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Research Article

Gene Cloning of γ-Glutamyltranspeptidase and Its Relationship to Endogenous Formaldehyde in Shiitake Mushroom (*Lentinus edodes*)

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Abstract: To reveal the relationship between theγ-Glutamyltranspeptidase (GGT) and endogenous formaldehydein shiitake mushroom based on gene level, we cloned the GGT gene from shiitake mushroom. The full-length sequence is 2063 bp contained a 5'-terminal Untranslated Region (UTR) of 36 bp, a 3'-terminal UTR of 113 bp and an ORF of 1914 bp encoding a polypeptide of 637 amino acids, which is high conserved in fungus. Quantitative real-time RT-PCR revealed the change of GGT expression along the growing process of shiitake mushroom, which increased first and then decreased ($p<0.05$). Simultaneously, the GGT activity and endogenous formaldehyde were determined and had the same trend with mRNA expression ($p<0.05$). In addition, at each growth stage the GGT expression, activity and formaldehyde were higher in stalks than in caps ($p<0.05$). The results confirmed the positive relationship between GGT and endogenous formaldehyde production, which hinted that endogenous formaldehyde could be controlled by regulating the expression of GGT and activity.

Keywords: γ-Glutamyltranspeptidase, formaldehyde, gene cloning, mushroom

INTRODUCTION

γ-Glutamyltranspeptidase (GGT) is a major enzyme of Glutathione (GSH) homeostasis and is the only peptidase catalyzing hydrolysis of peptide bonds involving a γ-glutamyl residue as well as the removal of γ-glutamyl moiety from glutathione and other related γ-glutamyl compounds to other amino acids and peptides (Tate and Meister, 1981; Taniguchi and Ikeda, 1998). GGTs are well characterized due to these important roles in anti-tumor (Kim *et al*., 2007), antioxidant (Hanigan and Ricketts, 1993; Harding *et al*., 1997), growth and development (Lieberman *et al*., 1996). Specifically, GGTs from onion (Lancaster and Shaw, 1994; Shaw *et al*., 2005) and garlic (Cho *et al*., 2012) have been studied well for their close relationship to characteristic odor formation and greening. Likely from alliums plants, GGT from shiitake mushroom is also found as one of key enzymes in the odor formation pathway, where it catalyzes the conversion of lentinic acid to methyl disulfide with the cooperation of S-alkyl-l-cysteine sulfoxidelyase (C-S lyase) and then methyl disulfide is polymerized to cyclic and linear sulfur compounds leading to the specific odor. Unfortunately, formaldehyde was found as a by-product in this odor formation path (Yasumoto *et al*., 1971a, 1971b; Iwami *et al*., 1975b, 1975c; Iwami, 1977; Chen and Ho, 1986). In addition, an investigation done by Hu (2008) shows endogenous formaldehyde in shiitake mushroom is much higher than that in most of other edible mushrooms.

To date, it is very clear about that GGT and C-S lyase are essential in the pathway of odor formation, however, only have a few literatures been published so far to report the properties of GGT (Iwami *et al*., 1975a) and its application to flavor enhancement (Iwami *et al*., 1974) and even no research of gene sequence and expression of shiitake GGT has been published yet. Although it has been revealed that formaldehyde can be produced in the odor formation pathway, there are few reports on the relationship between the GGT and endogenous formaldehyde and no effective methods of formaldehyde control have been found yet.

Thus in the present work, we cloned the gene of shiitake GGT by rapid amplification of cDNA ends (RACE). As the shiitake mushroom growing, mRNA expression of GGT was measured through the quantitative real-time RT-PCR. Simultaneously, the enzyme activity and endogenous formaldehyde were determined as well.

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MATERIALS AND METHODS

Shiitake mushroom: Shiitake mushroom used in this study was harvested from a farm in Hangzhou, China and was arranged in five growth stages. First, mark all newly formed fruiting bodies and harvest portion of them as the first stage and the rest were harvested at an interval of 12 h for the other four stages. During the whole sampling process, the temperature and the relative humidity were controlled at 20°C and 80%, respectively.

Rapid amplification of cDNA ends (RACE): Total RNA was extracted from maturated fruiting bodies with the TRIzol® reagent (Invitrogen, US) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 mg of total RNA using® II reverse transcriptase (Invitrogen, US) according to the manufacturer's instructions.

Based on the sequence of shiitake mushroom GGT, the 5' and 3' ends were obtained by using a GeneRacerTM RLM-RACE kit (invitrogen, US) according to the instruction manual. Gene-specific primer GGT-5' RACE-R1 (Table 1) and 5' RACE Outer Primer were used for the first-round 5' end PCR. The PCR program was 94°C for 4 min then 35 cycles of 30 s at 94°C, 30 s at 60°C, 2 min at 72°C. Nested PCR was conducted with the gene-specific primer GGT-5' RACE-R2 (Table 1) and the 5' RACE inner primer at 45 s amplification. The rest of the steps were identical. For the 3' end, PCR was done initially with the primer GGT-3' RACE-F1 (Table 1) and the 3' RACE outer primer. The cycling protocol of the first-round 3' RACE PCR was as follows: 94°C for 3 min then 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 2 min. Then nested PCR with the specific primer GGT-3' RACE-F2 (Table 1) and the 3' RACE inner primer under the same conditions. All PCR products were extracted from the gel and cloned into a pUCm-T vector. Five clones of each product were sequenced with the ABI PRISM® 3100 Genetic Analyzer (Applied Bio-systems, USA). The nucleotide sequences were assembled and analyzed with DNASTAR Lasergene® ver 7.1.

