Microbiology (1994), 140, 1161–1168 Printed in Great Britain

Nitrogen assimilating enzymes in the white button mushroom *Agaricus bisporus*

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Agaricus bisporus **has the enzymic potential to assimilate ammonia by the activities of glutamine synthetase (EC 6.3.1.2), NAD-dependent glutamate dehydrogenase (EC 1.4.1.2) and NADP-dependent glutamate dehydrogenase (EC 1.4.1.4). It also contains glutamate synthase (EC 1.4.7.1) and a number of transaminating activities like glutamate–oxaloacetate transaminase (EC 2.6.1.1), glutamate–pyruvate transaminase (EC 2.6.1.2) and alanine–glyoxylate transaminase (EC 2.6.1.44).** ***A. bisporus*** **showed good growth in a defined buffered medium on glucose as a carbon source and a number of organic nitrogen compounds or ammonia as a nitrogen source. No growth was observed using nitrate as a nitrogen source.** ***A. bisporus*** **was not able to use organic nitrogen containing substances as a sole nitrogen and carbon source. Specific activities of the ammonia assimilating enzymes showed some variation when mycelia were cultivated on different nitrogen sources. Highest specific activities for glutamine synthetase, NAD-dependent glutamate dehydrogenase and NADP-dependent glutamate dehydrogenase were found when mycelia were grown on glutamate as a nitrogen source. Lowest values were found when the mycelia were grown on ammonia or glutamine. The specific activities of the ammonia assimilating enzymes showed no variation during maturation of the sporophores.**

Keywords: *Agaricus bisporus*, glutamate dehydrogenase, glutamate synthase, glutamine synthetase, nitrogen metabolism

INTRODUCTION

Although a variety of nitrogen sources can be used for growth, the assimilation of ammonia into glutamate and glutamine is believed to play a central role in the nitrogen metabolism of several yeasts and fungi (Genetet *et al.*, 1984; Holmes *et al.*, 1989; Kusnan *et al.*, 1987; Lara *et al.*, 1982). Glutamate and glutamine play a prominent role in a number of vital metabolic pathways by serving as donors in transamination and amido nitrogen transfer reactions. Furthermore, glutamine and ammonia have been reported to influence nitrogen catabolism (Wiame *et al.*, 1985). Glutamine functions as a mediator in nitrogen catabolite repression, a process in which a number of nitrogen-related enzymes is repressed under conditions of sufficient nitrogen.

Ammonia assimilation in fungi is catalysed by NADP-specific glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4) with the formation of glutamate, or by glutamine synthetase (GS; EC 6.3.1.2) with the formation of glutamine. The amido group of glutamine can be incorporated into glutamate through a reaction catalysed by glutamate synthase (GOGAT; EC 1.4.7.1). Coupling of the GS and GOGAT reactions provides an irreversible route for ammonia assimilation.

Which route is used for the assimilation of ammonia seems to depend on the organism and on the growth conditions. The primary incorporation of ammonia into carbon skeletons has been shown to be catalysed by the concurrent activity of NADP-GDH and the GS/GOGAT pathway in *Cenococcum geophilum* (Martin *et al.*,

Abbreviations: CEA, compost extract agar; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; GOT, glutamate–oxaloacetate transaminase; GPT, glutamate–pyruvate transaminase; AGT, alanine–glyoxylate transaminase.

1988), *Aspergillus nidulans* (Kusnan *et al.*, 1987) and *Stropharia semiglobata* (Schwartz *et al.*, 1991). Studies with *Neurospora crassa* suggested that NADP-GDH and GS are responsible for the assimilation of ammonia when this compound is present in high concentrations (Hernandéz *et al.*, 1983). However, when the ammonia concentration is low assimilation mainly takes place by the operation of the GS/GOGAT pathway (Lara *et al.*, 1982; Lomnitz *et al.*, 1987).

Despite the fact that the common white button mushroom *Agaricus bisporus* is cultivated for economical purposes, its primary nitrogen metabolism has received relatively little attention. A review on the metabolism, biochemistry and physiology is given by Hammond & Wood (1985). Most studies that have been undertaken focussed on the post-harvest metabolism and the nutritional values of the sporophores. However, some studies have described parts of the enzymology of the nitrogen metabolism of this organism. Since the Krebs cycle appears to be blocked at 2-oxoglutarate dehydrogenase, the γ-aminobutyrate–succinate shunt is used (Hammond & Wood, 1985). Being part of this pathway, GDH appears to link the amino acid metabolism to the Krebs cycle. Moore & Al-Gharawi (1976) have found high specific activities of NAD-dependent and NADP-dependent glutamate dehydrogenase in sporophores of *A. bisporus*. Levels of amino acids and oxo-acids have been studied in mycelium grown on a defined medium using glutamate as a nitrogen source (Piquemal, 1970) and in sporophores (Latché, 1970). Both authors found high levels of transaminating activities, but they did not identify the corresponding enzymes. Glutamate appeared to be the principal donor of amino groups in both studies. However, the pathways leading to the production of glutamate have not been studied.

