Nitrogen metabolism in the commercial mushroom *Agaricus bisporus*

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General Introduction

The common white button mushroom (*Agaricus bisporus*) is one of the most well-known mushrooms since it is produced in large quantities for human consumption The long history of its cultivation is reviewed by Spencer (1985) Mushroom cultivation started about 1630 near Paris but only in recent decades it has developed into an industry Today the main mushroom producing countries are USA, France, the Netherlands, the United Kingdom and Italy In 1993 about 6000 people were employed by the Dutch mushroom growing industry and the annual production was estimated at 190 million kg of mushrooms (Van Horen, 1994) Annual productions in France and the United Kingdom in 1993 were estimated at 216 and 115 million kg, respectively (Desrumaux, 1994)

THE LIFE-CYCLE AND GENERAL BIOLOGY OF THE COMMON WHITE BUTTON MUSHROOM (*Agaricus bisporus*)

Features of the biology of *A. bisporus* have been reviewed by Elliott (1985) *A. bisporus* belongs to the class of Basidiomycetes and its life-cycle is shown in Fig 1 Raper *et al* (1972) used nutritionally deficient mutants to study the life cycle of *A. bisporus* carefully and found it to show a number of variations on the typical basidiomycete life-cycle In the typical basidiomycete life-cycle a network of hyphal filaments (the mycelium) arises from a germinating spore This mycelium (the monokaryon) is septate with uninucleate cells and is not normally capable of producing fruit bodies In order to generate mycelium which is capable of producing fruit bodies two genetically compatible homokaryotic mycelia must interact To be genetically compatible the two monokaryotic mycelia should bear a different mating type Interaction results in hyphal fusions between the two mycelia (plasmogamy) In the resultant mycelium (the dikaryon) the cells are typically binucleate and as the cells divide a special "clamp connection" is formed After cell division nuclei can migrate through these connections in order to maintain the binucleate nature of the mycelium Clamp connections are a characteristic feature of Basidiomycetes although the mycelia of many basidiomycete fungi lack clamp connections A second characteristic feature of Basidiomycetes is the formation of spores on differentiated hyphal tips, the basidia The basidium is the site of the meiotic nuclear division and each spore receives a single nucleus from this meiosis So a basidium of a "typical" basidiomycete fungus bears four spores

A major difference between the life-cycle of *A. bisporus* and of the "typical" basidiomycete is found in the fertility of the spores Only a small minority of germinating *A. bisporus* spores produce an infertile mycelium and the mycelium originating from these spores is the equivalent of the normal basidiomycete homokaryon After hyphal fusion with a mycelium bearing a different mating type a fertile mycelium (the equivalent of a heterokaryon) is produced In contrast, the majority of the *A. bisporus* spores form a mycelium which is capable of producing fruit bodies without the need for interaction with mycelium bearing another mating type After germination the mycelia colonize their substrate and when the correct environmental conditions are met the mycelia will develop fruit bodies, on which basidia are formed In the *A. bisporus* life-cycle no asexual spores are formed

The self-fertility of the mycelium originates from the special nature of *A. bisporus* spores, basidia of *A. bisporus* normally bear two spores During fruit body development the number of nuclei in the basidium is reduced to two nuclei per cell, one of each mating type These nuclei will fuse and undergo a meiosis, resulting in four post-meiotic nuclei The four nuclei are then distributed over

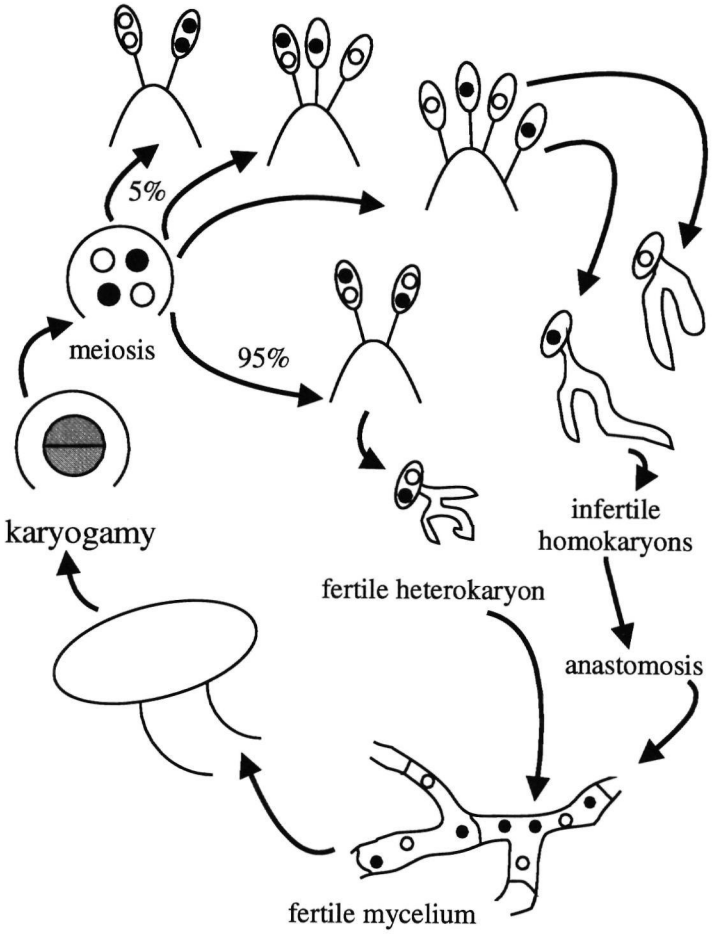


Fig. 1. The life-cycle of *A. bisporus* taking into account the typical frequencies of aberrant basidia (Adapted from Elliot, 1985)

two spores. Migration of the post-meiotic nuclei into the spores is non-random. Spores containing nuclei bearing a different mating type are about two orders of magnitude more common than spores containing nuclei of the same mating type (Evans, 1959, Kerrigan *et al.*, 1993). As a result the majority of these spores will, after germination, produce a fertile mycelium. This type of life cycle is called secondarily homothallic. The life cycle of the "typical" basidiomycete fungus is called heterothallic. Of the basidiomycetes studied so far about 90% are heterothallic.

Although 95% of the basidia bear two spores, three- and four-spored basidia are also found. Most of these spores will contain only nuclei of one mating type.

The number of nuclei per cell represents another variation on the typical basidiomycete life cycle.

As the *A. bisporus* spores germinate they will form a multinucleate mycelium instead of an uninucleate mycelium The exact amount of nuclei in an *A. bisporus* cell is highly variable with values ranging from 1 to 25 Because of the multinucleate nature of *A. bisporus* mycelium the terms monokaryon and dikaryon are not valid The mycelium can be better described as homokaryon and heterokaryon, respectively

A third distinguishing feature is the absence of clamp connections in the *A. bisporus* mycelium In the binucleate mycelium of typical basidiomycete fungi a precise one to one ratio is maintained with respect to the mating type of the nuclei The nuclei are associated in pairs and divide synchronously Such synchronous division of nuclei has not been observed in *A. bisporus* The nuclei within the *A. bisporus* mycelium are of two types with respect to mating type and it is assumed that they are present in approximately equal numbers However the young basidium contains just 2 nuclei, one of each mating type, which suggests that there must be a mechanism to maintain a 1 to 1 ratio of nuclear types

THE CULTIVATION OF THE COMMON WHITE BUTTON MUSHROOM (*Agaricus bisporus*)

Compost preparation

A. bisporus is grown commercially on a compost which is prepared from a mixture of horse manure, wheat straw, chicken manure and gypsum (Fermor *et al.*, 1985, Van Griensven, 1988) and the main characteristics of its cultivation are depicted in Fig 2 The composting process involves two phases Phase I is an outdoor composting process, lasting for 3 weeks, during which easily degradable substrates are consumed mainly by thermophilic bacteria Phase II is an indoor process a pasteurization period (56-60°C during 6 h) is followed by conditioning at approximately 45°C during 8-9 days During conditioning a flora of thermophilic fungi develops in the compost (Ross & Harris, 1983) Furthermore the conditioning phase is used to lower the levels of free NH_3 which is inhibitory to growth of *A. bisporus*

The basic nutritional demands of *A. bisporus* are largely known (Wood & Fermor, 1985) and the chemical composition of compost can easily fulfill these demands However the chemical composition of the compost by itself does not seem to guarantee proper growth of *A. bisporus* Successful colonization and use of the compost by *A. bisporus* strongly depends on ecological properties, usually referred to as "compost selectivity" It is not clear how this selectivity can be described in chemical or biological terms Compost selectivity was recently investigated by Straatsma *et al.* (1989) They reported that the linear growth rate of *A. bisporus* on sterilized compost is almost doubled by pre-incubation of the compost with the thermophilic fungus *Scytalidium thermophilum* Furthermore *S. thermophilum* protects against negative effects of compost bacteria on mycelial growth of *A. bisporus* The nature of the growth promoting effect is unknown *S. thermophilum* is the most dominant species at the end of Phase II and it is postulated that this fungus provides for compost selectivity In experimental composts a positive relation was found between the logarithm of mushroom yield of *A. bisporus* and the degree of colonization of the Phase II compost by *S. thermophilum*

Time table	Activities
Preparation of compost	
day 1 to 21	Phase I composting, easily degradable substrates are consumed
day 22 to 32	Phase II composting, pasteurization of the compost (6 hrs at 56-60°C) and conditioning (8-9 days at 45°C)
Spawning and colonization of compost	
day 32 to 46	Introduction into the compost and colonization of the compost by *A. bisporus* at 24-27°C
Casing and development of fruit bodies	
day 46 to 56	Application of the casing layer and subsequent colonization by *A. bisporus*
day 56 to 63/66	Initiation of fruitbody development, growth of fruit bodies and picking of the first flush
day 63/66 to 71/77	Development and picking of the second flush
day 71/77 to 80/86	Development and picking of the third flush
day 86	Killing the *A. bisporus* mycelium by heat-treatment

Fig. 2. Typical simplified sequence of events in the practice of commercially growing *A. bisporus*

Spawning and initiation of fruit body development

The composting process yields a selective substrate which can be inoculated with *A. bisporus* *A. bisporus* is introduced into the compost in the form of millet grains covered with mycelium (spawning) After inoculation the mycelium is allowed to fully colonize the compost at a temperature between 24 and 27°C and a relative humidity of 90-95% This takes about 14 days, after which the compost is covered with a 5 cm thick casing layer to induce the production of fruit bodies The casing layer consists of a mixture of peat and lime This casing layer is colonized by the mushroom mycelium in about 10 days Then the development of fruit bodies is stimulated by lowering the CO_2 concentration and temperature of the air in the growing room The initiation of pinheads and the subsequent development of mature fruit bodies are not fully understood A range of abiotic parameters including temperature, CO_2 concentration, humidity and pH, have been shown to influence fruit body initiation markedly (Flegg & Wood, 1985), but the key factor is thought to be the presence of bacteria normally found in soil (Eger, 1961, Wood, 1976) Bacteria are found in large numbers in the hyphal environment of *A. bisporus* and their population

composition is found to be affected by the presence of the hyphae (Stanek 1976) Scanning electron microscope studies have shown that the bacterial population in the hyphal environment consisted of several types, some attached to the hyphae with filament like structures (Masaphy *et al.*, 1987) The most prominent species has been identified as *Pseudomonas putida* (Hayes *et al* , 1969, Rainey & Cole, 1987) The exact effect of *P. putida* on the vegetative mycelium has yet to be determined, but it has been suggested that the mycelium produces "self inhibitory compounds" which are removed by the bacteria to below a certain threshold level

The first mushrooms of a crop can be picked 17 to 21 days after casing New batches of mushrooms appear at intervals of 6 to 12 days and are called flushes The successive flushes decline in yield In commercial mushroom growing, three to four flushes are harvested after which the mycelium is killed by heat-treatment Yields of 200 kg of mushrooms per tonne fresh compost can be obtained

Supplementation

Yields of mushrooms can be increased substantially by adding products with high protein contents to the colonized compost just before applying the casing layer (Gerrits, 1988) The increase in yield that is brought about by this supplementation is a result of more pinheads being formed instead of the mushrooms becoming larger Supplementation however increases the risk of development of weed mould and may lead to high temperature in the compost as a result of microbial activity In order to limit those risks compost supplements are treated in such a way that the proteins are denatured, making them less soluble and less readily available

Carroll and Schisler (1976) developed the use of delayed-release nutrients for mushroom composts Cotton-seed meal was blended with vegetable oil, spray dried and treated with formaldehyde At present good results (25-50% increase in yield) have been obtained with hydrolyzed feathermeal, maize gluten, toasted and formalin treated soymeal extractions and extracted and toasted soy concentrate (Overstijns 1989) Since the yield increase is due to the protein added with these substances, amino acids have to play an important role in the nutrition of mushrooms Overstijns (1989) reports that only 20% of the amino acids available from the compost are present in the mature fruit body and that the remaining 80% of the amino acids is fixed by the mycelium present in the compost and the casing soil and by competitive microorganisms He postulates that the amino acids present in the compost limit the yield in mushrooms Large differences in yield that occur in practice are explained by the selectivity of the compost In a less selective compost a larger part of the amino acids present are used by competitive microorganisms As a result supplementation will only produce good results if the mycelium grows intensively throughout the compost In such cases the amino acids from the supplements are used for the production of mushrooms with high efficiency (nearly 100 %) The efficiency is also influenced by the amino acid composition of the supplement Overstijns (1989) claims that the effect of soy-based supplements is superior because their amino acid composition strongly resembles the amino acid composition of mushrooms

However the amino acid composition of mushrooms is not a rigid feature It was shown that nutrient supplementation and type of compost can affect the amino acid composition of the fruit bodies (Kissmeyer-Nielsen *et al.*, 1966, Kosson & Bakowski 1984) It is possible that the decrease in yield between successive flushes is a result of the depletion of nutrients However yield increases as a result of supplementation tend to be confined to the first two flushes (Gerrits, 1982,

Randle *et al.*, 1983) Another possibility is that reduced mushroom yields in later flushes are the result of the accumulation of toxic metabolites (Schisler & Sinden, 1962)
Increased yields as a result of supplementation can only be reached on *A. bisporus* strains Strains of the closely related *A. bitorquis* do not react at all or react negatively to supplementing
As will be clear metabolism of nitrogen compounds has a large influence on the outcome of commercial mushroom growing However, the practice of modern mushroom cultivation has evolved from a process of trial and error Therefore, despite its long history of cultivation, knowledge of the basic biology of the common white button mushroom is rather limited Furthermore, the study of its biology is hampered by the unusual nature of its breeding system, its poor spore germination, its slow growth and its reluctance to fruit except on composted horse manure

NITROGEN METABOLISM IN FUNGI

Although nitrogen metabolism is studied in a large variety of organisms, nitrogen metabolism in fungi has received relatively little attention Research mainly has focused on the yeast *Saccharomyces cerevisiae* and the filamentous ascomycete fungi *Neurospora crassa* and *Aspergillus nidulans* In contrast to the relatively abundant amount of information from ascomycete fungi, there are only few reports on nitrogen metabolism in basidiomycete fungi
The supply and composition of nitrogen sources seldom meets the demands of the organism This means that nitrogen sources have to be degraded by catabolic enzymes like proteinases to simple molecules that can be used to synthesize cellular components Eventually such conversions result in the production of simple molecules like ammonium ions and amino
acids, which can be transported into the cell After they have been taken up by the cell these molecules can play vital roles in cellular metabolism These various aspects of nitrogen metabolism in fungi have been reviewed by a number of authors (Pateman & Kinghorn, 1976, Marzluf, 1981, Wiame *et al.*, 1985, Davis, 1986, Jennings, 1988, Klionsky *et al.*, 1990, Mora, 1990) The current ideas about cellular nitrogen metabolism are summarized in Fig 3
Whether the details of nitrogen metabolism are common to all filamentous fungi cannot even be guessed at present, but considerable similarity is suggested at least for ascomycete fungi Using the scheme presented in Fig 3 as a guide-line, a summary will be given of what is known about the nitrogen metabolism of *A. bisporus* If there is no information available on the nitrogen metabolism in *A. bisporus*, the information available from other fungi will be reviewed

Which nitrogen sources are present in mushroom compost?