Phylogenetic analysis: The distance-based tree reconstruction was performed with the Neighbor-Joining (NJ) method with the alignment generated by ClustalX 1.8, using the BLOSUM weight matrix. The observed distances were corrected for multiple substitutions using Matoo Kimura's formula. 1,000 bootstrap replicates were made. The reliable branch nodes are supported by bootstrap values higher than 50%. The deduced amino acid sequence was analyzed by Misc Protein Analysis (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm¼misc1) using the GARNIER method. The protein location is predicted by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

Table 1: Sequence for primers used in cDNA cloning and real-time PCR

Primers	Sequences
GGT-5'RACE-R1	CCCAGTGACGGGGTCAAGGATTT
GGT-5'RACE-R2	CCGAATCTCGTCCGCAAACTGT
GGT-3'RACE-F1	CCAGACCCTGGTACAAGCCACGTC
GGT-3'RACE-F2	CCCGTCACTGGGATAGTAATCAA
18s-F	CGGATCTCTTGGCTCTCCCAT
18s-R	CGCAAGGTGCGTTCAAAGATTCG
GGT-F	GGAATCAACGATTGCGGACGAGAC
GGT-R	CACCTGCACACAGGAAGACCCA

Real-time PCR analysis of GGT mRNA expression at different growth stages: The expression of GGT in shiitake mushroom was determined by real-time PCR. Total RNA was isolated from fruiting bodies in different growth phases using the TRIzol® reagent (Invitrogen, US) according to the manufacturer's instructions. After treatment for 20 min at 37°C with 1 unit of DNase I (TaKaRa) to prevent genomic DNA contamination, 1 mg of total RNA was reverse transcribed using the method described above. The shiitake 18S RNA (GenBank ID: JN234840.1) was used as an internal control. Real-time PCR was done with the iQTM5 Multicolor Real-Time PCR detection system (Bio-Rad, USA). Amplification was done in a total volume of 25 mL, containing 12.5 mL of SYBR® Premix Ex TaqTM (TaKaRa, Japan), 1 mL of diluted cDNA and 1 ml of each primer (18s-F and 18s-R, GGT-F and GGT-R, Table 1). The 18s real-time PCR program was 95°C for 1 min then 45 cycles of 95°C for 10 s, 65°C for 25 s (GGT was 62°C for 25 s). The melt curve analysis of amplification products was done at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The Ct values were determined automatically by the software. All of the PCR data were analyzed with Microsoft Excel 2007 and RKWard 0.5.3, according to the Bio-Rad real-time PCR application guide. The comparative Ct method (2-ΔΔCt method) was used to analyze the expression levels of GGT (Livak and Schmittgen, 2001). All data are given in terms of relative mRNA expressed as mean±SE and $p<0.05$ was considered statistically significant.

GGT activity measurement: The activity of GGT was measured according to a previous method (Iwami *et al*., 1975c) with some modifications. The assays were performed at 37°C in 96-well microtiter plate. Each well was filled with 200 µlTris-HCl buffer (0.05 M, pH 7.0) containing purified enzyme, 10 mM γ-GPNA and 10 mMglygly. Assays were initiated with γ-GPNA and activity was measured as the formation of p-nitroaniline at 410 nm. One unit of enzyme was defined as the amount of enzyme catalyzing 1 µmol of p-nitroaniline release per minute from γ-GPNA. Protein in the extract was measured by staining with Coomassie Brilliant Blue G-250 using bovine serum albumin as a standard.

Formaldehyde measurement: Formaldehyde was extracted from shiitake mushroom according to the method of Jianrong *et al*. (2008) with some modifications. Five grams fresh fruiting bodies were homogenized with 50 mL 2% Trichloroacetic Acid (TCA) and then centrifugal at 8000 g for 10 min. To 0.5 mL supernatant adjusted pH value around 7 with 4 M NaOH, 0.5 mL 2.0 mg/mL 2,4-dinitrophenylhydrazine was added, incubating at 60°C for 60 min for formaldehyde derivatization. Formaldehyde was measured by HPLC system equipped with an ODS column of 4.6 mm×25 cm (Agilent Co., USA) and an ultraviolet detector. Analytic conditions were as follows, flow rate: 1ml/min; eluant: acetonitrile/distilled water = 70/30; column temperature: 30 °C; wavelength: 355 nm. The running time of each sample was 7 min.

Data statistics: Data are expressed as mean values (n = 3) accompanied with standard deviation. Analysis was performed with the SPSS software (version 18.0) (SPSS, Chicago, IL, USA) and the significant level is set as $p<0.05$.

RESULTS AND DISCUSSION

Characterization of the full-length GGT: The presence of GGT activity has been confirmed from bacteria to mammals and the complete full-length ORF of GGT has been found in some mammals, plants, bacillus etc. However, the sequence of GGT in edible mushrooms has not been reported.

In the present study, the cDNA sequence containing the full-length Open Reading Frame (ORF) of GGT was obtained and deposited with GenBank under accession number JX123478. The full-length sequence is 2063 bp contained a 5'-terminal untranslated region (UTR) of 36 bp, a 3'-terminal UTR of 113 bp and an ORF of 1914 bp encoding a polypeptide of 637 amino acids, which is very similar to those of other GGT proteins from NCBI. Compared with our earlier work (Li *et al*., 2012), the estimated theoretical mass, 70 kDa and pI value, 6.16 are in accord with the counterpart of 68 kDa and 6.40 of GGT purified from shiitake mushroom. The result of second structure prediction showed GGT protein contained 167 helixes, 173 sheets, 137 turns and 160 coils with the percent of 26.9, 27.9, 22.1 and 25.8%, respectively. However, there is a large difference in the second structures between the predicted value and the measured value.

Predicting by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/), shiitake GGT is probably a membrane protein, which is similar to that of Arabidopsis thaliana (Storozhenko *et al*., 2002), tomato (Martin and Slovin, 2000) and human kidney (Tate and Khadse, 1986).

