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ORIGINAL ARTICLE

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The smell and odorous components of dried shiitake mushroom, *Lentinula edodes* V: changes in lenthionine and lentinic acid contents during the drying process

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Abstract This study on dried shiitake mushroom [*Lentinula edodes* (Berk.) Pegler] focuses on the behavior of the aromatic lenthionine compound and its precursor, lentinic acid, during the drying process. Only a small amount of lenthionine was present after the drying process, regardless of the drying temperature or time; however, the amount increased during the rehydration process using pH 8.0 buffer. The lenthionine content after incubation in the buffer was correlated with the lentinic acid content in the dried shiitake mushroom. The lentinic acid content increased significantly during the drying process at 40°C up to 4 h. The results show that the characteristic smell of dried shiitake mushroom was weak after drying, and that rehydration in a mild alkaline solution was necessary to increase the smell. The lentinic acid content of dried shiitake could serve as an indicator of the smell produced by rehydration and would be useful in screening to select odor-rich strains. Moderate heating likely caused the increase in lentinic acid content, which might be related to a reaction to heat.

Key words Dried shiitake mushroom · Lenthionine · Lentinic acid · Drying process

Introduction

Odor is an important factor in evaluating foods,[1–3] as in the case of the famous odorous dried shiitake mushroom [*Lentinula edodes* (Berk.) Pegler]. The causative agent appears to be 1,2,3,5,6-pentathiepane, also known as lenthionine (LT),[4] produced from 15-amino-10-carboxy-12-oxo-2,4,6,8-tetrathia-11-azahexadecan-16-oic acid-2,2,4,6,8-pentaoxide, also known as lentinic acid (LA). The reaction is catalyzed by γ-glutamyl transpeptidase (γ-GT) and *S*-alkyl-L-cysteine sulfoxide lyase (C-S lyase), followed by nonenzymatic polymerization of the resulting thiosulfinate.[5] The smell is absent in fresh shiitake mushroom, and the amount of LT in the dried fruiting bodies increases during the rehydration and cooking procedure.[6] This increase is the result of a reaction between the substrate and enzymes, which is caused by rehydration of the dried shiitake mushroom. This means that the drying process plays a crucial role in the production of odorous compounds.

The amount of savory compound 5′-guanylic acid, which is present in the dried shiitake mushroom, changes during the rehydration process.[7,8] The LT and LA contents in the fruiting bodies may also change during the drying and rehydration processes. However, the relationship between them and the effects of the drying process are uncertain. This study investigated the change in LT content with or without rehydration, and the LA content in the shiitake mushroom during the drying process, and the relationship between the LT content at rehydration of the dried shiitake mushroom and the LA content.

Materials and methods

Fruiting bodies

Fruiting bodies were purchased to determine the analysis conditions. We used the strain coded F103 (Fujisyukin) for the drying tests. The fruiting bodies, purchased from a producer in Tsukuba City, were inoculated on logs (*Quercus serrata*) in February 2009 and harvested in September 2009.

Drying process

The pilei and stipes of the fruiting bodies were separated, and the pilei were dried at 40° or 60°C. They were sampled after 1, 2, 4, 6, 12, 20, and 24 h at 40°C, and after 6, 12, 20, and 24 h at 60°C. The weight of pilei and the wet-bulb temperature were recorded at intervals of 15 min up to 6 h and at intervals of 15 min before and 30 min after sampling time. Thermal sensor was inserted into the center of the

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pileus and the inner temperature was recorded by a data logger (TR-71U,T&D Corp). Pilei with water content above 40% were subsequently lyophilized.

Lenthionine analysis

To determine the amount of LT after rehydration, 1 ml phosphate buffer (0.2 M, pH 6.5–7.5), tris(hydroxymethyl) aminomethane (Tris)-HCl buffer (0.2 M, 0.5 M, pH 7.5–8.5), or borate buffer (0.2 M, pH 8.5–10.0) was added to about 20 mg powdered pilei and incubated from 1 to 5 h at 4°, 15°, 25°, and 37°C. Meanwhile, the amount of LT without rehydration was measured without buffer. Next, 500 µl of methanol (MeOH) solution of butyrophenone (5 nl/ml, used as the internal standard) was added to the sample. After centrifugation, the precipitate was washed twice using MeOH. The supernatant and wash fluid were combined and then diluted with water until the MeOH content was below 30%. The solution was added to a 50-mg octadecyl silica (ODS) cartridge (Bond Elut, Varian), which was preconditioned according to the instruction manual. The cartridge was washed using 1 ml 30% MeOH, and LT was eluted using 1 ml 65% MeOH. Next, 20 µl of the eluate was injected into a high-performance liquid chromatograph (HPLC) system equipped with an ODS column of 4.6 mm$\varphi \times$ 15 cm (Mightysil RP-18 GP, Kanto Chemical) and an ultraviolet detector. Analytical conditions were as follows: flow rate 1 ml/min; eluant, water/MeOH = 35/65; column temperature 45°C; wavelength 230 nm.[9]