The aim of this study was to investigate in more detail the ammonia assimilating enzymes leading to the production of glutamate in *A. bisporus* mycelium and sporophores. Furthermore, the levels of these enzymes after growth on some selected nitrogen sources were determined.

METHODS

Organism and culture conditions. *Agaricus bisporus* strain Horst ®U1 was used throughout this study. Stock cultures were maintained at 4 °C on slants of wheat agar. Mycelium was grown at 24 °C either on agar plates with five to seven inoculation points or in static cultures with liquid medium.

Liquid medium contained 100 mM glucose, a variable amount of an appropriate nitrogen source, 20·5 mM MOPS, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 0·5 mM $CaCl_2$, 0·134 mM Na_2EDTA, 25 μM $FeSO_4$, 5 μM $ZnSO_4$, 5 μM $MnSO_4$, 4·8 μM H_3BO_3, 2·4 μM KI, 52 nM Na_2MoO_4, 4 nM $CuSO_4$, 4 nM $CoCl_2$, 0·5 μM thiamin.HCl and 0·1 μM D(+)-biotin. pH was adjusted to 6·8. The concentrations of the nitrogen sources were adjusted to the amount of nitrogen they contained so as to reach 20 mM concentrations of nitrogen atoms. Growth in a culture without an added nitrogen source was used as a control. The media were sterilized at 121 °C for 15 min and dispensed at 50 ml aliquots in 250 ml Erlenmeyer flasks. Glutamine, asparagine, cysteine, phenylalanine and glucosamine were suspected to be unstable on heating and were filter-sterilized.

The cultures were inoculated with mycelium grown for 7 d on agar plates containing compost extract medium (CEA) according to Rainey (1989) solidified with 1·5 % (w/v) Bacto-agar, overlayed with a cellophane disk and inoculated with seven inoculation points per Petri dish. The mycelium was scraped off the plates with a sterile spatula and fragmented in a Sorvall Omnimixer for 30 s. Aliquots of the homogenate were added to the liquid media.

After 21 d cultures were harvested on filter paper and dry weight was determined as well as the pH of the culture fluid. Sporophores of *A. bisporus* were obtained from mycelium cultivated on a commercially prepared compost and harvested at different stages of growth.

Preparation of cell-free extracts. Cell-free extracts were prepared by freezing the mycelium or sliced sporophores in liquid nitrogen followed by grinding the frozen material with glass beads (0·1–0·11 mm diam.) in a mortar. To 1 g of the frozen material 1 ml of extraction buffer was added. The composition of the extraction buffer depended on the enzyme studied. For determination of GDH activity a 100 mM potassium phosphate buffer pH 7·0, containing 10 % (v/v) glycerol and 2 mM β-mercaptoethanol was used. For determination of GS a 200 mM Tris/acetate buffer pH 7·4, containing 2 mM EDTA and 2 mM DTT was added. For the determination of transaminating enzymes both these buffers were applicable. For determination of GOGAT a 100 mM potassium phosphate buffer pH 7·0, containing 20 % (v/v) glycerol and 10 mM DTT was added. After addition of the extraction buffer the suspension was centrifuged at 40000 ***g*** (30 min, 4 °C). The clear supernatant was used as a cell-free extract. The protein concentration of the extact was determined using either the bicinchoninic acid protein assay kit (Sigma) as modified by Hill & Straka (1988) using bovine serum albumin as a standard or the Bio-Rad protein assay kit using bovine γ-globulin as a standard.

Assay of enzyme activities. The reductive amination activity of the glutamate dehydrogenases was determined by measuring the decrease in absorbance at 340 nm at 20 °C. For NAD-dependent glutamate dehydrogenase (NAD-GDH; EC 1.4.1.2) the reaction mixture contained 50 mM Tris/HCl buffer, pH 8·3, 0·25 mM NADH, 25 mM NH_4Cl, 50 mM 2-oxoglutarate and enzyme. The NADP-GDH activity was determined in a reaction mixture containing 50 mM Tris/HCl, pH 7·8, 0·25 mM NADPH, 25 mM NH_4Cl, 5 mM 2-oxoglutarate and enzyme.

GS activity was measured as transferase activity, synthetic activity and biosynthetic activity, using cell-free extracts desalted on an Econopac P6 column (Bio-Rad). The transferase reaction and the synthetic reaction were assayed by the formation of γ-glutamylhydroxamate as described by Ferguson & Sims (1971). The transferase reaction was measured at 20 °C in a solution containing 100 mM imidazole/acetate pH 6·5, 200 mM glutamine, 20 mM NH_2OH, 20 mM Na_2HAsO_4, 1 mM $MnCl_2$ and 0·2 mM ADP. The synthetase reaction was measured in a solution containing 100 mM imidazole/acetate pH 6·5, 40 mM glutamate, 4 mM NH_2OH, 50 mM $MgCl_2$ and 5 mM ATP.