Phylogenetic analysis: Based on the result of alignment from edible fungus to pathogenic fungus, the phylogenetic tree revealed the deduced amino acid sequence of GGT from shiitake mushroom had the closest relationship with GGT of tremellamesenterica and batrachochytriumdendrobatidis, but it is an independent branch (Fig. 1). The result of protein sequence alignment indicates the GGT is highly conserved in fungus (Fig. 2).

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1            ----------MTGQPDTPFELPLY----------------------------VDEKHEE
2            ------------------------------------------------------------
3            ----------MKR-GKHLPFPAD----------------------------VSEL-PL
4            ----------MKQDSVSLPSPAL----------------------------ASSK-NF
5            -------MASATYSDEKYPHQMKVN----------------------------IYNINDN
6            --MADDGFQASFREQSALNDPSSST----------------------------NQHRQPP
7            -MPSTRPPHRPSSLRSNTNSETSSI----------------------------IEPIQEE
8            -MTALRSAHRPSSLRSTANSEAGS----------------------------IEPIQEE
9            MPPIAASPPNHDENAPLLASPSLEAGTTGPPTPSPPWQFWNRPPFRRVHFADHDQFVSPA
10           ------MPSQEDESQPLLPPPSPSS----SPHPTPLWKFW-----RRISFPLPLNRVSLS
11           -----MRPNHDYEYQALIG----------------------------------------NQK
12           ------------------------------------------------------------
13           ----------MAFSHGVAASDSEK----------------------------QLLMPP

