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ORIGINAL ARTICLE

Molecular cloning, characterization, and expression analysis of a β-*N*-acetylhexosaminidase (*Le*Hex20B) from the shiitake mushroom, *Lentinula edodes*

Naotake Konno · Ayumi Obara · Yuichi Sakamoto

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Abstract We previously reported on a β-*N*-acetylhexosaminidase, *Le*Hex20A, belonging to glycoside hydrolase family 20 (GH20), from the fruiting body of *Lentinula edodes* (shiitake mushroom). In this study, we purified, cloned, and characterized another β-*N*-acetylhexosaminidase, *Le*Hex20B, from *L. edodes* fruiting bodies. The cDNA of *Le*Hex20B includes an open reading frame of 1,686 bp encoding a 20 amino acid signal peptide and a 541 amino acid mature protein. The amino acid sequence identity of *Le*Hex20A and *Le*Hex20B was 57 %, and *Le*Hex20B had high sequence identity to GH20 proteins; thus, *Le*Hex20B belongs to GH family 20. *Le*Hex20B showed β-*N*-acetylhexosaminidase activity and catalyzed degradation of chitooligosaccharides ($GlcNAc_{2\text{-}6}$) exolytically with *N*-acetylglucosamine (GlcNAc) production. The maximum *Le*Hex20B activity was observed at pH 5.0 and at 60 °C. *Le*Hex20B had highest catalytic efficiency (k_{cat}/K_m) for $GlcNAc_3$ and showed high affinity for $GlcNAc_{3\text{-}6}$. The transcript level of *Le*Hex20A was significantly increased in fruiting bodies after harvest, suggesting that *Le*Hex20A is mainly involved in fruiting body autolysis. On the other hand, *Le*Hex20B was highly expressed in young fruiting bodies and mycelia. Therefore, *Le*Hex20B seems to be mainly involved in elongation of fruiting bodies and mycelia.

N. Konno · A. Obara · Y. Sakamoto
Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami-Shi, Iwate 024-0003, Japan

N. Konno (✉)
Department of Applied Biological Chemistry, Utsunomiya University, 350 Mine-machi, Utsunomiya, Tochigi 321-8505, Japan
e-mail: konno@cc.utsunomiya-u.ac.jp

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Introduction

Chitin, a polysaccharide composed of β-1,4 linked *N*-acetylglucosamine (GlcNAc) residues, is one of the main cell wall components in fungi, together with glucans (mainly β-1,3-glucan and β-1,6-glucan). In fungal cell walls, chitin generally occurs as a highly crystalline microfibril (α-chitin) form and makes up approximately 2–8 % of the dry mass [1–3]. Most of the filamentous fungi, including ascomycetes and basidiomycetes, produce enzymes associated with chitin, and some of them act on the chitin in their own cell walls. These enzymes cause morphological changes involving enzymatic synthesis, reorienting, and lysis of the cell wall chitin [4, 5].

Enzymatic degradation of chitin requires two kinds of glycoside hydrolases (GHs), chitinases (EC 3.2.1.14) and β-*N*-acetylhexosaminidases (EC 3.2.1.52). Chitinases hydrolyze β-1,4 linkages in chitin polymers endolytically, and GlcNAc oligosaccharides (chitooligosaccharides) are produced [6, 7]. Subsequently, β-*N*-acetylhexosaminidases degrade the chitooligosaccharides formed by chitinases, especially chitobiose, into monomers [8, 9]. The enzymes hydrolyze nonreducing terminal monosaccharide residues of β-*N*-acetylgalactosaminides and β-*N*-acetylglucosaminides. Fungal β-*N*-acetylhexosaminidases belong to GH family 20 (GH20) in the CAZy database [10]. GH20 enzymes perform substrate-assisted catalysis; a glutamate of the enzyme acts as the catalytic acid/base and the acetamido group of the substrate acts as a nucleophile [11, 12]. The physiological function and role of fungal β-*N*-acetylhexosaminidases are mainly investigated in