The biosynthetic reaction was assayed by the formation of inorganic phosphate (Shapiro & Stadtman, 1970). The biosynthetic assay mixture contained 100 mM imidazole/acetate pH 6·5, 40 mM glutamate, 10 mM NH_4Cl, 50 mM $MgCl_2$ and 5 mM ATP. Glutamate and ammonia were omitted in the controls. GOGAT activity was determined at 30 °C by measuring the amount of glutamate formed, using desalted cell-free extracts (see above). The assay mixture contained 100 mM potassium phosphate pH 7·0, 20 mM 2-oxoglutarate, 10 mM L-

glutamine and 1 mM NADH. The reaction was stopped by heating at 90 °C for 5 min. Glutamate was analysed on a Hewlett Packard HP 1084 B liquid chromatography system, coupled to a HP 1046 A fluorimeter. After derivatization with *ortho*-phthaldialdehyde (OPA) according to the method of Jones & Gilligan (1983), separations took place at a flow of 0·8 ml min^{-1} on LiChrospher 100 RP-18 (5 μm) in LiChroCART 125-4. Oven temperature was 35 °C. Activities of glutamate–oxaloacetate transaminase (GOT; EC 2.6.1.1) and glutamate–pyruvate transaminase (GPT; EC 2.6.1.2) were determined at 20 °C by measuring the decrease in absorbance at 340 nm using coupled enzymic assays. The assay mixture for glutamate–oxaloacetate transaminase activity contained 200 mM Tris/HCl pH 8·5, 0·1 mM pyridoxal 5-phosphate, 20 mM aspartic acid, 5 mM 2-oxoglutarate, 0·3 mM NADH and 2 units of malate dehydrogenase (MDH; EC 1.1.1.37) in a volume of 1·0 ml. The assay mixture for glutamate–pyruvate transaminase activity contained 200 mM Tris/HCl pH 8·5, 0·1 mM pyridoxal 5-phosphate, 50 mM alanine, 10 mM 2-oxoglutarate, 0·3 mM NADH and 9 units of lactate dehydrogenase (LDH; EC 1.1.1.27) in a volume of 1·0 ml. The reactions were started by adding NADH. Alanine–glyoxylate transaminase activity (AGT; EC 2.6.1.44) was determined by the decrease in glyoxylate (Vogels & van der Drift, 1970). The assay mixture contained 200 mM Tris/HCl pH 8·5, 0·1 mM pyridoxal 5-phosphate, 40 mM alanine and 20 mM sodium glyoxylate in a volume of 1·0 ml. In all cases the amino acid was omitted in the controls.

One unit of enzyme activity (U) is defined as 1 μmol product formed min^{-1} under the incubation conditions used.

Partial purification of NAD-GDH and NADP-GDH using HPLC ion-exchange chromatography. Cell-free extract was prepared in buffer A (10 mM potassium phosphate buffer, pH 7·4, containing 10%, v/v, glycerol and 2 mM β-mercaptoethanol) and 3 ml of this solution was loaded on a Waters-Millipore Protein PAK DEAE-5PW column (0·75 × 7·5 cm). The column was washed with 47 ml of buffer A and thereafter the bound protein was eluted with a linear gradient of 10–100 mM potassium phosphate in buffer A.

Partial purification of GS using Fractogel DEAE-chromatography. A 100 ml volume of cell-free extract was saturated to 55% with $(NH_4)_2SO_4$ and centrifuged at 40000 *g* for 30 min. The precipitate was dissolved in 50 mM Tris/acetate pH 7·4, 1 mM EDTA, 1 mM β-mercaptoethanol, 5 mM glutamate and 5 μM ATP (buffer B) and the volume was adjusted to 10–15 ml. This solution was dialysed overnight against buffer B and loaded on a Merck Fractogel DEAE 650 column (1·5 × 21 cm) previously equilibrated with the same buffer. The column was washed with 50 ml of buffer B and thereafter the bound protein was eluted with a linear gradient of 0–300 mM NaCl in buffer B.

RESULTS

Demonstration of glutamate dehydrogenase activities

GDH activity could be readily detected in cell-free extracts of mycelia grown on CEA as well as in cell-free extracts of sporophores at different stage of growth. In cell-free extracts of mycelium the NAD-GDH activity was $0·235 \pm 0·144$ U mg^{-1} ($n = 5$), while in cell-free extracts of sporophores it was $0·145 \pm 0·039$ U mg^{-1} ($n = 12$). For NADP-GDH these activities were, respectively, $0·209 \pm 0·124$ U mg^{-1} ($n = 7$) and $0·106 \pm 0·034$ U mg^{-1} ($n = 13$). The values of both GDH activities were more or less constant in sporophores of different developmental stages as classified according to the system proposed by Hammond & Nichols (1976), Sporophores of developmental class 0 (primordia) to 6 (almost fully developed) were tested.