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2            ------------------------------------------------------------
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4            HPTWSLIWKW----------TLLALAV-CYASSSLAT------VFQRDTGK
5            AITTPLSVAA----------RPNARTKTFLRLLTVAG------LAVACTIA
6            RIASKPSLPP----------SRLSRSVSFVGGINHPD------ADDDYDPE
7            VVSNDGSISA----------GVEPSATETSPLLSSRRHPHSSKHHHHHLVP
8            VVSNDGQISA----------AATP-DTETSALLPSHR------HRTQLLAP
9            SAHSESELSADGNGGYGTNPKYPRQPLQQEEKMGQWMMYCLLVLVG--------MVFGAL
10           SDEADQEVRN----------KYQN----RTDRLVRILSYILILLVG--------LCIGAL
11           SAINRSSFNR----------KRWIIIIFIIFIITTS--------IAYQFV
12           ------------------------------------------------------------
13           SSAHEPNLLR----------SNRVTLIVKIAITTTAA--------ILYLAI
```


1 IVYRHTRSCG----------HHASEPEL----------
2 ----------
3 --NLISGTSS----------LTLKGP----------
4 PTSRISASVG----------AGITGIS----------
5 FIHGFTKGSMPPMEDGFYPNVVLKEPGE----------
6 REPLLSPDGQRRTKRRLTALLNSRRREERLRLRSEDREPRSWVAVVGILCSVFVVLFVIV
7 PS-RQSKPRSLCNSILVLLAIFTFAIATSIVLKN----------LLG
8 PSSHKSRSRTIWNSILVLLAIFTFAVTISIVLKN----------LLG
9 FSRNWSIKGDAGDMPMVPPVWTLPPPTG----------
10 LPRIPWRSRDVRDEPMVPPIYNLPPPTG----------
11 ISQHYN----------PETHSSFKKQ----------
12 ----------
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2 ----------
3 ----------RNPSYLVKARSGAVASENKLCSDIGVDVLKD-GGNAVD
4 ----------RNPAYLVKAYNGAVASENRLCSELGVSTLKQ-GGNAVD
5 ----------FTTMERNPAYLVEAQNGVVATENKRCSDMGVIALRK-GGSAVD
6 AAGLVVAQNPIPGSGPHHLPNGRNPSYLITAAHGAVASESETCSKVGVDILRD-GGNAVD
7 EFDDTVGD----DPGSTDPRGRRHPAVLASGRKAGVATENEVCSKVGMDVLLE-KGTAVD
8 EFNDPEDPSLPIDPPGTDPRGRRHPAVLATGRKAGVATENEICSRIGMDILLA-KGTAVD
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10 ----------LPRNPAYLINATTAAVASEDLTCSQLGVSILRDKNGTAVD
11 ----------HERNPSYLVTGWNGAVASEEARCSQIGLDVLKE-NGTATD
12 ----------MNGVVATEHPICSEVGVQVLKE-GGSAVD
13 ----------ANFGAVASESSICSTAGIDMLKK-GGNAAD
1 AAIATSLCIDVVNPFAS--GVSGGGIMTVRLPPKEDGEES----------EVWTIN
2 ----------MFSS--GIGGGGFMTVRIPPPSPGGSS----------EVYVVD
3 SAIATTFCIGVVNMFSS--GLGGGGFMTVRIPPSSPGKAS----------DMYTID
4 AAISTTFCIGVVNMFSASSGIGGGGFMTVRIPPASDNETS----------KVYTID
5 AAITATLCIGVVNSFSS--GIGGGGFMTIREPPARRGDKS----------KVFTID
6 AAISATLCIGVINMFSS--GIGGGGFMTIK-PPGE----------EAWTID
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10 AAITTTLCIGLLNGFAS--GIGGGGFLVLRAPEGTSRDVEVWKGL---EEVKEGGVVAVD
11 AAIATALCVGVVNCFST--GIGGGGFMVIKPAPCRSRQDCVSETP----------ISLD
12 AAVAAGLCIGVTNMYSS--GIGGGGFMVIRSKNGAAEY----------ID
13 AMVATVFCVGVIGMYHS--GIGGGGFALIRSPDGTYEH----------VD
: : *:.***: :: ::
1 YRETAPAAANTTMFIE-NPN---ASRFGGLASAVPGEILGLETAHQMWG-KLPWKRLVQP
2 FRETAPASASTTMYRN-NPM---GAMFGGLASGVPGELRGLEEAHKRWG-SLPWSRLVQP
3 FRETAPSLANATMYVG-RPE---SSKFGGLAVGVPGELRGLEEAHRRWG-TLSWKRLIEP
4 FRETAPTFSNKTMFKH-DPM---SARYGGLSVGVPGEIRGLEEAHRLWG-TLPWKDLIQP
5 FRGMAPAATTPTMFDNGKRN---LSESQGLSMTIPGELAGLQMAHNEWG-AMAWRDLVVP
6 FRETAPAASNATMFLK-NPL---SSTIGGLSVAVPGEIRGMAAAHDRWG-HLPWVRLFRE
7 FRETAPAAANKTMYVG-RVP---KAQFGGLAVGVPSELRGLQEAHKRYG-KLAWKRLVQP
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10 FRETSPEKVEREMYGISKAG-RVAAQVGGLAVGVPGELRGLEAAHQLYG-SLSWEELVMP
