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A novel immune-tolerable and permeable lectin-like protein from mushroom *Agaricus bisporus*

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ABSTRACT

A lectin like protein designated as LSMT is recently discovered in *Agaricus bisporus*. The protein adopts very similar structure to Ricin-B like lectin from *Clitocybe nebularis* (CNL) and HA-33 from *Clostridium botulinum* (HA-33), which both recognize sugar molecules that decorate the surface of the epithelial cells of the intestine. A preliminary study *in silico* pointed out potential capability of LSMT to perform such biological activity. Following that hypothesis, we demonstrated that LSMT is indeed capable of penetrating out from a dialysis tube of the mice intestine origin. Furthermore, the protein appeared not to evoke the immune response upon introduction into mice, unlike its structural homologs. This is the first report on the biological implication of LSMT that might lead to its application.

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1. Introduction

Light subunit of mushroom tyrosinase (LSMT) [1] or Mushroom tyrosinase associated Lectin-like protein (MtaL) [2] is one of the intrinsic components of the tetrameric complex of PPO3 [3], one of mushroom *Agaricus bisporus* tyrosinase isoforms. The isoform is normally addressed as the heavy subunit. LSMT was discovered serendipitously, during the structure elucidation of PPO3 [3] that employed the commercially available mushroom powder [4]. In the crystal structure of the tetrameric complex, LSMT occurs in its mature version because its structure is altered by means of the absence of one of the surface loops [3]. The missing loop is likely originated from proteolytic activity [5]. Henceforth, LSMT in the crystal structure of PPO3 complex is regarded as the mature LSMT (mLSMT).

LSMT is a protein of unknown function that is encoded by the *orf239342* [3], which is a rather specific gene in *A. bisporus* variant *bisporus* or *burnettii* [6]. The gene coding for LSMT was found clustered in the chromosome five as of genes encoding PPO2, PPO3,

PPO4, and PPO5 [6]. Thus, its association with the PPO3 appears to be consistent. Interestingly, LSMT is expressed only at a stage later than PPO3 during the growth of the mushroom, and its expression may not correspond to the expression level of PPO3 [6]. Furthermore, its relation to biological process in the cell is unknown. Apart from localization of the encoding gene and its three dimensional structure, very little is known about LSMT.

The crystal structure of mushroom tyrosinase showed that LSMT is proteolytically cleaved, and consists of two peptide fragments of ~2.2 and 12.8 kDa, respectively, that remain tightly associated [3]. However, this processing has only minor structural consequences as shown by the crystal structure of recombinantly produced intact LSMT (rLSMT) [2]. The N-terminal tail of the protein has relocated covering part of the tyrosinase-binding interface, and thus has to move away when binding to mature mushroom tyrosinase.

Most importantly, LSMT shares high structural homology to the agglutinating protein from *Clostridium botulinum* (HA-33) and Ricin-B like lectin from the mushroom *Clitocybe nebularis* (CNL), which both have been developed as drug carriers [7,8]. Assessment for use of these proteins as a drug carrier is based on their capability to recognize sugars on the surface of the epithelial cells in the intestine [9]. Mechanism of internalization of HA-33 in the intestine is not yet clear [7] but the protein has been demonstrated to pass

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through the intestinal epithelial cells barrier [8]; similar situation also applies to CNL [9]. Recent *in silico* study using the structure of mLSMT (deduced from the quaternary complex, PDB ID 2y9w) suggests that most of the amino acid residues responsible for binding of sugar in CNL may also be present [11]. Some residues elemental for sugar recognition in HA-33 were also found. Therefore, capability of HA-33 and CNL as drug carriers might also be the case for LSMT. However, recombinant HA-33 has been reported to be immunogenic because it evokes high titer of antibody in mice [10]. On the other hands, CNL is shown stimulate immune response *in vitro* [11]. As for LSMT, this property is not yet known but the protein has so far not been reported as toxin (as of CNL) or toxin component (as of HA-33), or immunogenic.

The aforementioned hypothesis requires experimental evidence, especially on the capability of LSMT to permeate through the epithelial cells barrier in the intestine. Unfortunately, efforts to isolate LSMT from the mushroom tyrosinase quaternary complex have so far been fruitless while producing the recombinant version would be hampered from validation of the protein entity, particularly in the absence of information on its characteristics and biological function [1]. However recently, the three dimensional structure of rLSMT has successfully been elucidated by means of X-ray crystallography [2]. The structure of rLSMT is identical to that of previously elucidated mLSMT, providing the undisputed proof for rLSMT to represent mLSMT. Most importantly, rLSMT can be employed in the experimental study.