ascomycetes. Mycoparasitic fungi such as *Trichoderma* spp. produce extracellular chitinolytic enzymes for hydrolysis of host cell walls during host invasion [13, 14]. López-Mondéjar et al. [15] reported that *T. atroviride* produces two kinds of β-*N*-acetylhexosaminidase, and the enzymes are essential for the use of chitin as a nutrient source. On the other hand, some fungal chitinolytic enzymes act on their own cell walls during morphological changes. For example, it has been reported that the filamentous fungi *Aspergillus nidulans* produce some chitinolytic enzymes used in autolysis at late stages of cultures, and a β-*N*-acetylhexosaminidase from this strain is suggested to have an important role in cell death [16]. Furthermore, chitinolytic enzymes containing β-*N*-acetylhexosaminidases seem to act in hyphal growth and branching in filamentous fungi [17, 18].

Most basidiomycetes form a fruiting body (mushroom) during sporulation as part of their usual life cycle. The cell walls of the fruiting body are also constructed mainly from chitin and β-glucans [19]. Therefore, GHs for chitin and β-glucans also act in morphological changes of the fruiting bodies. Kamada et al. [4] detected chitinase, β-1,3-glucanase, and β-1,6-glucanase activities in the stipe of *Coprinopsis cinerea*, and suggested that the enzymes act in stipe elongation. Furthermore, Iten and Matile [20] reported that the cell wall chitin of fruiting bodies undergoes autolysis by chitinolytic enzymes after harvesting. However, little is known about the physiological function or role of chitinolytic enzymes in basidiomycetes.

Recently, we purified a β-*N*-acetylhexosaminidase, *Le*HexA, from *L. edodes* fruiting bodies. *Le*HexA was the first cloned and characterized GH20 β-*N*-acetylhexosaminidase from basidiomycetes [21]. *Le*Hex20A has a molecular mass of 79 kDa, and the corresponding gene (*lehex20a*) has 1,659 nucleotides, encoding 553 amino acid residues. In this study, we purified and characterized another β-*N*-acetylhexosaminidase, *Le*Hex20B, from *L. edodes* fruiting bodies. The gene encoding *Le*Hex20B (*lehex20b*) was cloned, and expression patterns of *lehex20a* and *lehex20b* were analyzed to discuss their biological functions.

Materials and methods

Materials

L. edodes strain H600 (Hokken. Co., Ltd., Tochigi, Japan) was used in all experiments. Fruiting bodies were prepared using the method of Nagai et al. [22]. *L. edodes* samples for real-time PCR were prepared as described previously [23]. The mycelia were cultured in MYPG liquid medium (0.25 % malt extract, 0.1 % yeast extract, 0.1 % peptone, and 0.5 % glucose) for 2 weeks at 25 °C with shaking as described previously [24]. Young fruiting bodies were grouped by height (<1, 1–2, 2–3, and 3–5 cm). Mature fruiting bodies were separated into pileus, gill, and stipe parts. Harvested mature fruiting bodies were immediately transferred to a desiccator at 25 °C and 80 % humidity for post-harvest preservation. All samples were stored at −80 °C.

Purification of *Le*Hex20B

Proteins were extracted from gill parts of fresh fruiting bodies (400 g). Samples were crushed in liquid nitrogen, suspended in 400 ml 10 mM sodium phosphate buffer (pH 7.0), and incubated with rotation for 30 min at room temperature. Ammonium sulfate was added until the concentration reached 70 % saturation, and the resulting precipitates were dissolved in 10 mM sodium phosphate buffer (pH 7.0) containing ammonium sulfate at 30 % saturation. *Le*Hex20B was purified sequentially by column chromatography on a Phenyl-Toyopearl column (1.6 × 10 cm, Tosho Co., Ltd., Tokyo, Japan), a MonoQ 5/50 GL anion exchange column (0.5 × 5 cm, GE Healthcare, Little Chalfont, UK), a Toyopearl DEAE-650S anion exchange column (0.8 × 7.5 cm, Tosoh Co., Ltd.), and a Superdex 75 10/30 gel filtration column (GE Healthcare) using the same strategy described previously for *Le*HexA purification [21]. Purified *Le*Hex20B was analyzed by SDS-PAGE, and proteins were stained by Oriole fluorescent gel stain solution (Bio-Rad, CA, USA). The N-terminal amino acid sequence was analyzed as described in Sakamoto et al. [24].

