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Published in:
Biotechnic & histochemistry

DOI:
10.1080/10520295.2017.1339912

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

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Citation for published version (APA):
Ismaya, W. T., Efthyani, A., Retnoningrum, D. S., Lai, X., Dijkstra, B. W., Tjandrawinata, R. R., & Rachmawati, H. (2017). Study of response of Swiss Webster mice to light subunit of mushroom tyrosinase. *Biotechnic & histochemistry*, *92*(6), 411-416. https://doi.org/10.1080/10520295.2017.1339912

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Biotechnic & Histochemistry

ISSN: 1052-0295 (Print) 1473-7760 (Online) Journal homepage: https://www.tandfonline.com/loi/ibih20

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To cite this article: WT Ismaya, A Efthyani, DS Retnoningrum, X Lai, BW Dijkstra, RR Tjandrawinata & H Rachmawati (2017) Study of response of Swiss Webster mice to light subunit of mushroom tyrosinase, Biotechnic & Histochemistry, 92:6, 411-416, DOI: 10.1080/10520295.2017.1339912

To link to this article: https://doi.org/10.1080/10520295.2017.1339912

Published online: 11 Aug 2017.

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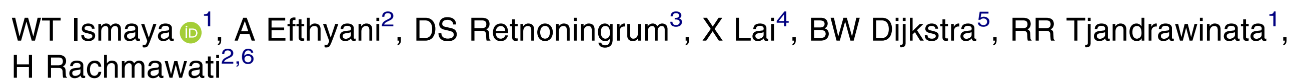

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Accepted May 29, 2017

Abstract

The light subunit of mushroom, *Agaricus bisporus*, tyrosinase (LSMT), has been identified as an extrinsic component of the enzyme. Its function is unknown, but it can cross an epithelial cell layer, which suggests that it can be absorbed by the intestine. A similar capability has been demonstrated for the HA-33 component of the progenitor toxin from *Clostridium botulinum*, which is the closest structural homolog of LSMT. Unlike HA-33, LSMT appears to be non-immunogenic as shown by preliminary tests in Swiss Webster mice. We investigated the immunogenicity and histopathology of LSMT in mice to determine its safety in vivo. LSMT did not evoke generation of antibodies after prolonged periods of intraperitoneal administration. Histopathological observations confirmed the absence of responses in organs after twelve weekly administrations of LSMT. We found that LSMT is not toxic and is less immunogenic than the *C. botulinum* HA-33 protein, which supports further research and development for pharmaceutical application.

Key words: histopathology, immune tolerance, lectin-like protein, light subunit, mushroom, ORF239342, tyrosinase

The light subunit of mushroom tyrosinase (LSMT) (Ismaya et al. 2016a,b) is a protein of unknown function found in the edible mushroom, *Agaricus bisporus*. The protein also has been called MtaL (mushroom tyrosinase associated lectin-like protein) (Lai et al. 2016). LSMT is associated with PPO3, one of the tyrosinase isoforms in the mushroom (Ismaya et al. 2011); however, it is encoded by an unrelated gene, *orf239342* (Ismaya et al. 2011) and it is not involved in tyrosinase activity, because the enzyme remains active in the absence of LSMT (Espin et al. 2000). Recently, LSMT has been cloned successfully and overproduced in *Escherichia coli* (Lai et al. 2016), which enables us to obtain the protein in a sufficient quantity for biological studies and to overcome difficulties in isolating the protein from its complex with PPO3.

Apart from its structure, the association of LSMT with PPO3 and the location of its gene, little is known about LSMT. The protein shares high structural homology with the HA-33 hemagglutinating protein from *Clostridium botulinum* (Ismaya et al. 2011). The latter protein has been developed for pharmaceutical applications (Fujinaga 2010, Ito et al. 2011), especially as a drug-carrying protein, because it has been shown to cross the epithelial layer of the intestine (Nakamura et al. 2008);

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Biotechnic & Histochemistry 2017, **92(6):** 411–416

DOI: https://doi.org/10.1080/10520295.2017.1339912

recombinant LSMT also has been shown to do this (Ismaya et al. 2016b).