The NAD-dependent and the NADP-dependent GDH activities could be separated completely on a Protein PAK DEAE 5PW column, indicating the presence of two different enzymes. The elution pattern is shown in Fig. 1. The NAD-GDH eluted at 54 mM with 40% recovery of its activity. The NADP-GDH eluted at 88 mM potassium phosphate with 94% recovery of its activity. The chromatographic separation resulted in a 10-fold increase of specific activity for NAD-GDH. For NADP-GDH, specific activity increased 45-fold. NADP-GDH proved to be a relatively stable enzyme, showing no loss of activity after storage overnight at 4 °C. NAD-GDH was rather unstable, showing complete loss of activity after the same treatment. Stability was influenced by the type of buffer used. Activity was lost very fast in 50 mM Tris/HCl buffer (70% in 4 h). NAD-GDH could be stabilized partially by using a 50 mM phosphate buffer, pH 7·0, supplemented with 10% (v/v) glycerol. Apparent K_m values for their substrates were determined after partial purification on Protein PAK-DEAE chromatography. The results are compiled in Table 1. Both enzymes proved to be strictly specific for their coenzymes. The pH optima of the amination reactions were determined by using different buffers covering a pH range from 6·0 to 10·0. Maximal amination rates were found at pH 8·3 for NAD-GDH and at pH 7·7 for NADP-GDH. No effect of different buffers could be found. Deaminating reaction rates of the enzymes have not been determined.

For NAD-GDH optimal activity was observed at a temperature of about 37 °C while NADP-GDH activity was optimal at 47 °C.

Demonstration of glutamine synthetase activity

GS activity was shown both in CEA-grown mycelia and in sporophores. Based on the transferase reaction a specific activity of $0·017 \pm 0·007$ U mg^{-1} ($n = 7$) and $0·034 \pm 0·020$ U mg^{-1} ($n = 5$) was observed in the extracts of CEA-grown mycelia and sporophores respectively. Based on the synthetase reaction these activities were $0·012 \pm 0·002$ U mg^{-1} ($n = 3$) for extracts of mycelia and $0·011 \pm 0·004$ U mg^{-1} ($n = 5$) for extracts of sporophores. The transferase and synthetase activity of extracts of sporophores of different stages of development was about identical. When stored for 4 d at 4 °C the cell-free extract lost 10–20% of transferase activity and 40–50% of synthetic activity. Measurement of the biosynthetic activity in cell-free extracts of sporophores was difficult. The most sensitive method, a coupled assay utilizing pyruvate kinase and lactate dehydrogenase (Shapiro & Stadtman, 1970) could not be used, owing to a high ATPase or phosphatase activity of the extracts. Probably for the same reason, measurement of liberated phosphate gave background values which were about 10 times higher than the biosynthetic activity of GS. Biosynthetic activity could only be measured after ion-exchange

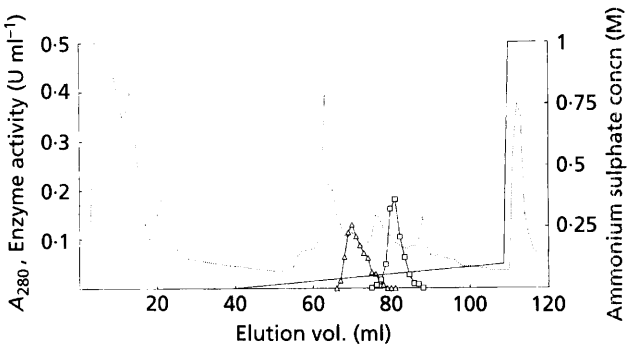

Fig. 1

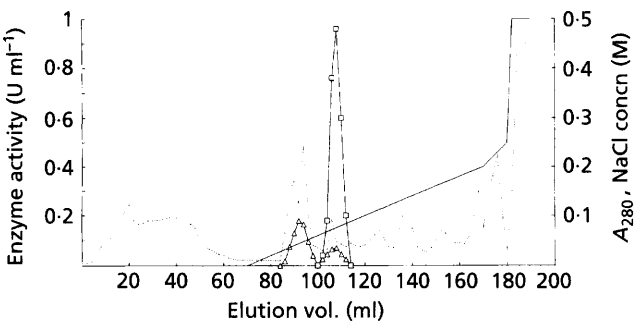

Fig. 2

Fig. 1. Separation of GDH activities on a Protein PAK DEAE column. Enzyme activities of NAD-GDH (△) and NADP-GDH (□) are indicated. ---, A_{280}; ——, ammonium sulphate gradient.

