Investigations into Enzymes of Nitrogen Metabolism of the Ectomycorrhizal Basidiomycete, *Suillus bovinus*

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Dedicated to Professor André Pirson on the occasion of his 90th birthday

Suillus bovinus, Glutamate Dehydrogenase, Glutamine Synthetase/Glutamate Synthase, Aminotransferases, Urease

Axenic mycelia of the ectomycorrhizal basidiomycete, *Suillus bovinus*, were grown in liquid media under continuous aeration with compressed air at 25 °C in darkness. Provided with glucose as the only carbohydrate source, they produced similar amounts of dry weight with ammonia, with nitrate or with alanine, 60–80% more with glutamate or glutamine, but about 35% less with urea as the respectively only exogenous nitrogen source.

In crude extracts of cells from NH_4^+-cultures, *NADH-dependent glutamate dehydrogenase* exhibited high aminating (688 nmol × mg protein^{-1} × min^{-1}) and low deaminating (21 nmol × mg protein^{-1} × min^{-1}) activities. Its K_m-values for 2-oxoglutarate and for glutamate were 1.43 mM and 23.99 mM, respectively. pH-optimum for amination was about 7.2, that for deamination about 9.3. *Glutamine synthetase* activity was comparatively low (59 nmol × mg protein^{-1} × min^{-1}). Its affinity for glutamate was poor (K_m = 23.7 mM), while that for the NH_4^+ replacing NH_2OH was high (K_m = 0.19 mM). pH-optimum was found at 7.0. *Glutamate synthase* (= GOGAT) revealed similar low activity (62 nmol × mg protein^{-1} × min^{-1}), K_m-values for glutamine and for 2-oxoglutarate of 2.82 mM and 0.28 mM, respectively, and pH-optimum around 8.0. *Aspartate transaminase* (= GOT) exhibited similar affinities for aspartate (K_m = 2.55 mM) and for glutamate (K_m = 3.13 mM), but clearly different K_m-values for 2-oxoglutarate (1.46 mM) and for oxaloacetate (0.13 mM). Activity at optimum pH of about 8.0 was 506 nmol × mg protein^{-1} × min^{-1} for aspartate conversion, but only 39 nmol × mg protein^{-1} × min^{-1} at optimum pH of about 7.0 for glutamate conversion. Activity (599 nmol × mg protein^{-1} × min^{-1}), substrate affinities (K_m for alanine = 6.30 mM, for 2-oxoglutarate = 0.45 mM) and pH-optimum (6.5–7.5) proved *alanine transaminase* (= GPT) also important in distribution of intracellular nitrogen.

There was comparatively low activity of the obviously constitutive enzyme, *urease*, (42 nmol × mg protein^{-1} × min^{-1}) whose substrate affinity was rather high (K_m = 0.56 mM). *Nitrate reductase* proved substrate induced; activity could only be measured after exposure of the mycelia to exogenous nitrate.

Routes of entry of exogenous nitrogen and tentative significance of the various enzymes in cell metabolism are discussed.

Introduction

In mycorrhiza, the symbiotic association of plant roots and fungal mycelia, both partners take various advantages from each other. A very important interdependence is the exchange of nutrients (see: e.g. Harley and Smith, 1983; Cairney and Chambers, 1997). The fungus profits by provision with photosynthetically produced carbohydrates, while the tree root benefits from a special supply with inorganic, but also organically bound nitrogen (e.g. Bowen and Theodorou, 1973; Lewis, 1976; Pateman and Kinghorn, 1976; Martin *et al.,* 1987; Tinker *et al.,* 1994).

Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEAE, triethanolamine hydrochloride; TRICINE, (N-Tris-[hydroxymethyl]methylglycine); TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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For growth of isolated mycorrhizal fungi, various exogenous nitrogenous sources have been found suitable. Among them there are inorganic molecules, such as ammonia and nitrate, and organic molecules, such as urea, proteins and several amino acids, as well (e.g.: Peeler and Mullins, 1981; France and Reid, 1984; Littke *et al.*, 1984; Abuzinadah *et al.*, 1986; Read *et al.*, 1989; Finley *et al.*, 1992; Sarjala, 1999). For *de novo* synthesis of cellular proteins, nitrogen of all these sources has to be incorporated into amino acids, of course, and amino acids have also been found to be transferred to the tree root. Among them, glutamine has been reported on first place repeatedly (Harley and Smith, 1983; Martin *et al.*, 1987). Also transfer of alanine, glutamate and aspartate has been observed (Melin and Nilsson, 1953; Martin *et al.*, 1986). From this, enzymes, such as glutamate dehydrogenase, glutamine synthetase, and also glutamate synthase (= glutamine-2-oxoglutarate aminotransferase), have been examined intensively by various authors in the past (e. g. Pateman and Kinghorn, 1976; Stewart *et al.*, 1980; Ahmad and Hellebust, 1991; Botton and Chalot, 1995). Beside these ammonia metabolizing enzymes, also those catalyzing processes which produce NH_4^+ for incorporation, such as nitrate and nitrite reductases and urease for hydrolytic cleavage of urea, have been under investigation (Ho and Trappe, 1980; Plassard *et al.*, 1984a,b; Sarjala, 1990).