The use HA-33 as a drug carrier is limited by its immunogenicity, as shown by an increase in the titer of antibody after its challenge to mice (Sayadmanesh et al. 2013). By contrast, preliminary data suggest that weekly administration of recombinant LSMT to Swiss Webster mice for four weeks caused no production of antibodies (Ismaya et al. 2016b), which suggests that LSMT is tolerated by the immune system. Therefore, LSMT may be an alternative to HA-33 as a drug carrier. In addition, our recent studies employing various cancer cell lines also demonstrated that administration of recombinant LSMT at concentrations below 100 µg/ml had no effect on cell proliferation (Ismaya et al. 2017). The non-immunogenic properties of LSMT and the potential negative effects of LSMT on tissues must be investigated further. Therefore, we challenged mice with repeated administration of recombinant LSMT to investigate its safe use.

Materials and methods

Animals and reagents

Our investigation was approved by the Animal Research Ethics Committee of the Institut Teknologi Bandung in February 2016 (Certificate no. 01/KEPHP-ITB/022016). We used 16 male and 16 female 8-week-old 25–30 g Swiss Webster mice. The mice were obtained from the in-house animal breeding facility at the School of Pharmacy, Bandung Institute of Technology. The animals were treated according to the approved protocol for animal handling in the animal laboratory facility at the School of Pharmacy, Bandung Institute of Technology. Prior to the experiment, the mice were acclimated for 7 days with food and water available *ad libitum*.

All chemicals and reagents were purchased from Sigma (St. Louis, MO) or Merck AG (Darmstadt, Germany) except where specifically noted otherwise.

Production and purification of recombinant LSMT

Production and purification of recombinant LSMT was performed as described previously (Ismaya et al. 2017). Briefly, *Escherichia coli* BL21 (DE3) carrying the LSMT gene was grown in LB broth containing 100 µg/ml ampicillin at 37° C for 12 h. LSMT expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.05 mM and the bacterial growth was continued for 4 h at 25° C. Bacterial cells were harvested by centrifugation at 5,000 × *g* at 4° C for 15 min and washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4). The cells were re-suspended in low-salt PBS (27.5 mM NaCl, 0.5 mM KCl, 10 mM phosphate buffer, pH 7.4) prior to cell disruption by sonication (Misonix; QSonica, Newtown, CT). Soluble recombinant LSMT was recovered by centrifugation at 10,000 × *g* at 4° C for 15 min and loaded onto a cOmplete™ Ni-NTA affinity column (Roche, Singapore) and washed with low-salt PBS buffer prior to elution using the same buffer containing 200 mM imidazole. The protein subsequently was purified over an Enrich™ SEC70 size exclusion column (BioRad, Singapore) equilibrated with low-salt PBS. The same buffer was used for elution. The His-tag was removed during incubation with TEV protease (Sigma) on ice for 12 h and the tag-free recombinant LSMT was purified using the Enrich™ SEC70 column. All purification steps were conducted at 20° C using an NGC Scout Plus purification system (BioRad, Singapore).

Immunogenicity

Mice of both sexes were given 0.5 ml recombinant LSMT in PBS by a weekly intraperitoneal (i.p.) injection of 25 µg/mouse on the same day for 12 weeks. As a control, another set of mice was administered PBS on the same schedule. The recombinant LSMT sample solutions were prepared as described previously (Ismaya et al. 2016b). Blood was withdrawn from the tail vein and immediately centrifuged at 1,000 × *g* at room temperature (25° C) for 10 min to obtain the serum. The presence of IgG in the serum was analyzed by the dot-blot method (Vera-Cabrera et al. 1999) using HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, the serum sample (control solution) was spotted onto a square cut polyvinylidene difluoride (PVDF) membrane. Then the anti-mouse IgG was spotted onto the sample (or control) spot on the membrane and the membrane was incubated at room temperature for 1 h. Prior to visualization, the membrane was washed briefly with PBS buffer containing 0.05% Tween-20 solution (PBST). The immunoprecipitate was visualized using the Opti-4CN substrate (BioRad). Briefly, 10 ml diluted Opti-4CN solution (1 ml concentrate in 9 ml distilled water) was mixed with 0.2 ml Opti-4CN

