Journal of Bioscience and Bioengineering
VOL. 121 No. 4, 420–423, 2016

www.elsevier.com/locate/jbiosc

Lentiavidins: Novel avidin-like proteins with low isoelectric points from shiitake mushroom (*Lentinula edodes*)

Yoshimitsu Takakura,[1,*,§] Kozue Sofuku,[1] Masako Tsunashima,[1] and Shigeru Kuwata[2]

Plant Innovation Center, Japan Tobacco, Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan[1] *and Graduate School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan*[2]

Received 21 April 2015; accepted 5 September 2015
Available online 20 October 2015

A biotin-binding protein with a low isoelectric point (p*I*), which minimizes electrostatic non-specific binding to substances other than biotin, is potentially valuable. To obtain such a protein, we screened hundreds of mushrooms, and detected strong biotin-binding activity in the fruit bodies of *Lentinula edodes*, shiitake mushroom. Two cDNAs, each encoding a protein of 152 amino acids, termed lentiavidin 1 and lentiavidin 2 were cloned from *L. edodes*. The proteins shared sequence identities of 27%–49% with other biotin-binding proteins, and many residues that directly associate with biotin in streptavidin were conserved in lentiavidins. The p*I* values of lentiavidin 1 and lentiavidin 2 were 3.9 and 4.4, respectively; the former is the lowest p*I* of the known biotin-binding proteins. Lentiavidin 1 was expressed as a tetrameric protein with a molecular mass of 60 kDa in an insect cell-free expression system and showed biotin-binding activity. Lentiavidin 1, with its p*I* of 3.9, has a potential for broad applications as a novel biotin-binding protein.

[**Key words:** Biotin-binding protein; Cell-free extract; Isoelectric point; *Lentinula edodes*; Non-specific binding]

Biotin-binding proteins have been found in a diverse array of organisms. For example, in vertebrates, avidin (1) and avidin-related proteins (AVR1, AVR2, AVR3, AVR4/5, AVR6, AVR7) (2) were found in the chicken (*Gallus gallus*), xenavidin (3) in *Xenopus tropicalis*, and zebavidin (4) in *Danio rerio*. In bacteria, streptavidin (5) was found in *Streptomyces avidinii*, bradavidin (6) and bradavidin II (7) in *Bradyrhizobium japonicum*, rhizavidin (8) in *Rhizobium etli*, and burkavidin (9) in *Burkholderia pseudomallei*. In fungi, tamavidin 1 and tamavidin 2 (10) were found in tamogitake mushroom (*Pleurotus cornucopiae*).

Avidin and streptavidin have been recognized for a long time and are well characterized. The dissociation constant (K_d) of biotin is 6×10^{-16} M for avidin and 4×10^{-14} M for streptavidin (11). The extraordinarily high affinities of the avidin- and streptavidin-biotin interactions are of great scientific value and have been exploited in diverse applications in medicine, biochemistry, and biotechnology (12,13). Among biotin-binding proteins, tamavidin 2 is unusual in that can be expressed at high levels in its soluble form in *Escherichia coli*, and it has a high affinity for biotin (10). Tamavidin 2 could, therefore, be produced industrially in *E. coli* and engineered to exhibit distinct characteristics such as a lower isoelectric point (14), reversible biotin-binding (15), or extremely high thermal stability (16).

The detection or separation of biotinylated biomolecules from crude cell extracts is one of the most important applications of biotin-binding proteins, where the non-specific binding of proteins to substances other than biotin can be extremely detrimental. In this regard, both avidin and streptavidin may show undesirable characteristics. Avidin, which is a basic glycoprotein, has shown a high level of non-specific binding to various biological components at physiological pH, resulting in high background levels (17). This high non-specific binding is thought to be due to its high isoelectric point (pI > 10). Although streptavidin has a lower pI (6.1–7.5), it contains an Arg-Tyr-Asp (RYD) tripeptide that apparently mimics the Arg-Gly-Asp (RGD) cell adhesion domain of fibronectin (18), thereby causing background binding in histochemical and cytochemical applications.