The various literature data available result from examinations of several different organisms, i. e., as far as we know, there is no comprehensive information yet on the respective enzymatic equipment of one species. Therefore, we decided to take up over-all investigations into the enzymatic equipment for basic carbohydrate and protein metabolism of the ectomycorrhizal partners, *Suillus bovinus* and *Pinus sylvestris*. Starting with the isolated fungus, we proved existence and elucidated some kinetic properties of all glycolytic enzymes, recently (Kowallik *et al.*, 1998). Studies on the fate of pyruvate, the endproduct of glycolysis, are under way. In this paper, we present data on ammonia liberating and ammonia incorporating enzymes of *Suillus bovinus*, sustaining the fungus' growth, but also producing nitrogenous compounds for potential transfer to its mycorrhizal partner.

Material and Methods

Axenic cultures of *Suillus bovinus* (L. ex Fr.) O. Kuntze, Boletaceae, were used. Mycelia, isolated from fruiting bodies collected at Sennefriedhof/Bielefeld by Dr. U. Röder, Department of Ecology, University Bielefeld, Germany, were kept on 1.2% agar plates prepared with nutrition solution (see below) at room temperature in the dark (= stock cultures).

Growth conditions

Mycelia were grown in liquid media. Samples of stock cultures were transferred to nutrition solution after Kottke *et al.*, (1987) containing 10 g/l glucose as carbon source: $(NH_4)_2HPO_4$, 500 mg/l; KH_2PO_4, 500 mg/l; $MgSO_4 \times 7\ H_2O$, 150 mg/l; $CaCl_2$, 50 mg/l; KCl, 37.28 mg/l; NaCl, 25 mg/l; H_3BO_3, 15.46 mg/l; $MnSO_4 \times H_2O$, 8.45 mg/l; $ZnSO_4 \times 7\ H_2O$, 5.75 mg/l; $CuSO_4 \times 5\ H_2O$, 1.25 mg/l; $FeCl_3 \times 6\ H_2O$, 1 mg/l; $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$, 0.18 mg/l; thiamine hydrochloride, 0.1 mg/l. Nitrogen was supplied either as an inorganic (NH_4^+ or NO_3^-), or as an organic (alanine, glutamate, glutamine or urea) molecule. pH of the media was set to 5.0 in the beginning. It dropped with time in all cases except of glutamate. The autoclaved (5 min at 1.2 bar) media were inoculated with suspended hyphae and the resulting suspensions filled in sterilized (4 h at 150 °C) culture tubes with an inlet for aeration at their bottom. Length of tubes was 45 cm, ∅ 4 cm. For growth, tubes were placed in a water bath of 25 °C in the dark and the suspensions were aerated continuously with compressed air.

Preparation for enzymatic analysis

3–4 days after inoculation, mycelia were harvested on filter paper in a Buchner funnel. The resulting pellet was resuspended in 0.1 M phosphate-buffer pH 7.5 and cells were broken by grinding with sea sand in a mortar under cooling with liquid nitrogen. After separation from sand and large fragments by filtration through 4 layers of cheese cloth, the resulting homogenates were centrifuged for 20 min at 20 000 x *g* and 4 °C (Sorvall RC-5 Superspeed Refrigerated Centrifuge). The resulting supernatants were used as crude extracts.

Enzyme assays

All enzyme assays, based on literature as indicated below, were optimized for the *Suillus* crude extract; i.e., maximum *in vitro* activities (= capacities) were determined. Optimum concentrations for substrates and cofactors, optimum pH, and appropriate concentrations of auxiliary enzymes and of artificial electron donors and acceptors had to be determined. These data will not be presented here in detail. It shall only be mentioned that activities of all enzymes could be improved over those determined with literature tests developed for different living materials.

Glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase, EC 1.4.1.3) (after Schmidt, 1974; Smith *et al.*, 1975)

Enzyme activity has been determined in both directions. Aminating action was analyzed by following decrease in absorbance at 334 nm resulting from NADH oxidation due to reductive amination of 2-oxoglutarate by the enzyme. Deaminating action was analyzed by following increase in absorbance at 334 nm resulting from NAD^+ reduction due to 2-oxoglutarate oxidation resulting from oxidative deamination of glutamate by the enzyme.

Assay 1: 50 mM HEPES-buffer pH 7.25, 200 mM NH_4Cl, 10 mM 2-oxoglutarate (start), 110 µg crude cell extract protein/ml test volume, 0.4 mM NADH.

Assay 2: 100 mM TRIS-glycine-buffer pH 9.35, 100 mM glutamate (start), 145 µg crude cell extract protein/ml test volume, 0.3 mM NAD^+.

Glutamine synthetase (glutamate-ammonia ligase [ADP-forming], EC 6.3.1.2) (after Rowe *et al.*, 1970; Meister, 1974)

The so-called „synthetic reaction" (see Miflin and Lea, 1982; Brun *et al.*, 1992) using hydroxylamine instead of NH_4^+ and resulting in γ-glutamylhydroxamic acid instead of glutamine formation by the enzyme has been used. γ-glutamylhydroxamic acid was determined colorimetrically at 546 nm after formation of a yellow-brown complex with $FeCl_3$ (see Mecke, 1985).

Assay: 100 mM HEPES-TRICINE-buffer pH 7.0, 12.5 mM $MgSO_4$, 137.5 mM L-glutamate, 10 mM ATP, 150 µg crude cell extract protein/ml test volume, 12 mM hydroxylamine (start). Reaction was stopped with equal volume as assay volume of stop reagent (0.2 M trichloroacetic acid, 0.67 N HCl, 0.37 M $FeCl_3$) after 30 min at 30 °C. Within 30 min, 5 min centrifugation at 4 300 x *g* and determination of absorbance at 546 nm in supernatant had to be performed.

Glutamate synthase (NADH) (= glutamine-2-oxoglutarate aminotransferase)

(L-glutamate:NAD^+ oxidoreductase [transaminating], EC 1.4.1.14) (after Miller and Stadtman, 1972; Kowallik and Neuert, 1984)

Absorbance changes at 334 nm resulting from NADH oxidation due to reductive transfer of the amido group of glutamine to 2-oxoglutarate by the enzyme were determined.

Assay: 220 mM phosphate-buffer pH 8.0, 100 mM glutamine, 2.0 mM 2-oxoglutarate (start), 110 µg crude cell extract protein/ml test volume, 0.2 mM NADH.

Aspartase (= fumaric aminase) (L-aspartate-ammonia lyase, EC 4.3.1.1) (after Tokushige, 1985)

Absorbance changes at 240 nm resulting from fumarate produced by deamination of aspartate by the enzyme were determined.

Assay: 100 mM TRIS-HCl-buffer pH 7.9, 66 mM Na-aspartate, 27 µg crude cell extract protein/ml test volume (start). For control, accumulation of the second reaction product, NH_4^+, was also determined at up to 300 µg crude cell extract protein/ml test volume.

Procedure: Enzyme reaction was stopped by addition of 40 µl 3 M trichloroacetic acid/ml test volume. After centrifugation (10 min, 15 000 x *g*), supernatant was neutralized with NaOH and 0.5 ml samples were tested for NH_4^+ by absorbance changes at 334 nm dependent on reductive amination of 2-oxoglutarate with glutamate dehydrogenase (Boehringer test, cat. no. 1112732).

Aspartate transaminase (= glutamate-oxaloacetate transaminase [GOT]) (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) (after Bergmeyer and Bernt, 1974a)

Action of the enzyme has been examined in both directions. Transfer of the amino group of aspartate to 2-oxoglutarate – leading to oxaloacetate and glutamate – was followed by decrease in absorbance at 334 nm resulting from NADH oxidation by oxaloacetate reduction catalyzed by added malate dehydrogenase. The opposite reaction, transfer of the amino group of glutamate to oxaloacetate, was analyzed by increase in absor-

bance at 334 nm resulting from NAD^+ reduction by oxidation of 2-oxoglutarate by added 2-oxoglutarate dehydrogenase. Formation of NADH by oxidative deamination of glutamate by endogenous glutamate dehydrogenase and concomittant consumption of NADH from reduction of oxaloacetate by endogenous malate dehydrogenase had to be considered.