11 FRESIPG---GDLYSEKFLNDPKSSQVGGLAIGIPGELSGFDLAFRKYGGGVSWSRIFEP
12 FREEAPARSSKDMFKS-DPS---KARTGGLAVGVPGELYGYWTAHQKYG-KLPWARLVQP
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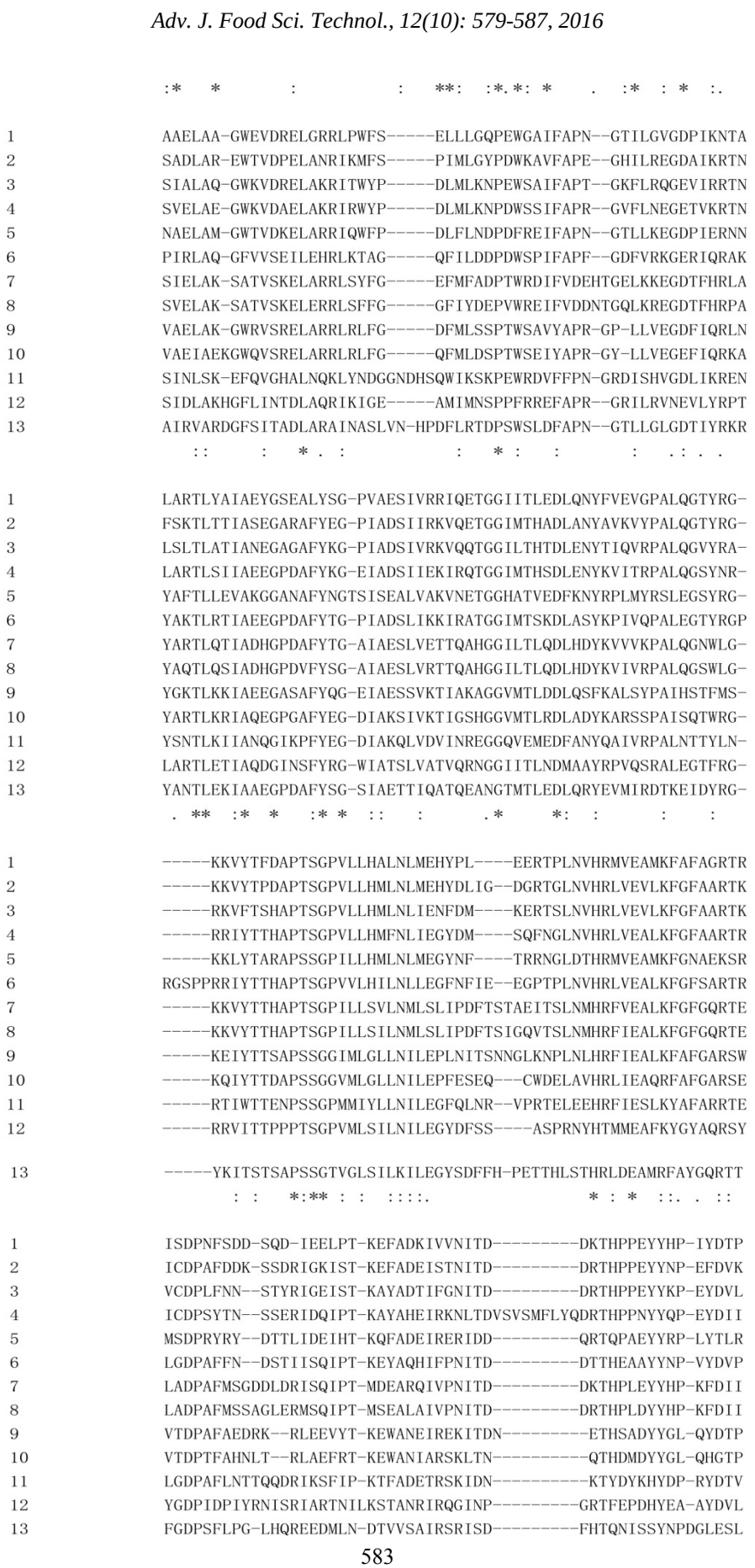
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3 SIALAQ-GWKVDRELAKRITWYP-----DLMLKNPEWSAIFAPT--GKFLRQGEVIRRTN
4 SVELAE-GWKVDAELAKRIRWYP-----DLMLKNPDWSSIFAPR--GVFLNEGETVKRTN
5 NAELAM-GWTVDKELARRIQWFP-----DLFLNDPDFREIFAPN--GTLLKEGDPIERNN
6 PIRLAQ-GFVVSEILEHRLKTAG-----QFILDDPDWSPIFAPF--GDFVRKGERIQRAK
7 SIELAK-SATVSKELARRLSYFG-----EFMFADPTWRDIFVDEHTGELKKEGDTFHRLA
8 SVELAK-SATVSKELERRLSFFG-----GFIYDEPVWREIFVDDNTGQLKREGDTFHRPA
9 VAELAK-GWRVSRELARRLRLFG-----DFMLSSPTWSAVYAPR-GP-LLVEGDFIQRLN
10 VAEIAEKGWQVSRELARRLRLFG-----QFMLDSPTWSEIYAPR-GY-LLVEGEFIQRKA
11 SINLSK-EFQVGHALNQKLYNDGGNDHSQWIKSKPEWRDVFFPN-GRDISHVGDLIKREN
12 SIDLAKHGFLINTDLAQRIKIGE-----AMIMNSPPFRREFAPR--GRILRVNEVLYRPT
13 AIRVARDGFSITADLARAINASLVN-HPDFLRTDPSWSLDFAPN--GTLLGLGDTIYRKR
:: : * . : : * : : : . : . .
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3 LSLTLATIANEGAGAFYKG-PIADSIVRKVQQTGGILTHTDLENYTIQVRPALQGVYRA-
4 LARTLSIIAEEGPDAFYKG-EIADSIIEKIRQTGGIMTHSDLENYKVITRPALQGSYNR-
5 YAFTLLEVAKGGANAFYNGTSISEALVAKVNETGGHATVEDFKNYRPLMYRSLEGSYRG-
6 YAKTLRTIAEEGPDAFYTG-PIADSLIKKIRATGGIMTSKDLASYKPIVQPALEGTYRGP
7 YARTLQTIADHGPDAFYTG-AIAESLVETTQAHGGILTLQDLHDYKVVVKPALQGNWLG-
8 YAQTLQSIADHGPDVFYSG-AIAESLVRTTQAHGGILTLQDLHDYKVIVRPALQGSWLG-
9 YGKTLKKIAEEGASAFYQG-EIAESSVKTIAKAGGVMTLDDLQSFKALSYPAIHSTFMS-
10 YARTLKRIAQEGPGAFYEG-DIAKSIVKTIGSHGGVMTLRDLADYKARSSPAISQTWRG-
11 YSNTLKIIANQGIKPFYEG-DIAKQLVDVINREGGQVEMEDFANYQAIVRPALNTTYLN-
12 LARTLETIAQDGINSFYRG-WIATSLVATVQRNGGIITLNDMAAYRPVQSRALEGTFRG-
13 YANTLEKIAAEGPDAFYSG-SIAETTIQATQEANGTMTLEDLQRYEVMIRDTKEIDYRG-
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11 -----RTIWTTENPSSGPMMIYLLNILEGFQLNR--VPRTELEEHRFIESLKYAFARRTE
12 -----RRVITTPPPTSGPVMLSILNILEGYDFSS----ASPRNYHTMMEAFKYGYAQRSY
13 -----YKITSTSAPSSGTVGLSILKILEGYSDFFH-PETTHLSTHRLDEAMRFAYGQRTT
: : *:** : : ::::. * : * ::. . ::
1 ISDPNFSDD-SQD-IEELPT-KEFADKIVVNITD---------DKTHPPEYYHP-IYDTP
2 ICDPAFDDK-SSDRIGKIST-KEFADEISTNITD---------DRTHPPEYYNP-EFDVK
3 VCDPLFNN--STYRIGEIST-KAYADTIFGNITD---------DRTHPPEYYKP-EYDVL
4 ICDPSYTN--SSERIDQIPT-KAYAHEIRKNLTDVSVSMFLYQDRTHPPNYYQP-EYDII
5 MSDPRYRY--DTTLIDEIHT-KQFADEIRERIDD---------QRTQPAEYYRP-LYTLR
6 LGDPAFFN--DSTIISQIPT-KEYAQHIFPNITD---------DTTHEAAYYNP-VYDVP
7 LADPAFMSGDDLDRISQIPT-MDEARQIVPNITD---------DKTHPLEYYHP-KFDII
8 LADPAFMSSAGLERMSQIPT-MSEALAIVPNITD---------DRTHPLDYYHP-KFDII
9 VTDPAFAEDRK--RLEEVYT-KEWANEIREKITDN--------ETHSADYYGL-QYDTP
10 VTDPTFAHNLT--RLAEFRT-KEWANIARSKLTN---------QTHDMDYYGL-QHGTP
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12 YGDPIDPIYRNISRIARTNILKSTANRIRQGINP---------GRTFEPDHYEA-AYDVL
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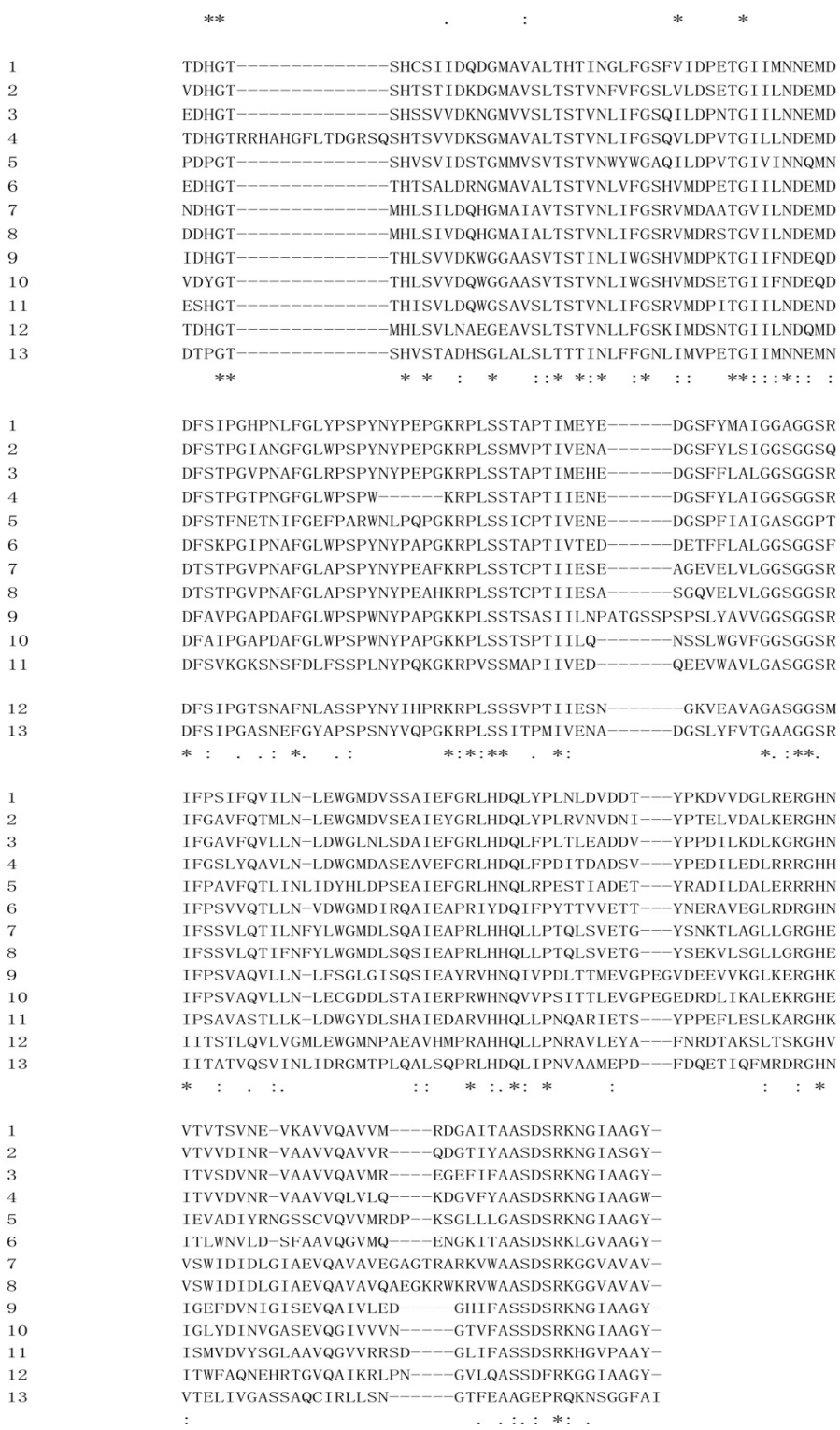