substrate. Immediately, 0.5 ml of the Opti-4CN reaction solution was added onto the previously treated PVDF membrane immersed in PBST. Generation of color originated from the reaction between the HRP-conjugated antibody and the Opti-4CN substrate. The Opti-4CN reaction mixture was monitored for 30 min unless the color developed faster. Finally, the membrane was washed with distilled water for 15 min, then air dried at room temperature for visual analysis.

Body weight and histopathology

The mice were weighed each week. For histopathology, mice were sacrificed in a CO_2 chamber (3–4 min at 100% CO_2). A mid-sagittal incision in the abdomen and thorax permitted removal of the liver, heart, spleen, lungs and kidneys. Each organ was weighed individually and its ratio to the body weight was calculated to obtain the organ index value (organ weight percentage of body weight). These data were determined prior to sampling for microscopy.

We prepared slides of organ samples for histopathological evaluation according to the protocol provided by Abcam (Abcam 2016). Briefly, after dividing organ samples into left and right halves, they were immersed in buffered 10% formalin for one week at 25° C. The organs were flushed with water, then dehydrated in 70, 80, 90%, ethanol and twice in absolute ethanol for 4 h each. The organs then were soaked twice in xylene for 30 min and three times in melted paraffin wax (58° C) for 30 min each. After the paraffin solidified at room temperature, the block was sectioned at 3 µm using a microtome and the sections were transferred to warm water before mounting on microscope slides. Slides were dried in an oven for 24 h prior to staining with hematoxylin and eosin (H & E). The slides were soaked successively in 100, 95 and 80% ethanol for 2 min each, then in hematoxylin solution for 5 min. After flushing with water twice for 12 min each, the slides were stained with 0.25% eosin for 2.5 min. Finally the slide was flushed with water for 5 min. This procedure was repeated a second time. The slides were dipped in 95% ethanol ten times, soaked in absolute ethanol for 2 min and soaked three times for 2 min each in xylene. Finally, the sample was mounted with a drop of Entellan and coverslipped for observation under the microscope.

Statistical analysis

The body weights and the organ indexes of the treatment groups were compared using the independent-samples *t*-test. Differences were considered significant at $p \leq 0.05$.

Results

Body and organ weights

The body weights of for both male and female mice, with or without LSMT treatment were similar (Fig. 1a). There also were no significant differences in organ index values between treated and untreated mice for either male or female mice (Fig. 1b)

Immunogenicity

Repeated doses of 25 µg LSMT for 12 weeks appeared not to induce formation of specific antibodies (Fig. 2). Evaluation of the serum after 8 (Fig. 2a) and 12 (Fig. 2b) weeks using anti-mouse IgG antibody revealed no signals originating from an immune cross reaction in either male or female mice.