Fig. 2. Separation of GS from other γ-glutamylhydroxamate producing activities on a Fractogel DEAE column. GS transferase activity (□) and synthetase activity (△) are shown. ---, A_{280}; ——, NaCl gradient.

Table 1. Apparent K_m values for the substrates of the glutamate dehydrogenases of *Agaricus bisporus*

Values are means obtained with two different partly purified enzyme preparations.

Substrate	K_m value (mM)	
	NAD-GDH	NADP-GDH
NH_4Cl	6·5	0·5
2-Oxoglutarate	10	1·0
L-Glutamate	20	10
NAD(P)H	0·1	0·1
NAD(P)	0·5	0·7

Table 2. Apparent K_m values for the substrates of glutamine synthetase of *Agaricus bisporus*

Values are means obtained with two different partly purified enzyme preparations.

Substrate	K_m value (mM)		
	Transferase reaction	Synthetic reaction	Biosynthetic reaction
Glutamate	–	3·5	12·1
Glutamine	39	–	–
Hydroxylamine	1·5	0·2	–
Ammonia	–	–	0·15

chromatography on Fractogel DEAE. The elution profile of such a separation of cell-free extract of sporophores is shown in Fig. 2. Two different peaks of enzyme activity were detected after separation. One, which eluted at 45 mM NaCl, had a negligible transferase activity and a high synthetase activity. The other eluted at higher ionic strength (75 mM NaCl) and had both transferase and synthetase activity as well as biosynthetic activity. The ratio of activity of the latter between transferase and synthetase was 10:1. The ratio of synthetase activity and biosynthetic activity was 1:1. Obviously this enzyme is GS. Apparent K_m values for some of its substrates are shown in Table 2. The enzyme which eluted at lower ionic strength was capable of producing γ-glutamylhydroxamate in a synthetic reaction. Next to hydroxylamine this enzyme was also capable of using aminophenol as a substrate. This reaction was coupled to the hydrolysis of ATP.

Demonstration of glutamate synthase activity

The occurrence of GOGAT activity was tested in cell-free extracts of CEA-grown mycelia. First, the method proposed by Meers *et al.* (1970), using NADH as a cofactor was tried. This method measures GOGAT activity by the rate of decrease in A_{340}. A change of absorbance in both control and the reaction mixture was observed. However, the differences in the rates were very small and addition of azaserine, an inhibitor of GOGAT activity, had only minor effects. Since monitoring the change in A_{340} can give rise to misleading conclusions, we measured changes in the production of glutamate by HPLC analysis. The results are summarized in Table 3. Most glutamate was produced in the reaction mixture that contained 2-oxoglutarate, glutamine and NADH. Omitting either one of these substances from the reaction mixture resulted in a drop of the amount of glutamate formed. Furthermore, the formation of glutamate was inhibited by the addition of the glutamine analogue azaserine. Based on its substrate requirements and the inhibiting effect of azaserine we assume the glutamate-producing activity to be GOGAT. From the data obtained a specific activity of 0·019 μmol glutamate produced min^{-1} (mg protein)$^{-1}$ ($n = 2$) was

Table 3. Production of glutamate in cell-free extract as a measure of GOGAT activity

A complete reaction mixture contained 100 mM potassium phosphate buffer pH 7·0, 10% (v/v) glycerol, 5 mM dithiothreitol, 20 mM 2-oxoglutarate, 10 mM glutamine, 1 mM NADH and 200 µl desalted cell free extract (2·65 mg protein ml^{-1}) in a final volume of 1 ml. HPLC analyses were performed in duplicate and a typical result is shown.

Reaction mixture	Glutamate produced (µmol ml^{-1})
Complete	1·8
Complete + azaserine	0·3
− NADH	0·6
− Glutamine	0·0
− 2-Oxoglutarate	0·45

calculated. Using cell-free extracts we were not able to show a stoichiometric decrease of glutamine. This is probably due to the participation of glutamine in concurrent reactions like glutaminases, which also yield glutamate, or transamidases and transaminases.

Demonstration of aminotransferase activities

Transaminating activities could easily be demonstrated in cell-free extracts of both CEA-grown mycelia and sporophores, using coupled assays. The transaminating activities tested were glutamate–oxaloacetate transaminase (GOT), glutamate–pyruvate transaminase (GPT) and alanine–glyoxylate transaminase (AGT). In cell-free extracts of CEA-grown mycelia the specific activities of the transaminating enzymes were 0·233 U mg^{-1} ($n = 2$) for GOT, 0·326 U mg^{-1} ($n = 2$) for GPT and 0·030 ± 0·009 U mg^{-1} ($n = 4$) for AGT. In cell-free extracts of sporophores GOT activity was 0·185 ± 0·059 U mg^{-1} ($n = 10$), GPT activity 0·358 ± 0·099 U mg^{-1} ($n = 10$) and AGT activity 0·020 U mg^{-1} ($n = 5$). The transaminating activities were measured in sporophores at different stages of development ranging from stage 0 to stage 6 (Hammond & Nichols, 1976). Both GOT, GPT and AGT showed more or less the same specific activity at all stages of development.