The nitrogen sources available to *A. bisporus* in compost have been discussed by Wood & Fermor (1985) Electron microscopy studies (Atkey & Wood, 1983) and chemical analysis (Eddy & Jacobs, 1976) have shown that during composting the straw surfaces become coated with an amorphous polysaccharide matrix in which microbial cells and debris are embedded This coating may correspond to the nitrogen-rich lignin-humus complex described by Gerrits *et al.* (1967) Since these surface layers on straw disappear during colonization of the compost by mushroom mycelium they have been proposed as a nutrient source for the fungus (Wain, 1981) This polysaccharide matrix, contains a large percentage of the compost nitrogen The microbial cells,

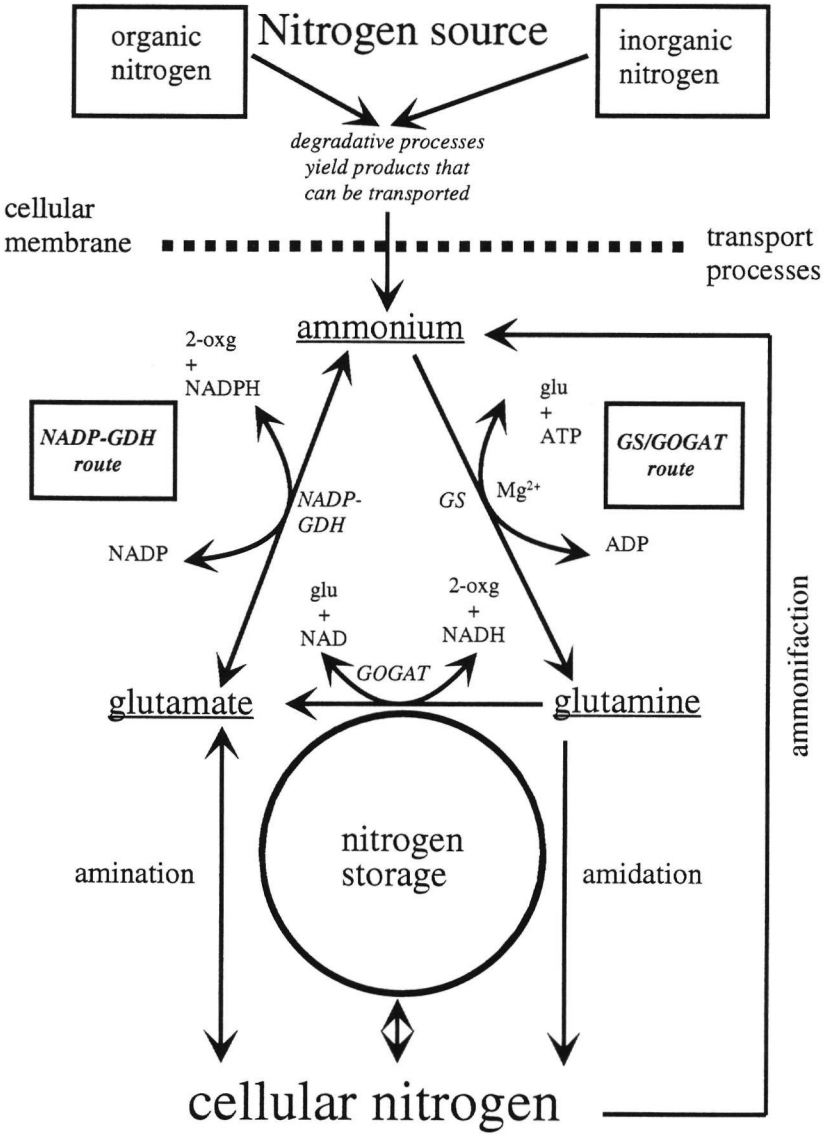


Fig. 3. Flow diagram of nitrogen metabolism in fungi.
Abbreviations: NADP-GDH; NADP-dependent glutamate dehydrogenase, GS; glutamine synthetase, GOGAT; glutamate synthase, 2-oxg; 2-oxoglutarate, glu; glutamate.

mycelium and spores embedded in it contain 40% carbohydrates, 12% protein and 4% phenolic compounds (Wain, 1981) Furthermore it was shown that *A. bisporus* is able to use heat-killed bacteria (Fermor & Wood, 1981) as well as fungal and actinomycete mycelium (Fermor & Grant, 1985) for growth Fermor *et al.* (1991) observed an efficient mineralization of microbial biomass from ^{14}C-labelled compost This compost was prepared by using [^{14}C]-protein hydrolysate as a component in the preparation of compost Based on an estimation of the contribution of microbial biomass to the production of *A. bisporus* biomass, they concluded that *A. bisporus* obtains the bulk of its carbon nutrition from the plant polymers within straw They suggested that the microbial biomass acts as a concentrated source of nitrogen and minerals Barron (1988) showed that *A. bisporus* is sensitive to the presence of bacterial colonies and develops specialized directional hyphae that grow towards the bacterial colony Subsequently these hyphae penetrate the colony and proliferate to produce coralloid, haustorial-like masses of absorptive hyphae The bacteria in the colony are lysed and the contents are taken up and used as a nutrient source This is not a common property of fungi About 100 fungal species belonging to the Basidiomycota, Oomycota, Zygomycota, Deuteromycota and Ascomycota were tested, and only *A. bisporus*, *Coprinus quadrifidus*, *Lepista nuda* and *Pleurotus ostreatus* showed this behavior

Degradative enzymes

The enzymes responsible for lysis of microbial cells have been studied by Grant *et al.* (1984) Using cultures of *A. bisporus* on freeze-dried *Bacillus subtilis* as sole carbon and nitrogen source, it was shown that extracellular ß-N-acetylmuraminidase (EC 3 2 1 17) was the main bacteriolytic enzyme present Fermor & Wood (1981) showed that ß-N-acetylglucosaminidase is also produced by *A. bisporus* during growth on *B. subtilis* A summary of the extracellular degradative capacities of *A. bisporus* is given by Wood & Fermor (1985) Next to ß-N-acetylmuramidase and ß-N-acetylglucosaminidase which degrade bacterial cell wall polymers, *A. bisporus* also produces DNAase and RNAase when grown in phosphate-free medium Furthermore *A. bisporus* produces three types of proteinases acid proteinases, neutral proteinases and alkaline proteinases (Fermor & Wood, 1981) In defined media *A. bisporus* has been shown to use protein readily as sole source of carbon, nitrogen and sulfur (Kalisz *et al.*, 1986a) Utilization of protein was not affected by the presence of glucose or ammonium in the media Regulation of proteinase activity in the presence of protein was studied by Kalisz *et al.* (1987) Extracellular proteinase in cultures of *A. bisporus* was observed only under conditions of induction, no activity was found in media lacking a protein source In media containing protein as sole carbon source, the addition of ammonium and sulfate did not inhibit expression of the proteinase activity Best growth and the highest proteinase activity was observed in media containing both protein and glucose
Major increases of proteinase activity in the culture fluid occurred when most of the protein had been consumed (i e stationary growth/death phase) This suggests that the proteinases were immobilized on the cell wall or extracellular layers On depletion of the protein substrate the wall-bound proteinases are released into the culture fluid The regulation of proteinase production appears to differ considerably from that described for fungi such as *Aspergillus* species and *Neurospora crassa* (Cohen, 1980) In summary, in *A. bisporus* proteinase production only depends on the presence of an inducer, regardless the presence of more easily available sources of nitrogen, carbon or sulfur In *Aspergillus* species and *N. crassa* proteinases are only produced if no easily available source of either nitrogen, carbon or sulfur is available In such cases

Aspergillus species do not need any inducer, whereas *N. crassa* needs an inducer in the form of protein

A review on the biochemistry of proteinases from eukaryotic organisms is given by North (1982) Proteinases (the endopeptidases) can by classified in a number of ways, like on the basis of the pH range over which they are active (acid, neutral or alkaline) or on the basis of their ability to hydrolyze specific proteins (collagenase etc) The most satisfactory classification is based on the sensitivity of proteinases to various inhibitors According to this classification four different types can be distinguished and there is some overlap with the classification based on pH optimum Aspartic proteinases (EC 3 4 23) are usually active at acidic pH's, metalloproteinases (EC 3 4 24) are active at neutral pH's, serine proteinases (EC 3 4 21) usually have an alkaline pH optimum and cysteine proteinases (EC 3 4 22) are active at acid or neutral pH's

There have been few investigations of the proteinases of *A. bisporus* An extracellular prolyl-endopeptidase (EC 3 4 21 26) has been purified from the culture filtrate of *A. bisporus* growing on a complex medium and an intracellular prolyl-endopeptidase was purified from fruit bodies of *A. bisporus* (Sattar *et al.*, 1990) The enzyme was a monomeric 78 kD protein and was most active at or around pH 7 5 Kizuki & Moriya (1982) purified and partially characterized a proteinase from fresh fruit bodies of *A. bisporus* This proteinase is probably a cysteine proteinase and has a molecular mass of 67 kD Burton *et al.* (1993) purified a serine proteinase from the stipes of senescent fruit bodies This proteinase had a molecular mass of 27 kD and a broad pH optimum between pH 6 5 and 11 5 The intracellular proteinases probably function in the maturation of the fruit body

Fermor *et al.* (1991) postulated that the ability to efficiently release nutrients from biopolymers in an environment rich in phenolic compounds may be one of the factors selecting for growth of *A. bisporus* mycelium in compost Phenolic compounds combine with proteins reversibly by hydrogen bonding, and irreversibly by oxidation followed by covalent condensations (Loomis & Battaile, 1966) Therefore they are likely to interfere with protein degradation Although no special attention was paid to enzymatic performance in the presence of phenolic compounds, no large differences were found between the extracellular prolyl-endopeptidase and the prolyl-endopeptidase from the fruit body (Sattar *et al.*, 1990) The pH optima, isoelectric points, molecular weights, substrate specificity and susceptibility towards inhibitors of both *A. bisporus* prolyl-endopeptidases resembled those of mammalian, plant and microbial enzymes However both *A. bisporus* enzymes showed no immunological cross reaction with bovine prolyl-endopeptidase antiserum

Uptake of nitrogen sources.

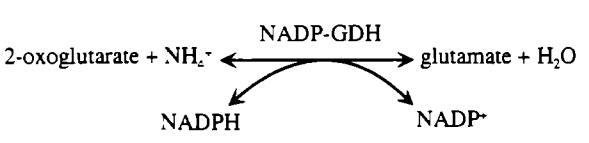
With regard to nitrogen metabolism, the main function of the catabolic enzymes described is to produce small solute molecules that can be transported across the cellular membrane *A. bisporus* is able to use both inorganic and organic nitrogen sources (Treschow, 1944, Casimir & Heinemann, 1953, Bohus, 1959) Although slight differences have been reported by these authors, best growth was observed on the amino acids alanine, asparagine, glycine, and glutamine Also urea and ammonium salts support good growth Nitrate can not be used as a nitrogen source

Among the substances that are transported across the fungal membranes amino acids represent one of the largest and most diverse groups Amino acid transport in fungi is studied most extensively in *A. nidulans*, *N. crassa* and *Penicillium chrysogenum* and appears to represent a

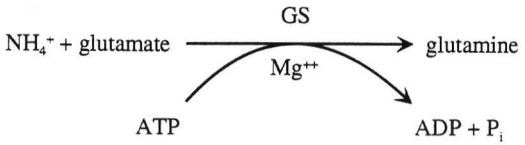
compromise between bacterial transport systems, with specificities for individual amino acids, and animal cell systems, with broad specificities for classes of structurally related amino acids (Whitaker, 1976, Wolfinbarger, 1980, Garraway & Evans, 1984) Furthermore, different transport systems are expressed at different stages of development of the fungi and under different environmental conditions In general four groups of transport systems can be recognized in fungi, a single system of broad specificity, systems of broad but differing specificities, systems specific for acidic, neutral or basic amino acids and specific systems for single amino acids
Affinity coefficients for amino acids differ between transport systems and between individual amino acids and vary between 10^{-7} and 10^{-4} M. Common to most amino acid transport systems is that they are repressed by the presence of ammonium ions This type of regulation is most prominent in the case of the general and acidic amino acid transport systems The models proposed for ammonium ion regulation of amino acid transport systems collectively involve NAD-and NADP-dependent glutamate dehydrogenases A number of mutants of *Aspergillus-Neurospora-* and *Saccharomyces* species, altered in glutamate dehydrogenase activity have been reported, each providing different responses to ammonium repression of transport
As is the case for amino acids, ammonium ions are transported actively across the cellular membrane Kleiner (1981) describes transport systems with very high affinity for ammonium in *P. chrysogenum* (K_m 0 25 µM), *A. nidulans* (K_m 2 8 µM), *N. crassa* (K_m 7 µM) and *S. cerevisiae* (K_m 1 µM) Nonspecific diffusion of NH_3 was only observed when repression of ammonium-transport systems had occurred
Next to exogenous ammonium, ammonium ions are also produced intracellularly as a result of the turn-over of cellular components (ammonification)

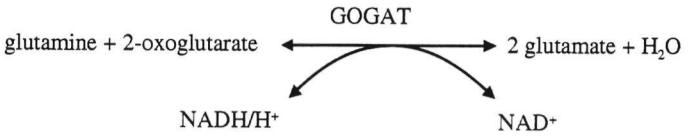
Ammonium assimilation

The assimilation of ammonium occupies a central position in nitrogen metabolism (Brown, 1980, Marzluf, 1981, Jennings, 1988, Mora, 1990) Once inside the cell, ammonium can be incorporated into the amino acids glutamate and glutamine by either of two basic reactions (Fig 3) Synthesis of glutamate is catalyzed by the enzymes glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT), while synthesis of glutamine is catalyzed by glutamine synthetase (GS) Many yeasts and filamentous fungi possess two types of glutamate dehydrogenase, a NADP-dependent glutamate dehydrogenase (NADP-GDH) and a NAD-dependent glutamate dehydrogenase (NAD-GDH) Sanwal & Lata (1961, 1962) suggested that NADP-GDH functions in the formation of glutamate in *N. crassa* Studies using double mutants lacking both NADP-GDH and NAD-GDH demonstrated that also in *A. nidulans* NADP-GDH is involved in the formation of glutamate, while NAD-GDH appeared to be involved in catabolism of glutamate (Arst *et al.*, 1975) The catabolic role of NAD-GDH in yeast was confirmed using mutants of *S. cerevisiae* (Miller & Magasanik, 1990)
On basis of this knowledge two different pathways have been postulated for the assimilation of ammonium One pathway is catalyzed by NADP-GDH in the following reversible reaction



The affinity of NADP-GDH for ammonium is usually relatively low, so this enzyme is likely to function efficiently at high intracellular ammonium concentrations.
The other pathway is formed by the action of GS and GOGAT according to the following reactions;





The high affinity of GS for ammonium allows this pathway to operate at low intracellular concentrations of ammonium. Coupling of the two reactions allows the formation of glutamate in a physiologically irreversible manner.
Catabolism, transport and assimilation of nitrogen appear to be repressed by ammonium ions and the products of its assimilation (Marzluf, 1981; Wiame *et al.*, 1985; Jennings, 1988). This phenomenon is called nitrogen catabolite repression. Even activities not involved in nitrogen source utilization can be affected by nitrogen catabolite repression. Buswell *et al.* (1982) reported suppression of ligninolytic activity by ammonium in *Sporotrichum pulverulentum* (the imperfect stage of *Phanerochaete chrysosporium*). In *A. bisporus* constitutive laccase activity was reported (Kalisz *et al.*, 1986b). However, *Agaricus* laccase, though constitutive, was induced by protein and repressed by ammonium. No regulation by carbon sources was observed (Wood, 1980).
Glutamine, the final product of ammonium assimilation, is proposed as the central compound in nitrogen catabolite repression (Wiame *et al.*, 1985; Jennings, 1988; Mora, 1990). Glutamine donates its nitrogen by an irreversible transamidation, in contrast to glutamate, which donates nitrogen by reversible transamination reactions. Therefore the intracellular level of glutamine can be regulated more precisely. The different pathways concerned with amino acid biosynthesis have been reviewed by Pateman & Kinghorn (1976). All other amino acids derive their nitrogen atoms from glutamate and glutamine by transamination and transamidation reactions . Nucleic acids derive their nitrogen mainly from glutamine (Meister, 1980)In *A. bisporus* the central role of glutamate was demonstrated in both mycelium and fruit bodies (Latché, 1970; Piquemal, 1970; Piquemal *et al.*, 1972). Glutamate was found to act as the most prominent nitrogen donor in transamination reactions. The main transaminating activities were glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT). Another major transaminating activity involved the production of glycine from glyoxylate, in which alanine was the preferential nitrogen donor.
Next to taking part in numerous transamination reactions, glutamate is also an intermediate in the 4-aminobutyrate shunt (Fig. 4). It was suggested that in *A. bisporus* the Krebs cycle is blocked at the 2-oxoglutarate dehydrogenase complex (Le Roux, 1966; Rast *et al.*, 1976).