Fig. 1: Comparison of the deduced amino acid sequence of GGT from shiitake mushroom with that of other 13 fungus; The number "1,2,3,4,5,6,7,8,9,10,11,12,13" represents the Schizophyllum commune (XP_003029869.1), Serpulalacrymans (EGO00269.1), Laccaria bicolor (XP_001877715.1), Coprinopsiscinerea (XP_001830153.2), shiitake mushroom (JX123478), Piriformosporaindica (CCA67579.1), Sporisoriumreilianum (CBQ71021.1), Ustilagomaydis (XP_758438.1), Cryptococcus neoformans (XP_775747.1), Tremellamesenterica (ADO17674.1), Melampsoralaricipopulina (EGG07461.1), Batrachochytriumdendrobatidis (EGF82436.1), Penicilliummarneffei (XP_002148446.1), respectively

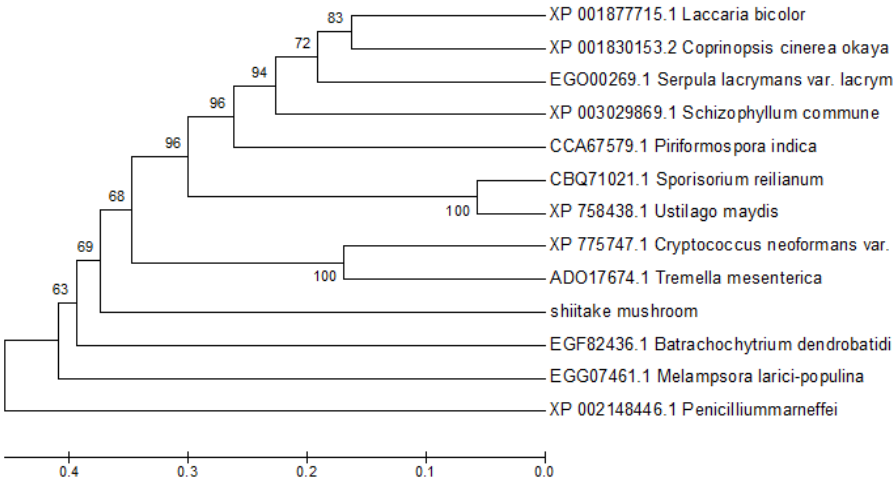

Fig. 2: Phylogenetic tree of 13 GGT complete amino acid sequences were aligned by using MegAlign

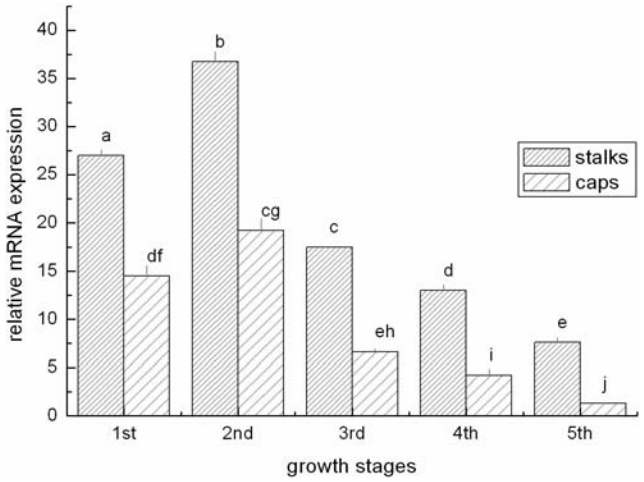

Fig. 3: Relative expression of GGT from shiitake mushroom at different growth stages; The columns with different superscript lowercase letters are significantly different ($p<0.05$)

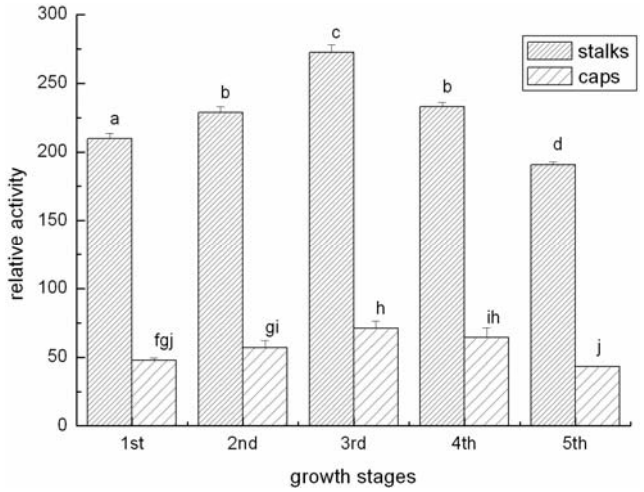

Fig. 4: Relative activity of GGT from shiitake mushroom at different growth stages; The columns with different superscript lowercase letters are significantly different ($p<0.05$), the vertical axis represents the enzyme relative activity defined as the enzyme activity per mg protein

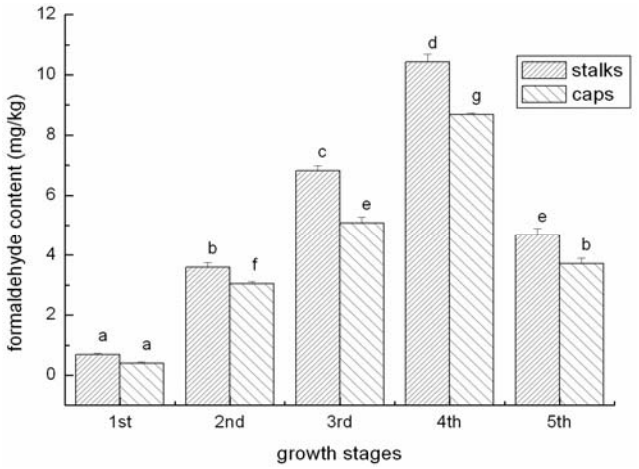

Fig. 5: Formaldehyde content in shiitake mushroom at different growth stages; The columns with different superscript lowercase letters are significantly different ($p<0.05$)

Expression of GGT: The expression of GGT mRNA in stalks and caps of shiitake mushroom at five growth stages was assessed by real-time PCR, which aims to find the change of GGT in transcription level and to deep confirm the relationship between formaldehyde production and odor formation path. Generally, GGT was expressed much more in stalks than in caps. Specifically either in stalks or in caps, the expression of GGT was increased significantly from first stage to second stage ($p<0.05$) and then decreased gradually as the fruiting body matured ($p<0.05$), with 4.8-fold maximal decline (Fig. 3). A previous work of Sakamoto *et al*. (2009) found the mRNA expression of GGT decreased gradually along the shiitake mushroom storage. When Cho *et al*. (2012) changed the garlic growth temperature from 0°C to 20°C; GGT mRNA expression was changed identically with the greening trend. All these studies indicated that the GGT expression was influenced by growth development process and environment conditions.

GGT activity: In addition to GGT mRNA expression, its activity was determined which was higher in stalks than in caps. At different growth stages, the activity changed with the same trend in stalks and caps, where it increased from the first stage to third stage and then declined gradually as the fruiting body matured (Fig. 4).

Formaldehyde content: To understand whether a correlation between the GGT and the endogenous formaldehyde exists or not, the formaldehyde content was determined. Generally at each stage, the formaldehyde in stalks was significantly higher than that in caps ($p<0.05$). In stalks, the formaldehyde increased from the minimum of 0.68 mg/kg at first stage to the maximum of 10.40 mg/kg at the fourth stage and in caps, it increased from the minimum of 0.39 mg/kg at first stage to the maximum of 8.68 mg/kg at the fourth stage (Fig. 5). This result was similar to that of two previous studies by Lin *et al*. (2002) and LeQin *et al*. (2009) and they both found that the endogenous formaldehyde increased constantly along the shiitake mushroom growing.