Lentinic acid standard

Standard LA was extracted and recrystallized according to the methods described by Yasumoto et al. and Aoyagi et al. with some modifications.[10,11] Fresh fruiting bodies were homogenized with cold methanol and centrifuged. The supernatant was condensed under vacuum to remove MeOH, and lipids were removed by diethyl ether extraction. The resulting solution was passed through an Amberlite IR-120B column (H^+ form) and LA was eluted using 3 N ammonium water. After removing the ammonia from the LA fraction, the resulting solution was passed through a DEAE-Sephadex column (acetate form), and LA was eluted by stepwise elution using acetic acid. To obtain an LA crystal, crystallization was repeated four times and the crystal was dried under vacuum. The crystal gave an MH^+ ion at m/z 483 by fast atom bombardment mass spectrometry (JMS-HX110A, JEOL). The ^{1}H, ^{13}C, and two-dimentional (2D) nuclear magnetic resonance (NMR) spectrums of the crystal in dimethy sulfoxide-d_6 were recorded by a JEOL Alpha 500 spectrometer. ^{1}H NMR δ: 1.87–1.97 (2H, m, 14-H_2), 2.32 (2H, ddd, J = 15.0, 11.3, and 6.7 Hz, 13-H_2), 3.15 (3H, s, 1-CH_3), 3.25 (1H, t, J = 12.5 Hz, 9A-H), 3.45 (1H, t, J = 6.7 Hz, 15-H), 3.66 (1H, dd, J = 12.5 and 3.1 Hz, 9B-H), 4.47 (1H, ddd, J = 12.5, 7.9, and 3.1 Hz, 10-H), 4.55 (1H, d, J = 13.4 Hz, 7A-H), 4.75 (1H, d, J = 13.4 Hz, 5A-H), 4.82 (1H, d, J = 13.4 Hz, 7B-H), 4.90 (1H, d, J = 13.4 Hz, 5B-H), 4.97 (1H, d, J = 13.4 Hz, 3A-H), 5.20 (1H, d, J = 13.4 Hz, 3B-H), 8.72 (1H, d, J = 7.9 Hz, 11-H). ^{13}C NMR δ: 26.77 (14-C), 31.61 (13-C), 43.42 (1-C), 47.54 (10-C), 52.92 (15-C), 53.63 (9-C), 66.12 (5-C), 66.25 (7-C), 69.60 (3-C), 170.25 (16-C), 171.69 (1′-C), 172.03 (12-C). These results confirm that the crystal was LA.[10–12]

Lentinic acid analysis

First, 1 ml of 0.1 N HCl was added to about 50 mg powdered pilei and was agitated for at least 30 min. After centrifugation, 25 µl supernatant was mixed with 75 µl acetonitrile (ACN). To the 10 µl solution, 50 µl of boric buffer (0.4 M, pH 9.0), including 10 nmol/ml homoserine (used as the internal standard) and 40 µl of 9-fluorenylmethyloxycarbonyl chloride solution (3 mM in ACN), was added and heated at 40°C for 10 min. Then 40 µl of adamantanamine solution (50 mM in 40% ACN) was added; this mixture was heated again under the same conditions. The mixture was diluted with 60 µl 40% ACN and 10 µl of the solution was injected into the HPLC system equipped with an ODS column (same column as used for the LT analysis) and a fluorescence detector.[13,14] Analytical conditions were as follows: flow rate 1 ml/min; eluant (liner gradient), 0.1 M CH_3COOH/Na buffer (pH 3.8)/ACN = 78/22 (0 min) → 69/31 (16 min) → 58/42 (23 min) → 55/45 (37 min) → 20/80 (45 min) → 10/90 (55 min); column temperature 38°C; excitation wavelength 263 nm, emission wavelength 313 nm.