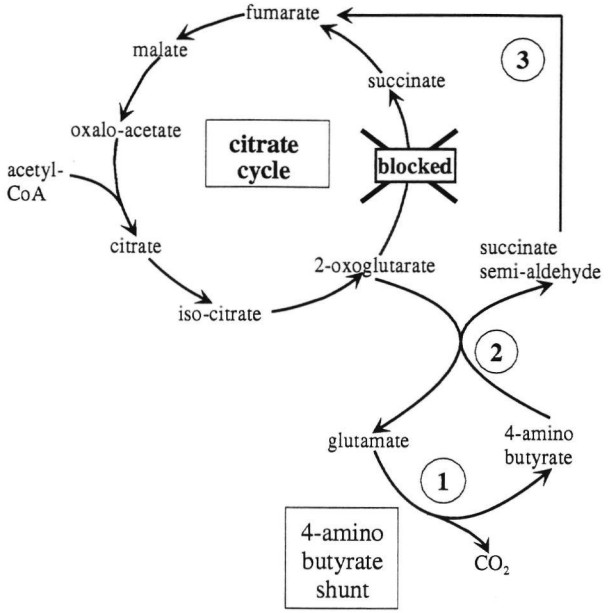


Fig. 4. The enzymes of the 4-aminobutyrate shunt.
1: glutamate decarboxylase, 2: 4-aminobutyrate aminotransferase
3: succinate-semialdehyde dehydrogenase
(Baldy, 1975; 1976; 1977)

This complex may be functionally replaced by the enzymes of the 4-aminobutyrate shunt: glutamate decarboxylase, 4-aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase. The enzymes involved in the shunt were characterized by Baldy (1975, 1976, 1977). Also in fruit bodies of *Coprinus cinereus* the inoperative 2-oxoglutarate dehydrogenase is by-passed through the 4-aminobutyrate shunt (Moore & Ewaze, 1976).

Amino acid biosynthesis

In *S. cerevisiae*, *N. crassa* and many other fungi the enzymes of many amino acid biosyntheses are controlled by a complex regulatory system (Hinnebusch, 1988; Barthelmess & Kolanus, 1990). This "general amino acid control" causes derepression of many of the enzymes of the arginine, lysine, tryptophan, histidine, isoleucine, valine and leucine biosynthetic pathways upon starvation of cells for any of these amino acids. However, the full range of amino acids whose limitation would lead to enzyme derepression is not known in any organism. In many pathways amino acid

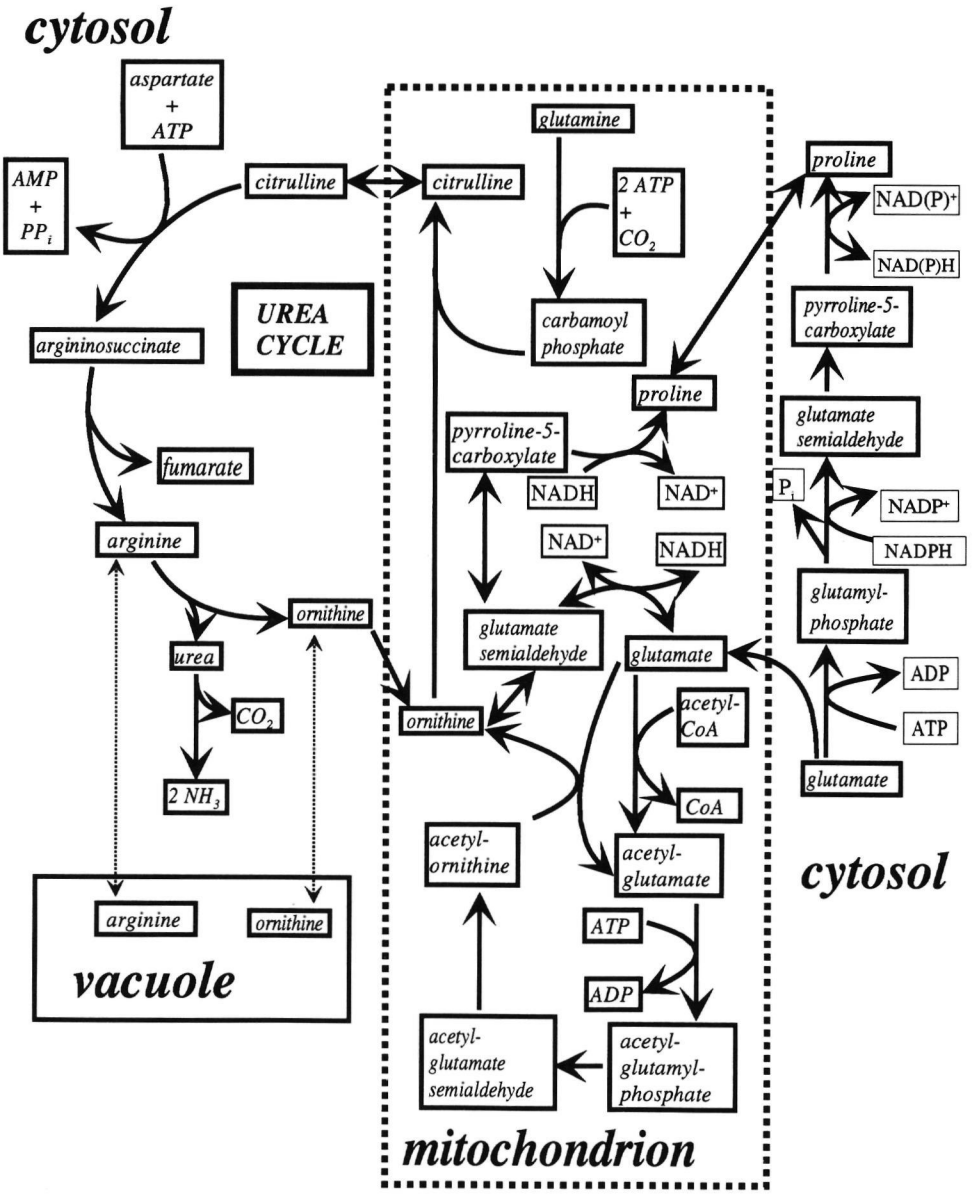


Fig. 5. Compartmentalization of arginine and proline metabolism in *Neurospora crassa* and *Saccharomyces cerevisiae* (Davis (1986); Jennings (1988)).

specific regulatory systems operate to override derepression by the general control system when the end products of those pathways are not limiting

Compartmentalization of nitrogen metabolism

Compartmentalization plays an important role in the nitrogen metabolism of the fungal cell This is illustrated in *N. crassa* and *S. cerevisiae* by the way in which the various parts of the arginine and proline metabolism are divided over several cellular compartments (Davis, 1986, Griffin, 1994) As illustrated in Fig 5 the metabolism of arginine consists of the biosynthesis of arginine and its subsequent degradation into ornithine and urea through the urea cycle The synthesis of arginine has three main components, the synthesis of ornithine, the synthesis of carbamoyl-phosphate and the conversion of these compounds to arginine The biosynthesis of ornithine is located in the mitochondria The conversion of ornithine to citrulline, including the biosynthesis of carbamoyl-phosphate, is mitochondrial in *N. crassa*, but cytosolic in *S. cerevisiae* The conversion of citrulline to arginine, which completes the biosynthesis of arginine is cytosolic in both fungi

As is the case for arginine metabolism, proline metabolism may be located in different cellular compartments Glutamic semialdehyde and Δ^1-pyrroline-5-carboxylate are intermediates in both the biosynthesic and degradative pathways In *S. cerevisiae* the biosynthesis of proline takes place in the cytosol, while the proline-degradative pathway is located in the mitochondria It is not clear whether the same system is also operative in other fungi

Furthermore the vacuole contains large amounts of arginine, ornithine and some other amino acids, leaving only a small pool in the cytosol with a large metabolic turnover Transfer of metabolites between the different cellular compartments offers an additional method of metabolic regulation

An active urea cycle has been demonstrated in *A. bisporus* (Reinbothe & Tschiersch, 1962) $^{14}CO_2$ fed to fruit bodies was preferentially transferred to urea, while ^{14}C-amidine labelled arginine was cleaved yielding ^{14}C-urea as the only radioactive product Uniformly labelled arginine gave rise to ^{14}C-urea and ^{14}C-proline as the only radioactive products Proline is presumably formed very effectively from ornithine To a minor extent urea was also formed by aerobic purine degradation Levenberg (1962) studied the formation of carbamoyl-phosphate, which serves as a precursor for citrullline, in cell free extracts from fruit bodies He demonstrated that carbamoyl-phosphate synthase from *A. bisporus* uses glutamine instead of ammonium ions as nitrogen donor

A. bisporus fruit bodies contain a high level of urea Hammond (1979) reported an urea content of 0 1 % (dry matter) at harvest, which increased tenfold during storage at room temperature for 4 days Production of urea is not limited to the fruit bodies Piquemal (1970) reported urea levels up to 30% of the soluble nitrogen pool of vegetative mycelium Urea is found frequently in basidiomycetes but very little is known about its physiological role (Levenberg, 1962, Foret, 1990) No studies have been made of compartimentalization of nitrogen metabolism in *A. bisporus*

The accumulation of arginine into the vacuole offers distinct advantages *N. crassa* and *S. cerevisiae* often contain large pools of basic amino acids These amino acids have the highest nitrogen content and may therefore act as a nitrogen storage (Davis, 1986, Klionsky *et al.*, 1990) Release of amino acids from the vacuole in case of nitrogen starvation is triggered by the cytosolic glutamine pool

Next to being a location for nitrogen storage, the fungal vacuole has a number of other functions (Klionsky *et al.*, 1990) It contains a variety of hydrolase activities, like proteinase, trehalase, α-mannosidase and alkaline phosphatase Its function as a storage compartment involves, next to amino acids, inorganic ions like Ca^{++}, Mg^{++} and Zn^{++}, and polyphosphates Furthermore the fungal vacuole is involved in the precise homeostatic regulation of cytosolic ion concentration and pH In growing hyphal tips of *Pisolithus tinctorius* the vacuoles are connected by extensible tubular elements (Shepherd *et al.*, 1993) The tubular system is proposed to have the potential for intracellular transport of solutes in the hyphal tips without concomitant transport of large amounts of membrane as is the case in intracellular transport using vesicles

A relation between nitrogen metabolism and morphogenesis?

In the basidiomycete fungus *Coprinus cinereus* an active urea cycle was detected in the cap of the developing sporophore (Ewaze *et al.*, 1978, Moore, 1984) The production of urea is accompanied by an increase of the activity of the enzymes of the Krebs cycle and the urea cycle, as judged from the increased activities of succinate dehydrogenase, NADP-GDH, GS, ornithine acetyltransferase and ornithine carbamyltransferase Urease activity was absent in the cap, while being present in stipe and mycelium So the urea formed was not degraded but accumulated in the cap tissue It is proposed that urea is one of a number of metabolites that function as osmotic solutes which drive water into the cells of the gill hymenium This would provide a driving force by which the cap is able to expand itself

As in most fungi both NAD-GDH and NADP-GDH can be found in *C. cinereus* However, based on its kinetic characteristics NAD-GDH is believed to perform both amination and deamination of glutamate as the nutritional conditions require A specific role is postulated for NADP-GDH in the development of the fruit body of *C. cinereus* NADP-GDH may act in cooperation with GS as an ammonium-scavenging system Microscopic studies of basidia involving activity staining demonstrated that the increase in activity of NADP-GDH was initiated as karyogamy became evident (Moore *et al.*, 1987) Furthermore it was shown *in vitro* that ammonium and glutamine and their analogues are able to inhibit *C. cinereus* basidium differentiation (Chiu & Moore, 1990) Also in *S. cerevisiae* sporulation is inhibited by ammonium and glutamine (Delavier-Klutchko *et al.*, 1980)

Unfortunately the detailed model of fruit body development in *C. cinereus* does not apply to all Agaricales (Moore & Al-Gharawi, 1976) In *A. campestris* (= *A. bisporus*) high activities of NAD-GDH and NADP-GDH were found in both cap and stipe of the fruit bodies In contrast, a number of fungi showed no NADP-GDH activity and even among different dikaryons of *C. cinereus* large differences in NADP-GDH activity in cap and stipe can be found

Fruit body development and the formation of spores depends on the translocation of metabolites from the parent mycelium (Gruen, 1982) Furthermore it has been demonstrated that in a number of basidiomycete fungi fruit body development is accompanied by proteinase activity (Chao & Gruen, 1987, Schwalb, 1977, Terashita *et al.*, 1981) In *A. bisporus* the nature of the metabolite(s) transported from the mycelium to the developing fruit bodies is not known However the distribution of proteins over the different parts of the fruit body (stipe, pileus and gills) at different stages of fruit body development strongly suggest transport of cellular material to the gills (Foret, 1990) Proteinase activity has been studied in *A. bisporus* during flushing and fruit body development (Burton *et al.*, 1994) In *A. bisporus* fruit body primordia are produced before the

first flush with little or no further production thereafter (Flegg, 1979) During initiation of the first flush a number of primordia develop, some die while a number stay dormant until release for growth in subsequent flushes Proteinases are expected to perform several roles in the regulation of flushing and the subsequent development of a fruit body They may act as part of the control for regulating enzyme levels in the mycelium and fruit bodies It has been shown that tyrosinase activity which is responsible for tissue browning (Burton 1988) and chitin synthase activity necessary for cell wall growth (Hanseler *et al.*, 1983) can be proteolytically activated Furthermore proteinase activity may be involved in the formation of low molecular weight nitrogenous compounds for nutrition In the mycelium present in the casing layer proteinase activity was found to show little variation during flushing In the primordia (pinhead-stage) proteinase activity oscillated with about 10-fold difference between maximum and minimum activities, with maximum activity occurring during interflush periods In the developing mushrooms highest proteinase activity was found at 3-4 days after flush initiation Furthermore it was found that proteinase activity was not distributed homogeneously over the fruit body Highest proteinase activity was found in the gills The oscillations of the proteolytic activity were synchronous with the activities of fruit body trehalase, glycogen phosphorylase and mycelial extracellular endo-glucanase This suggests that these enzymes may have analogous physiological functions, trehalase and glycogen phosphorylase releasing stored carbohydrate and protease possibly releasing amino acids from storage proteins However, because of the multiplicity of proteinases usually present in tissues conclusions on their physiological role are uncertain

This introduction does not describe all aspects of nitrogen metabolism into detail However it will be clear that nitrogen metabolism is closely connected with many aspects of the life cycle of fungi Furthermore the ammonium assimilating enzymes and the early products of ammonium assimilation, glutamate and glutamine have been shown to play a central role in nitrogen metabolism In the case of *A. bisporus*, much research effort has concentrated on the study of the degradative capacities and on the development and storage of fruit bodies In many other aspects, the knowledge of basic nitrogen metabolism in *A. bisporus* is quite meager So, many aspects of nitrogen metabolism are still open for investigation

OUTLINE OF THIS THESIS

Because of its central role in nitrogen metabolism and its significant regulatory influence on catabolic processes, we focussed our research on ammonium assimilation in *A. bisporus* Chapter 2 gives an inventory of the main nitrogen assimilating enzymes, while Chapter 3 describes the effect of growth conditions on the levels of nitrogen assimilating enzymes Chapter 4 describes the study of ammonium assimilation using ^{15}N nuclear magnetic resonance Chapters 5 and 6 describe the purification and characterization of glutamine synthetase and NADP-dependent glutamate dehydrogenase , respectively Chapter 7 describes the NADP-dependent glutamate dehydrogenase gene and studies on the expression of its messenger RNA The general conclusions are compiled in the summary

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

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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 $0{\cdot}235 \pm 0{\cdot}144$ U mg^{-1} ($n = 5$), while in cell-free extracts of sporophores it was $0{\cdot}145 \pm 0{\cdot}039$ U mg^{-1} ($n = 12$). For NADP-GDH these activities were, respectively, $0{\cdot}209 \pm 0{\cdot}124$ U mg^{-1} ($n = 7$) and $0{\cdot}106 \pm 0{\cdot}034$ 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 $0{\cdot}017 \pm 0{\cdot}007$ U mg^{-1} ($n = 7$) and $0{\cdot}034 \pm 0{\cdot}020$ 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 $0{\cdot}012 \pm 0{\cdot}002$ U mg^{-1} ($n = 3$) for extracts of mycelia and $0{\cdot}011 \pm 0{\cdot}004$ 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

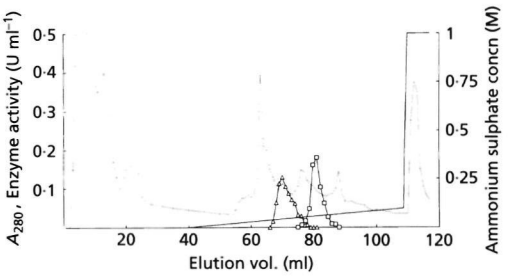

Fig. 1

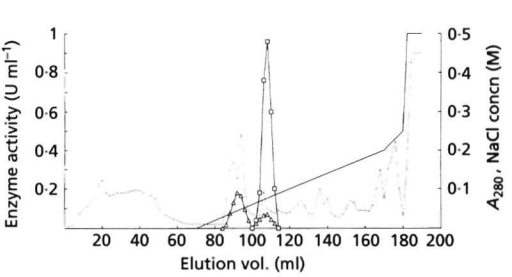

Fig. 2

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

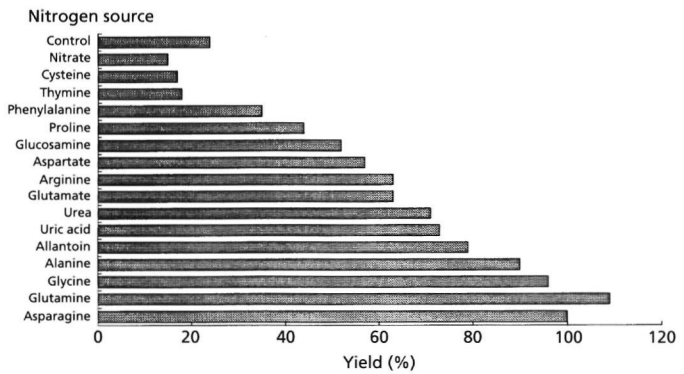

Fig. 3. Yields on monomeric nitrogen sources. Mycelia were cultured on several monomeric nitrogen sources as described in Methods. In order to correct for differences between experiments, yield on asparagine (181 ± 61 mg dry wt, $n = 3$) was set at 100 %. All values shown are measurements of at least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

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Regulation of nitrogen metabolising enzymes in the commercial mushroom *Agaricus bisporus*.