The charge of a biotin-binding protein largely participates in non-specific binding to biological macromolecules. Since many biological macromolecules including DNA, RNA, acidic proteins or biomembranes are minus-charged in physiological pH, a biotin-binding protein with a lower pI value, which is minus-charged in such pH, may be effective to reduce the electrostatic charge-dependent non-specific binding, and therefore be of value in various applications. Indeed, the mutant avidin Avm-pI4.7, with an acidic pI of 4.7, showed low non-specific binding to DNA and cells (17). A mutant tamavidin 2 with a pI of 5.8–6.2 also showed lower non-specific binding to DNA and glycoproteins from human sera compared with wild-type tamavidin 2 with a pI of 7.4–7.5 (14). Since mutations introduce the risk of disturbing the biotin-binding activity, an alternative approach could be to modify the proteins chemically or enzymatically, but such modification introduces the risk of creating heterogenous molecular species. Therefore, searching for novel biotin-binding proteins from unstudied sources has become an attractive approach. We screened various kinds of mushrooms for such proteins and here report the identification of

* Corresponding author. Tel.: +81 285 34 2655; fax: +81 285 25 4460.
E-mail address: yoshimitsu.takakura@jt.com (Y. Takakura).
§ Present address: Leaf Tobacco Research Center, Japan Tobacco, Inc., 1900 Idei, Oyama, Tochigi 323-080, Japan.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.09.003

two avidin-like proteins with p*I*s of 3.9 and 4.4, respectively, in shiitake mushroom (*Lentinula edodes*).

MATERIALS AND METHODS

Extraction of proteins and nucleic acids Mushrooms including *L. edodes* and *P. cornucopiae* were purchased from local grocery stores. Total soluble proteins were extracted in 0.1 M HEPES-KOH pH 7.5, precipitated by using ammonium sulfate (75% saturate), then dissolved in and dialyzed against 20 mM HEPES pH 7.5 according to the procedure described by Takakura and Kuwata (19). Genomic DNA was isolated by the standard SDS-phenol method, and mRNA was purified according to the procedure described by Takakura and Kuwata (19).

Biotin-binding assay The procedure for non-denaturing SDS-PAGE and staining of biotin-binding activity with biotinylated horseradish peroxidase (HRP) was described previously by Takakura et al. (16). Briefly, the protein sample was dissolved in 1× SDS gel-loading buffer (20) without reducing agent at room temperature for 10 min, separated by SDS-PAGE on a 10% gel, and then blotted onto a PVDF membrane (Millipore). The membrane was blocked with Tris-buffered saline (TBS) containing 3% BSA and 0.2% Tween 20, incubated in TBS containing 3% BSA with biotinylated HRP (Vector Laboratories) (1:1000 dilution) at room temperature for 1 h, and washed three times with TBS containing 0.2% Tween 20. The biotin-binding proteins were visualized by using ECL Detection Reagents (Amersham Biosciences). Streptavidin from *S. avidinii* (60 kDa as a tetramer, from Sigma) was used as a control.

cDNA cloning PCR was performed with *L. edodes* genomic DNA as a template. The 50-μl reaction contained 100 ng of genomic DNA and 5 pmol of each degenerate primer in 1× GC buffer (Takara Bio). The forward primer was 5′-GGN ACI TGG TAT/C AAT/C G/CAA/G C/TTN GG-3′ and the reverse primer was 5′-TAT/C TGN CCI G/CA/TC CAN GTN GT-3′, where N represents A, T, G, or C, and I represents inosine. Ex-taq (0.5 U) (Takara Bio) was incubated with Taq Start Antibody (Clontech) at room temperature for 10 min and then added to the reaction, which then underwent one cycle of 96 °C for 3 min, 40 cycles of 96 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and one cycle of 72 °C for 6 min. Amplified DNA was cloned into the pCR2.1Topo vector (Invitrogen) and sequenced. The PCR product was used as a hybridization probe to screen the *L edodes* cDNA library, which was constructed from 5 μg of mRNA by using a λZAP cDNA synthesis kit (Stratagene). cDNA clones were rescued as pBluescript plasmids from λ phages by using *in vivo* excision according to the manufacturer's instructions. DNA and amino acid sequences were analyzed by using GENETYX-WIN ver. 8 software (Genetyx Co.).

Cell-free protein synthesis The PCR product was amplified from each cDNA clone (*leav 1* or *leav2*) by using the primers 5′-ATGGCTCCTACGACATTGA-CATCGAGGCAA-3′ and 5′-TGATCAGGATCCCTACGCAATCTCAGCACGAG-3′ for *leav 1* or the primers 5′-ATGGCTCCTACGACATTGACATCAAGGCAA-3′ and 5′-CAATGAG-GATCCTTACGCAGTCTCAGCACGAA-3′ for *leav2*. The termini of the products were filled in by using the Klenow fragment of DNA polymerase I (Takara Bio). Each product was then digested with *Bam*HI and cloned into the expression vector pTD1 (Shimadzu) predigested with *Eco*RV and *Bam*HI. RNA was synthesized from 10 μg of the linearized expression vector by using the T7 RiboMAX Express Large Scale RNA Production System (Promega) in a 200-μl reaction. Protein was synthesized from 1 mg of the RNA by using Transdirect *insect cell* (DTT+) (Shimadzu) in a 1-ml reaction, which consisted of 500-μl of insect cell extract, 300-μl of reaction buffer, 20-μl of 4 mM methionine, 200-μl of RNA at 25 °C for 5 h, according to the manufacturer's instructions. A protein synthesis reaction without RNA was run as a negative control. A 50-μl scale synthesis was performed by using the FluoroTect GreenLys in vitro Translation Labeling System (Promega), and protein expression was verified by means of SDS-PAGE and fluorescence detection.

