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THE BIOSYNTHESIS AND POSSIBLE FUNCTION OF γ-GLUTAMINYL-4-HYDROXYBENZENE IN *AGARICUS BISPORUS*

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Key Word Index—*Agaricus bisporus*; Agaricaceae; common mushroom; biosynthesis and function; phenols; γ-glutaminyl-4-hydroxybenzene; γ-glutaminyl-3,4-dihydroxybenzene; melanogenesis.

Abstract—The occurrence of γ-L-glutaminyl-4-hydroxybenzene (GHB) and γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB) has been studied in the different developmental stages of *Agaricus bisporus*. In addition, the biosynthetic pathway of GHB has been established, GHB melanin synthesized *in vitro*, melanin isolated from mushroom spores, and an electropherogram made of spore enzymes possibly involved in melanogenesis. Whereas GHB occurs in the mycelium (if the fungus is grown on compost) as well as in the fruiting body, GDHB appears to be specific to the reproductive hyphae. The contents of GHB and GDHB in the fruit body increase with age; the spore, however, only contains a trace of GHB and no GDHB. The fruit body is autonomous with respect to the synthesis of both moieties of GHB; the phenol originates via the shikimic acid pathway. Benzoic acid and phenol are not precursors of the aromatic residue of GHB, but can be cleaved and then incorporated into the γ-glutamyl residue. GHB melanin and the *A. bisporus* melanin have similar elemental composition, almost the same IR spectrum and identical solubility properties. Hence, it is suggested that the physiological role of GHB is as a precursor of the spore wall melanin.

INTRODUCTION

The fruit body of the common mushroom contains γ-glutaminyl-4-hydroxybenzene (GHB*; [1,2]). In aqueous fungal extracts, this is readily oxidized [3,4] to γ-glutaminyl-3,4-dihydroxybenzene (GDHB [5]) and γ-glutaminyl-3,4-benzoquinone (GBQ [6]). GHB contributes 1–2% to the dry matter of the gills [2,3] and appears to be the only phenol of the *A. bisporus* fruit body occurring in any quantity [2]. It has been suggested that aromatic compounds present in fungal fruit bodies might have arisen by translocation from outside or been derived from exogenous aromatics by minor structural alterations, especially in those Basidiomycetes that degrade lignins to simpler phenolics [7]. Since the natural nutrient supply of *A. bisporus* studied here (short-composted horse manure on wheat straw basis) does not contain GHB [8] a foreign source of the phenol can be excluded. Three possibilities remain, therefore, for the origin of GHB in the fruit body of the fungus: (i) the compound might be translocated from the substrate hyphae; (ii) it might be derived from an imported aromatic compound that is generated during lignin breakdown by the vegetative mycelium [9–11]; or (iii) it could be synthesized from a simple non-aromatic compound, such as acetate or glucose. Involvement of the shikimate–chorismate pathway has been suggested [12] for the related *A. bisporus* metabolite γ-glutaminyl-4-hydroxymethylaniline (agaritine; [13–15]). In the absence of any experimental evidence, however, the possibility cannot be ruled out that GHB may originate via the acetate–malonate pathway, as holds true for the majority of the fungal phenolics [16] (though these are mostly of Deuteromycetes origin: cf. ref. [17]). Pyrrolidone carboxylate (5-oxoproline), a common constituent of Basidiomycetes [18] which also accumulates in the sporocarp of *A. bisporus* [19], or glutamic acid could yield the γ-glutamyl residue of GHB.

Several possibilities can be thought of as to the physiological function of GHB in the fruit body. Since mushroom mycelium has not been analysed in respect of GHB the question remains open whether it is translocated from the substrate hyphae and is without any function except, perhaps, antisepsis after wounding [20]. On the other hand, GHB could represent a detoxification product of a phenol resulting from the breakdown of plant lignins and transported into the fruit body, since such simple phenols are prone to be quickly oxidized by phenolase — of which a variety is present in the sporocarp [21], whereas compost-grown mycelium does not show tyrosinase activity [22] — and the melanins thus formed known to interfere with enzymes [23] specifically involved in hyphal growth (cf. ref. [24]). Furthermore, GHB might be a precursor of the mushroom spore wall melanin [25], as indicated by the changes observed in the pool size of the phenol during fruit body development and by its distribution pattern within the sporocarp [3]. Finally, the hypothesis has been put forward that GHB may be the causal agent of dormancy in the mushroom spore, several sulfhydryl-dependent enzymes thought to be the target of GBQ and of further oxidation products of the latter [20,26]. For a number of reasons given