Growth on monomeric nitrogen sources

To assess growth of *A. bisporus* on different nitrogen sources, mycelium was cultured in static liquid cultures for a period of 21 d at 24 °C. The medium used contained 100 mM glucose as a carbon source. The results are summarized in Fig. 3. Good growth was obtained with the amino acids asparagine, glutamine, glycine and alanine. Glutamate, arginine and aspartate were less suited as a nitrogen source. Urate, allantoin and urea also gave good yields. After having established which substances could serve as a nitrogen source, we also attempted to culture *A. bisporus* on these substances as a sole carbon and nitrogen source. None of the organic nitrogen compounds supported growth when given as sole source of nitrogen and carbon. Growth on ammonia/glucose gave a drop in pH of the culture fluid to about 2·5. In all other cases pH dropped to about 5·8–6·0. Growth yields on ammonia therefore depended strongly on the buffering capacity of the medium. Table 4 shows the effect of culturing *A. bisporus* on different ammonium salts with respect to the pH of the medium. The yield after growth on ammonium salts was comparable to growth on glutamate. Best results were obtained with $(NH_4)H_2PO_4$. Growth was also tested on different carbon sources, using 20 mM $(NH_4)H_2PO_4$ as a nitrogen source. Good growth was obtained on 100 mM glucose and 120 mM xylose. Some growth was observed with 200 mM glycerol as a carbon source. No growth was found with citrate, acetate, pyruvate, succinate, 2-oxoglutarate, mannitol and oxalate. Specific activities of NADP-GDH, NAD-GDH and GS

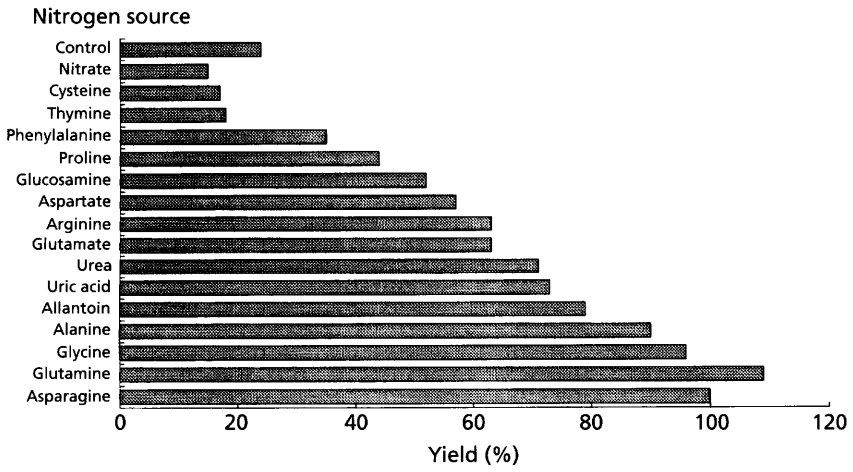

Fig. 3. Yields on monomeric nitrogen sources. Mycelia were cultured on several monomeric nitrogen sources as described in Methods. In order to correct for differences between experiments, yield on asparagine (181 ± 61 mg dry wt, $n = 3$) was set at 100%. All values shown are measurements of at least duplicate experiments.

Table 4. Growth of *Agaricus bisporus* on different ammonium salts

The values are means ± SD ($n = 3$). All cultures contained 20 mM NH_4^+, except for the control. Cultures were harvested after 12 d.

Nitrogen source	Yield (mg dry wt)	pH of culture fluid
NH_4Cl	75 ± 10	3·4
$(NH_4)_2SO_4$	74 ± 10	3·7
$(NH_4)H_2PO_4$	152 ± 15	4·1
NH_4-acetate	142 ± 17	6·4
None	15 ± 1	7·0

Table 5. Activities of ammonia-assimilating enzymes in mycelia grown on different nitrogen sources

Values are means ± SD for $n = 3$ to 9 or means of duplicate experiments ($n = 2$).