In addition, the highest formaldehyde content was observed at the fourth stage of shiitake mushroom, while mRNA expression and activity of GGT reached the highest level at the second stage and the third stage, respectively.

CONCLUSION

It is the first time to confirm the relationship of the GGT to endogenous formaldehyde from its transcription and translation levels in different parts and growth stages of shiitake mushroom. The results showed a positive relationship between the GGT and endogenous formaldehyde formation, which hinted us that the formaldehyde could be controlled by regulating the expression and activity of GGT in shiitake mushroom. Thus the next work will be carried out to study the regulating factors of GGT in shiitake mushroom.

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Conflict of interest: How to regulate the expression of GGT gene is the next research work we will do.

REFERENCES

Chen, C.C. and C.T. Ho, 1986. Identification of sulfurous compounds of shiitake mushroom (*Lentinus edodes* Sing.). J. Agr. Food Chem., 34(5): 830-833.

Cho, J., M. Park, D. Choi and S.K. Lee, 2012. Cloning and expression of γ-glutamyl transpeptidase and its relationship to greening in crushed garlic (*Allium sativum*) cloves. J. Sci. Food Agr., 92(2): 253-257.

Hanigan, M.H. and W.A. Ricketts, 1993. Extracellular glutathione is a source of cysteine for cells that express gamma-glutamyl transpeptidase. Biochemistry, 32(24): 6302-6306.

Harding, C.O., P. Williams, E. Wagner, D.S. Chang, K. Wild, R.E. Colwell and J.A. Wolff, 1997. Mice with genetic gamma-glutamyl transpeptidase deficiency exhibit glutathionuria, severe growth failure, reduced life spans, and infertility. J. Biol. Chem., 272(19): 12560-12567.

Hu, Z.H., 2008. Correlations between the endogenous formaldehyde and characteristic-related components in *Lentinus edodes*. TS255.2-34 Thesis, Zhejiang Gongshang University, Hangzhou, Zhejiang, China.

Iwami, K., 1977. Enzymatic development of flavor in *Lentinus edodes*. Nippon Nōgeikagaku Kaishi, 51(6): R39-R46. (In Japanese)

Iwami, K., K. Yasumoto and H. Mitsuda, 1974. γ-Glutamyltranspeptidase in edible mushrooms and its application to flavour enhancement. J. Jpn. Soc. Food Nutr., 27(7): 341-345.

Iwami, K., K. Yasumoto, K. Nakamura and H. Mitsuda, 1975a. Reactivity of Lentinus γ-glutamyltransferase with lentinic acid as the principal endogenous substrate. Agr. Biol. Chem. Tokyo, 39(10): 1941-1946.

Iwami, K., K. Yasumoto and H. Mitsuda, 1975b. Enzymatic cleavage of cysteine sulfoxide in *lentinus edodes*. Agr. Biol. Chem. Tokyo, 39(10): 1947-1955.

Iwami, K., K. Yasumoto, K. Nakamura and H. Mitsuda, 1975c. Properties of γ-glutamyltransferase from *Lentinus edodes*. Agr. Biol. Chem. Tokyo, 39(10): 1933-1940.

Jianrong, L., H. Zihao and J. Yueming, 2008. Improvement of the sampling preparation method for determining formaldehyde content in raw *Lentinus edodes*. T. Chinese Soc. Agr. Eng., 24(10): 252-254.