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Abstract.

Mycelium of *Agaricus bisporus* strain Horst U1 was grown in batch cultures on different concentrations of ammonium, glutamate and glucose to test the effect of these substrates on the activities of NADP-dependent glutamate dehydrogenase (NADP-GDH, EC 1 4 1 4), NAD-dependent glutamate dehydrogenase (NAD-GDH, EC 1 4 1 2) and glutamine synthetase (GS, EC 6 3 1 2) When grown on ammonium, activities of NADP-GDH and GS were repressed NAD-GDH activity was about 10 times higher than the activities of NADP-GDH and GS At concentrations below 8 mM ammonium, NADP-GDH and GS were slightly derepressed When glutamate was used as the nitrogen source, activities of NADP-GDH and GS were derepressed, compared to growth on ammonium activities of these two enzymes were about 10 times higher Activities of GDH's showed no variation at different glutamate concentrations Activity of GS was slightly derepressed at low glutamate concentrations Growth of *A. bisporus* on both ammonium and glutamate as nitrogen sources, resulted in enzyme activities comparable to growth on ammonium alone Activities of NADP-GDH, NAD-GDH and GS were not influenced by the concentration of glucose in the medium In mycelium starved for nitrogen, activities of NADP-GDH, NAD-GDH and GS were derepressed while in carbon-starved mycelium the activity of GS and both GDH's was repressed

Keywords: *Agaricus bisporus* - glutamate dehydrogenase (GDH) - glutamine synthetase (GS) - amino transferases - regulation - nitrogen

Introduction.

Ammonium occupies a central position in the nitrogen metabolism of yeasts and filamentous fungi It is the form of inorganic nitrogen converted to organic nitrogen, it is the product of assimilatory nitrate reduction and it is produced intracellularly by the breakdown of cell components [4]

Assimilation of ammonium has been shown to be catalysed by two different pathways, both resulting in the formation of glutamate [4] One route is catalysed by NADP-dependent glutamate dehydrogenase (NADP-GDH, EC 1 4 1 4), aminating 2-oxoglutarate directly to glutamate in a reversible reaction The K_m of NADP-GDH for ammonium is usually high, so this enzyme is likely to function efficiently only at high intracellular ammonium concentrations Many yeasts and fungi also possess a NAD-dependent glutamate dehydrogenase (NAD-GDH, EC 1 4 1 2), but the main function of this enzyme appears to be the catabolism of glutamate The alternative route involves two enzymes, glutamine synthetase (GS, EC 6 3 1 2) and glutamate synthase (GOGAT, EC 1 4 7 1) Glutamate is aminated to glutamine by GS in an ATP-depedent reaction, this glutamine, in turn, is used to aminate 2-oxoglutarate to yield 2 glutamate molecules in a reaction catalysed by GOGAT These enzymes catalyse the formation of glutamate in a physiologically irreversible manner The higher affinity of GS for ammonium allows this pathway to operate at low intracellular concentrations of ammonium, thus compensating the investment of one mole of ATP

Apart from being the principal nitrogen-containing compound incorporated into biomass, ammonium and/or the products of ammonium assimilation are also involved in the regulation of uptake and utilization of other nitrogen sources This phenomenon is called nitrogen catabolite repression and has been reviewed by a number of authors [4, 12, 13, 14, 18, 24]

Nitrogen catabolite repression probably acts as the key mechanism for the adaptation of fungi to changes in their environment with respect to the availability of organic and inorganic nitrogen [24]

Worldwide, the industrial cultivation of edible mushrooms, mainly *Agaricus bisporus*, amounts to about 1 5 million tonnes annually Despite this economic importance fundamental knowledge of the physiological and biochemical processes in *Agaricus bisporus* is only fragmentary In a study of the primary nitrogen metabolism we recently demonstrated the presence of NADP-GDH, NAD-GDH, GS and GOGAT in the white button mushroom *Agaricus bisporus* [1] To define more precisely the participation of each of these enzymes in the assimilation of ammonium, we determined their behaviour under a number of different nutritional conditions

Materials and methods

Organism and growth conditions.

Agaricus bisporus strain Horst ®U1 was used throughout this study. Stock cultures were maintained at 4°C on slants of wheat agar Mushroom spawn on sterile grain was obtained from Somycel (Langeais, France) Mycelium was grown at 24°C in liquid media Two slightly different media were used Medium A contained 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 thiamine.HCl and 0.1 µM D(+)-biotine In some experiments a modification of the medium described by Dijkstra et al. [8] was used (medium B) In this medium the amounts of amino acids were omitted Both media were buffered at pH 6 8 using 10 g l^{-1} of either 3-(N-morpholino)propanesulfonic acid (MOPS) or 2-(N-morpholino)ethanesulfonic acid (MES) and contained 100 mM glucose and a variable amount of nitrogen source Apart from glutamate either ammoniumacetate or $(NH_4)_2HPO_4$ were used as nitrogen sources As described by Baars et al [1] these ammonium-salts gave good yields with only a moderate drop in the pH of the culture fluid

Liquid cultures were inoculated with mycelium grown for 7 days on agar plates containing compost extract medium (CEA) prepared according to Rainey [19] and solidified with 1.5% (w/v) of bacto-agar The plates were overlayed with a cellophane disk and inoculated at 7 inoculation points per petri dish After growth, the mycelium was scraped off the plates with a sterile spatula and fragmented in a Sorvall Omnimixer or a Waring blender for 30 sec. Aliquots of the homogenate were used as inoculum for the liquid media Unless stated otherwise, cultures were harvested after 21 days by filtration over nylon gauze (100 µm pore size)

For some experiments the medium was solidified with 1.5% (w/v) of bacto-agar. These agar plates were overlayed with a cellophane disk and inoculated with grains covered with mycelium After 21 days of cultivation mycelium was harvested by scraping off the agar plates

Enzyme extraction and assays.

Preparation of cell-free extracts, assay of enzyme activities and determination of protein were performed as described previously [1] When GDH activity and transaminase activity had to be determined a 100 mM potassium phosphate buffer pH 7 0, containing 10% (v/v) glycerol and 2 mM ß-mercaptoethanol was used for extracting the enzymes, for extraction of GS a 200 mM

Tris/acetate buffer pH 7.4, containing 2 mM EDTA and 2 mM DTT was added.

Both NAD-GDH and NADP-GDH activities were assayed as reductive amination by measuring the decrease in absorbance of NAD(P)H at 340 nm. Reactions were started by addition of the enzyme. NH_4Cl was omitted in the controls.

Activities of glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1.) and glutamate pyruvate transaminase (GPT; EC 2.6.1.2.) were both determined as described by Baars et al. [1] by measuring the decrease in absorbance at 340 nm in coupled enzymatic assays. Reactions were started by adding the enzyme. For both enzyme activities the amino acid was omitted in the controls. GS activity was determined by measuring γ-glutamylhydroxamate produced in the transferase assay.

All enzyme activities were determined at 20°C. One unit of enzyme activity (U) is defined as 1 μmole product formed per min under the incubation conditions used.

Analytical methods.

Glucose was determined using dinitrosalicylic acid as described by Miller [16]. Ammonium was determined using either the method described by Weatherburn [23] or the method described by Bergmeyer [2]. Glutamate was determined by a modification of the method described by Beutler [3]. Instead of using diaphorase and (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT), we used 0.75 mM N-methyldibenzopyrazine methyl sulfate (PMS) and 3.75 mM (3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to visualise the amount of NAD formed.

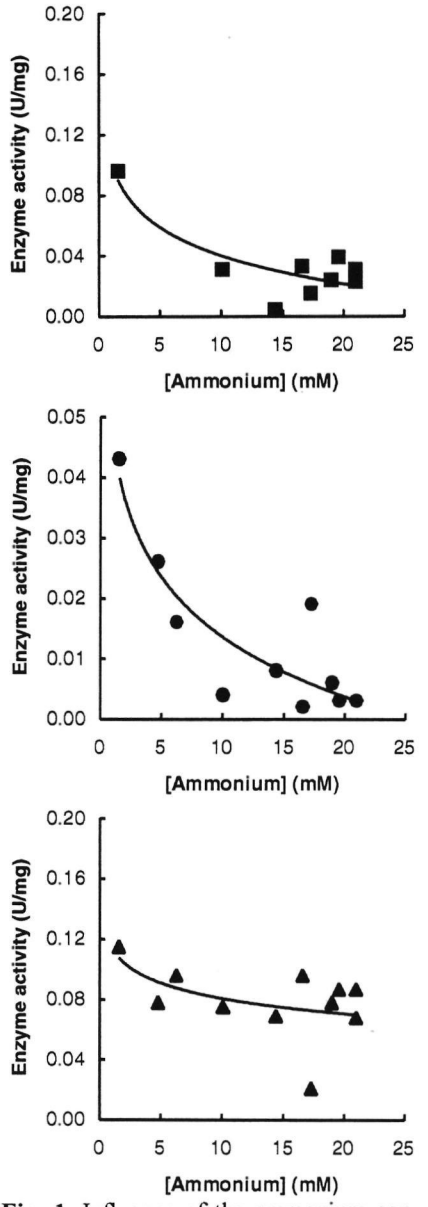

Fig. 1. Influence of the ammonium concentration of the medium on the activity of GS (■), NADP-GDH (●) and NAD-GDH (▲). This figure contains the compiled results from four independent experiments.

Results

Effect of the nitrogen and carbon source on activities of glutamate dehydrogenases and glutamine synthetase.

To study the effect of different ammonium concentrations on enzymes involved in ammonium assimilation, *A. bisporus* was cultured in liquid media containing 100 mM glucose and 1 to 20 mM ammonium acetate At higher concentrations of ammonium no growth was observed After a period of 2 to 4 weeks the cultures were harvested and activities of NADP-GDH, NAD-GDH and GS were determined in cell-free extracts of the mycelium (Fig 1) Similar results were obtained with medium A and medium B In all cell-free extracts NADP-GDH exhibited the lowest activity, while NAD-GDH showed the highest specific activity Intermediate values were found for the activity of GS The activity of NAD-GDH showed hardly any variation over the complete range of ammonium concentrations, while the activities of NADP-GDH and GS were slightly derepressed at ammonium concentrations below 8 mM The effect of the glucose concentration (25, 50 and 100 mM) on the activity of both GDH's and GS was tested in mycelium grown either on 10 mM glutamate or 20 mM ammonium Mycelia were harvested after 16 days of growth in medium B Enzyme activities were determined in cell-free extracts and residual glucose was determined in the culture fluids When ammonium was used as a nitrogen source, enzyme activities were 0 004 ± 0 002 U mg^{-1} (n = 3), 0 078 ± 0 009 U mg^{-1} (n = 3) and 0 026 ± 0 004 U mg^{-1} (n = 3) for NADP-GDH, NAD-GDH and GS, respectively When glutamate was used as a nitrogen source NADP-GDH activity was 0 119 ± 0 029 U mg^{-1} (n = 3), NAD-GDH activity was 0 297 ± 0 020 U mg^{-1} (n = 3) and GS activity was 0 307 ± 0 017 U mg^{-1} (n = 3) Residual glucose concentrations were 4, 17 and 75 mM for both nitrogen sources The enzyme activities showed no variation in response to different glucose concentration but when glutamate was used as nitrogen source, activities of both GDH's and GS were much higher as compared to ammonium (see also Table 1)

The effect of culture age on the activity of GDH's and GS was tested by growing mycelium for a period of 20 days on medium B, with 17 mM ammonium acetate as nitrogen source and 100 mM glucose as carbon source The ammonium concentration in the culture fluid dropped from 17 mM to 14 4 mM over the 20 days period Activities of NADP-GDH (0 015 ± 0 006 U mg^{-1}, n = 8) and GS (0 005 ± 0 002 U mg^{-1}, n = 4) were more or less the same over the whole period, whereas the NAD-GDH activity increased slightly from 0 044 U mg^{-1} to 0 069 U mg^{-1}

Effect of nitrogen or carbon starvation on activities of glutamate dehydrogenases and glutamine synthetase.

Liquid cultures with either low concentrations of ammonium or glutamate or low concentrations of glucose were allowed to grow until either the carbon or nitrogen source was exhausted At that point mycelium was harvested and activities of NAD-GDH, NADP-GDH and GS were determined Furthermore, ammonium, glutamate and glucose concentrations were determined in the culture fluids Results are compiled in Table 1 Depletion of either the carbon or nitrogen source had a marked effect on the activity of the enzymes In mycelium starved for nitrogen activities of GS and GDH's are derepressed As compared to growth on

ammonium, in nitrogen starved mycelium NAD-GDH activity increased 2 5-fold, while the activities of NADP-GDH and GS increased 14-fold and 10-fold, respectively In carbon starved mycelium activity of GS and GDH's is repressed, independent whether ammonium or glutamate was used as sole nitrogen source for growth

Activity	Culture condition			
	Nitrogen starved	NH_4^+-grown[a]	Glutamate grown[a]	Carbon starved
NAD-GDH	0 247 ± 0 057	0 094 ± 0 030	0 336 ± 0 104	0 014 ± 0 009
	(n = 5)	(n = 8)	(n = 5)	(n = 4)
NADP-GDH	0 176 ± 0 041	0 013 ± 0 008	0 193 ± 0 070	0 012 ± 0 007
	(n = 5)	(n = 9)	(n = 5)	(n = 4)
GS	0 277 ± 0 233	0 028 ± 0 020	0 152 ± 0 024	0 008 ± 0 006
	(n = 5)	(n = 6)	(n = 3)	(n = 4)

Table 1. Effect of nitrogen or carbon starvation on the activities of glutamate dehydrogenases and glutamine synthetase of *A. bisporus.* [a] Adapted from [1] Mycelia were grown on either 20 mM ammonium or 20 mM glutamate as a nitrogen source and 100 mM glucose as a carbon source

Influence of ammonium on activities of nitrogen metabolising enzymes in glutamate grown mycelium.

To test the effect of ammonium on the activity of nitrogen metabolising enzymes, mycelium was grown on medium A, solidified with 1 5 % agar Ammonium and glutamate were added in different quantities to yield a total nitrogen concentration of 20 mM Furthermore, 100 mM glucose was added as a carbon source After 19 days of cultivation mycelium was harvested and activities of NAD-GDH, NADP-GDH, GS, GOT and GPT were determined (Fig 2) The activities of all five enzymes were high if glutamate was used as sole nitrogen source Adding ammonium, however, caused a drop in the activity of the enzymes tested The repression by ammonium was most pronounced for NADP-GDH Effects were already evident in mycelium cultivated in the presence of 5 mM ammonium and 15 mM glutamate Similar results were obtained when the experiment was performed in liquid medium or when aspartate was used instead of glutamate (results not shown)

Discussion

Studies on *Aspergillus nidulans, Neurospora crassa* and *Saccharomyces cerevisiae* represent a large portion of our knowledge about nitrogen metabolism in fungi It is generally believed that ammonium assimilation is mediated by the enzymes glutamine synthetase (GS) and NADP-dependent glutamate dehydrogenase (NADP-GDH) NAD-dependent glutamate

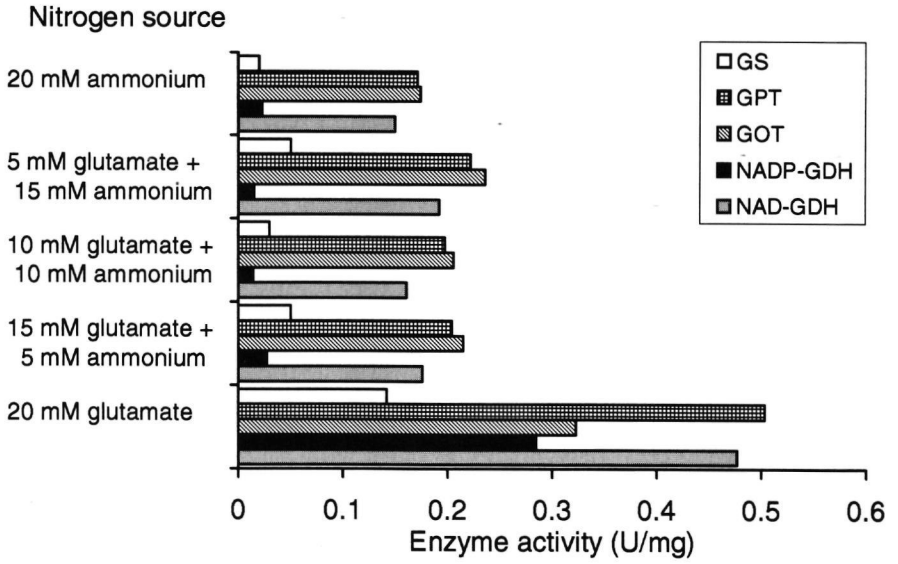

Fig. 2. Influence of combinations of ammonium and glutamate as a nitrogen source on the activities of glutamate dehydrogenases, glutamine synthetase and amino transferases. Mycelial cultures were harvested after 19 days of growtth. Typical results are shown.

dehydrogenase (NAD-GDH) is believed to be involved in the degradation of glutamate [4, 12]. Knowledge on basidiomycete fungi, including economically important mushroom species, is scarce compared to ascomycetes.