Biotin-binding assay with fluorescent biotin Binding to biotin-4-fluroescein was assayed according to the protocol described by Kada et al. (21) and Takakura et al. (10). Briefly, various quantities of the cell-free extracts were incubated with biotin-4-fluorescein (Molecular Probe, 25 pmol per 200-μl assay) at room temperature for 30 min. Fluorescence was measured by using an Infinite M200 microplate reader (Tecan).

RESULTS AND DISCUSSION

Biotin-binding activity in *L. edodes* Hundreds of mushroom species were screened for biotin-binding activities, which were assayed after total soluble proteins were separated by non-denaturing SDS-PAGE and immobilized onto a membrane. This assay method is based on the characteristic of biotin-binding proteins that they retain their activities (16) as well as their oligomeric structures (22) in non-denaturing SDS-PAGE in which the heat-denature of protein sample is omitted. This characteristic seems to come from the property of biotin-binding proteins that their inter-subunit associations are tolerant to SDS, but not to heat-denature.

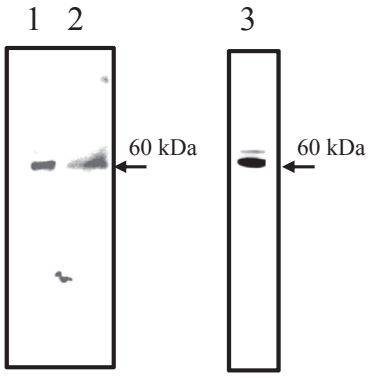

FIG. 1. Biotin-binding activity from mushrooms. Proteins from *Pleurotus cornucopiae* (lane 1) and *Lentinula edodes* (lane 2), and streptavidin (lane 3) were separated on non-denaturing SDS-PAGE and transferred onto a PVDF membrane. Biotin-binding activity was then visualized by using a biotinylated peroxidase.

A clear signal of approximately 60 kDa was detected in the extract from *L. edodes* (Fig. 1), indicating that a biotin-binding protein was present in *L. edodes*. The size of the biotin-binding protein from *L. edodes* was similar to those of tamavidins from *P. cornucopiae* (10) and streptavidin. Some other mushrooms were also positive in the screen, but the signals were not as strong as that in *L. edodes*. We therefore focused on the protein from *L. edodes* in this study.

Cloning of cDNAs for biotin-binding proteins from *L. edodes* Degenerate primers based on consensus sequences among the amino acid sequences of three biotin-binding proteins—streptavidin, tamavidin 1, and tamavidin 2—were designed (Fig. 2). A DNA segment was amplified from the genomic DNA of *L. edodes* by PCR using these primers. The sequence was similar to those of known biotin-binding proteins. By using the PCR product as a probe, we obtained eight cDNA clones from the screen of 88,000 plaques in the *L. edodes* cDNA library.

The eight cDNA clones were sequenced and classified into two types. The longest clones of both types encoded polypeptides consisting of 152 residues (Fig. 2). We termed the encoded proteins lentiavidins (*L. edodes* avidin). Lentiavidin 1 showed 30%, 46%, 48%, and 49% homology to avidin, streptavidin, tamavidin 2, and tamavidin 1, respectively. Lentiavidin 2 showed 27%, 39%, 44%, 45%, and 85% homology to avidin, streptavidin, tamavidin 2, tamavidin 1, and lentiavidin 1, respectively. The cDNA encoding lentiavidin 1 is termed *leav1* and that encoding lentiavidin 2 *leav2*. Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB675684 for *leav1* and AB675685 for *leav2*.

Primary structures of lentiavidins The 19 amino acids at the N-termini of lentiavidin 1 and lentiavidin 2 were characteristic of secretion signal peptides (Fig. 2), which are not present in tamavidins (10). The calculated molecular mass of the putative mature lentiavidin 1 and lentiavidin 2 was 14,415.4 Da and 14,403.6 Da, respectively. The calculated p*I* values were 3.9 and 4.4, respectively, which are very low (Table 1). Remarkably, lentiavidin 1 has the lowest in p*I* among the known biotin-binding proteins. It is, therefore, highly likely that the lentiavidins would show low levels of charge-driven non-specific binding to substances other than biotin.

Eleven of 12 residues that directly associate with biotin in streptavidin were conserved in lentiavidin 1, and nine in lentiavidin 2 (Fig. 2), suggesting that lentiavidin 1 might have a high affinity for