Proliferation potency against heating

We used the strain coded FMC 140 (stock culture of the Mushroom Science Lab, Forestry and Forest Products Research Institute). A mycelial mat on potato dextrose agar media (Oxoid) was punched using a 2-mm-diameter cork borer and inoculated into 100 µl potato dextrose broth media (Becton Dickinson) in a 250 µl tube. The tube was heated at 30°–60°C for 10–360 min using a thermal cycler (TP3000, Takara Bio). Mycelial growth was checked after incubation for 5 days at 25°C under dark conditions.

Results

Lenthionine analysis

In this experiment, quantification of the odorous compounds was performed by HPLC, as the use of gas chromatography requires an extremely high volume of samples. LT was used as the indicator of smell instead of 1,2,4-trithiolane, which was used in previous studies.[15–18] In the preliminary tests, the impurity peaks from the fruiting bodies could not be separated from those of 1,2,4-trithiolane, whereas LT could be detected as a single peak in the same tests. The positive correlation between the amount of odorous compounds including 1,2,4-trithiolane and LT was previously clarified.[15]

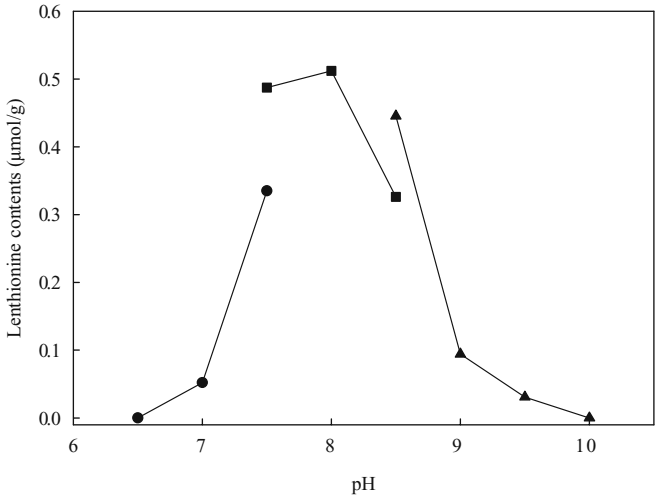

Fig. 1. Effects of pH on amount of lenthionine produced. The *circles*, *squares*, and *triangles* indicate the lenthionine content of dried shiitake incubated in phosphate buffer, Tris-HCl buffer, and borate buffer, respectively

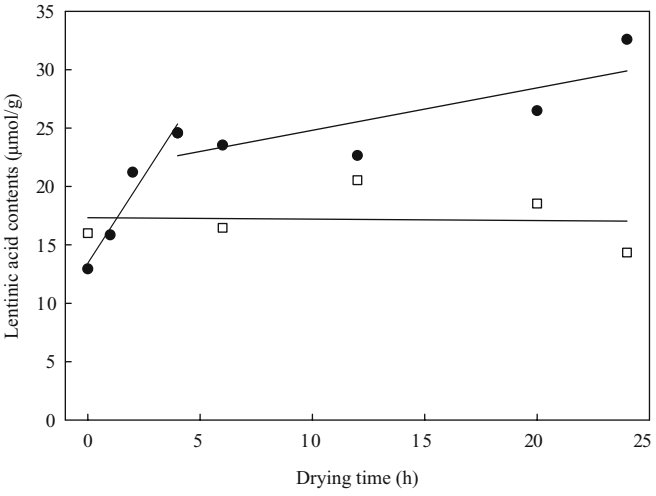

Fig. 2. Lentinic acid content change during drying process. The *circles* indicate the lenthionine content during drying at 40°C, and the *squares* indicate that at 60°C. The correlation coefficient (r) between lentinic acid content and drying time from 0 to 4 h and for 4 to 24 h at 40°C, was 0.97 and 0.82, respectively. The P values were 0.03 and 0.09, respectively. The r and P values between the lentinic acid content and the drying time at 60°C were 0.05 and 0.94, respectively

The amount of LT produced by rehydration was measured under different pH conditions using the three kinds of 0.2 M buffer at 25°C after 3 h incubation; the optimal pH was 8.0 (Fig. 1). The amount of LT incubated at 4°–25°C for 1 h was almost the same, but that at 37°C was a little low. When the incubation time was extended from 1 to 5 h, the content gradually decreased. The amount of LT produced by 0.2 and 0.5 M Tris-HCl buffer was compared, because the relationship between the pH and amount of LT produced was similar to that between the pH and γ-GT activity, and γ-GT activity was influenced by the buffer concentration.[19] The LT content using 0.2 and 0.5 M buffer was 0.54 and 0.53 μmol/g, respectively. Therefore, rehydration for the production of LT was performed using 0.2 M Tris-HCl buffer (pH 8.0) at 25°C for 1 h of incubation.