Assay 1: 220 mM phosphate-buffer pH 8.0, 100 mM aspartic acid, 20 mM 2-oxoglutarate (start), 25 µg crude cell extract protein/ml test volume, 5 units/ml malate dehydrogenase (GOT-free!), 0.2 mM NADH.

Assay 2: 100 mM phosphate-buffer pH 7.0, 100 mM glutamate, 2.0 mM oxaloacetate (start), 117 µg crude cell extract protein/ml test volume, 0.4 units/ml 2-oxoglutarate dehydrogenase, 0.3 mM NAD^+.

Alanine transaminase (= glutamate-pyruvate transaminase [GPT]) (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) (after Bergmeyer and Bernt, 1974b)

Absorbance changes at 334 nm resulting from NADH oxidation due to pyruvate reduction by added lactate dehydrogenase were determined. Pyruvate resulted from transfer of the amino group of alanine to 2-oxoglutarate by the enzyme.

Assay: 100 mM phosphate-buffer pH 7.5, 207 mM alanine, 20 mM 2-oxoglutarate (start), 25 µg crude cell extract protein/ml test volume, 0.4 units/ml lactate dehydrogenase, 0.2 mM NADH.

Urease (urea amidohydrolase, EC 3.5.1.5) (after Schlegel and Kaltwasser, 1974)

Absorbance changes at 334 nm resulting from NADH oxidation due to reductive amination of 2-oxoglutarate by ammonia liberated from urea by the enzyme were determined.

Assay:100 mM TEAE-buffer pH 8.0, 500 mM urea, 26 mM 2-oxoglutarate, 9 units/ml glutamate dehydrogenase + 0.4 mM ADP (activator), 0.2 mM NADH, 110 µg crude cell extract protein/ml test volume (start).

Nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.3) (after Hagemann and Reed, 1980)

Enzyme activity was determined by 2 methods - 1. by absorbance changes at 334 nm resulting from NADH oxidation due to nitrate reduction to nitrite by the enzyme; 2. by absorbance at 540 nm of a coloured product resulting from a reaction of nitrite produced with Griess-Ilosvay's reagent.

Assay: 100 mM K-phosphate-buffer pH 7.4, 1.8 mM KNO_3 (start), 0.2 mM NADH, 300 µg crude cell extract protein/ml test volume. For nitrite determination, above assay mixture was incubated for 15 min at 30 °C. Thereafter, reaction was stopped by rapid addition of 1.25 ml sulfanilamide-N-(1-naphthyl)ethylenediamine reagent (1 vol. 0.5 g sulfanilic acid in 100 ml 2 N acetic acid plus 2 vol. 100 mg naphthylethylenediaminedichloride in 200 ml 2 N acetic acid) to 0.5 ml of assay mixture. After 10 min, absorbance of colour developed was determined.

Soluble protein

Soluble protein in crude extracts was determined according to Lowry *et al.* (1951) with bovine serum albumin as reference.

Fast performance liquid chromatography (FPLC)

0.5 ml of crude cell extract, filtered through a 0.2 µm sterile filter (Sartorius GmbH, Göttingen Germany), were applied to a Superose 6 (glutamine synthetase) or Superose 12 (glutamate synthase) column (HR 10/30, Pharmacia, Uppsala Sweden). The column was equilibrated and eluted with 100 mM HEPES-buffer pH 7.5 in case of glutamine synthetase and with 220 mM phosphate-buffer pH 8.0 in case of glutamate synthase, respectively. Fractions of 250 µl were collected at a flow rate of 1 ml $\times$ min^{-1} at room temperature. For molecular weight determination, the column was calibrated with following standards: thyroglobulin dimer 1338 kDa, thyroglobulin 669 kDa, ferritine 440 kDa, catalase 232 kDa, aldolase 158 kDa, albumin 67 kDa, hemoglobin 64.5 kDa, chymotrypsin 25 kDa, myoglobin 17.2 kDa, and cytochrome c 12.2 kDa.

Ion exchange chromatography

Crude cell extract was separated on DEAE Sephacel (column 2.2 $\times$ 8 cm) using 50 mM TRIS-HCl-buffer pH 7.8 + 6 mM dithiothreitol + 1 mM EDTA for elution. The column was washed with 2 bed volumes of buffer and developed with a linear KCl gradient (0–0.6 M). 1.6 ml fractions were collected at a flow rate of 45 ml $\times$ h^{-1}. KCl densitiy in fractions was calculated from index of refraction

(Abbe universal refractometer, Schmid and Haensch, Berlin Germany).