*Abbreviations: GHB, γ-L-glutaminyl-4-hydroxybenzene; GDHB, γ-L-glutaminyl-3,4-dihydroxybenzene; GBQ, γ-L-glutaminyl-3,4-benzoquinone; agaritine, γ-L-glutaminyl-4-hydroxymethylaniline.

elsewhere [3], it is, however, unlikely that GHB and its metabolites could play this alleged role.

The present study was undertaken to establish the pathway of GHB synthesis and to, thus, also gather further evidence for the likelihood of either of the possibilities mentioned for the function of GHB in *A. bisporus*.

RESULTS

Occurrence of GHB in different developmental stages of Agaricus bisporus

Apart from the fruiting body, mycelia feeding on compost also contain GHB, regardless of whether fruiting has occurred or not. The GHB content of the hyphae is considerably higher than that of young sporocarps (Table 1). On the other hand, when grown on an artificial medium (YPD [37]) the organism does not synthesize more than a trace of GHB (*ca* 0.04 mg/g dry wt). No GHB at all was found in mycelia cultivated on a fully synthetic medium containing fructose, ammonium salts, thiamine and mineral salts (Dütsch, Schatzmann and Rast, unpublished results). At the present, this situation obviously precludes the study of GHB biosynthesis using mycelia.

The GHB content of the fruit body increases with age, reaching a maximum at the time of spore shedding, but only a trace of GHB is present in the spores. GDHB appears to be specific for the reproductive stage of the fungus. The concentration of the diphenol is always considerably smaller than that of the monophenol, though its relative proportion increases with the age of the sporocarp and reaches about one-fifth of that of GHB in the fully-opened fruit body. The spores, however, contain not even a trace of GDHB (Table 1).

Biosynthesis of GHB in the fruit body

The tracer compounds were administered in the smallest possible concentration to prevent possible induction of enzymes under the influence of the exogenous substances and so as not to interfere with the steady-state of the internal precursor pools. Tables 2 and 3 demonstrate that the fruit body can synthesize GHB and that shikimic acid proved to be an excellent precursor, being incorporated exclusively into the aromatic part of the molecule. The dilution factor of glucose was fairly high, but over two-thirds of the radioactivity found in GHB after application of the sugar was present in the phenolic moiety. The other compounds tested are metabolically quite remote from the aromatic ring of

Table 1. Occurrence of γ-glutaminyl-4-hydroxybenzene and γ-glutaminyl-3,4-dihydroxybenzene in different developmental stages of *A. bisporus*

Material analysed		GHB (mg/g dry wt)	GDHB (mg/g dry wt)	GDHB/GHB (%)
Mycelia				
	Growing on YPD	*	0.0	0
	Growing on compost			
	before fruiting	8.2	0.0	0
	after fruiting	7.0	0.0	0
Fruit body				
	Cap diam. 20 mm	4.4	0.2	5
	50 mm	5.3	0.6	11
	100 mm	6.7	1.2	18
Spores		0.2	0.0	0

* Trace (cf. text).

Table 2. Incorporation of labelled precursors into γ-glutaminyl-4-hydroxybenzene (GHB) in the *A. bisporus* fruit bodies

	Precursor fed			
	Amount (μmol)	Sp. act. (μCu/μmol × 10^3)	Sp. act. of GHB isolated (μCu/μmol)	Dilution factor* (×)
$NaH^{14}CO_3$	5.00	60	5.84	51 282
[U-^{14}C]Acetic acid	1.72	58	116.29	858
[U-^{14}C]-L-Glutamine	1.16	43	110.89	499
[U-^{14}]-L-Glutamic acid	1.32	38	101.80	493
[U-^{14}C]-L-Pyrrolidone carboxylic acid	1.25	40	106.56	469
[U-^{14}C]Glucose	0.31	327	133.17	761
[U-^{14}C]Phenol	0.52	58	4.33	6963
[(ring)U-^{14}C]Benzoic acid	0.28	108	48.95	618
[U-^{14}C]Shikimic acid	0.48	21	627.45	16

* Corrected for different precursor amounts; averages of two experiments.