Cloning of cDNA-encoding *Le*Hex20B

Total RNA were extracted from the fresh fruiting bodies using a MasterPure Yeast RNA Purification Kit (EPICENTRE, WI, USA). cDNA was synthesized from total RNA extracted using a SMART PCR RACE kit (BD Biosciences, CA, USA), according to the manufacturer's protocol. Based on *L. edodes* genome information [25], *lehex20b*-specific primers (5′-ATCTGGCCGATACCCCGTTCTCTG-3′ and 5′-TTCATCGCACATCTGCGGCCTCAAAG-3′) were designed. PCR was performed as described previously [26]. The presence of a signal peptide in the deduced amino acid sequence was predicted using the SignalP server [27]. Comparative analysis of homology with the enzymes registered in the GenBank databases was carried out using the NCBI BLAST algorithm [28] with the default parameters.

Analysis of mRNA levels by real-time PCR

Total RNA from mycelia, young fruiting bodies during development, and fruiting bodies after harvest were

extracted as described above, and then reverse transcribed using a QuantiTect Reverse Transcription kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR was performed with *lehex20a*-specific primers (5′-TCTCGTGGTCGCTACCGTTAT-3′ and 5′-CGTCGAAAAGTCCGTAGGAAGA-3′) and *lehex20b*-specific primers (5′-AAATGACCCAACCGGTA ATAGCT-3′ and 5′-TGGCGAGTGGATTGAAAGTG-3′) on a Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA) [23]. The real-time PCR data were normalized against the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) expression [29] detected with *gpd*-specific primers (5′-CCGCTACCCAGAAGACTGTTG-3′ and 5′-GAACGACCTCCACGCCAAT-3′). The expression patterns were analyzed by $\Delta\Delta C_T$ method [30], and the expression level of gills of fresh fruiting bodies was used as a calibrator.

Enzyme assays

β-*N*-Acetylhexosaminidase activity was measured in 20 mM sodium acetate buffer (pH 4.2) at 37 °C for 15 min. During purification, activity was determined using 0.5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-GlcNAc) (Sigma-Aldrich Inc., St. Louis, MO, USA) as a substrate. The reaction was quenched with 0.4 M Na_2CO_3, and the amount of *p*NP released was determined spectrophotometrically at 405 nm. The extinction coefficient of *p*NP was assumed to be 17,100 M^{-1} cm^{-1}. To elucidate substrate specificity of the enzyme, assays were performed using the following substrates: *p*NP-GlcNAc, *p*-nitrophenyl-*N*-acetyl-beta-D-galactosaminide (*p*NP-GalNAc), *p*-nitrophenyl-D-glucoside (*p*NP-Glc) (Sigma-Aldrich), chitooligosaccharides ($GlcNAc_{2\text{-}6}$, Seikagaku Biobusiness Co., Tokyo, Japan), chitin (Wako Pure Chemicals Co., Osaka, Japan), and colloidal chitin, which were prepared according to [31]. The amounts of GlcNAc released from chitin oligomers were measured by the Morgan-Elson assay according to the method of [8]. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol GlcNAc per minute. To determine the kinetic properties of *Le*Hex20B, the reactions were performed with 0.05–0.5 mM of substrate [21]. To analyze the effect of pH on the activity, assays were performed using the following buffers: 50 mM citric acid-sodium phosphate at pH 3–5, 50 mM sodium acetate at pH 4.0–6.0, 50 mM sodium phosphate at pH 6.0–8.0, and 50 mM Tris/HCl at pH 8.0–9.0. The pH stability of the enzyme was estimated by measuring residual activity after incubation in the buffer at 4 °C for 20 h. The effect of temperature on the activity was determined by conducting the assays for 15 min at temperatures in the range of 10–80 °C. To analyze thermostability, aliquots of the enzyme were treated in 20 mM sodium phosphate buffer (pH 4.2) at 10–80 °C for 30 min. The effects of pH and temperature on the enzymatic activities were measured using *p*NP-GlcNAc, $GlcNAc_2$, and $GlcNAc_4$.