Lentinic acid amount

Five fruiting bodies cultivated on different bed logs were separated into pilei and stipes, and the amount of LA in both parts was measured. The amount of LA in pilei was 74.7% (±3.7%) of the amount in whole fruiting bodies. This shows that the LA content in the pilei can be substituted for that of the fruiting bodies.

A correlation did not existed between LA content and drying time at 60°C [correlation coefficient (r) = 0.05, and P = 0.94, Fig. 2]. Moreover, the P value obtained by analysis of variance was 0.194. These results show that the LA content was constant during the drying process at 60°C. On the other hand, there was a significant positive correlation between LA content and drying time at 40°C up to 4 h (r = 0.97, P = 0.03). However, no significant correlation was seen after 4 h (r = 0.82, P = 0.09), i.e., LA content increased during drying at 40°C up to 4 h, after which it remained almost constant.

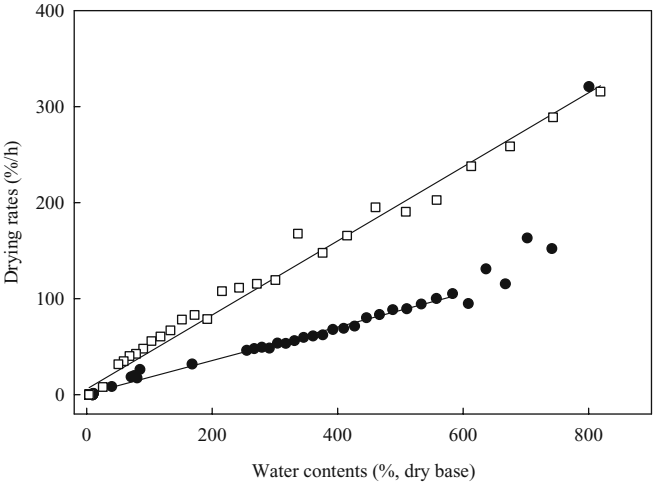

Fig. 3. Drying characteristic curve for shiitake mushroom. The *circles* indicate the drying rate at 40°C, and the properties of the regression line (water content < 600) were as follows: $y = 0.17\ x + 0.70$, $r = 1.00$, $P < 0.001$. The *squares* show the drying rate at 60°C, and the properties of the regression line were as follows: $y = 0.39\ x + 5.90$, $r = 1.00$, $P < 0.001$

Water content and temperature in the drying process

The water content of pilei decreased exponentially along with the drying time at both drying temperatures. Constant-rate drying was not seen, and clear decreasing-rate drying was observed in both drying processes except in the early drying stage at 40°C (Fig. 3). The regression lines between water content and the drying rate during both drying processes were highly significant (r = 1.00, $P < 0.001$ at 40°C; r = 1.00, $P < 0.001$ at 60°C), and the gradients of the decreasing-rate drying (K_d) for 40° and 60°C drying processes were

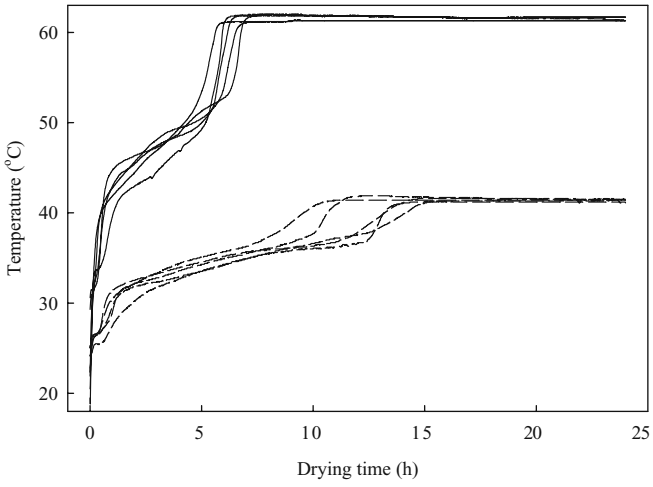

Fig. 4. Inner temperature of pilei during drying process. The *solid lines* show the temperature change during the 60°C drying process, and the *broken lines* show that during the 40°C drying process