Ammonium had a marked effect on the activity of nitrogen assimilating enzymes in *A. bisporus*. Low activities of NADP-GDH and GS were found after growth of mycelium on ammonium (2.5 to 20 mM). The activity of NAD-GDH was about 10 times higher. At low ammonium concentrations a slight derepression of NADP-GDH and GS was observed. With glutamate, instead of ammonium, as a nitrogen source a profound effect on the activity of both NADP-GDH and GS was found, their activities showed a ten-fold increase. The activities of NAD-GDH, NADP-GDH and GS were not influenced by the concentration of the carbon source in the medium. Feeding cultures with both ammonium and glutamate, resulted in enzyme activities typical for growth on ammonium alone. Such effects were readily detectable even if ammonium was present in low concentrations compared to glutamate. Similar effects were observed in *C. utilis* [10] and in *A. nidulans* and *N. crassa* [17].

With regard to GS, the situation in *A. bisporus* resembles the situation in most fungi studied thus far. Pateman [17] observed an increased activity of GS in *A. nidulans* and *N. crassa* in response to glutamate instead of ammonium as a nitrogen source. Furthermore, the concentration of the nitrogen source did not affect the activity of GS. No derepression of GS activity was found in the basidiomycete fungus *Stropharia semiglobata* at low ammonium concentrations [22]. In *Candida utilis* GS activity is 4.5 times higher when grown on glutamate as compared to growth on ammonium [10].

The role of NADP-GDH in ammonium assimilation has received a lot of attention. The

observation that in several yeasts [5] and in *N. crassa* and *A. nidulans* [17] a marked derepression of NADP-GDH activity occurs at low ammonium concentrations in the medium led to the conclusion that NADP-GDH is the primary enzyme involved in glutamate formation in fungi A similar derepression was found for *S. semiglobata* at ammonium concentrations lower than 10 mM [22] Pateman [17] proposed that the presence of ammonium would decrease the intracellular concentration of glutamate as a result of GS activity In *A. nidulans* and *N. crassa* this would then result in a derepression of NADP-GDH This hypothesis, however, cannot be applied to *A. bisporus* Our results indicate only a slight derepression of NADP-GDH activity at low ammonium concentrations and substantial differences were found in response of NADP-GDH to ammonium and glutamate as nitrogen sources Pateman [17] demonstrated derepressed activity of NADP-GDH in *N. crassa* and *A. nidulans* when growth took place on ammonium and repressed activity when growth took place on glutamate In *A. bisporus* the opposite situation occurs, NADP-GDH activity being repressed when grown on ammonium and derepressed when grown on glutamate Such differences in response to various nitrogen sources have also been found for other basidiomycete fungi The NADP-GDH in *Schizophyllum commune* is repressed during growth of mycelium on glucose-containing media with ammonium as sole nitrogen source and derepressed when glutamate is the nitrogen source [7] Also in the ectomycorrhizal fungus *Hebeloma cylindrosporum* NADP-GDH appears to be induced by glutamate as nitrogen source [6] In *Coprinus lagopus* NADP-GDH activity was not influenced much by use of either ammonium or glutamate, nor by their concentrations [9] In *Pleurotus ostreatus* NADP-GDH was even found to be absent [15]

In *A. bisporus* the activity of NAD-GDH is not influenced by the concentration of neither the nitrogen source nor the carbon source NAD-GDH activity in ammonium-grown mycelium is three times lower compared to that in glutamate-grown mycelium Again, there are marked differences with other fungi In *N. crassa* [21] and *C. lagopus* [9] high activities of NAD-GDH usually coincide with low activities of NADP-GDH Such a relation was not found for *A. bisporus* Furthermore, in *S. commune* NAD-GDH had the highest activity in ammonium-grown mycelium and lowest in glutamate-grown mycelium [7] Both activities differed by a factor 2 In *A. bisporus* the opposite situation occurs

In *A. niger* [11], *Fusarium oxysporum* [20], *C. lagopus* [9] and *S. commune* [7] it was found that the levels of the two GDH's were related to the age of the cultures In *A. bisporus* such a relation was not found over a period of 20 days, after which cultures were usually harvested

Our results clearly show that the responses of the main ammonium assimilating enzymes in *A. bisporus* differ markedly from the results obtained for the ascomycete fungi *A. nidulans*, *N crassa* and the yeast *C. utilis* To investigate the ammonium assimilation pathway and its regulation in more detail, further studies will make use of ^{15}N-labeled compounds as tracers Use of mutants is not yet possible because they are not available An alternative is the development of probes on the basis of DNA sequences of nitrogen assimilatory genes These probes can be used to study expression at a molecular level

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^{15}N-NMR study of ammonium assimilation in *Agaricus bisporus*.

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Summary

Ammonium assimilation was studied by feeding [^{15}N]ammonium to actively growing mycelium of *Agaricus bisporus* Products of ammonium assimilation were analysed using ^{15}N-NMR Participation of glutamine synthetase, glutamate synthase and NADP-dependent glutamate dehydrogenase was determined by inhibiting glutamine synthetase with phosphinothricin and glutamate synthase with azaserine Our results clearly indicate that, under the conditions used, ammonium assimilation is mainly catalysed by the enzymes of the glutamine synthetase/glutamate synthase pathway No indications were found for participation of NADP-dependent glutamate dehydrogenase Furthermore ^{15}N-labelling shows that transamination of glutamate with pyruvate to yield alanine is a major route in nitrogen metabolism Another major route is the formation of N-acetylglucosamine Compared to the formation of N-acetylglucosamine there was only a limited formation of arginine

Key words: ^{15}N-NMR, Mushroom, *Agaricus*, Ammonium assimilation

Introduction

A large number of studies [1,2] have demonstrated the existence of two systems for nitrogen assimilation in microorganisms the reductive amination of 2-oxoglutarate catalyzed by NADP-dependent glutamate dehydrogenase (NADP-GDH, EC 1 4 1 4) with the synthesis of glutamate (GDH-pathway), and the amidation of glutamate catalysed by glutamine synthetase (GS, EC 6 3 1 2) with the formation of glutamine Consequently, the amino acids glutamine and glutamate are primary products of nitrogen assimilation and serve as donors of amide nitrogen and in transamination reactions

An alternative to the NADP-GDH pathway for glutamate production is offered by the reaction catalysed by glutamate synthase (GOGAT, EC 1 4 7 1) This reaction involves the reductive transfer of the amide nitrogen of glutamine to 2-oxoglutarate By coupling the GOGAT reaction with GS an essentially irreversible pathway (GS/GOGAT-pathway) for the formation of glutamate is achieved

Until 1980 ammonium assimilation in fungi was believed to be catalysed by NADP-GDH [1] However more recent studies on *Neurospora crassa* have shown that ammonium assimilation in fungi can also be mediated by the joint operation of GS and GOGAT [3,4,5] Furthermore studies on *Cenococcum geophilum* [6], *Aspergillus nidulans* [7] and *Stropharia semiglobata* [8] have shown that ammonium assimilation in fungi can be catalysed by the concurrent activity of the NADP-GDH pathway and the GS/GOGAT pathway

Although the cultivation of the commercial mushroom *A. bisporus* has developed into an industry over the past decades, only a limited amount of basic knowledge on its nitrogen metabolism is available Recently Baars et al [9] made an inventory of the nitrogen assimilating enzymes in *A. bisporus* Besides high transaminating activities, NADP-GDH, GS and GOGAT activities could be demonstrated in cell-free extracts So *A. bisporus* has the enzymic potential to produce glutamate by either the NADP-GDH pathway or the GS/GOGAT pathway In order to determine the relative contribution of both pathways for ammonium assimilation in more detail the incorporation of [^{15}N]ammonium by *A. bisporus* was studied by nuclear magnetic resonance spectroscopy (^{15}N-NMR)

Materials and Methods

Organism and culture conditions.

Agaricus bisporus strain Horst® U1 was used throughout this study Stock cultures were stored at 4°C on slants of wheat agar Mycelium was grown at 24°C in static cultures using Fernbach flasks containing 100 ml of liquid medium Two slightly different media were used Medium A contained 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 thiamine HCl and 0 1 µM D(+)-biotine In most cases a modification of the medium described by Dijkstra et al [10] was used (medium B) In this medium the amino acids were omitted Both media contained 100 mM glucose As a nitrogen source either 20 mM glutamate or 10-20 mM $(NH_4)H_2PO_4$ was used

Liquid cultures were inoculated with mycelium grown for 7 days on agar plates containing compost extract medium prepared according to Rainey [11] and solidified with 1 5% (w/v) of bacto-agar The plates were overlayed with a cellophane disk and inoculated at seven points per petri dish After growth the mycelium was scraped off the plates with a sterile spatula and fragmented in a Waring blender for 30 sec Aliquots of the homogenate were used as an inoculum for the liquid media Unless stated otherwise, cultures were harvested after 22 days by filtration over nylon gauze (100 µm pore size)

[^{15}N]ammonium feeding procedure.

Mycelium harvested from liquid cultures was dried by pressing gently between nylon gauze layered upon filter paper and parafilm After weighing, equal portions (2 to 4 g fresh weight) were incubated in 50 or 100 ml medium B in which the nitrogen source was replaced by 5 or 10 mM $^{15}NH_4Cl$ (99% enriched in ^{15}N, ICN Biomedicals, Cleveland, Ohio, USA) In addition, penicillin G (50 µg ml^{-1}) and streptomycin (50 µg ml^{-1}) were added to prevent bacterial growth The cultures were shaken at 50 rpm for specified periods in 1000 ml baffled flasks at room temperature Where indicated, azaserine (AZS) or phosphinothricin (PPT) were added to the culture to final concentrations of 1 and 5 mM, respectively

Harvest and extraction of nitrogen compounds

After [^{15}N]ammonium feeding mycelia were harvested either by filtration over nylon gauze (100 µm pore size) or by centrifugation (12000 x *g* for 10 min) After washing the mycelia with 0 15 M NaCl and demineralised water, nitrogen compounds were extracted as described by Martin et al [12] Briefly, mycelia were ground and extracted in 25 ml of ice cold methanol/chloroform/water (12 5 3, v/v/v) The homogenate was centrifuged (10000 x *g* for 15 min) and the pellet was extracted a second time The supernatants were combined and dried at 40°C using a rotavapor The samples were dissolved in 0 5 ml of 0 01 M HCl The acidic solution was washed with 1 ml of ice cold chloroform to remove compounds interfering with the ^{15}N-NMR measurement and stored at -20°C

When amino acid compositions of the mycelia were to be determined, 1 µmol norleucine was

added to the mycelia as an internal standard before the extraction procedure. The extracted pellet was dried overnight at 80°C to estimate the amount of mycelium extracted

^{15}N-nuclear magnetic resonance.

The ^{15}N-NMR spectra were obtained with a Bruker AMX-600 spectrometer operating at 60.816 MHz Proton decoupling by the WALTZ-16 composite pulse sequence was used The ^{15}N-NMR spectra were obtained with the following settings 90° pulse, 2 s recycle delay, and 16 K data points were recorded per free induction decay (FID) The number of scans accumulated per FID differs from one sample to the other and is given in the legend of the figures The FIDs were zerofilled once and multiplied by exponential window function with 10 Hz line broadening prior to Fourier transformation leading to spectra with 16 K real data points Spectra were recorded in 20% D_2O to provide a lock signal Chemical shifts were reported relative to liquid ammonia at 25°C, where 0 ppm has been obtained by multiplying the 0 ppm ^{1}H TSP frequency by 0 10132914 [13] Temperature was maintained at 298 K Assignments of resonances were made by comparison with published ^{15}N-NMR data [14,15,16,17] and by analysing spectra of extracts from [^{15}N]-glutamate-fed mycelia

Enzyme assays.

Laccase (EC 1 14 18 1) activity of culture supernatants was used as a measure of mycelium growth and was determined spectrophotometrically according to a modification of the method of Wood [18] Briefly, up to 0 5 ml of culture fluid was added to 2 ml substrate buffer (0 1 M Na-acetate, pH 5.6, containing 1 mg ml^{-1} N,N-dimethyl-*p*-phenylenediamine sulfate) in a total volume of 2.5 ml. Absorbance of the red quinone product was measured at 552 nm. A change in absorbance of 0 1 min^{-1} was defined as one unit of enzyme activity (U) Activities of NADP-GDH and GS were determined according to the methods described by Baars et al [9]. GS activity was measured by the transferase reaction

Analytical procedures.

Ammonium was determined by the method described by Bergmeyer & Beutler [19] Glutamate was determined by a modification of the method described by Beutler [20] Instead of using diaphorase and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), we used 0.75 mM N-methyldibenzopyrazine methyl sulfate (PMS) and 3 75 mM (3-[4,5-dimethylthiazol]-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

After recording the NMR spectra amino acid composition of the extracts was analysed on a Varian LC-5060 Liquid Chromatograph equipped with a Varian 9095 autosampler, coupled to a Varian 9070 fluorescence detector. After derivatization with 9-fluorenyl-methyl chloroformate (FMOC) the amino acids were separated on a TSK gel ODS 80 T_M column (250 x 4 mm, TosoHaas, Montgomeryville, PA, USA) according to the method described by Einarsson et al [21] using a flow rate of 1 1 ml min^{-1}

Results

Growth with [^{15}N]ammonium as a nitrogen source

Mycelium was grown on medium A with 20 mM $^{15}NH_4Cl$ as a nitrogen source to study the major pools of ^{15}N-labelled compounds. Actively growing mycelium (3.4 g fresh weight) was harvested after 9 days of growth. Soluble compounds were extracted and subjected to NMR spectrometry (Fig. 1). Several peaks can be identified from the spectrum. Resonances were found at 32.7 ppm (lysine, ornithine or γ-aminobutyrate); 38.9 ppm (α-amino nitrogens of glutamine, glutamate, lysine and arginine); 40.7 ppm (alanine); 71 ppm (arginine ω,ω'-N);

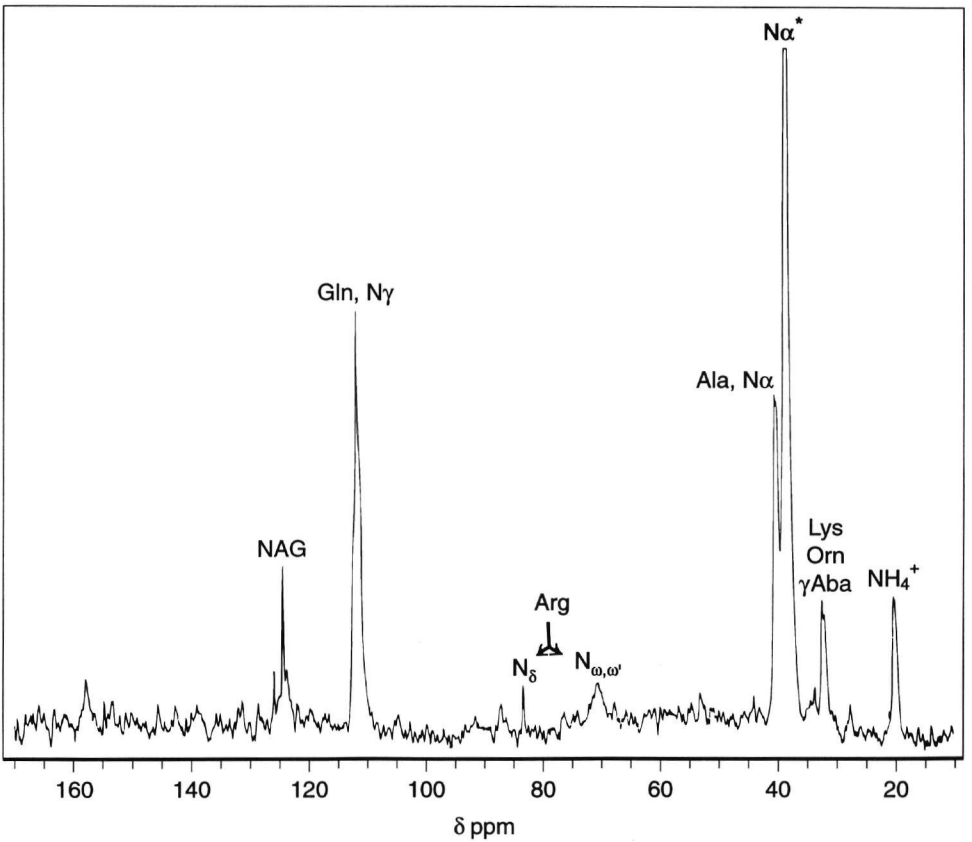

Fig. 1. ^{15}N-NMR spectrum of nitrogen compounds extracted from mycelia of *A. bisporus* growing actively on [^{15}N]-ammonium as a nitrogen source (27059 scans).
Resonance frequencies: NH_4^+, 20.6 ppm; lysine, ornithine or γ-aminobutyrate (γ-ABA), 32.7 ppm; α-amino nitrogens (Nα*) of glutamine, glutamate, lysine and arginine, 38.9 ppm; alanine, 40.74 ppm; arginine ω,ω'-N, 71.04 ppm and 83.61 ppm; glutamine γ-N, 112.18 ppm and N-acetyl-D-glucosamine (NAG) 124.75 ppm.