Thin-layer chromatography (TLC) of reaction products

$GlcNAc_{2\text{-}6}$ (0.7 mM) were incubated with *Le*Hex20B (0.46 nM) in 20 mM sodium acetate buffer (pH 4.2) at 37 °C, and the reaction mixtures were applied to a TLC plate (Silica 60 F254, Merck Co., Darmstadt, Germany). The reaction products were developed with *n*-butanol/methanol/25 % ammonia solution/water (5:4:2:1 by volume), and detected using aniline-diphenylamine reagent [32].

Nucleotide sequence accession number

The nucleotide sequence encoding *Le*Hex20B has been deposited in the DDBJ/EMBL/GenBank databases under the accession number [DDBJ: AB981197].

Results and discussion

Purification of *Le*Hex20B and cloning of its gene, *lehex20b*

*Le*Hex20B (0.92 μg) was purified from fresh fruiting bodies of *L. edodes* (400 g) by a four-step column chromatographic method in a similar manner to *Le*HexA purification. In the first step, a (Phenyl-Toyopearl column), *Le*Hex20A, and *Le*Hex20B were separable as distinct peaks with activity toward *p*NP-GlcNAc. Although *Le*Hex20A went through the Phenyl-Toyopearl column in buffer containing ammonium sulfate at 30 % saturation, *Le*Hex20B was absorbed to this column and was eluted with buffer containing ammonium sulfate at 10 % saturation. As a result, a single major band was obtained by SDS-PAGE, and the deduced molecular mass of the purified *Le*Hex20B was 75 kDa (Fig. 1). The N-terminal amino acid sequence of the protein was IWPIPRSLDSG. A search for this sequence in the *L. edodes* genome database yielded one matching region in the sequence designated AUGUSTUS01_g3889.t1.

Based on the nucleotide sequence of AUGUSTUS01_g3889.t1, primers were designed. cDNA-encoding *Le*Hex20B was cloned from total RNA extracted from the gills of fresh fruiting bodies. The cDNA contained an open reading frame of 1,686 bp, encoding 561 amino acid residues. According to SignalP analysis, the first 20 amino acid residues in the N-terminal region are expected to be a signal peptide. This expected N-terminal region was

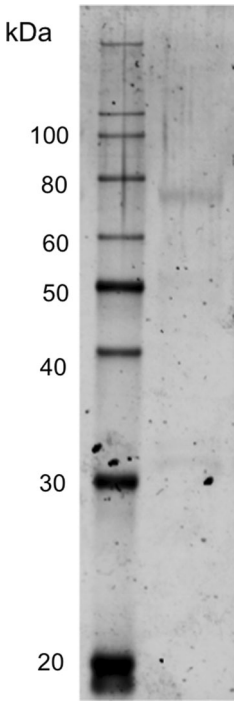

Fig. 1 SDS-PAGE of purified *Le*Hex20B. Approximately, 1 μg of sample was separated on a 10 % (w/v) polyacrylamide gel. Lane 1, molecular mass standards (kDa); lane 2, purified *Le*Hex20B

identified as a result of N-terminal analysis. Therefore, mature *Le*Hex20B, consisting of 541 amino acids, is an extracellular or cell wall protein. The deduced amino acid sequence was analyzed using the blastp algorithm of the NCBI protein database. Search results indicated that the amino acid sequence contains GH20 domains. The amino acid sequence identity of *Le*Hex20A and *Le*Hex20B was 57 %. The BLAST search found that *Le*Hex20B has high sequence identity to putative GH20 proteins (containing putative β-*N*-acetylhexosaminidase sequences) from basidiomycetous species such as *Gloeophyllum trabeum* (sequence ID, EPQ54817; identity, 65 %), *Serpula lacrymans* (EGN97893; 63 %), *Phanerochaete carnosa* (EKM49790; 62 %), and *Fomitiporia mediterranea* (EJC97794; 62 %). These results support *Le*Hex20B belonging to GH20. The sequence was further analyzed using the blastp algorithm in genome sequence databases of basidiomycetes [33]. These searches revealed that *Le*Hex20B has high levels of similarity to proteins of basidiomycetes including *Coprinopsis cinerea* (DOE Joint Genome Institute ID number 2961; similarity 53 %), *Postia placenta* (112369; 54 %), *Heterobasidion annosum* (61259; 58 %), *Agaricus bisporus* (186352; 51 %), *Polyporus arcularius* (649991; 59 %), *Schizophyllum commune* (66483; 59 %), and *Phanerochaete chrysosporium* (140587; 62 %). Thus, homologs of *lehex20b* seem to be conserved in basidiomycetes.