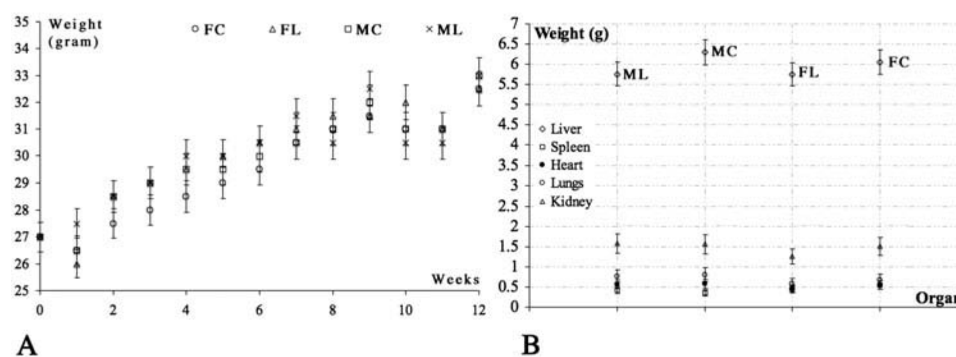

Fig. 1. Body (A) and organ (B) weights. ML, male mice LSMT; MC, male mice control; FL, female mice LSMT; FC, female mice control.

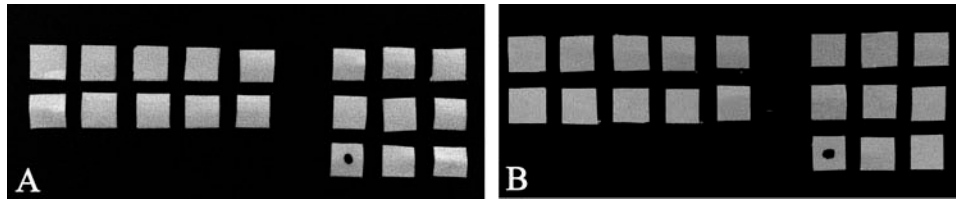

Fig. 2. Immuno-dot blot profile of blood serum of mice after 8 (A) and 12 (B) weeks. The first row includes samples from five male mice with LSMT treatment and three male mice without LSMT treatment. The second row contains the samples from five female mice with LSMT treatment and three female mice without LSMT treatment. The third row contains samples (from left to right) of an IgG positive control, a negative control with PBS, and LSMT in the absence of mice serum.

Histopathology

We found no significant differences in the organs of treated or untreated mice (Fig. 3). The number of Kupfer cells in the liver and the amounts of red and white pulp in the spleens were similar. Cardiac muscle morphology exhibited no alteration after treatment with repeated doses of LSMT. Similarly, neither lung nor kidney structure exhibited differences between groups.