Table 3. Distribution of radioactivity in ^{14}C-labelled γ-glutaminyl-4-hydroxybenzene after administration of various precursors in *A. bisporus* fruit bodies

^{14}C-Labelled precursor	Label found in the Glutamyl moiety (%)	Aromatic moiety (%)
$NaHCO_3$	99	1
Phenol	99	1
L-Glutamine	98	2
L-Glutamic acid	98	2
L-Pyrrolidone carboxylic acid	98	2
Acetic acid	95	5
Benzoic acid	94	6
Glucose	31	69
Shikimic acid	1	99

For details, see Table 2. These are the averages from two experiments.

GHB though they all contributed to its synthesis in yielding the γ-glutaminyl side chain. Pyrrolidone carboxylate was as good a precursor of the γ-glutamyl moiety of GHB as glutamine and glutamic acid. Interestingly, most of the label of benzoic acid—fed as a model compound for soluble lignin breakdown products (cf. ref. [27]) that might be transported from the hyphae into the fruit body—was not found in the aromatic moiety of GHB, but in the side chain. The very low incorporation rate of phenol into GHB is probably an experimental artefact as a large proportion of the tracer substance was converted very quickly into a dark-brown pigment, the process most certainly being catalysed by phenol oxidases leaking out at the cut base of the stipe. Colouring of the tracer solution before uptake was weak with benzoic acid and nil with the other precursors tested.

Synthesis of GHB melanin in vitro

Melanin was synthesized using GHB and a crude commercial preparation of *A. bisporus* tyrosinase (that displays also peroxidase activity: Stüssi and Rast, unpublished data), and compared with melanin isolated from mushroom spores. As judged from the C, H and N contents, solubility properties and IR spectra (Table 4 and Fig. 1), the two pigments are very similar. In the spores, phenolase as well as peroxidase is present (Fig. 2).

Table 4. Properties of *A. bisporus* melanins

Property	Spore wall melanin	GHB melanin
Solubility in		
KOH	+	+
HCl	−	−
Organic solvents	−	−
Elemental composition*	C 57.9; H 3.8 N 8.8; S 3.1	C 53.4; H 5.2 N 10.3

* Calculated on ash-free basis.

DISCUSSION

The dilution rates observed in GHB synthesis from ^{14}C-labelled shikimate, glucose, and acetate (Table 2), and the distribution of radioactivity in the two moieties of the phenol after administering these tracers (Table 3) demonstrate that GHB is derived from an intermediate of the shikimate–chorismate pathway. It is, thus, probably a metabolite of *p*-aminobenzoic acid (cf. ref. [16]), as has been suggested also for the closely related compound agaritine [12]. *p*-Aminobenzoic acid is likely to be decarboxylated and hydroxylated to yield *p*-aminophenol. There is no reason to consider the synthesis of GHB from acetate via a polyketide as an additional, though minor, pathway of GHB synthesis since the small proportion of radioactivity found in the phenolic residue of GHB after feeding labelled acetate (Table 3) is probably a result of the gluconeogenetic activity of *A. bisporus* [28]. As benzoic acid undergoes ring fission (Table 3) and, thence, is most probably converted to succinate and acetyl-CoA (via β-ketoadipate: cf. ref. [16]), the slight labelling of the aromatic residue of GHB after feeding benzoic acid might—like in the case of acetate (see above)—be due to the tracer substance being in part metabolized to the GHB precursor glucose. Whether benzoic acid is first decarboxylated and then transformed into catechol, or converted to protocatechuic acid—as in the closely related Basidiomycete *Schizophyllum commune* [29]—cannot be established on account of the present evidence. Phenol (and thus catechol) being a much poorer GHB precursor than benzoic acid (Table 2) would suggest that, as expected, the metabolism of benzoic acid to β-ketoadipate in *A. bisporus* proceeds through *p*-hydroxybenzoate and protocatechuate. However, the very low incorporation rate of the phenol might be an experimental artefact (see Results).