Here we report the test result for capability of LSMT to pass through the epithelial cells barrier of the intestine *ex vivo* and preliminary immunogenicity *in vivo* studies of rLSMT employing Swiss Webster mice. The protein was able to cross the intestinal border as evidenced by its escape from a dialysis bag made of the small intestine and did not generate immune response. Thus, LSMT appears to behave similarly to HA-33 and CNL, but without their immunogenicity. This is the first report on biological implication of LSMT and moreover reveals the prospect of LSMT application.

2. Material and methods

Chemicals and reagents used were purchased from Sigma–Aldrich (St. Louis, MO–USA), Merck (Darmstadt, Germany), except when specifically mentioned. The plasmid pET21d+ harboring the gene coding for the full length LSMT was prepared and generously provided by Xuelei Lai of the ESRF.

2.1. Approval for experiments involving animal

Research plans and protocols for the study involving the use of animal has been reviewed and approved by the Animal Research Ethics Committee – Institut Teknologi Bandung in March 2015 (Certificate No. 08/KEPHP-ITB/032015).

2.2. Production of rLSMT

Expression and purification of rLSMT was performed according to previously described procedure [2] with some modifications. Briefly, a single colony of *Escherichia coli* BL21 (DE3) carrying the gene coding for rLSMT was grown in LB broth containing 100 µg/ml ampicillin at 37 °C for 12 h at an agitation rate of 200 rpm. Protein expression was induced with addition of isopropyl β-D-thiogalactopyranoside to a total concentration of 0.05 mM for 4 h at 25 °C. Bacterial cells were harvested by cold centrifugation for 15 min at 6000 g. The cell pellet was washed with phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4) and then resuspended in low-salt PBS (27.5 mM NaCl, 0.5 mM KCl, and 10 mM phosphate buffer, pH 7.4) at a

concentration of 50 mg cells per ml buffer. The cell suspension was sonicated (Misonix, QSonica, Newtown, CT–USA) on ice for 15 times of 10 s pulse (15 RMS) with 10 s intervals. Afterward, the cell debris and other insoluble fraction were separated from the crude extract by cold centrifugation for 30 min at 14,000 g.

The crude extract was loaded onto a five ml cComplete Ni-NTA affinity column (Roche, Singapore) and washed with low-salt PBS buffer prior to elution with the respective buffer containing 200 mM imidazole. Partially purified rLSMT was then loaded onto an Enrich™ SEC70 size exclusion column (BioRad, Singapore) that has been equilibrated and eluted with low-salt PBS. All the purification steps were done at ±20 °C on an NGC Scout Plus purification system (BioRad, Singapore). The His-tag was removed during incubation with TEV protease (Sigma, St. Louis, MO–USA) on ice for 12 h and the tag-free rLSMT was cleaned up by re-purification with the size exclusion column eluted with low-salt PBS. The final solution of rLSMT (with or without the His_{6x}-tag) was buffer exchanged to PBS.

2.3. Test for permeability

Ability of LSMT to penetrate out of dialysis bag made of the intestine was tested using non-everted gut sac method [12]. Briefly, mice were sacrificed in a CO₂ chamber (3–4 min at 30% CO₂) after 12 h of fasting. Mid-sagittal incision of the mice abdomen was performed to obtain the small intestine, from which short pieces of (±3 cm) jejunum were prepared. These jejuna were washed by elution through internal jejunum with PBS. After one end of the jejunum was knotted and the sample was inserted into the jejunum, the other end was knotted. This intestine bag (regarded as the donor) was dialyzed against PBS buffer (regarded as the acceptor) for 1 h at 37 °C. The remaining sample in the jejunum and the dialysis buffer were independently collected and their protein content was then recovered using trichloroacetic acid – acetone method [13]. The protein content of each fraction was evaluated by an SDS PAGE analysis with silver nitrate staining. This experiment was carried out with rLSMT free of His_{6x}-tag. Meanwhile, immunoblot analysis was done with the His_{6x}-tagged rLSMT and employing antibody raised against the histidine tag.