Multiple sequence alignment of GH20 members indicates that a glutamate, Glu316 in *Le*Hex20B, acts as the catalytic acid/base [34, 35]. GH20 enzymes from bacteria, plants, insects, mammals, and ascomycetes have a consensus H–x-G–G motif preceding the catalytic residue [36, 37]. We reported that *Le*Hex20A and its homologs from basidiomycetes have the sequence S-x-G–G in the corresponding position [21]. *Le*Hex20B and the homologs shown above commonly have the S-x-G–G motif, suggesting that this sequence is unique for basidiomycete GH20 enzymes.

Enzymatic properties of *Le*Hex20B

The enzymatic characteristics of purified *Le*Hex20B were investigated. Effects of pH and temperature on enzyme activity were examined using *p*NP-GlcNAc and chitooligosaccharides ($GlcNAc_2$ and $GlcNAc_4$) as substrates. The maximum *Le*Hex20B activity was observed at pH 5.0 in 50 mM sodium acetate buffer, and the enzyme was stable across a pH range from 5 to 8 when incubated at 4 °C for 20 h. *Le*Hex20B showed maximum activity at 60 °C (incubation time, 15 min). However, the enzyme was inactivated after incubation at 60 °C for 30 min. These properties were similar to those of *Le*Hex20A.

The substrate specificity of purified *Le*Hex20B was also investigated. *Le*Hex20B showed hydrolytic activity toward *p*NP-GlcNAc, *p*NP-GalNAc, and $GlcNAc_{2\text{-}6}$, but none toward *p*NP-Glc or crystalline chitin. *Le*Hex20B had no hydrolytic activity toward colloidal chitin, while *Le*HexA did. The enzymatic reaction products of $GlcNAc_{2\text{-}6}$ ($GlcNAc_2$, $GlcNAc_4$, and $GlcNAc_6$) were analyzed by TLC (Fig. 2). $GlcNAc_2$ was rapidly degraded to GlcNAc monomers. When $GlcNAc_4$ and $GlcNAc_6$ were used as substrates, the initial (30 min) products were monomers and oligomers that were shorter than the original substrate.

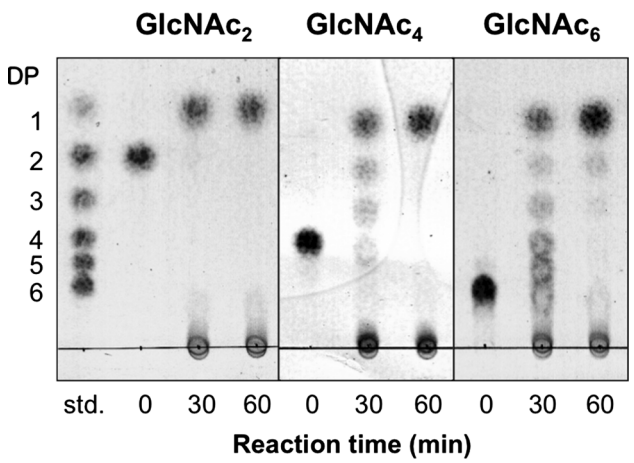

Fig. 2 TLC analysis of *Le*Hex20B action on chitooligosaccharides ($GlcNAc_{2\text{-}6}$). $GlcNAc_{2\text{-}6}$ (0.7 mM) were incubated with *Le*Hex20B (0.46 nM) in 20 mM sodium acetate buffer (pH 4.2) at 37 °C. Standards (Std.) were GlcNAc and $GlcNAc_{2\text{-}6}$. *DP* degree of polymerization