Glutamine, glutamic acid and pyrrolidone carboxylate display about the same dilution rate with respect to GHB synthesis (Tables 2 and 3) and this result can best be explained by assuming that they are freely interconvertible and constitute a common GHB precursor pool. Two possibilities may be considered for the formation of the peptide bond of GHB: (i) a reaction involving free glutamic acid or pyrrolidone carboxylate, or (ii) group transfer from another γ-glutamyl derivative. The occurrence in the *A. bisporus* fruit body of agaritine and of a γ-glutamyltransferase with unusual substrate specificity [30] that catalyses the formation of GHB from *p*-aminophenol in the presence of agaritine would suggest (ii) being the main route. However, the reverse may also hold: GHB could function as the γ-glutamyl donor for the synthesis of agaritine [30]. The question as to the primary origin of the γ-glutamyl residue of GHB, therefore, remains open.

Since mushroom hyphae do not synthesize GHB in media other than on compost (Table 1), the latter must contain a substance—either originally present or generated during the growth of the fungus—that represents an obligate GHB precursor or induces the formation of enzymes specifically required for its synthesis. In view of the fact that the fruit body hyphae can synthesize GHB from glucose (Tables 2 and 3) the second possibility appears more likely. Attempts for the large-scale production of GHB, which has been recently proposed as an agent for the chemotherapy of melanomacarcinoma [31], by the submerged liquid culture of mushroom

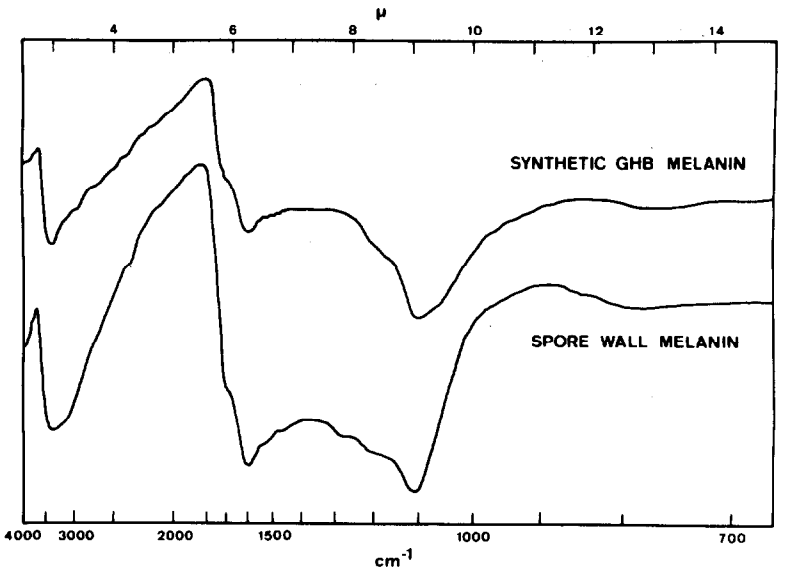

Fig. 1. Infra-red spectra of native and synthetic *A. bisporus* melanins. GHB, γ-glutaminyl-4-hydroxybenzene.

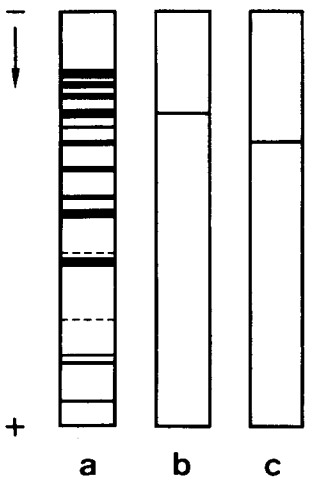

Fig. 2. Polyacrylamide gel electrophoresis of an enzyme extract from *A. bisporus* spores. a, Protein pattern; b, phenolase; c, peroxidase.

mycelium may, therefore, have to await the identification of that inducer. Despite this shortcoming, mushroom mycelium would still appear to be a material better suited for the purpose than the fruit body, because the vegetative hyphae do not oxidize GHB (Table 1).