83.6 ppm (arginine δ-N); 112.2 ppm (glutamine γ-N) and 124.7 ppm (N-acetyl-D-glucosamine).

Time-dependent incorporation of [^{15}N]ammonium

To study the uptake of $^{15}NH_4^+$ and its time-dependent incorporation, mycelium was grown in medium B, using 150 mM glucose and 10 mM $(NH_4)H_2PO_4$ as carbon and nitrogen source, respectively. After 20 days of culture the medium was replaced by fresh medium and laccase activity was determined to measure mycelial growth [18]. From day 20 to 23 a linear increase in laccase activity from 10 to 25 U.ml^{-1} was observed. Thereafter laccase activity hardly increased and the mycelium was harvested for the incorporation experiment at day 24. In the freshly harvested mycelium activities of NADP-GDH and GS were 0.038 U.mg^{-1} and 0.032 U.mg^{-1}, respectively. Equal portions of mycelium (2.8 g fresh weight) were incubated in 100 ml nitrogen-free medium B supplemented with 5 mM $^{15}NH_4Cl$ and antibiotics for a limited number of time intervals. Uptake of ^{15}N was followed by enzymatic determination of residual $^{15}NH_4^+$ in the incubation medium. After 27 h about 1 mM was taken up by the mycelium, while after 48 h the $^{15}NH_4^+$ concentration in the incubation medium was decreased to 0.3 mM. Mycelia were harvested at different time intervals from the incubation medium, their soluble compounds were extracted and ^{15}N-NMR spectra were recorded (Fig. 2). Spectra of samples

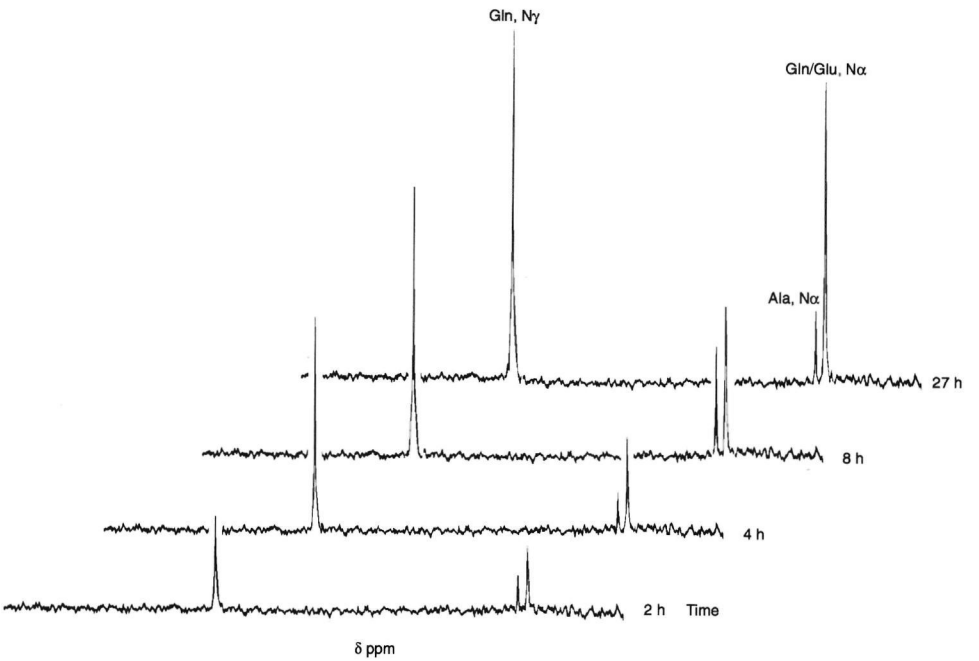

Fig. 2. ^{15}N-NMR spectra of extracts from *A. bisporus* obtained after feeding [^{15}N]-ammonium chloride (99% ^{15}N) at time zero (3072 scans). Resonance frequencies: α-amino nitrogens (Nα*) of glutamine/glutamate, 40.6 ppm; alanine 42.7 ppm; glutamine γ-N, 111.8 ppm.

taken over a period of 27 h show the gradual appearance of a number of labelled compounds Three peaks are observed a resonance at 111 8 ppm due to glutamine γ-N and resonances at 42 7 and 40 6 ppm due to α-N from alanine and glutamate/glutamine, respectively The resonance from glutamine γ-N is the most prominent over the complete period of labelling and increases with time The signal to noise ratio of the glutamine γ-N peak increases from 19 8 after 2 h of incubation to 39 8, 47 6 and 57 4 after 4, 8 and 27 h, respectively Labelling of glutamate/glutamine α-N lags behind but in time becomes almost as intense as the resonance of glutamine γ-N

Inhibition of [^{15}N]ammonium incorporation by phosphinothricin and azaserine

Ammonium assimilation can be influenced *in vivo* by adding specific inhibitors of GS and GOGAT [7,22] The biosynthetic reaction catalysed by GS purified from *A. bisporus* is inhibited strongly by phosphinothricin (PPT, K_i= 17 μM, [23]) GOGAT activity in cell-free extracts was inhibited completely by 1 mM azaserine (AZS, [9]) *In vitro* NADP-GDH activity was not affected by addition of PPT (5 mM) to the NADP-GDH assay mixture, while addition of AZS (1 mM) caused about 25% inhibition of NADP-GDH activity So it is unlikely that these inhibitors exert a significant effect on NADP-GDH activity *in vivo* The effect of inhibitors on the ammonium assimilatory pathway was tested with mycelium pre-grown on medium B with 100 mM glucose and 20 mM $(NH_4)H_2PO_4$ After 22 days of cultivation actively growing mycelium, as followed from laccase activity, was harvested At

Table 1. Effect of addition of azaserine or phosphinothricin on the free amino acid pool of ammonium grown mycelium

Mycelium was pre-grown on medium B with 100 mM glucose and 20 mM $(NH_4)H_2PO_4$ After 22 days of cultivation actively growing mycelium was harvested and divided in 3 equal portions (4 1 g fresh weight) and resuspended in 50 ml of medium B with 10 mM $^{15}NH_4Cl$, antibiotics and additions After 19 5 h soluble nitrogen pools were extracted from the mycelium and amino acids were analysed by HPLC

Amino acid	No inhibitor	1 mM AZS	5 mM PPT
	μmol (g dry matter)$^{-1}$		
Glutamine	13 7	24 6	1 4
Asparagine	1 0	1 9	0 5
Glutamate	6 7	8 3	10 2
Aspartate	1 2	1 5	1 5
Alanine	12 6	231 4	20 4
Serine	1 2	5 4	2 0
Glycine	2 0	4 7	3 3
γ-Aminobutyrate	3 7	4 6	3 8

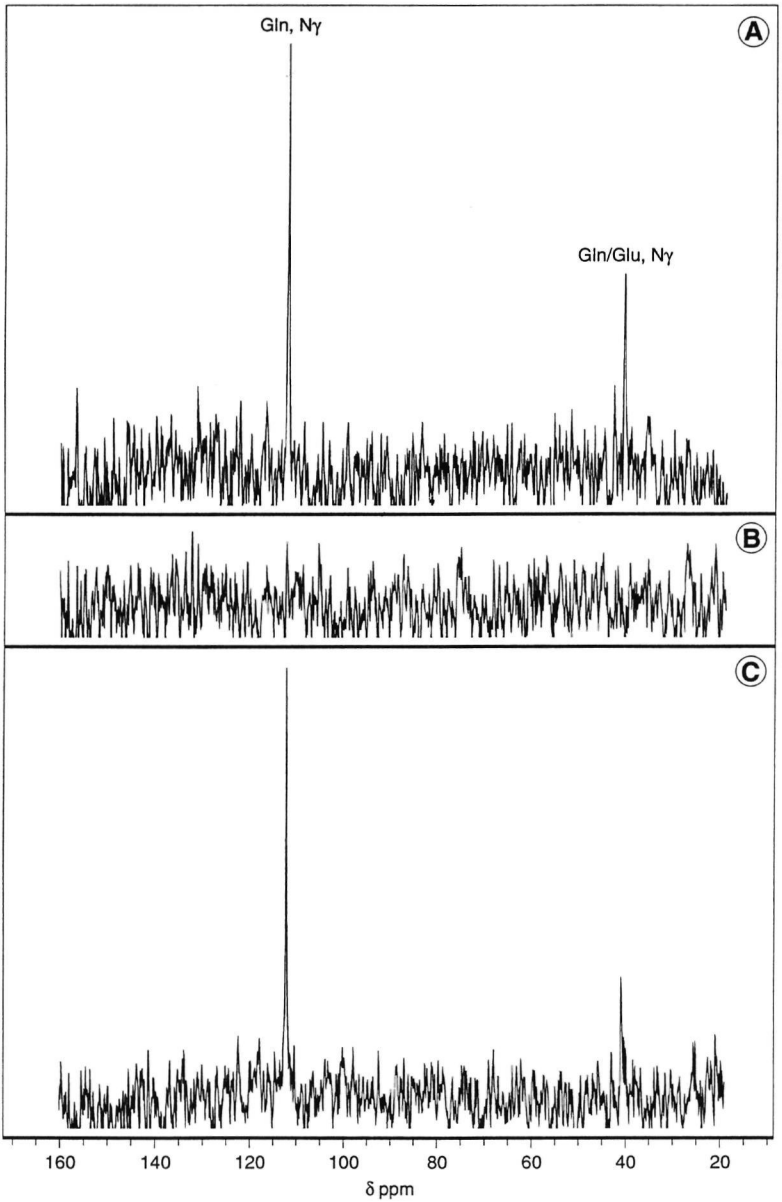

Fig. 3. Effect of 1 mM azaserine or 5 mM phosphinothricine on the incorporation of [^{15}N]-ammonium by *A. bisporus* analysed by ^{15}N-NMR (5000 scans).
Resonance frequencies: α-amino nitrogens (Nα*) of glutamine/glutamate, 40.6 ppm; glutamine γ-N, 111.8 ppm. A) no addition; B) phosphinothricine added; C) azaserine added. Extracts for ^{15}N-NMR were prepared after incubation for 19.5 h.

this time the mycelium the residual ammonium concentration of the medium was 10 mM For incubation with $^{15}NH_4^+$ the harvested mycelium was divided in 3 equal portions (4 1 g fresh weight per portion) and resuspended in 50 ml of medium B with 10 mM $^{15}NH_4Cl$ and antibiotics After 19 5 h soluble nitrogen pools were extracted from the mycelium

The addition of inhibitors of ammonium assimilation had marked effects on the uptake of $^{15}NH_4$ Addition of 1 mM AZS to ammonium-grown mycelium inhibited uptake by 60% Addition of 5 mM PPT completely inhibited uptake of $^{15}NH_4^+$ and even resulted in an increase of extracellular ammonium of 0 05 mM When inhibitors were absent, peaks could be observed at 40 62 ppm, 42 75 ppm and 111 84 ppm in the spectra (Fig 3A) Inhibition by AZS resulted in the accumulation of labelled glutamine γ-N, at the expense of the intensities of the α-N peaks at 40 62 ppm and 42 75 ppm (ratio γ-N/α-N changed from 2 2 to 3 7, Fig 3C) No incorporation of $^{15}NH_4^+$ was observed when PPT was added (Fig 3B) Analysis of the free amino acid pools of the mycelium also reflects the influence of addition of inhibitors (Table 1) Levels of glutamine and alanine were influenced most by the addition of AZS or PPT Addition of 1 mM AZS to the incubation medium increased the levels of glutamine and alanine by a factor 2 and 18, respectively Inclusion of 5 mM PPT in the incubation medium caused a drop in the level of glutamine by a factor 10

Discussion

Growth conditions of the mycelium can have a large influence on the pathway used for the assimilation of ammonium in fungi In *N. crassa* it has been found that NADP-GDH and GS are responsible for ammonium assimilation when there is a large supply of nitrogen However when the ammonium concentration is low, assimilation is mainly catalysed by the GS/GOGAT-pathway [4,5] In *C. geophilum* ammonium assimilation takes place via the concurrent activity of NADP-GDH and GS However in the N-starved mycelia N-flux through GDH is higher than the flux through GS, while in rapidly growing mycelia the opposite situation occurs [6, 24]

This study describes the use of ^{15}N-NMR to study ammonium assimilation in *A. bisporus* The main signals of the NMR spectra of extracts from [^{15}N]ammonium-grown mycelium (Fig 1) corresponded with α-N of several amino acids and γ-N of glutamine Some minor signals were located at 32 7, 71, 83 6 and 124 7 ppm The resonance at 32 7 ppm presumably corresponds with γ-aminobutyrate Since the Krebs cycle appears to be blocked at 2-oxoglutarate dehydrogenase, there is an active γ-aminobutyrate shunt [25] The resonance at 124 7 ppm corresponds with the formation of N-acetylglucosamine Jennings [26] proposed that a nitrogen reserve could be formed by polymerization of N-acetylglucosamine This could be the reason why the cell wall of *A. bisporus* contains about 45% N-acetylglucosamine The intensity of the resonances at 71 and 83 6 ppm, corresponding with ω,ω'-N and δ-N from arginine, respectively, is very low as compared to arginine resonances in *C. graniforme* [16] and *N. crassa* [14] These fungi appear to accumulate arginine as a nitrogen reserve into their vacuoles [16, 27]

Time-dependent incorporation showed that glutamine, glutamate and alanine are labelled first (Fig 2) Nitrogen incorporated into glutamate is quite rapidly transaminated to alanine This is consistent with the high activity of glutamate-pyruvate transaminase found in cell-free extracts of *A bisporus* [9] However, transamination to aspartate was not observed, despite a high activity of glutamate oxaloacetate transaminase in cell-free extracts [9] The activities of

the primary enzymes of ammonium assimilation have been determined by Baars et al [9] In ammonium-grown mycelium activities of GS and NADP-GDH were 0 028 and 0 013 U mg^{-1}, respectively To determine their relative contribution in ammonium assimilation inhibitor studies, including effects on $^{15}NH_4^+$ uptake/incorporation and on amino acid pools, were performed (Table 1, Fig 3)

Inhibitory effects of PPT and AZS on actively growing mycelium were reflected in the pools of free amino acids Inhibition by PPT resulted in a 10-fold decrease of the glutamine pool and a moderate increase of the pools of glutamate and alanine Incorporation of $^{15}NH_4^+$ was completely blocked Moreover an excretion of ammonium was observed This indicates a rapid intracellular turn-over of unlabelled nitrogen-containing compounds Release of ammonium was also found by Lea et al [28] upon incubation of cyanobacteria and a number of higher plants with PPT Inhibition by AZS resulted in a marked accumulation of alanine and a 2-fold increase of glutamine Inhibition by AZS apparently is not absolute since labelling of glutamate is still found No labelled alanine was observed which indicates that the increase in alanine must be derived from the abovementioned turn-over These results are compatible with the presence of a ω-amidase pathway in which unlabelled α-N of glutamine is transaminated Such a pathway was described in *N. crassa* [29] Based upon the complete inhibition of $^{15}NH_4^+$-incorporation by PPT, a specific inhibitor for GS from *A. bisporus* [23], it can be concluded that ammonium assimilation in *A. bisporus* mainly proceeds via the GS/GOGAT pathway NADP-GDH is not or only moderately affected by PPT and AZS, respectively

Ammonium assimilation in *A. bisporus* shares a number of characteristics with ammonium assimilation in the basidiomycete fungus *S. semiglobata* [8] The results of ^{15}N-labelling showed that, despite the high activity of NADP-GDH, ammonium assimilation is catalysed mainly by the action of GS/GOGAT Martin et al [12] concluded from their ^{15}N-labelling studies with ammonium-fed beech ectomycorrhizal fungi, that ammonium assimilation mainly occurs via the GS/GOGAT pathway and that NADP-GDH plays little, if any, role in this process In other fungi such as *Cenococcum graniforme* [6, 24], *A. nidulans* [22], *N. crassa* [5] and the yeasts *Candida albicans* and *Saccharomyces cerevisiae* [30], the concurrent operation of NADP-GDH and GS/GOGAT is reported Relative participation of the GS/GOGAT pathway is calculated to be 1 6% in *S. cerevisiae*, 78% in *C. albicans* [30] and about 50-60% in *A. nidulans* [7,22]

In the past, the elucidation of the pathway of ammonium assimilation in fungi has relied heavily on the use of mutants and on measurement of the levels and kinetic properties of the enzymes involved, particularly NADP-GDH and GS In recent years ^{15}N-NMR has been added to these methods of studying metabolism (for reviews see Martin [31] and Lundberg et al [32] Present results have shown a distinct role for GS/GOGAT in nitrogen assimilation in *A. bisporus* NADP-GDH appears to play a minor role in the assimilation of ammonium Studies with mutant strains of *A. bisporus* may help in obtaining insight in the precise role of this enzyme in nitrogen metabolism More detailed information could further be obtained from ^{15}N-labelling experiments followed by gas chromatography-mass spectrometry (GC/MS) analysis

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Purification and characterization of glutamine synthetase from the commercial mushroom *Agaricus bisporus*.