Nitrogen source	Enzyme activity (U mg^{-1})		
	NAD-GDH	NADP-GDH	GS transferase
20 mM NH_4^+	0·094 ± 0·030 ($n = 8$)	0·013 ± 0·008 ($n = 9$)	0·028 ± 0·020 ($n = 6$)
20 mM Glutamate	0·336 ± 0·104 ($n = 5$)	0·193 ± 0·070 ($n = 5$)	0·152 ± 0·024 ($n = 3$)
20 mM Glutamine	0·106 ± 0·027 ($n = 4$)	0·016 ± 0·014 ($n = 4$)	0·026 ± 0·014 ($n = 4$)
5 mM Allantoin	0·149 ($n = 2$)	0·194 ($n = 2$)	Not determined
20 mM Glycine	0·155 ($n = 2$)	0·068 ($n = 2$)	0·067 ($n = 2$)
20 mM Arginine	0·080 ($n = 2$)	0·007 ($n = 2$)	0·027 ($n = 2$)

as measured in mycelia grown on some selected nitrogen sources are shown in Table 5. With regard to NAD-GDH, the specific activities are about the same on the different organic nitrogen sources and NH_4^+, except when grown on glutamate. The specific activities of NADP-GDH and GS show large variations on the different nitrogen sources tested.

DISCUSSION

The presence of both a NAD-dependent (NAD-GDH) and a NADP-dependent glutamate dehydrogenase (NADP-GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT) in *Agaricus bisporus* is clearly demonstrated by our results. Furthermore, major transaminating activities like glutamate–oxaloacetate transaminase (GOT), glutamate–pyruvate transaminase (GPT) and alanine–glyoxylate transaminase (AGT) were detected.

LeJohn (1971) studied a large number of fungi and concluded that most higher fungi possess both NAD-GDH and NADP-GDH. Using mutant strains the crucial role of NADP-GDH in anabolism was demonstrated for *Neurospora crassa* (Fincham, 1962, and references therein) and *Aspergillus nidulans* (Kinghorn & Pateman, 1973). The participation of NADP-GDH in the assimilation of ammonia into glutamate has been described for several fungi (Hernandéz *et al.*, 1983; Kusnan *et al.*, 1987; Martin *et al.*, 1988; Schwartz *et al.*, 1991). Assessment of the phenotype of double mutants of *A. nidulans* lacking both NAD-GDH and NADP-GDH (Arst *et al.*, 1975) demonstrated for the first time the relative anabolic and catabolic roles of NADP-GDH and NAD-GDH, respectively. Miller & Magasanik (1990) recently confirmed this view for yeasts. Using mutants of *Saccharomyces cerevisiae* they showed that normally NAD-GDH is not involved in glutamate biosynthesis. According to their results NAD-GDH catalyses the major pathway for generation of ammonia from glutamate.

Our results do not allow firm conclusions regarding the metabolic role of NAD-GDH and NADP-GDH. Comparison of the K_m values for the different substrates of the glutamate dehydrogenases (Table 1) shows that NAD-GDH has a lower affinity for its substrates. Furthermore, these values do not suggest any preference for a certain reaction direction. The K_m values for the substrates of NADP-GDH might suggest that this enzyme catalyses the aminating reaction.

Measurement of GS activity in cell-free extracts of mycelium and sporophores of *A. bisporus* using spectrophotometric methods proved to be complicated. Estimations of the transferase activity and the synthetic activity of GS were hampered by the presence of other enzymes also capable of catalysing γ-glutamylhydroxamate formation. The presence of GS could only be established after partial purification of the enzyme on ion-exchange chromatography. Our results show that there are two fractions showing synthetic activity. The fraction which was eluted at 75 mM NaCl appeared to be GS. The identity of the enzyme which eluted at 45 mM NaCl is not clear. Although not able to catalyse the biosynthetic reaction, it was capable of catalysing the synthetic reaction. Apart from GS other enzymes like glutaminase and glutamine amidotransferases (Meister, 1980) as well as γ-glutamyltranspeptidase (Orlowski & Meister, 1970) have been reported to catalyse formation of γ-glutamylhydroxamate. Also, some of the enzymes involved in the synthesis of agaritine in sporophores of *A. bisporus* (Levenberg, 1970) can catalyse the formation of γ-glutamylhydroxamate. Since the unknown enzyme could use aminophenol as a substrate, a role in agaritine metabolism is suggested.

When compared to the GS from *S. cerevisiae*, the K_m values for glutamine, glutamate and hydroxylamine are more or less the same (Mitchell & Magasanik, 1983; Kim & Rhee, 1987). To allow a more precise comparison of the catalytic capacities of GS from *A. bisporus* with other fungi more detailed investigations of the enzyme are needed. GOGAT has been described to play an important role in ammonia assimilation in fungi (Schwartz *et al.*, 1991;

Kusnan *et al.*, 1987). GOGAT activity is usually detected by measuring the rate of NADH consumption from the decrease of A_{340}. This, however, may lead to wrong conclusions. For instance, concurrent action of a glutaminase and NAD-GDH may also yield production of 2 mol glutamate at the expense of 1 mol NADH. For this reason, we used a direct measurement of the glutamate formed to determine GOGAT activity. From our results we calculate a specific activity of 0·019 μmol glutamate min^{-1} mg^{-1}. This would give a specific activity of 9 nmol NADH consumed min^{-1} (mg protein)$^{-1}$. This activity is 2–4 times lower compared to the specific activities found in *Aspergillus nidulans* grown on either ammonia, glutamine or nitrate (Kusnan *et al.*, 1987) and the specific activity found by Holmes *et al.* (1989) for a number of *Candida* species and *Saccharomyces* strains.

