8 ^b	131.4 ± 4.2 ^a	41.3 ± 1.2 ^a
	6	123.3 ± 2.5 ^a	66.1 ± 2.5 ^a	127.1 ± 2.5 ^a	139.3 ± 6.1 ^a	44.2 ± 2.3 ^a
	7	119.5 ± 2.4 ^a	58.5 ± 2.4 ^b	124.2 ± 2.1 ^a	135.3 ± 3.8 ^a	39.8 ± 2.3 ^a
Extracts using pH 4.5 water	3	96.9 ± 2.8 ^c	41.2 ± 1.3 ^d	97.3 ± 1.2 ^d	115.7 ± 1.4 ^c	29.3 ± 1.8 ^d
	4	101.6 ± 2.5 ^c	45.3 ± 1.5 ^c	105.7 ± 1.4 ^c	123.9 ± 2.7 ^b	34.5 ± 1.2 ^c
	5	110.7 ± 1.7 ^b	47.3 ± 2.1 ^c	113.5 ± 2.7 ^b	129.2 ± 1.2 ^{ab}	38.3 ± 2.2 ^{ab}
	6	118.5 ± 2.1 ^a	55.5 ± 2.9 ^b	123.1 ± 2.6 ^a	138.9 ± 1.3 ^a	42.9 ± 1.3 ^a
	7	115.3 ± 2.8 ^{ab}	46.5 ± 1.6 ^c	119.3 ± 2.7 ^{ab}	134.7 ± 1.1 ^a	39.2 ± 1.9 ^a
Extracts using 80% ethanol	3	96.2 ± 3.8 ^c	37.5 ± 2.5 ^d	96.8 ± 1.5 ^d	113.6 ± 3.9 ^c	27.5 ± 1.2 ^d
	4	101.2 ± 1.3 ^c	41.3 ± 1.8 ^d	104.3 ± 3.1 ^c	121.9 ± 2.1 ^b	32.8 ± 1.5 ^c
	5	106.9 ± 2.2 ^b	46.4 ± 2.2 ^c	112.2 ± 2.9 ^{bc}	126.5 ± 3.1 ^b	37.2 ± 1.3 ^b
	6	112.6 ± 2.7 ^{ab}	49.5 ± 1.3 ^c	121.6 ± 2.5 ^a	138.8 ± 1.9 ^a	41.5 ± 1.2 ^a
	7	109.4 ± 2.5 ^b	48.1 ± 1.5 ^c	118.6 ± 1.9 ^{ab}	134.2 ± 1.9 ^a	38.5 ± 1.3 ^{ab}
Extracts using 40% ethanol	3	95.9 ± 2.9 ^c	37.1 ± 2.7 ^d	95.3 ± 2.8 ^d	112.7 ± 3.9 ^c	26.1 ± 1.7 ^d
	4	98.7 ± 2.4 ^c	39.2 ± 2.3 ^d	101.4 ± 3.9 ^c	120.4 ± 2.1 ^b	31.9 ± 1.5 ^c
	5	103.3 ± 1.1 ^c	41.5 ± 1.2 ^d	109.2 ± 3.8 ^c	124.6 ± 1.3 ^b	35.6 ± 1.5 ^b
	6	109.1 ± 2.2 ^b	48.9 ± 1.1 ^c	118.6 ± 2.5 ^{ab}	134.4 ± 1.5 ^a	39.6 ± 1.2 ^{ab}
	7	101.8 ± 1.8 ^c	45.7 ± 1.7 ^c	111.2 ± 1.7 ^c	129.7 ± 3.2 ^a	36.3 ± 1.7 ^b

Different lowercase letters after the values as the superscripts in same column indicate that one-way ANOVA of the mean values is different significantly ($p < 0.05$). The measured IL-1β, IL-6, TNF-α, iNOS, and LZM secretion in control were 71.4, 23.1, 45.7, 88.6 pg/mL, and 18.7 ng/mL, respectively

NA not applicable

both showed impacts on the activities of these extracts. In general, the most appropriate extracting solvent also was pH 6.5 water because it generated higher activities for the respective extracts, followed by pH 4.5 water, 80% ethanol, and 40% ethanol solutions. A fermentation time other than 6 days led to lower activities of the extracts, which

demonstrated again that insufficient or excessive fermentation (i.e., protein degradation) might lead to decreased immune potentials of Mao-tofu or its extracts. As the result, pH 6.5 water and fermentation time of 6 days ensured the respective extract with the highest immune activities to activate macrophages.