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Purification and Characterization of Glutamine Synthetase from the Commercial Mushroom *Agaricus bisporus*

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Abstract. *Agaricus bisporus* glutamine synthetase, a key enzyme in nitrogen metabolism, was purified to apparent homogeneity. The native enzyme appeared to be a GS-II type enzyme It has a molecular weight of 325 kDa and consists of eight 46-kDa subunits. Its pI was found at 4.9 Optimal activity was found at 30°C. The enzyme had low thermostability. Stability declined rapidly at temperatures above 20°C. The enzyme exhibits a K_m for glutamate, ammonium, and ATP of 22 mM, 0.16 mM and 1.25 mM respectively in the biosynthetic reaction, with optimal activity at pH 7. The enzyme is slightly inhibited by 10 mM concentrations of L-alanine, L-histidine, L-tryptophan, anthranilic acid, and 5′-AMP and was strongly inhibited by methionine sulfoximine and phosphinothricine. For the transferase reaction K_i-values were 890 μM and 240 μM for methionine sulfoximine and phosphinothricine respectively. For the biosynthetic reaction K_i was 17 μM for both methionine sulfoximine and phosphinothricine

Glutamine synthetase (GS; EC 6 3 1.2.) is a key enzyme in nitrogen metabolism. Glutamine produced by GS is essential for protein synthesis, and its amide nitrogen is used to synthesize many essential metabolites such as nucleic acids, amino sugars, histidine, tryptophan, asparagine, and various cofactors Furthermore, glutamate can be produced from glutamine through the action of glutaminases, glutamate synthase (GOGAT; EC 1.4.7.1.), and other transamidases [18].

Most organisms contain only one form of GS. However, in plants, algae, and rhizobia various isozymes of GS are found which are thought to serve specialized functions in addition to the central metabolic role [2, 6, 17, 19, 27].

In fungi, studies on GS have focused on its role in ammonium assimilation. Tracer studies with $^{15}NH_4^+$ have shown that GS participates in combination with GOGAT and/or NADP-dependent glutamate dehydrogenase in ammonium assimilation in a number of fungi [13, 15, 16, 25]

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Although much attention has been paid to the physiological role of GS, the enzyme has been purified from relatively few fungi. Structure and regulation of GS have been studied most extensively in *Neurospora crassa* [22, 24]. GS from *N. crassa* contains two different subunits with different catalytic properties. Presence of these subunits was related to the nitrogen or carbon source used for growth [22]. In contrast, *Laccaria laccata* [5], *Candida utilis* [28], and *Saccharomyces cerevisiae* [21] possess a GS with one type of subunit. As part of a study concerning the primary nitrogen metabolism of the commercial mushroom *Agaricus bisporus*, GS activity was reported to be present in cell-free extracts of vegetative mycelium and fruit bodies [3]. For determination of its physiological role more precisely, detailed information on the enzyme is of great value. This paper reports the purification and characterization of the GS of *A. bisporus*.

Materials and Methods

Organism and culture conditions. Mycelium from *Agaricus bisporus* strain Horst*U1 was grown either in static cultures or in a 10-L fermentor (CHEMAP AG, Volketswil, Switzerland) A modifica-

tion of the medium described by Dijkstra et al [7] was used in which the trace amounts of amino acids were omitted Glutamate (10 mM) was used as a nitrogen source This growth condition resulted in a high specific activity of GS [3] Static cultures were performed in Fernbach flasks, containing 100 ml of the same medium When mycelium was grown in a fermentor, 0.05% (vol/vol) Antifoam emulsion C (Sigma Chemical Co , St Louis, Missouri, USA) was added to the medium Aeration was performed by supply of 1 5 L air/min, and the medium was stirred at a low rate (100 rpm in the beginning, gradually increased to 500 rpm) Media were inoculated as described by Baars et al [3] Static cultures were harvested after 21 days of growth, fermentor cultures after 14 days

Preparation of cell-free extracts. Harvested mycelium was frozen in liquid nitrogen and subsequently ground with glass beads (Ø 0 10–0 11 mm) in a mortar The powdered frozen material was suspended in an equal amount of extraction buffer (0 2 M Tris-acetate pH 7 0, containing 2 mM EDTA and 2 mM dithiothreitol) The suspension was centrifuged at 40,000 *g* (30 min, 4°C), and the clear supernatant was used as a cell-free extract and stored at −80°C until use

Assay of enzyme activity and protein concentration. Determination of the transferase and synthetase activities of GS was performed as described by Baars et al [3] The biosynthetic activity was determined as described by Shapiro and Stadtman [26], either measuring the formation of inorganic phosphate or assaying the formation of ADP in a coupled assay One unit of enzyme activity (U) is defined as 1 µmol product formed per minute under the incubation conditions used

Protein concentrations were determined with the BioRad protein micro assay kit with bovine γ-globulin as a standard

Purification of glutamine synthetase. All procedures were carried out at 4°C, unless stated otherwise Cell-free extract (8 ml) was dialyzed against three volume changes of 500 ml buffer A (25 mM sodium phosphate pH 7 0 containing 10% (vol/vol) glycerol, 2 mM dithiothreitol, and 20 mM $MgCl_2$) After dialysis the cell-free extract was loaded on a Dyematrex™ Green A column (Amicon, Inc , Beverly, Massachusetts, USA, 2 × 5 cm) pre-equilibrated with buffer A After protein was allowed to bind to the column for 30 min, unbound protein was washed from the column with 40 ml of buffer A Subsequently the column was equilibrated with 50 ml of buffer B (50 mM Tris-acetate pH 7 0, containing 1 mM EDTA, 1 mM dithiothreitol, 5 mM glutamate, and 5 µM ATP) to which 50 mM $MgCl_2$ was added Protein was eluted with 55 ml of 1 M NaCl in buffer B The pooled active fractions were dialyzed overnight against 1 L of buffer B The desalted enzyme preparation was applied on a Protein Pak DEAE 5-PW column (Waters-Millipore, Milford, Massachusetts, USA, 0 75 × 7 5 cm) pre-equilibrated with buffer B After the column was washed with 10 ml buffer B, bound protein was eluted with a 70-ml linear gradient of 100–200 mM NaCl in buffer B Pooled active fractions were desalted and concentrated by ultrafiltration (Centriprep 30 Amicon, Inc) Thereafter the enzyme preparation was applied to the Protein Pak DEAE 5-PW column for a second time Again the protein was eluted with a 70-ml linear gradient of 100–200 mM NaCl in buffer B

For partially purified preparations of GS, 1 5 ml of cell-free extract was loaded on a Fractogel EMD-DEAE 650 S column (Merck, Darmstadt, Germany, 2 × 5 cm) that was pre-equilibrated with buffer B The column was washed with buffer B until A_{280} was close to zero, and subsequently the enzyme was eluted with a linear gradient of 100–200 mM NaCl in buffer B

Gel permeation chromatography. After partial purification by ion-exchange chromatography, concentrated active fractions (0 5–1 0 ml) were applied on a Sephacryl S-300 column (1 5 × 96 cm) pre-equilibrated in buffer B, containing 0 15 M NaCl and eluted at a flow rate of 0 5 ml min^{-1} The Sepharose S-300 column was calibrated in separate runs with the following proteins as standards thyroglobulin (M_r 669,000), apoferritin (M_r 443,000), β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), and carbonic anhydrase (M_r 30,000) The apparent molecular weight of GS was interpolated from the position on a plot of log molecular weight versus elution volume on Sephacryl S-300

Electrophoretic methods. Gel-electrophoresis methods were performed on precast slabgels using Pharmacia Phastsystem® equipment (Pharmacia, Uppsala, Sweden) Native PAGE was performed either on gradient gels (8–25%) or on homogeneous gels (7 5%, 12 5% and 20%) Urease (M_r 545,000 and 272,000), bovine serum albumin (M_r 132,000 and 66,000), chicken egg albumin (M_r 45,000) and α-lactalbumin (M_r 14,200) were used for calibration

For SDS-PAGE a 10–15% gradient gel was used A mixture of rabbit muscle phosphorylase B (M_r 97,400), bovine serum albumin (M_r 66,000), chicken egg albumin (M_r 45,000), bovine carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and hen egg white lysozyme (M_r 14,400) was used for calibration

Isoelectric focusing was performed at 15°C on precast IEF gels (pH range 3–9), with phycocyanin (pI 4 65), β-lactoglobulin B (pI 5 1), bovine carbonic anhydrase (pI 6 0), human carbonic anhydrase (pI 6 5), equine myoglobin (pI 7 0), human hemoglobin A (pI 7 1), human hemoglobin C (pI 7 5), lentil lectin (three bands, pIs 7.8, 8.0, and 8 2), and cytochrome C (pI 9 6) as markers

Gels were stained with Coomassie brilliant blue (PhastSystem® Development Technique File No 200, Pharmacia, Uppsala, Sweden) GS activity was detected by the transferase assay as described by Barratt [4]

Results

Purification of glutamine synthetase. Mycelia from both static and fermentor cultures were used for purification of GS. In some preliminary experiments fruitbodies harvested from commercial compost were used. No differences were found between GS from these three sources. Cell-free extracts could be stored for long periods. GS activity decreased about 20% after 8 months of storage at −80°C

A Dyematrex™ testkit pre-equilibrated in buffer A was used for screening dye ligands for affinity for GS. Apart from binding to the dye Green A, GS also had affinity for the dye Blue A. However, recovery of the GS activity from the latter dye was much lower. Cibacron blue had no affinity towards GS from *A bisporus*. Binding of GS to the dye ligands depended on the presence of $MgCl_2$ in the buffer. Biospecific elution of GS from the Green A column was attempted with 10 mM of either ATP, ADP, AMP, NAD, NADH, NADPH, or EDTA. GS activity (transferase assay) could be eluted from the column only with ATP or with buffer in which $MgCl_2$ was replaced

Table 1. Purification of GS from *Agaricus bisporus*

	Protein (mg)	Total activity[a] (U)	Specific activity[a] ($U.mg^{-1}$)	Recovery %
Cell-free extract	73.4	11.2	0.15	100
Dialysis	73.2	6.2	0.08	55
Green A chromatography	20.5	4.1	0.20	37
Protein Pak DEAE	0.72	0.53	0.74	4.7
Protein Pak DEAE	0.04	0.003	0.07	0.03

[a] Activity was measured using the transferase-assay.

by EDTA. In both cases recovery of the activity was low (8%).

After optimalization of the method, the column was used in a purification scheme as presented in Table 1. GS was eluted from the dye-affinity column with 1 M NaCl in buffer B with a recovery of 66%. Elution of GS by a gradient of 0–1 M NaCl in buffer B gave inferior results. In the following step the enzyme was applied to a Protein Pak DEAE 5 PW anion-exchange column. GS eluted from this column at 140 mM NaCl. Active fractions were pooled and the Protein Pak DEAE 5 PW column step was repeated. The purification procedure resulted in a pure GS preparation. However, during purification, activity of the enzyme was lost rapidly. Recovery of enzyme activity, measured by the transferase reaction, was only 0.03%. Biosynthetic activity was lost even more rapidly. After dye-affinity chromatography no biosynthetic activity could be shown. On the basis of protein measurements, the enzyme was purified about 1800-fold. Electrophoresis of the final enzyme preparation on native PAGE showed a single protein band (Fig. 1). From Ferguson plot analysis of the electrophoresis pattern on homogeneous 7.5%, 12.5%, and 20% slab gels, a molecular weight of 325 kDa was estimated. From the elution pattern of GS on a Sephacryl S-300 column a molecular weight of 345 kDa was estimated. After SDS-PAGE only one band with a molecular weight of 46 kDa was found. The pI of the pure GS was estimated to be 4.9 ± 0.06 (n = 4). The purified protein migrated similarly on native PAGE to a band of GS activity obtained on a similar gel after electrophoresis of cell-free extract. The results of the activity staining are not shown. Owing to rapid diffusion of the γ-glutamylhydroxamate formed during the activity staining, results were hard to record.

Attempts were made to determine the N-terminal sequence of the protein. Purified GS (24 μg) was blotted onto PVDF membrane (BioRad, Richmond, California, USA) according to the manufacturer's instructions and sent in for sequencing. Attempts

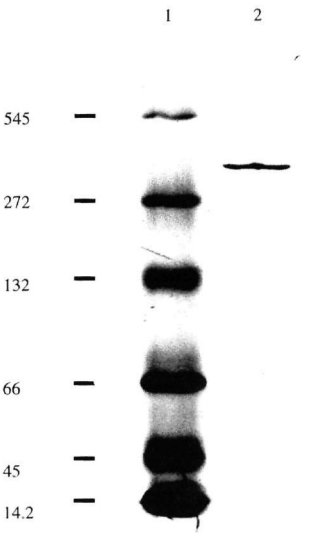

Fig. 1. Separation of purified GS by native PAGE on an 8–25% gradient gel. Protein was analyzed by staining with Coomassie brilliant blue. Lane 1, marker lane (molecular weight given in kDa); lane 2, purified GS (1.2 μg).

to sequence were unsuccessful. The N-terminus appeared to be blocked.

For kinetic experiments, 8.4 U of GS activity (measured as transferase activity) was partially purified with Fractogel EMD-DEAE. The enzyme eluted at 140 mM NaCl with 40% recovery of activity. Specific activity increased by a factor of 9. In this partially purified GS preparation biosynthetic activity could still be detected.

Stability. During the purification procedure a large part of GS activity was lost. Attempts to stabilize the activity were performed with partially purified GS. Fractogel EMD-DEAE fractions containing GS activity were incubated at 4°C with up to 10 mM of either glutamate, ATP, 2-mercaptoethanol, $MgCl_2$, or $MnCl_2$. None of these substances, either alone or in combination with each other, were able to prevent inactivation. Also, 0.5 M trehalose, 1 M sucrose, 10% mannitol, or 20% glycerol were ineffective for preservation of the enzyme activity.

pH and temperature optima. The effect of pH of the assay mixture on the biosynthetic and transferase activity of GS is shown in Fig. 2. The pH optimum for the transferase activity, determined with cell-free

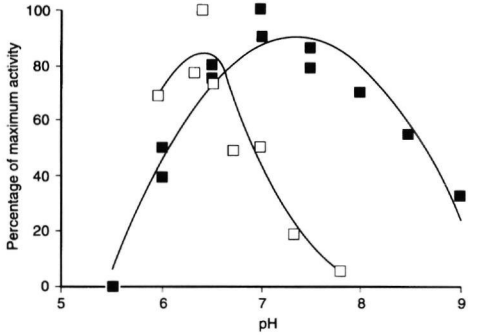

Fig. 2. Effect of pH on the biosynthetic and transferase activity of glutamine synthetase. For the biosynthetic reaction (-■-) and the transferase reaction (-□-), 100% activity represents 0.01 U.ml^{-1} and 0.246 U.ml^{-1} respectively.

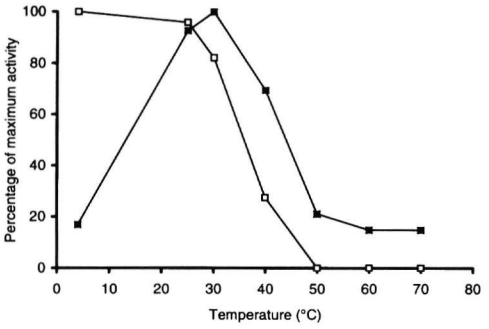

Fig. 3. Temperature optimum and temperature stability of the glutamine synthetase. At the optimum temperature (-■-) and at maximum stability (-□-) 100% activity represents 0.01 U.ml^{-1} and 0.007 U.ml^{-1} respectively.

extract, was found to be 6.4. To determine the pH optimum and temperature optimum for the biosynthetic reaction, we used partially purified GS. For the biosynthetic reaction the pH optimum was 7.0.