With regard to transaminating activities, we measured high activities of GOT, GPT and AGT. Piquemal (1970) and Latché (1970) also investigated transaminating activities in *A. bisporus*. In their elaborate studies these authors found that glutamate was the principal donor of amino groups in transaminating reactions. They describe GOT, GPT and AGT to be the main transaminases, next to a number of less active transaminating activities. However, they do not report any specific activities. Using $^{15}NH_4^+$ as a tracer Schwartz *et al.* (1991) also found high activities for these transaminases in the basidiomycete fungus *Stropharia semiglobata* grown on ammonia as a nitrogen source. Our findings concerning growth on different nitrogen sources are generally in good agreement with previous authors (Treschow, 1944; Casimir & Heinemann, 1953; Bohus, 1959). These authors found good growth of *A. bisporus* on alanine, asparagine, glycine, glutamine and urea. They also found nitrate to be a poor nitrogen source. Fraser & Fujikawa (1958) reported good growth on phenylalanine and tyrosine. However, in our experiments these amino acids proved to be poor nitrogen sources. Furthermore, Casimir & Heinemann (1953) found poor growth on glutamate and aspartate. In our experiments these amino acids proved to be good nitrogen sources. Strain differences as have been noticed by Bohus (1959) can be an explanation. Such strain differences may perhaps extend to carbon nutrition. In contrast with the findings of Treschow (1944) and Bohus (1959) we found no growth on organic acids as a carbon source, while using $(NH_4)H_2PO_4$ as a nitrogen source.

Acidification of the medium while using ammonia as a nitrogen source has also been described by Treschow (1944) and Bohus (1959). This acidification might be associated with the uptake of ammonium by the cells. Huth *et al.* (1990) describe a similar acidification of the medium while culturing *Candida maltosa* and *Candida albicans*, using ammonia as a nitrogen source. In their experiments, they show that the uptake of ammonia is accompanied by the release of a proton from the cell. Excessive acidification is often avoided by use of ammonium (+)-tartrate as nitrogen source (Pateman, 1969; Pateman & Kinghorn, 1976), stabilizing the pH at about 5. However, optimum growth of *A. bisporus* occurs at a pH of 6·5. Therefore use of ammonium acetate (this study) or $(NH_4)_2HPO_4$ (Treschow, 1944) is more suitable for this fungus. The preference shown by *A. bisporus* towards asparagine, glutamine, alanine, glycine and to a lesser extent ammonia as a nitrogen source is not uncommon to fungi. Lundeberg (1970) investigated the utilization of various nitrogen sources by mycorrhiza-forming fungi and some litter-decomposing fungi. He concluded that asparagine and ammonia were most readily utilized as a nitrogen source. He also claims that although many groups of fungi are able to use nitrate as a nitrogen source, most of the higher basidiomycetes are not able to do so.

In *A. bisporus* the activities of NAD-GDH, NADP-GDH and GS are clearly influenced by the nitrogen source used for cultivation of the mycelium. NADP-GDH and GS appear to be repressed by ammonia and glutamine, while being derepressed by glutamate. NAD-GDH activity is less influenced by the nitrogen source.

Responses of the glutamate dehydrogenases from *N. crassa*, *A. nidulans*, *A. niger*, *Fusarium oxysporum*, *Coprinus lagopus* and *Schizophyllum commune* towards different nitrogen sources have been reviewed by Smith *et al.* (1975). The derepression of NAD-GDH activity by glutamate is shown by most fungi. With regard to NADP-GDH the responses can be very different. High levels of NADP-GDH in response to growth on ammonia have been reported for *N. crassa*, *A. niger* and *F. oxysporum*. In these organisms NADP-GDH is repressed by glutamate. In *S. commune* however, like in *A. bisporus*, NADP-GDH seems to be repressed by ammonia and derepressed by glutamate. With regard to the responses of GS activity towards different nitrogen sources *A. bisporus* resembles most other fungi. GS activity in *A. nidulans* and *N. crassa* (Pateman, 1969) and in *Candida utilis* (Ferguson & Sims, 1974) is derepressed when grown on glutamate and repressed when grown on glutamine or ammonia.

Summarizing, the nitrogen assimilating enzymes of *A. bisporus* have been clearly identified. Further study will focus on regulatory mechanisms and more detailed characterization of the nitrogen metabolizing enzymes.

ACKNOWLEDGEMENTS

We thank Mr Jenniskens from the Mushroom Experimental Station for a continuous supply of commercially grown mushrooms.

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Received 13 October 1993; revised 19 November 1993; accepted 25 November 1993