Results

Growth of mycelia

In axenic liquid cultures of mycelia of *Suillus bovinus*, various exogenous nitrogen sources led to significant, but clearly different increases in dry weight. Table I shows the data obtained 4 days after inoculation. They are slightly (see Kowallik *et al.*, 1998) affected by different changes in pH during growth, but probably also by different access to nutrients and oxygen because of variable aggregation. The earlier described (Kowallik *et al.*, 1998) yellowish-white, soft „balls" of hyphae existed at growth with NH_4^+, with nitrate and with alanine, but were replaced by fine white downy flakes at growth with glutamate and with glutamine.

Table I. Increase in dry weight of axenic cultures of mycelia of *Suillus bovinus* supplied with different nitrogen sources and pH of media at harvest. Growth conditions: liquid inorganic medium with glucose as sole carbon source and with indicated nitrogen sources, initial pH 5.0, continuous aeration with compressed air, 25 °C, darkness, (n = 6).

Nitrogen source	Relative increase in dry weight [%]	pH at harvest
$(NH_4)_2HPO_4$	100	3.11 ± 0.06
Nitrate	116 ± 8	3.96 ± 0.08
Alanine	107 ± 7	4.67 ± 0.05
Glutamate	182 ± 10	5.55 ± 0.09
Glutamine	160 ± 18	3.65 ± 0.04
Urea	65 ± 9	4.57 ± 0.17

Enzymes of nitrogen metabolism

Table II shows maximum *in vitro* activities (= capacities) and kinetic data, such as K_m- and $S_{0.5}$-values indicating half maximum substrate concentration for enzymes with and without Michaelis Menten kinetics, respectively and Hill coefficients for indication of cooperativities, for several enzymes involved in incorporation and metabolism of the various nitrogen sources.

Glutamate dehydrogenase exhibits largest aminating activity. Its affinity for 2-oxoglutarate is rather high, while that for NH_4^+ is about 4 times lower. Neither substrate seems to be converted cooperatively. Deaminating activity of the enzyme is found extremely low and its affinity for glutamate is more than 15 times smaller than that for 2-oxoglutarate. In both directions, the enzyme is strictly dependent on NADH/NAD^+.

Glutamine synthetase, determined by the so-called „synthetic reaction", shows high affinities for ATP and for hydroxylamine, but low affinity for glutamate. Similar results for glutamate and for NH_4^+ have already been reported by Stewart *et al.* (1980) for another fungus. There is indication for positive cooperativity of hydroxylamine (n = 1.67), but this does not necessarily include positive cooperativity of the true substrate, NH_4^+. There are no indications for isoforms, known to exist in higher plants and in algae, (e.g. Hirel and Gadal, 1980; Meya and Kowallik, 1995; Mäck, 1998). Fig. 1 (upper) shows only one peak of glutamine synthetase activity after separation on DEAE-Sephacel column, Fig. 1 (lower) the same after FPLC on Superose 6 of crude cell extracts. The

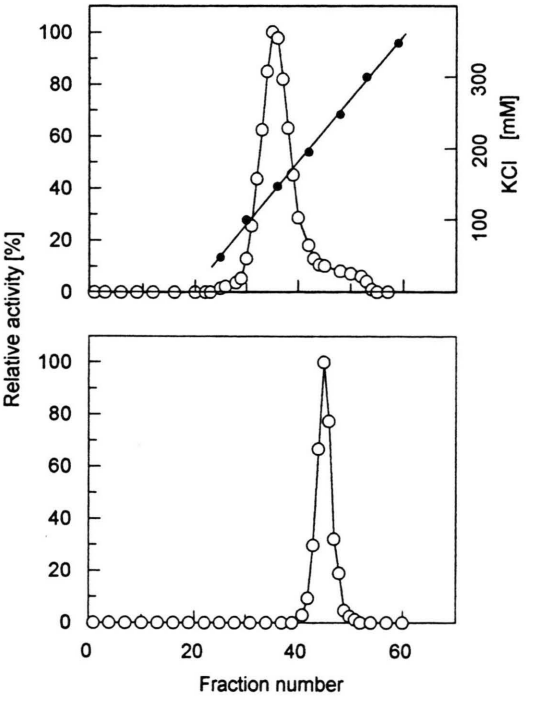

Fig. 1. Distribution of glutamine synthetase activity in crude extracts of *Suillus bovinus* after separation on (upper) DEAE-Sephacel (elution buffer: 50 mM TRIS-HCl + 6 mM DTT + 1 mM EDTA pH 7.8), and (lower) on Superose 6 (elution buffer: 0.1 mM HEPES pH 7.5).