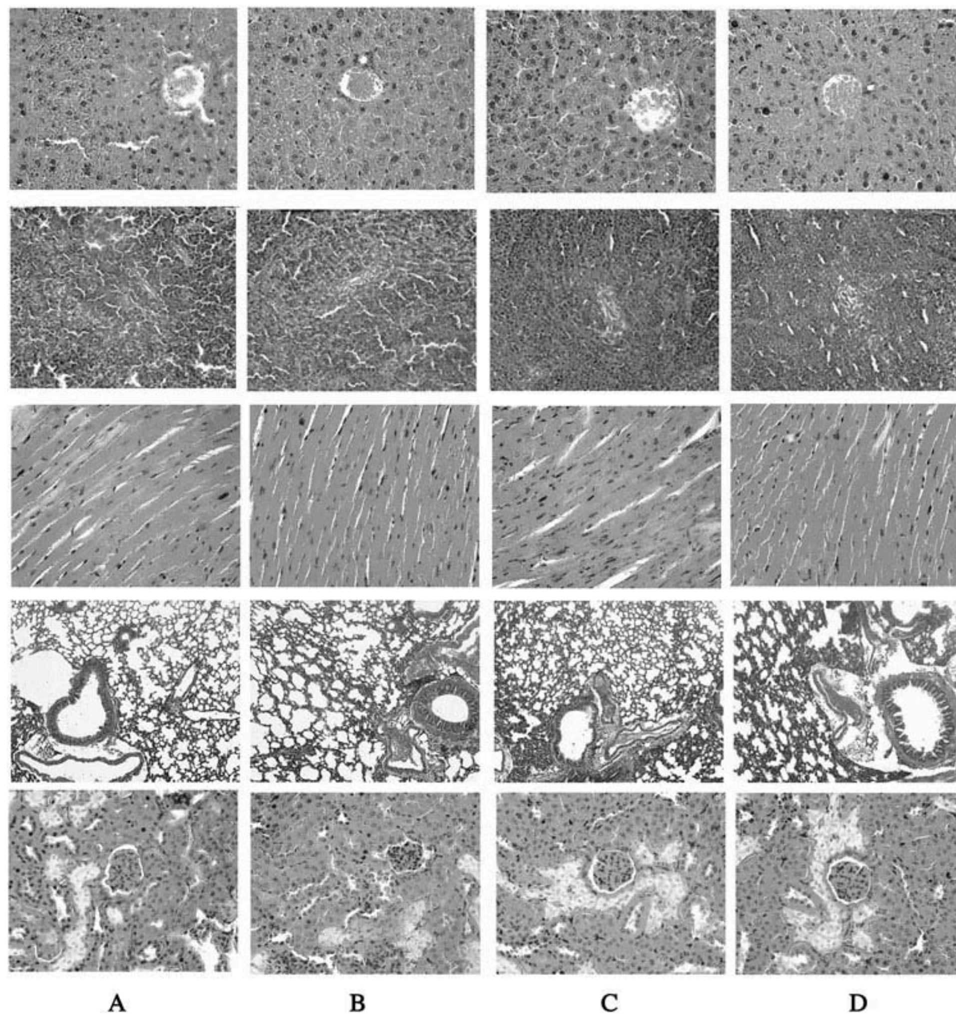

Fig. 3. Microscopy of organs from male mice with (A) and without (B) LSMT administration, and female mice with (C) and without (D) LSMT administration. From the top row down are the staining profiles of the liver, spleen, heart, lung, and kidney.

Discussion

We investigated LSMT treatment of Swiss Webster mice to assess its safe use for pharmaceutical applications. We measured body weight as an overall indication of LSMT toxicity, because changes in body weight reflect food consumption and efficiency of food utilization (Hoffman et al. 2002). The organ index also was used as a biological marker for toxicity (Schlenk et al. 2008). We found no significant differences in the body and organ weights after repeated injection of LSMT. The overall appearance of organs was normal.

LSMT has been reported to be non-immunogenic after weekly administration for 28 days (Ismaya et al. 2016b). Because the adaptive immune system is activated after 4–5 days of antigen presentation, administration for 28 days spanned the time for immune system activation (Delves et al. 2011). Assessment of a compound's immunogenicity is critical for protein based pharmaceutics or therapy to prevent reduced efficacy, anaphylaxis and autoimmune responses (Baker et al. 2010). Lack of an immune response indicates that the substance is not immediately cleared by the immune system, which enables the protein to circulate longer in the blood. Our findings provide further evidence that LSMT is non-immunogenic.

Histopathology often can be used to assess organ damage owing to treatment with exogenous agents (Singh and Wasson 2008). Overall, the organs of the mice treated with LSMT appeared normal, including the spleen, an immune organ that is involved in initiation of response to antigens (Cesta 2006).

LSMT appears to be safe as evidenced by the lack of significant changes in body weight, organ index and organ profile, and the absence of an immune response after administration of 25 µg of the protein for an extended period. LSMT appears not to be toxic or immunogenic for Swiss Webster mice. Furthermore, the non-toxicity of LSMT applies to both sexes. Our findings provide support for development of LSMT as an alternative to HA-33 as a pharmaceutical delivery agent.

Acknowledgments

This work was supported by the Ministry of Research, Science, Technology, and Higher Education, Republic of Indonesia through Excellent Research Program 2015–2018 (Contract No. 1059.10/I1.C03/KU/2015) and Dexa Laboratories of Biomolecular Sciences, Dexa Medica.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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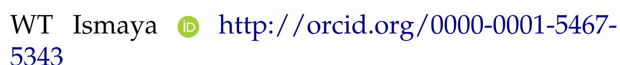
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