2.4. Preliminary immunogenicity test

Female Swiss Webster mice of eight weeks old were immunized by intraperitoneal injection with 0.5 ml of 50 µg/ml LSMT protein solution (with and without His-tag) every seven days (boost). The mice blood was collected after 28 days and the serum was isolated and analyzed for generation of IgG by dot-blot method [14], using horse radish peroxidase (HRP) conjugated anti-mouse IgG (Abcam, Hongkong) for detection. Extended immunization was also done to check for subchronic conditions, independent to the first 28 days period trial. The visualization was done with Pierce ECL western blotting substrate (ThermoFisher Scientific, Singapore) on an X-ray film developed in an XR 24Pro (Dürndental, Bietigheim-Bissingen, Germany).

3. Results and discussion

Similar to the previous report [2], two-step purification using sequentially Nickel affinity and size exclusion columns was sufficient to obtain pure protein. Current production and purification scheme were sufficient to obtain pure recombinant LSMT with a yield of about 10 mg per liter culture. After removal of the C-terminal His_{6x}-tag with TEV protease about 8.5 mg of protein remained. After purification, the protein was diluted in PBS system to create isotonic and pH conditions (salt concentration about

Fig. 1. Electrophoregram of the fractions collected after non-everted gut sac challenge of rLSMT. In the first lane is the protein molecular weight marker, while 1 and 2 are the content of the donor and acceptor compartments of the negative control, respectively. Lanes 3 and 4 are the content of the donor and acceptor compartments, respectively, upon introduction of rLSMT, while 5 is pure rLSMT as the protein marker.

150 mM and pH about 7.4) suitable for the permeability study *ex vivo* and for intraperitoneal injection *in vivo*.

3.1. Permeability study

To demonstrate that LSMT is able to cross the intestinal border, its ability to migrate out of a bag made of fresh small intestine was tested. The experiment was carried out within the first hour after the intestine was collected to ensure that the cells are still alive and active. Upon visualization of proteins in an electrophoresis gel, protein bands of ~18 kDa and of lower molecular weights (~12 kDa and ~5 kDa) were observed in the acceptor solution (Fig. 1). These species were absent in the control solution, suggesting that they originated from the rLSMT solution fed. While the presence of ~18 kDa species in the acceptor solution indicates the success of rLSMT to pass through the intestinal cell barrier, species of lower molecular weights might indicate that rLSMT was disintegrated during the transfer. However, the identity of these smaller fragments is still to be confirmed by e.g. mass finger printing or

sequencing.

Proteins of lectin family is known to induce transcytosis, in which the size and type of lectin is less important than density of the lectin loaded onto a transported particle [15]. Endocytosis takes place upon recognition of sugar molecules such as galactose, *N*-acetyl glucosamine, glucose, *N*-acetyl galactosamine, *N*-acetyl sialic acid, that decorate the surface of epithelial cells of the intestine [16]. Our experiment indicated that rLSMT is taken up by the cells and transported across the intestinal border, without binding to nano-particle or any other carrier agents.

3.2. Preliminary immunogenicity study

As further comparison to HA-33 and CNL, effect of LSMT administration to the immune system was tested. Development of HA-33 as a drug carrier has been hampered since the protein was shown to evoke high titer of antibody in mice [10]. Our preliminary *in silico* study based on the structure of mLSMT also indicated the presence of epitopes in its amino acid sequence, that potentially

Fig. 2. Output of the dot-blot analysis using rLSMT in the presence (A) and absence (D) of the His_{6x}-tag, as compared to the PBS buffer (negative controls, B and E) and to anti-mouse IgG antibody (positive controls, C and F).

may elicit an immune reaction [1]. That *ab initio* finding is anticipated because of similarity in their structures. However, upon challenging the mice with rLSMT, both in the presence and absence of the C-terminal His_{6x}-tag, no antibodies were detected after weekly immunization for 28 days (Fig. 2). As the adaptive immune response emerges approximately four to five days after activation of the innate immune response [17], the result suggested the absence of antibody that recognize rLSMT in the serum of mice and that no anti-LSMT antibodies had been raised. Furthermore, no antibody against IgG was raised during the repeated but extended experiment of weekly immunization to conform with sub-chronic toxic conditions (data not shown). Thus, rLSMT appears to be non-immunogenic.

Our results demonstrate that rLSMT shares similar capability as HA-33 and CNL to permeate through intestinal cell barrier without being immunogenic.

Declaration conflict of interest

The authors declare that they have no conflict of interest.

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