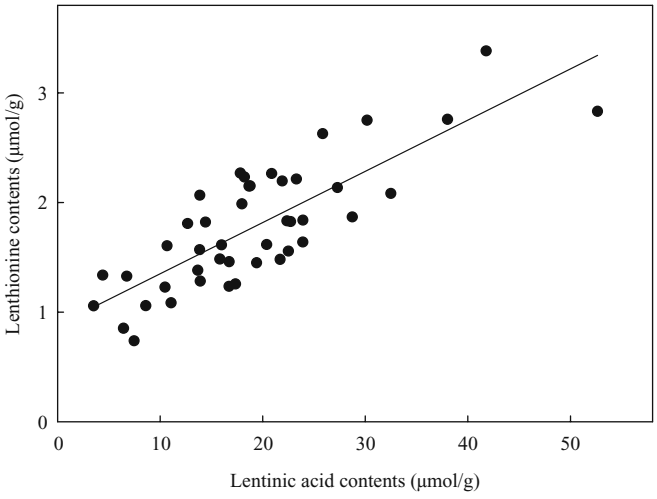

Fig. 5. Relationship between the amount of lenthionine produced on rehydration and the lentinic acid content in dried pilei. The correlation coefficient and *P* value between lenthionine and lentinic acid contents were 0.80 and <0.001, respectively. The equation for the regression line was $y = 0.046\ x + 0.833$

0.17 and 0.39, respectively. The average wet-bulb temperatures for the 40° and 60°C drying processes were 27.6° and 33.0°C, respectively. The drying characteristic curve shows that the drying of the shiitake mushroom was dominated by capillary desiccation.[20]

The inner temperature of the pilei rose rapidly from room temperature in the early stages of both drying processes (Fig. 4). The temperature in the 40°C drying process reached 32°C after 1 h, slowly rose to around 36°C after 6–11 h, and reached 40°C 2 h later. The temperature in the 60°C drying process increased to 43°C after 30 min, rose to around 52°C after 3–6 h, and reached 60°C 2 h later.

Proliferation potency against heating

Mycelial growth was detected in all tubes up to 360 min at 30°C, 240 min at 35°C, 120 min at 40°C and 20 min at 45°C (Table 1). However, growth was not detected after heating at 45°C for 40 min or at 50°C for 10 min.

Relationship between lenthionine and lentinic acid contents

The amount of LT in pilei at 40° and 60°C for 0–24 h was measured with or without rehydration. The maximum amount of LT without rehydration was 0.20 μmol/g, but the average was about 0.03 μmol/g. These results show that the amount of LT produced by drying was vary small. The amount of LT on rehydration was relatively larger than that without rehydration, and there was a highly significant positive correlation between the amount of LT on rehydration and the LA content of the dried shiitake ($r = 0.80$, $P < 0.001$, Fig. 5). The average conversion rate from LA to LT was 46 mmol/mol. The LA content could serve as an indicator of the degree of smell developed by rehydrated dried shiitake mushroom.

Table 1. Proliferation potencies of shiitake mycelia

Temperature (°C)	Heating time (min)							
	10	20	30	40	60	120	240	360
60	0							
55	0							
50	0	0						
45	100	100		0	0			
40		100	100		100	100		
35					100	100	100	
30						100	100	100

The values indicate the percentages of tubes in which mycelial growth was detected among 16 tubes

Discussion

The dried shiitake mushroom is well known for its characteristic smell, which is absent from the fresh mushroom. Odorous compounds were produced in the drying process, but the amount of LT without rehydration was very small at around 0.03 μmol/g. The smell of the dried shiitake mushroom was assessed as weak in the sensory evaluation done in the same manner as in previous studies.[17,18] To supply dried shiitake mushroom adapted to consumer preference, the amount of odorous compounds produced by the drying process alone was insufficient.

For LT production, the substrate, LA, and enzymes related to the synthesis, γ-GT and C-S lyase, had to be mixed and afterwards they had to react. γ-GT might be present in some subcellular organelles and be separated from the LA and/or C-S lyase, since the γ-GT in shiitake mushroom was present in the microsomal fraction during the purification process and could not be solubilized by ordinary methods.[19] Allicin synthesis compared well with odorous compound synthesis of dried shiitake mushroom; alliin and alliinase, a C-S lyase, were present in the cytoplasm and the vacuole,