Table 1 Kinetic parameters of *Le*Hex20B. The activity was measured in 20 mM sodium acetate buffer (pH 4.2) at 37 °C for 15 min. To determine the kinetic properties of *Le*Hex20B, the reactions were performed with 0.05–0.5 mM of substrate

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
*p*NP-GlcNAc	410 ± 10	91.0 ± 12.0	222
*p*NP-GalNAc	662 ± 43	66.8 ± 4.7	101
Chitobiose ($GlcNAc_2$)	340 ± 13	41.3 ± 1.1	122
Chitotriose ($GlcNAc_3$)	211 ± 18	49.1 ± 0.8	233
Chitotetraose ($GlcNAc_4$)	180 ± 1	42.2 ± 0.3	235
Chitopentaose ($GlcNAc_5$)	190 ± 2	38.4 ± 0.3	202
Chitohexaose ($GlcNAc_6$)	192 ± 14	36.5 ± 1.1	190

The enzyme further degraded these oligomers, and the accumulation of monomers was eventually observed, suggesting typical exo-type activity. These results indicate that *Le*Hex20B is a β-*N*-acetylhexosaminidase in the EC 3.2.1.52 classification.

The kinetic parameters of *Le*Hex20B for *p*NP-GlcNAc, *p*NP-GalNAc, and $GlcNAc_{2\text{-}6}$ were investigated (Table 1). *Le*Hex20B showed values for the kinetic constant k_{cat}/K_m as follows: $GlcNAc_3 > GlcNAc_4 >$ *p*NP-GlcNAc $> GlcNAc_5 > GlcNAc_6 > GlcNAc_2 >$ *p*NP-GalNAc. *Le*Hex20B showed the highest k_{cat} toward *p*NP-GlcNAc, but almost half the value toward $GlcNAc_{2\text{-}6}$. The k_{cat} values of *Le*Hex20B toward $GlcNAc_{2\text{-}6}$ were equivalent, while the k_{cat} value of *Le*Hex20A for $GlcNAc_2$ was 2.0-fold higher than for $GlcNAc_4$ and 2.5-fold higher than for $GlcNAc_6$, On the other hand, *Le*Hex20B showed high affinity for $GlcNAc_{3\text{-}6}$: the K_m value for $GlcNAc_3$ was 1.6-fold lower than for $GlcNAc_2$ and 1.9-fold lower than for $GlcNAc_6$. This tendency in the kinetic parameters of *Le*Hex20B was similar to that of *Le*Hex20A. Generally, β-*N*-acetylhexosaminidases hydrolyze nonreducing terminal monosaccharide residues of substrates with binding between a subsite (−1) of the enzymes and the nonreducing terminal residue of the substrate. In our last paper [21], we noted that some β-*N*-acetylhexosaminidases containing *Le*HexA might have other GlcNAc-binding subsites. The biggest reduction in the K_m value of *Le*Hex20B was observed between $GlcNAc_2$ and $GlcNAc_3$, implying that a subsite (+2) of the enzyme strongly binds to the substrate [38].

Expression pattern of *lehex20a* and *lehex20b*

The expression patterns of *lehex20a* and *lehex20b* in mycelia, young fruiting bodies during development, and mature fruiting bodies were analyzed by real-time PCR (Fig. 3). The *lehex20a* expression was observed at every stage and in every part of fruiting bodies. In contrast, no expression was detected at the mycelial stage. These results indicate that *lehex20a* was specifically expressed in the