Table II. Activities, apparent K_m- or $S_{0.5}$-values and Hill coefficients of enzymes in nitrogen metabolism of *Suillus bovinus*. Growth conditions: liquid inorganic medium with glucose as sole carbon source and ammonia as sole nitrogen source, initial pH 5.0, continuous aeration with compressed air, 25 °C, darkness. For preparation of crude cell extracts and enzyme assays see Material and Methods.

Enzyme	Activity [nmol × mg protein^{-1} × min^{-1}]	$K_m(S_{0.5})$ [mM]		Hill coefficient
Glutamate dehydrogenase (NADH):		2-oxoglutarate:	1.43 ± 0.09	1.08
aminating	688.4 ± 33.3	NH_4^+:	5.98 ± 0.40	0.94
deaminating	21.3 ± 3.2	glutamate:	23.99 ± 1.28	1.04
Glutamate dehydrogenase (NADPH)	no activity detected			
Glutamine synthetase		glutamate:	23.71 ± 1.96	1.19
synthetic reaction	59.4 ± 5.1	hydroxylamine:	0.19 ± 0.01	1.67
		ATP:	1.53 ± 0.18	1.03
Glutamate synthase (=GOGAT)	62.3 ± 3.7	glutamine:	2.82 ± 0.18	1.00
(NADH)		2-oxoglutarate:	0.28 ± 0.01	1.02
Glutamate synthase (NADPH)	no activity detected			
Glutamate synthase (Ferredoxin)	no activity detected			
Aspartase	no activity detected			
Aspartate transaminase (=GOT)		aspartate:	2.55 ± 0.17	0.99
NH_2-donor = aspartate	506.2 ± 28.7	2-oxoglutarate:	1.46 ± 0.08	1.08
NH_2-donor = glutamate	39.1 ± 4.8	glutamate:	3.13 ± 0.08	1.04
		oxaloacetate:	0.13 ± 0.02	0.99
Alanine transaminase (=GPT)	599.4 ± 36.0	alanine:	6.30 ± 0.64	0.97
		2-oxoglutarate:	0.45 ± 0.03	1.10
Urease	41.5 ± 2.7	urea:	0.56 ± 0.03	0.97
Nitrate reductase	no activity detected			

enzyme of *Suillus bovinus* exhibits an apparent molecular mass of 595 ± 25 kDa.

Glutamate synthase could only be detected dependent on NADH. The, in general, additionally known electron donors NADPH or reduced ferredoxin never revealed any measurable activity in crude extracts of *Suillus*. Total maximum activity of the NADH-dependent enzyme corresponds with that of glutamine synthetase, indicating the generally assumed close connection of both these enzymes also in this organism. Separation on Superose 12 revealed two peaks of active protein showing molecular masses of about 300 kDa and of about 1000 kDa. Both of them exhibited comparable sensitivity towards the glutamate synthase inhibitor, azaserine. Whether they represent native species or whether the heavier form is an aggregation product of preparation, cannot be decided.

Aspartate transaminase (= glutamate-oxaloacetate transaminase) exhibits more than 10 times higher activity with aspartate than with glutamate as NH_2-donor. At no big differences in the affinities and no indications for any homotropic cooperativity, this corresponds with the generally assumed preferred aspartate converting function of the enzyme (but see Discussion). Comparable results have been obtained by Khalid *et al.* (1988) for *Cenococcum geophilum*.

Alanine transaminase (= glutamate-pyruvate transaminase) shows also rather high activity

and – as in all other cases – lower affinity for its nitrogenous substrate than for the NH_2 accepting 2-oxocarbonic acid.

Activity and affinity of *urease* are comparatively low. Both were not improved by prolonged cultivation with urea. The enzyme, therefore, appears to be constitutive.

Absence of *nitrate reductase* activity – inspite of growth with this nitrogen source (see Table I) – is puzzling on first view. However, as in higher plants and also in some fungi, the enzyme turned out to be induced by its substrate. When grown with nitrate as the only nitrogen source, crude extracts of *Suillus bovinus*, revealed activity of nitrate reductase which was exclusively NADPH-dependent.