respectively, and allicin was synthesized when the vacuoles were disrupted by physical damage.[21,22] LT production might also involve some type of physical damage, and the shiitake cells would be damaged in terms of LT synthesis initiation during the drying process, since the smell was recognized at that time. K_d is influenced by the difference between dry-bulb and wet-bulb temperature ($T_{d\text{-}w}$).[20] The K_d of the dried shiitake mushroom was 0.17 at 40°C drying ($T_{d\text{-}w}$ = 12.4°C) and 0.39 at 60°C drying ($T_{d\text{-}w}$ = 27.0°C). When the $T_{d\text{-}w}$ was 12.4° and 27.0°C, the K_d of other agricultural products, such as oats, ranged from 0.4 to 5.5 and from 1.0 to 15.7, respectively.[20] These results show that the shiitake mushroom dried at a slower rate compare to the other agricultural products. This shiitake cell disruption is seemed to proceed slowly, that consequently causes a small amount of LT production while water remained in the cells. Rehydration causes the mixture of LA, γ-GT, and C-S lyase and the consequent activation of the LT synthesis system with sufficient water.

The pH value of the rehydrating solution affected LT production, and the optimal value was 8.0.[5] The cytoplasmic pH value of shiitake mushroom was thought to be around neutral, because a neutral value has been reported for many organisms, e.g., that of *Neurospora crassa* ranged from 7.1 to 7.4.[23,24] With regard to the pH value, LT production occurred in live cells and was found during the drying process; however, only a small amount of LT was produced when the dried mushroom was rehydrated in water, because the pH value was 5.5 in 50-fold water and was reported as 6.0–6.3 regardless of the rehydrating conditions.[6] These results show that rehydration of dried shiitake mushroom should be conducted in mild alkaline solution. In home cooking, sodium hydrogen carbonate is a good reagent because it is used as a food additive and it is the main component in baking powder; the pH value of its solution is around 8.3.

The amount of LT incubated in pH 8.0 buffer was correlated with the LA content in dried shiitake. LA is a precursor of LT, and not only LT but also other sulfur-containing compounds such as 1,2,4-trithiolane are produced from LA.[4,5,9,25,26] Thus, it is reasonable that the amount of LT produced increased as the LA content increased. Interestingly, these results show that the enzyme activity was maintained in the drying process, and the amount of LT produced was defined by the LA content. LT content is a suitable indicator of the rehydrated dried shiitake mushroom smell; the LA content is higher than the amount of LT produced and could be quantified without incubation. Moreover, the amount of LT increased along with the LA content for other fruiting bodies cultivated on sawdust media (data not shown); thus the LA content in dried samples could be used in the preliminary screening to select odor-rich strains.

The decreasing water content and/or the heating may affect the increase in LA content in the drying process. The water content in the 40°C drying process changed from 89.6% to 79.3% after 4 h, when the LA content stopped increasing. If the decrease in water content was a factor in the increase in LA content, then the LA content dried at 60°C would have increased up to 2 h, because the water content reached 79.8% after 2 h. Therefore, the water content decrease did not affect the LA content increase.

The drying process usually started at 40°C. One reason was that the growth of shiitake mushroom must be stopped as soon as possible, because a live fruiting body scatters spores, which result in a soiled appearance.[27] The exact temperature at which the shiitake cells die is not known. In this experiment, proliferation potency was not lost at 40°C at least up to 120 min, but it was lost when heated at 45°C for 40 min or at 50°C for 10 min. Proliferation potency is theoretically lost at 50.4°C after 10 min; the growth of hyphae stopped at 35°C and rapidly decreased above 30°C.[28–30] The average inner temperature after 4 h in the 40°C drying process reached 33.5°C. On the other hand, it took 13 min to reach the same temperature in the 60°C drying process. Afterwards, the temperature of the pilei reached 35°C after 18 min and 45°C after 2 h. Then, the cells in the 60°C drying process would be damaged, in as much as the growth was stopped instantly and the proliferation potency was definitely lost at around 2 h 40 min. An increase in LA content would have been detected in the 60°C drying process rather than in the 40°C drying process if it were caused by random reactions originating from the break in cellular homeostasis. Therefore, moderate heating to just below the point at which mycelial growth stopped might be a factor in the increase in LA content, and LA production might be related to the response to heat.

The drying of shiitake mushrooms usually starts at 40°C; the temperature is raised to 45°C after 3–5 h, 50°C after 6–8 h, and finally to 58°–60°C after 12–16 h, and the final water content is about 8%. Water content must be below 13% according to the Japanese Agricultural Standard (Ministry of Agriculture, Forestry and Fisheries of Japan, Notice No. 1416, 1996), and the dried shiitake mushroom is highly hygroscopic.[31] These procedures were set to produce dried shiitake mushrooms of excellent quality in terms of color and shape. To increase the LA content, drying at 40°C for 4 h in the early stage is recommended.

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