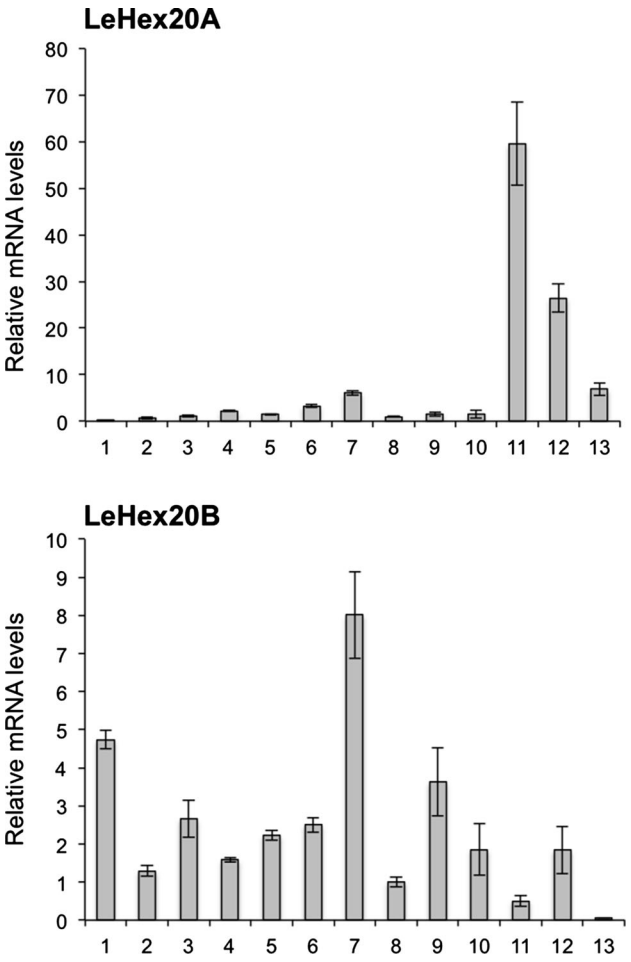

Fig. 3 Analysis of the transcription level of *lehex20A* and *lehex20b* using real-time PCR. Quantities of mRNAs were relative to the level in gills of fresh fruiting bodies. All data points are mean ± SD ($n = 3$). *1* mycelia grown in liquid culture for 2 weeks; *2* primordia less than 1 cm high; *3* primordia 1–2 cm high; *4* pilei of young fruiting bodies 2–3 cm high; *5* stipes of young fruiting bodies 2–3 cm high; *6* pilei of young fruiting bodies 3–5 cm high; *7* stipes of young fruiting bodies 3–5 cm high; *8* gills of fresh fruiting bodies (calibrator); *9* pilei of fresh fruiting bodies; *10* stipes of fresh fruiting bodies; *11* gills of fruiting bodies 3 days after harvesting; *12* pilei of fruiting bodies 3 days after harvesting; *13* stipes of fruiting bodies 3 days after harvesting

fruiting body. Moreover, expression was particularly high in the fruiting bodies 3 days after harvesting, (59.6 times higher in gills, 17.5 times in pilei, and 4.6 times in stipes, compared with the level in fresh fruiting bodies), suggesting that *Le*Hex20A is mainly involved in fruiting body autolysis after harvest. On the other hand, *lehex20b* was expressed at almost all stages and in mycelia and fruiting bodies. Expression was high in young fruiting bodies, particularly in the stipes of young fruiting bodies 3–5 cm high, a stage showing remarkable stipe elongation. High expression of *lehex20b* was also detected at the mycelial stage (8 times higher than in gills of fresh fruiting bodies). These results suggested that *Le*Hex20B is mainly involved in growth of hyphae and fruiting bodies.

The cell walls of fungi are constructed mainly from chitin and β-1,3/1,6-glucans. We reported four β-1,3-glucanases (EXG1, EXG2, TLG1, and GLU1) and one β-1,6-glucanase (*Le*Pus30A) from *L. edodes* fruiting bodies [23, 24, 26, 39–41]. EXG1 is suggested to have a role in stipe elongation of young mushrooms during development. In contrast, TLG1, GLU1, and LePus30A are mainly involved in cell wall autolysis during fruiting body senescence after harvesting. EXG2 also seems to operate on both of the processes described above. Thus, sharing of roles in the degradation of cell wall polysaccharides seems to occur commonly in fungi. In the cell wall of *L. edodes* mushrooms, chitin exists as a highly crystalline microfibril. Sakamoto et al. [40] reported two *L. edodes* genes showing homology with GH family 18 endochitinases. Kang et al. [42] cloned GH family 18 endochitinases from the mushroom tissues of *Coprinellus congregatus*, and the endochitinase was suggested to be involved in the degradation of mushroom cell walls during autolysis. Therefore, *Le*Hex20A and *Le*Hex20B may act in cooperation with other chitinolytic enzymes, especially endochitinases, on cell walls.

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