Table 2 Amino acid compositions (gram per 100 g protein) of soybean protein isolate and two extracts obtained from Mao-tofu with a fermentation time of 6 days

Amino acids	SPI	Extract using 40% ethanol	Extract using pH 6.5 water
Ala	3.28	3.91	3.79
Arg	7.93	5.88	6.14
Asp	12.18	12.93	12.70
Cys	1.22	1.90	2.17
Glu	20.06	26.04	23.89
Gly	3.62	3.67	3.66
His	2.61	2.89	2.83
Ile	4.36	2.82	3.06
Leu	8.07	5.08	5.56
Lys	6.31	6.17	6.14
Met	1.22	0.74	0.83
Phe	4.31	3.69	3.99
Pro	6.29	6.58	6.31
Ser	5.36	4.16	4.49
Thr	3.73	3.22	3.48
Tyr	2.97	3.15	3.64
Val	3.76	3.45	3.59
Total	97.27	96.29	96.26

IL-1 β , IL-6, and TNF- α all are key mediators in the response to inflammatory stimuli. Activation of macrophages via IL-1 β , IL-6, TNF- α , and other inflammatory mediators is important in innate immunity. The release of these cytokines is essential for the host survival from infection and is required for the repair of injured tissue. iNOS in macrophages can catalyze NO production from Arg and molecular oxygen [34]. LZM is part of the innate immune system and secreted by cells such as epithelial cells and macrophages. Activation of macrophages induces enhanced secretion of various immune molecules, including IL-1 β , IL-6, IL-12, TNF- α , iNOS, and LZM [34]. These assayed extracts were all capable of activating macrophages through increasing the secretion of the three cytokines and two enzymes. This finding is partly supported by Duarte et al. [35] as they found that fermented fish protein can enhance IL-6 secretion. Moreover, the results by Leea et al. [36] demonstrate that *Cheonggukjang* polysaccharides with immune activities can up-regulate iNOS and TNF- α expression in macrophages.

Amino acid compositions of two extracts

Two soluble extracts were prepared from Mao-tofu fermented for 6 days using pH 6.5 water and 40% ethanol solution, as they had the highest and lowest activities than those extracts obtained using other extracting solvents. In comparison with SPI, the two extracts showed different amino acid profiles (Table 2), which were attributed by two factors: degradation of soybean protein by the enzymes

from *Mucor* as well as different solubility of the degraded products in pH 6.5 water and 40% ethanol solution. The underlying mechanisms by which the two factors resulted in these amino acid differences are not discussed here. The extract using pH 6.5 water was clearly rich in several amino acids, such as Arg, Cys, Ile, Leu, and Tyr, than that using 40% ethanol solution. Ethanol has lower polarity than water and therefore 40% ethanol solution shows lower polarity than pH 6.5 water. That is, pH 6.5 water was a better solvent and more efficient than 40% ethanol solution to extract peptides (particularly, those peptides with immune significance) from Mao-tofu. Polarity differences of pH 6.5 water and 40% ethanol solution were thus accounted for the compositional and thereby immunomodulatory differences of the two extracts.

Some amino acids (particularly Arg, Cys, and Met) play important roles in immune regulation of the body. For example, Cys can influence the functions of lymphocytes and macrophages [37]. Arg is considered to be involved in body immune system to favor these biological indices such as lymphocyte proliferation and NK cell activity (e.g., NO production) [34]. Different amino acid compositions of the two extracts would partially govern these assessed immune activities. The extract using pH 6.5 water was rich in these amino acids (e.g., Arg and Cys) with definite immune significance and undoubtedly, would have higher immune activities than the extract using 40% ethanol solution.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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