In the artificial buffer systems used, all enzymes exhibited pH-optima between pH 7 and pH 8. Only deamination by glutamate dehydrogenase took place best at pH 9.35. In most cases, pH-dependences exhibited rather sharp optima, i. e. enzyme activity dropped by 50% at pH changes of less than one order. Less pronounced optima existed for alanine transaminase and – towards alkaline values – for glutamine synthetase (Fig. 2).

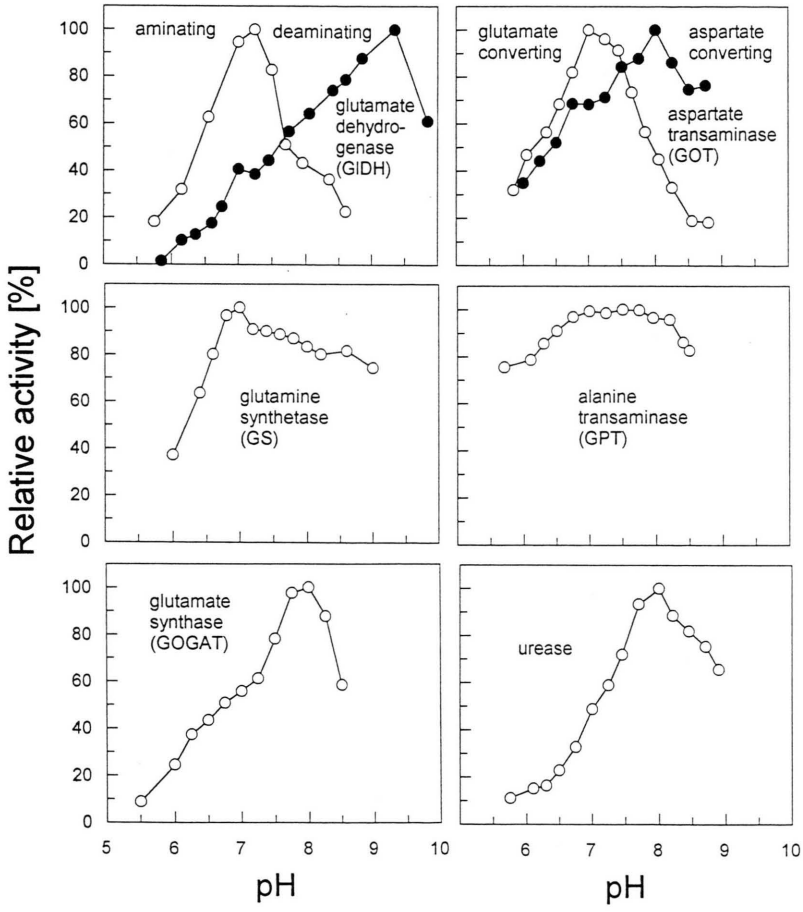

Fig. 2. pH-dependences of the activities of enzymes in nitrogen metabolism of axenic mycelia of the ectomycorrhizal basidiomycete, *Suillus bovinus*. Growth conditions: liquid inorganic medium with glucose as sole carbon source and $(NH_4)_2HPO_4$ as sole nitrogen source, initial pH 5.0, continuous aeration with compressed air, 25 °C, darkness. For enzyme assays see Material and Methods. Buffers used: HEPES = glutamate dehydrogenase (aminating), HEPES-TRIS = glutamine synthetase, phosphate = glutamate synthase, aspartate transaminase, alanine transaminase, nitrate reductase, phosphate and TRIS-glycine = glutamate dehydrogenase (deaminating), TEAE = urease.

Discussion

The strict dependence on NADH/NAD^+ of glutamate dehydrogenase of our *Suillus bovinus* isolates is most remarkable different from respective reports in literature which describe either NADH- plus NADPH-dependent or only NADPH-dependent glutamate dehydrogenases for various mycorrhizal fungi (*Sphaerostilbe repens*, Botton and Msatef, 1983; *Cenococcum graniforme*, Martin *et al.*, 1983, 1988; *Laccaria bicolor*, Ahmad *et al.*, 1990; Chalot *et al.*, 1991; *Stropharia semiglobata*, Schwartz *et al.*, 1991). Rudawska *et al.* (1994) report the NADPH-dependent enzyme species even for *Suillus bovinus*. Using isolates from Poland, this discrepancy can only be taken as another example of the repeatedly discussed metabolic differences in various isolates of presumably identical fungal species.