The GHB concentration of the vegetative mycelium is higher than that of the fruit body hyphae (Table 1) and hence part of it is likely to arrive in the stipe by translocation. The decrease observed in the GHB concentration of the substrate hyphae after development of the sporocarp (Table 1) is in line with this suggestion. The mycelium, however, is most probably not the only source of the GHB present in the plectenchyme since this can synthesize the phenol itself (Tables 2 and 3). Though these results do not disclose the possibility that substances transported from the vegetative into the reproductive hyphae confer GHB-synthesizing ability to the fruit body, they do represent evidence against the hypothesis that GHB would be a detoxification product of a phenol resulting from lignin breakdown and being transported into the stipe. The observation that such compounds undergo ring cleavage in the plectenchyme (see Table 3) further underlines this statement. The mushroom fruit body disposes of both enzymes [11] supposedly involved in the cleavage of the aromatic ring (cf. ref. [27]), i.e. of peroxidase and laccase. As, besides in vegetative mycelium, the latter is only present in the stipe of the sporocarp, and as the two peroxidase isoenzymes of the fruit body are identical with the peroxidases of compost-grown mycelium, whereas hyphae grown in other media do not synthesize peroxidase [11], this may be considered responsible for ring fission.

The following observations strongly suggest that GHB has a precursor role in melanogenesis which—unless there is mechanical injury of the tissue—takes place in the lamellae only: (a) the GHB content of the fruit body increases with its age (Table 1, [3]); (b) GHB accumulates in the lamellae where, at the time of sporulation, its concentration is more than four times that of the other parts of the fruit body [3]; (c) the spores are almost devoid of GHB (Table 1, [3]); (d) GDHB—not only an intermediate in the oxidation of GHB to GBQ but also an activator of phenolase [4, 8, 32]—occurs exclusively in the hyphae of the fruit body, where its concentration increases greatly during the maturation of the carpophore (Table 1); (e) the spore disposes of both types of enzymes, i.e. of phenolase and peroxidase (cf. ref. [32]) capable of initiating the oxidative polymerization of GHB to melanin (Fig. 2); (f) the native *A. bisporus* melanin has a similar elemental composition, the same solubility properties and practically the same IR spectrum as GHB melanin (Table 4 and Fig. 1).

The *A. bisporus* melanin can, thus, be regarded as a product derived from the shikimate–chorismate pathway, as holds also for the *Aspergillus nidulans* melanin [33], whereas the corresponding pigments of *Verticillium dahliae* and *Thielaviopsis basicola* are polyketide

metabolites [34, 35]. In *Alternaria* sp., finally, acetate as well as shikimate seems to be involved in melanogenesis [36].

EXPERIMENTAL

Fungus material. Unless otherwise stated, mycelium of *Agaricus bisporus* (Lange) Sing. ATCC 382581 was grown in four-baffled shaken flasks (250 ml) containing 50 ml YPD medium [37]. Cultivation of mushrooms was as described previously [3]. Compost-free vegetative mycelium grown under natural conditions was obtained by inclusion of a 2 cm layer of glass beads (3 mm diam.) into the substrate, the insert being separated from the compost by nylon nets [8, 38]. Mushrooms designed for the simultaneous quantitation of GHB and GDHB (Table 1) were carefully collected (i.e. not cut), and immediately used for analysis in order to prevent conversion of the mono- into the diphenol and into GBQ by tyrosinase set free by wounding (cf. ref. [3]). Collection of spores was as described previously [39].

Administration of radioactive compounds. The cut stipes of freshly harvested fruit bodies (cap diam. 1.5 cm, *ca* 1.2 g fr. wt) were dipped in 0.3 ml aq. tracer soln, and the mushrooms kept in H_2O-satd atmosphere. After uptake of the soln, the labelled substance was 'washed in' completely by repeatedly adding small amounts of H_2O to the beaker. The metabolic time was 24 hr.