Kim, K.M., S.G. Lee, M.G. Park, J.Y. Song, H.L. Kang, W.K. Lee, M.J. Cho, K.H. Rhee, H.S. Youn and S.C. Baik, 2007. γ-Glutamyltranspeptidase of *Helicobacter pylori* induces mitochondria-mediated apoptosis in AGS cells. Biochem. Bioph. Res. Co., 355(2): 562-567.

Lancaster, J.E. and M.L. Shaw, 1994. Characterization of purified γ-glutamyl transpeptidase in onions: Evidence for *in vivo* role as a peptidase. Phytochemistry, 36(6): 1351-1358.

LeQin, K., C. JingRong and W. QiuShuang, 2009. Effects of cultural practices on formaldehyde content of *Lentinula edodes*. Acta Agr. Shanghai (China), 25(4): 78-81.

Li, J., J. Huang, J. Yin, N. Wu, J. Song, L. Zhang and T. Jiang, 2012. Rapid purification and characterization of γ-glutamyl-transpeptidase from shiitake mushroom (*Lentinus edodes*). J. Food Sci., 77(6): C640-645.

Lieberman, M.W., A.L. Wiseman, Z.Z. Shi, B.Z. Carter, R. Barrios, C.N. Ou, P. Chévez-Barrios, Y. Wang, G.M. Habib, J.C. Goodman, S.L. Huang, R.M. Lebovitz and M.M. Matzuk, 1996. Growth retardation and cysteine deficiency in gamma-glutamyl transpeptidase-deficient mice. P. Natl. Acad. Sci. USA, 93(15): 7923-7926.

Lin, S.Q., S.Z. Wang and Z.S. Lin, 2002. Development of formaldehyde in the shiitake mushroom in the growing, storing and drying process. Edible Fungi China, 21(3): 26-28.

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 25(4): 402-408.

Martin, M.N. and J.P. Slovin, 2000. Purified gamma-glutamyl transpeptidases from tomato exhibit high affinity for glutathione and glutathione S-conjugates. Plant Physiol., 122(4): 1417-1426.

Sakamoto, Y., K. Nakade and T. Sato, 2009. Characterization of the post-harvest changes in gene transcription in the gill of the *Lentinula edodes* fruiting body. Curr. Genet., 55(4): 409-423.

Shaw, M.L., M.D. Pither-Joyce and J.A. McCallum, 2005. Purification and cloning of a gamma-glutamyl transpeptidase from onion (*Allium cepa*). Phytochemistry, 66(5): 515-522.

Storozhenko, S., E. Belles-Boix, E. Babiychuk, D. Hérouart, M.W. Davey, L. Slooen, M. Van-Montagu, D. Inzé and S. Kushnir, 2002. Gamma-glutamyl transpeptidase in transgenic tobacco plants. Cellular localization, processing, and biochemical properties. Plant Physiol., 128(3): 1109-1119.

Taniguchi, N. and Y. Ikeda, 1998. Gamma-Glutamyl transpeptidase: Catalytic mechanism and gene expression. Adv. Enzymol. RAMB., 72: 239-278.

Tate, S.S. and A. Meister, 1981. Gamma-Glutamyl transpeptidase: Catalytic, structural and functional aspects. Mol. Cell. Biochem., 39: 357-368.

Tate, S.S. and V. Khadse, 1986. Renal gamma-glutamyl transpeptidases: Influence of glycosylation on the electrophoretic behavior and molecular weights of their subunits. Biochem. Bioph. Res. Co., 141(3): 1189-1194.

Yasumoto, K., K. Iwami and H. Mitsuda, 1971a. A new sulfur-containing peptide from *Lentinus edodes* acting as a precursor for lenthionine. Agr. Biol. Chem. Tokyo, 35(13): 2059-2069.

Yasumoto, K., K. Iwami and H. Mitsuda, 1971b. Enzyme-catalyzed evolution of lenthionine from lentinic acid. Agr. Biol. Chem. Tokyo, 35(13): 2070-2080.