Proof of constitutive existence of urease appears to be novel, too. At least, we are not aware of any respective detailed report in literature.

In contrast, observation of the inductive character of nitrate reductase is not new; it is just another example for this property also in mycorrhizal fungi (Plassard *et al.*, 1984a,b; Sarjala 1990). In our experiments, 2–3 days exposure to nitrate yielded marked increase in cell mass and also nitrate reductase activity. Absence of isoforms of glutamine synthetase has also been reported before (*Laccaria bicolor*, McNally and Hirel, 1983). The molecular mass of the *Suillus* enzyme corresponds to that of some bacteria (Miller and Stadtman, 1972) and cyanobacteria (Srivastava and Amla, 1997); it is however almost two times larger than that reported for higher plants (Hirel and Gadal, 1980; Mäck, 1998), unicellular algae (Meya and Kowallik, 1995) and the fungus *Neurospora crassa* (Kapoor *et al.*, 1969).

Concerning respective significances of the various enzymes *in vivo*, the data obtained do not lead to unequivocal conclusions. Being determined in the same crude cell extract, i.e. based on identical total protein contents however, they should allow relative comparison of enzyme actions. A widely and controversially discussed question concerns incorporation of inorganic nitrogen, NH_4^+, into organic molecules of an organism. Reductive amination of 2-oxoglutarate and amidation of glutamate are the respective reactions.

The high aminating (688 nmol × mg $protein^{-1}$ × min^{-1}) and low deaminating (21 nmol × mg $protein^{-1}$ × min^{-1}) activities of glutamate dehydrogenase – together with the enzyme's good affinity for 2-oxoglutarate (K_m = 1.43 mM) and poor affinitiy for glutamate (K_m = 23.99 mM), clearly point – although its affinity for NH_4^+ is rather poor (5.98 mM) – to NH_4^+ incorporating rather than glutamate degrading action of the enzyme in *Suillus*. pH optima of about 7.2 for amination and of about 9.3 for deamination support this assumption. Glutamine synthetase exhibits much better affinity for the NH_4^+ replacing NH_2OH (K_m = 0.19 mM), indeed. Therefore, calculating arbitrary numbers for intracellular binding of ammonia of both enzymes by deviding activities into K_m-values, leads with relative numbers of about 100 for glutamate dehydrogenase and about 300 for glutamine synthetase to preferred involvement of the latter. However, respective NH_4^+ acceptors might not equally be available in both cases. While 2-oxoglutarate ought to be produced sufficiently by tricarboxylic acid cycle (unpublished data), glutamate might be scarce because of the rather low activity of glutamate synthase at the presumably intracellular pH of about 7. Since the affinity for glutamate of glutamine synthetase is rather poor (K_m = 23.71 mM), efficiency of the latter might be adversely affected. Summing up, there is good reason to assume that in *Suillus bovinus*, NADH-dependent glutamate dehydrogenase is mainly responsible for glutamate formation and not for glutamate degradation, as discussed in literature (Ahmed and Helleburst, 1991). Glutamate ought to be additionally produced by the glutamine synthetase/glutamate synthase system. Distribution of incorporation of exogenous nitrogen among both enzymes cannot reliably be decided.

The comparatively high aspartate-dependent activity of aspartate transaminase (506 nmol × mg $protein^{-1}$ × min^{-1}) certainly points to the already mentioned preferred action of aspartate, not glutamate conversion by the enzyme. But search for enzymes producing aspartate as product of NH_4^+ incorporation was unsuccessful. Neither amination of fumarate, nor reductive amination of oxaloacetate could be detected. Therefore, inspite of the much smaller glutamate-dependent activity (39 nmol × mg $protein^{-1}$ × min^{-1}), we have to assume mainly glutamate as NH_2-donor for the en-

zyme, *in vivo*. The much better affinity for oxaloacetate (K_m = 0.13 mM) than for 2-oxoglutarate (K_m = 1.46 mM), and the pH-optimum for glutamate conversion at about 7.0, and for aspartate conversion at about 8.0, certainly support this assumption which is also not contradicted by similar K_m-values for aspartate and for glutamate.

Unfortunately, technical reasons prevented us from examination of glutamate dependence of alanine transaminase. We, therefore, can only assume, that also this enzyme may function preferrably glutamate converting. A central position of glutamate in basic nitrogen metabolism of *Suillus bovinus* – as it is known for various other organisms – is certainly indicated by all data obtained.

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