Isolation and quantitation of GHB and GDHB. Mycelium or plectenchyme was minced in a mortar containing liquid N_2 and extracted with 0.1 M HCl in a Sorvall Omni-Mixer, a procedure immediately inactivating tyrosinase which, *in vivo*, is normally sequestered from its substrate. Using the same extractant, spores were treated in a vibrating mill to assure sufficient breakage of the cells. After centrifugation (15000 *g*, 20 min), washing of the sediment with 0.1 M HCl, a further centrifugation, recovery of the supernatant and evaporation of the solvent, the clear extract obtained after adding H_2O was chromatographed on Sephadex G-25 and the material corresponding to the GHB and GDHB peaks collected. Details of the extraction and working-up procedures were as described elsewhere [3]. All manipulations were carried out at 0–4°. The purity of the isolated fractions was checked by UV spectrophotometry and TLC (A: Si gel Merck 60 F_{254} with *n*-BuOH–HOAc–H_2O (4:1:1) or B: EtOH–H_2O (7:3), and C: cellulose Merck F_{254} with PhOH–H_2O (3:1; w/v); 0.2% ninhydrin in EtOH, 2% $FeCl_3$ in 0.5 M HCl, and diazotized *p*-nitroaniline). GHB and GDHB amounts were determined by measuring A_{254} and A_{248}, respectively, and using calibration curves. For the isolation of GHB from fruit bodies after application of tracers (Tables 2 and 3) an additional purification step was included: prior to Sephadex chromatography the extract was passed through a Dowex 50W-X8 column (25 ml), the resin washed with H_2O (700 ml), the amino acid fraction eluted with 2 M HCl (500 ml), the acid removed *in vacuo*, and the residue dissolved in H_2O (10 ml).

Determination of labelling pattern of [^{14}C]*GHB.* The substance was isolated as described above and its purity checked by TLC (system C) followed by radioactivity scanning. Hydrolysis of the compound was by refluxing in azeotropic HCl for 1 hr, and the acid removed in a rotary evaporator at 35–40°. The products, *p*-aminophenol and glu, were separated by TLC (system A) and radioactivities determined by scanning. The corresponding zones were cut out from the plates and the compounds eluted with H_2O. Si gel was removed with a glass filter (D4) and the radioactivity of the filtrate measured by conventional liquid scintillation counting using Bray's soln [40]; (for details see ref. [8]), 90–95% of the radioactivity present in the educt was recovered in the hydrolysis products.

Polyacrylamide gel electrophoresis of spore proteins. Spores (1 g) were suspended in chilled 95% EtOH (10 ml) and disrupted at 2° in a cell mill. After centrifugation (9000 *g*, 15 min) the residue was taken up in H_2O (2 ml), the suspension stirred for 1 hr in the cold room, and centrifuged. The supernatant was subjected to PAGE using gel system No. 1 of Maurer [41] and aliquots of 0.1–0.2 ml. Tyrosinase was located using GHB (0.8 mM) in Pi buffer (0.05 M, pH 6.8), peroxidase according to ref. [11]. Proteins were made visible with Coomassie BB.

Preparation of GHB melanin. Synthetic GHB (300 mg) was dissolved in Pi buffer (300 ml, 50 mM, pH 6.8), mushroom tyrosinase (500 mg, 4000 U/mg; Sigma) added, and the mixture aerated with a bubbler, and stirred for 30 hr at 22°. The dark-pigmented, fine suspension was centrifuged (10000 *g*, 20 min) and the supernatant acidified with 1 M HCl. The black ppt. obtained after centrifugation was taken up in 0.1 M HCl, the suspension centrifuged, the washing repeated (2 ×), the sediment extracted with 20% HCl under reflux for 120 hr, and the pigment collected on a glass filter (D4), washed with 5% HCl (10 ×) and H_2O (20 ×). The product was dried *in vacuo* over P_2O_5 at 80° for 48 hr; yield, 32 mg.

Isolation of A. bisporus *melanin.* The pigment was obtained by exhaustive extraction of the fungus material with hot organic solvents and HCl according to ref. [42]. Five g spores yielded 180 mg of a black amorphous powder, corresponding to 4% (dry wt) of the spore, or to 11% of the wall in which the melanin of the cell is deposited [25].

Test and tracer compounds. GHB was synthesized and its purity checked according to ref. [2], the *p*-benzyloxyaniline being, however, of commercial origin (Fluka, Switzerland). GDHB was isolated from lamellae of sporulating mushrooms using basically the same extraction and working-up procedure as for GHB (see above), purified as described previously [3], and its identity established by TLC, colour reagents, oxidation to GBQ by tyrosinase, and determination of mp, as well as by UV and NMR: for details, see ref. [8]. In every respect, the compound behaved like agaridoxin [5]. Tracer substances were purchased from the Radiochemical Centre, Amersham.

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