The effect of temperature on the biosynthetic reaction is shown in Fig. 3. A temperature optimum was found at 30°C. Temperature stability was determined by incubation of partially purified GS at different temperatures for 20 min. After being brought to room temperature, biosynthetic activity was measured as liberated phosphate (Fig. 3). Stability of GS decreased rapidly at temperatures above 20°C. Inactivation was not influenced by the presence of glutamate (up to 30 mM), ATP (up to 10 mM), $MnCl_2$ (up to 10 mM), $MgCl_2$ (up to 50 mM), 2-mercaptoethanol (up to 20 mM), ethylene glycol (up to 20%) or sucrose (up to 2 M).

Table 2. Inhibition of GS from *Agaricus bisporus* by metabolites

Metabolite	Remaining activity (%)
None	100
10 mM L-alanine	85
10 mM L-histidine	62
10 mM L-tryptophan	65
10 mM L-anthranilic acid	87
0.1 mM AMP	101
1.0 mM AMP	87
10 mM AMP	88
0.1 mM L-glutamine	104
1.0 mM L-glutamine	102
10 mM L-glutamine	95
0.1 mM glycine	111
1.0 mM glycine	113
10 mM glycine	97

Assays were conducted with GS partially purified by Fractogel EMD-DEAE. GS activity was determined as liberated phosphate in a biosynthetic reaction. (100% activity corresponded with 0.017 U/ml.)

Substrate affinity. The activity of the partially purified GS was determined as a function of the substrate concentrations. The apparent K_m-values were extrapolated from Lineweaver-Burk plots of the data obtained. In the biosynthetic reaction the K_m-values for L-glutamate, ammonium, and ATP were 22, 0.16, and 1.25 mM respectively. The K_m values for L-glutamine and hydroxylamine measured in the transferase assay were 91 and 0.42 mM respectively. The K_m values for glutamate and ammonia appeared to vary and were proportional to the concentration of each. The K_m for ATP was affected much less by the concentration of either ammonia or glutamate.

Effect of metabolites and specific inhibitors on enzyme activity. A number of metabolites known to affect GS activity in other organisms were tested for their effect on *A. bisporus* GS. Results are shown in Table 2. Glutamine and glycine at concentrations up to 10 mM did not affect the biosynthetic activity. Minor inhibitory effects were observed in the presence of 10 mM L-alanine. The biosynthetic activity of GS was clearly inhibited by 10 mM L-histidine and L-tryptophan. Anthranilic acid and AMP showed minor inhibitory effects.

Biosynthetic activity of GS, measured as liberated phosphate, was not affected by the presence of 10 mM of the glutamine analogs azaserine or albizzine. The glutamate analogs L-methionine-S-sulfoximine (MSO) and phosphinothricin (PPT) [2-amino-4-(methylphosphinyl)-butanoic acid], however, were potent inhibitors of GS. Inhibitory effects were tested both on the transferase and the biosynthetic reaction

(coupled assay). Both MSO and PPT were competitive inhibitors with respect to glutamate (biosynthetic reaction) and glutamine (transferase reaction). For the transferase reaction K_i was 890 μM for MSO and 240 μM for PPT. The biosynthetic reaction was affected much more by MSO and PPT. The K_i value for both compounds was 17 μM.

Discussion

Affinity chromatography techniques have proved to be very useful instruments in the purification of GS [5, 21, 23, 27]. The attempts to purify GS of *A. bisporus* were started with an anthranilate-Sepharose column as described by Palacios [23]. GS bound tightly to this column and could be eluted only with 100 mM AMP. Even 1 M NaCl was not effective. Since this column gave a low recovery, Green A was used as an alternative. GS from *A bisporus* was purified to apparent homogeneity by a combination of Green A chromatography and ion-exchange chromatography. The enzyme has an octameric structure with 46 kDa subunits, and a native molecular weight of about 325 kDa

Recently Woods and Reid [32] classified the different GS enzymes. They recognized three different types of GS. GS-Is are generally found in bacteria and are relatively heat-stable polymeric enzymes consisting of 12 identical subunits. GS-IIs are typical of eukaryotes but are also found in some bacteria. They are composed of eight subunits and are heat labile. GS-IIIs are hexameric enzymes found in bacteria. On the basis of its molecular structure and its heat lability (Fig. 3), GS from *A bisporus* is a GS-II type enzyme. The pH optimum for the biosynthetic reaction of *A. bisporus* GS is similar to the pH optima found for other organisms [30]. Although quite low, the K_m of *A. bisporus* GS for ammonium is somewhat higher than reported for GS from other sources, as is the K_m for glutamate [5, 20, 21, 28, 31]. Because of its high affinity for ammonium, GS would be capable of assimilating ammonium at low intracellular concentrations. According to Stewart and Rhodes [29], the high K_m value of GS for glutamate may be physiologically important. Since glutamate is both a substrate and a product of the GS-GOGAT pathway, there is a possibility of excessive cycling of glutamate at the expense of ATP, particularly when the ammonium concentration is high. The relatively low affinity of GS for glutamate could minimize this possibility and reduce competition for glutamate between GS and other glutamate-dependent reactions.

The way in which the K_m values of the substrates depend on each other appears to confirm a reaction mechanism as proposed by Meister [18] for mammalian GS. According to this mechanism, substrates are bound in an ordered sequence with an enzyme-bound γ-glutamylphosphate as an intermediate. MSO and PPT are suggested to represent structural analogs of the γ-glutamylphosphate and may therefore inhibit GS [14, 18]. This inhibition was also found for GS from *A. bisporus* with K_i values similar to those previously reported [10, 14]. The different response of the transferase reaction and the biosynthetic reaction of GS to both MSO and PPT has also been observed for yeast GS [12].

A number of metabolites like AMP, ADP, and the amino acids alanine, glycine, serine, histidine, and tryptophan may be involved in the regulation of the GS activity [11]. The response of GS activity to these feedback inhibitors seems to vary in GS types. GS-I is usually more susceptible to feedback inhibition than GS-II [32] GS from *A. bisporus* was not affected by glycine and glutamine, while histidine and tryptophan were clearly inhibitory. Minor effects were observed with alanine, AMP, and anthranilic acid. It is not clear if feedback inhibition by metabolites plays a major role in the regulation of GS in vivo. Other mechanisms for regulation of GS activity are known. For example, studies in *C utilis* [8, 9] have shown that GS activity can be regulated in response to environmental factors by dissociation of the octameric enzyme to a tetrameric structure with a reduced biosynthetic activity. Furthermore, Aguirre and Hansberg [1] have proposed a model for the regulation of *N. crassa* GS involving oxidative inactivation of the enzyme In summary, GS from *A bisporus* resembles GS-II type glutamine synthetases with respect to its structural and kinetic properties. Further study will focus on the physiology of GS activity in *A. bisporus*.

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Purification and characterization of NADP-dependent glutamate dehydrogenase from the commercial mushroom *Agaricus bisporus*.

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Purification and Characterization of NADP-Dependent Glutamate Dehydrogenase from the Commercial Mushroom *Agaricus bisporus*

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Abstract. The nicotinamide adenine dinucleotide phosphate (NADP)-dependent glutamate dehydrogenase (NADP-GDH) of *Agaricus bisporus*, a key enzyme in ammonia assimilation, was purified to apparent electrophoretic homogeneity with 27% recovery of the initial activity. The molecular weight of the native enzyme was 330 kDa. The enzyme is probably a hexamer, composed of identical subunits of 48 kDa. The isoelectric point of the enzyme was found at pH 4.8. The N-terminus appeared to be blocked. The enzyme was specific for NADP(H). The K_m-values were 2.1, 3.2, 0.074, 27.0, and 0.117 mM for ammonia, 2-oxoglutarate, NADPH, L-glutamate, and NADP respectively The pH optima for the amination and deamination reactions were found to be 7.6 and 9.0, respectively. The temperature optimum was 33°C. The effect of several metabolites on the enzyme's activity was tested. Pyruvate, oxaloacetate, ADP, and ATP showed some inhibitory effect. Divalent cations slightly stimulated the aminating reaction. Antibodies raised against the purified enzyme were able to precipitate NADP-GDH activity from a cell-free extract in an anticatalytic immunoprecipitation test. Analysis of a Western blot showed the antibodies to be specific for NADP-GDH

It is generally accepted that the assimilation of ammonia into glutamate and glutamine plays a central role in the nitrogen metabolism of yeasts and other fungi [1]. Ammonia assimilation is either catalyzed by NADP-specific glutamate dehydrogenase (NADP-GDH; EC 1.4.1 4) or by glutamine synthetase (GS; EC 6.3.1.2).

As a class, glutamate dehydrogenases catalyze the interconversion of 2-oxoglutarate and glutamate, using NAD^+ or $NADP^+$ as a cofactor. Excellent reviews on their molecular and kinetic properties have been provided by Goldin and Frieden [11], Smith et al. [25], and Hudson and Daniel [14] LeJohn [18] studied a large number of fungi and concluded that most higher fungi possess two glutamate dehydrogenases, one specific for NAD^+ (NAD-GDH) and the other specific for $NADP^+$ (NADP-GDH).

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Strong indications on the metabolic role of NADP-GDH were provided by studies of mutant strains of *Neurospora crassa* [10] and *Aspergillus nidulans* [16]. These studies showed that NADP-GDH is involved in the assimilation of ammonia into glutamate. Tracer studies with $^{15}NH_4^+$ have confirmed this view for *Cenococcum geophilum* [21], *A. nidulans* [17], and *Stropharia semiglobata* [24]. Furthermore, studies of mutant strains of *A nidulans* [3] and *Saccharomyces cerevisiae* [22] showed that NAD-GDH mainly generates ammonia from glutamate, thus serving a catabolic function

Even though the white button mushroom *Agaricus bisporus* has long been cultivated on an industrial scale, knowledge about its primary nitrogen metabolism is relatively scarce Recently, we inventoried the nitrogen-assimilating enzymes in *A. bisporus* [4]. Apart from GS, *A. bisporus* was shown to contain both NADP-GDH and NAD-dependent glutamate dehydrogenase (NAD-GDH; EC 1.4.1.2.).

For elucidation of the physiological role of nitrogen-metabolizing enzymes, detailed information on pure enzymes and their regulation is indispensable. This paper reports the purification and characterization of the NADP-GDH of *A bisporus*.

Materials and Methods

Organism and culture conditions. Fruit bodies of *A bisporus* strain Horst® U1 were obtained from mycelium cultivated on a commercially prepared compost and were harvested at stages 3 or 4 of growth (according to the classification of Hammond and Nichols [12])

Preparation of cell-free extracts. Cell-free extracts were prepared by freezing sliced fruit bodies in liquid nitrogen followed by grinding the frozen material with glass beads (ϕ 0 10–0 11 mm) in a mortar To 1 g of the frozen material 1 ml of extraction buffer (100 mM potassium phosphate buffer pH 6 5, containing 20% (vol/vol) glycerol) was added After addition of the extraction buffer, the suspension was centrifuged at 40 000 *g* (30 min, 4°C) The clear supernatant then was used as a cell-free extract

Protein determination. Protein concentrations were determined with the bicinchoninic acid-protein assay kit (Sigma Chemical Co , St Louis, Missouri, USA), with bovine serum albumin as a standard For very low protein concentrations the BioRad protein micro assay kit (BioRad, Richmond, California USA) was used In this case bovine gamma globulin served as a standard

Assay of enzyme activity. Enzyme activity was measured spectrophotometrically by following the changes in absorbance at 340 nm The reductive amination activity of NADP-GDH was determined in a reaction mixture containing 50 mM Tris-HCl (pH 7 8), 0 25 mM NADPH, 25 mM NH_4Cl and 5 mM 2-oxoglutarate Oxidative deamination activity was determined in a reaction mixture containing 100 mM glycine-NaOH (pH 9 0), 1 0 mM NADP, and 50 mM L-glutamic acid Reactions were measured at 33°C and were started by addition of the enzyme One unit of enzyme activity (U) was defined as 1 μmol product formed per minute under the incubation conditions used

Dye-affinity chromatography. Selection and preparation of an appropriate dye-affinity matrix was performed as described previously [13] Briefly, small amounts of cell-free extract were applied to about 90 different dye adsorbents, which were packed in a 96 well transplate cartridge previously equilibrated in 50 mM potassium phosphate buffer pH 6 5, containing 10% (vol/vol) glycerol After biospecific elution with 1 mM NADP in the equilibration buffer, the presence of enzyme activity in the eluate was tested with an activity staining as described by Vallejos [30] For testing optimal binding and recovery of the enzyme, 0 3 1, or 3 g of the selected dye was coupled to 25 g Sepharose CL-6B

Purification of glutamate dehydrogenase. All procedures were carried out at 4°C, unless otherwise stated Cell-free extract (50 ml) was desalted by dialysis against 2 volumes of 2 L buffer A [50 mM potassium phosphate pH 6 5, containing 5% (vol/vol) glycerol] After desalting, 31 ml of the cell free extract was loaded on the dye affinity column (2 × 5 cm), previously equilibrated with buffer A The dye affinity step was performed at 22°C Unbound protein was washed from the column with 25 ml of buffer A, and thereafter protein was eluted with an 80-ml linear gradient of 0–250 mM KCl in buffer A The fractions containing enzyme activity were pooled and chilled to 4°C After concentration and buffer change to buffer B (10 mM potassium phosphate pH 7 4, containing 10% glycerol), by use of a Centriprep YM-30 membrane (Amicon, Inc , Beverly, MA, USA), the enzyme preparation was applied on a Protein Pak DEAE 5-PW column (Waters-Millipore, Milford USA, 0 75 × 7 5 cm), previously equilibrated with buffer B The column was washed with 10 ml buffer B, and thereafter bound protein was eluted with a 90-ml linear gradient of 0–150 mM potassium phosphate in buffer B

Gel permeation chromatography. Cell-free extract (2 ml) was applied on a Sephacryl S 300 column (1 5 × 96 cm) previously equilibrated with 50 mM potassium phosphate pH 7 0, containing 10% (vol/vol) glycerol The Sepharose S-300 column was calibrated in separate runs with the following proteins as standards thyroglobuline (M_r 669,000), apoferritin (M_r 443,000), β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), and carbonic anhydrase (M_r 30,000) The apparent molecular weight of the NADP GDH was interpolated from the position on a plot of log molecular weight versus the elution volume on Sephacryl S-300

Electrophoretic methods. All gel-electrophoresis methods were performed on precast slabgels with Pharmacia PhastSystem® equipment (Pharmacia, Uppsala, Sweden) Native PAGE was performed either on homogeneous 7 5%, 12 5%, or 20% gels or on gradient gels (8–25% or 10–15%), with urease (M_r 545,000 and 272,000), bovine serum albumin (M_r 132,000 and 66,000), and chicken egg albumin (M_r 45,000) for calibration SDS-PAGE was performed on a 10–15% gradient gel, with albumin (M_r 66,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400) for calibration

Isoelectric focusing was performed on precast IEF gels with a pH range of 3–9 according to the manual, with phycocyanin (pI 4 65) β-lactoglobulin B (pI 5 1), bovine carbonic anhydrase (pI 6 0), human carbonic anhydrase (pI 6 5), equine myoglobin (pI 7 0), human hemoglobin A (pI 7 1), human hemoglobin C (pI 7 5), lentil lectin (three bands, pIs 7 8, 8 0 and 8 2) and cytochrome C (pI 9 6) as markers

Gels were stained with Coomassie brilliant blue (PhastSystem® Development Technique File No 200, Pharmacia, Uppsala, Sweden) In case of very low protein concentrations, a silver stain was used (PhastSystem® Development Technique File No 210)

Enzyme activity was stained on gels by the tetrazolium salt method of Vallejos [30]

Immunological techniques. After purification, NADP-GDH was used to elicit antibodies according to the method described by Dunbar and Schwoebel [9] In short, a New Zealand White rabbit was immunized by intracutaneous injection of 100 μg NADP-GDH protein emulsified in Freund's complete adjuvant Two subcutaneous booster injections of 100 μg NADP-GDH protein, emulsified in Freund's incomplete adjuvant, were given at 3-week intervals Bleedings were taken from the marginal ear vein Serum was obtained by incubating the blood at room temperature for 1 h After subsequent incubation at 4°C for 18 h, the serum was separated by centrifugation The serum was kept frozen at −20°C until use

The titer of the antiserum was determined by an ELISA method [15], with 96-well polystyrene plates (Nunc, GIBCO Laboratories, Grand Island, New York, USA) Sensitization was achieved with 2 5 μg/ml purified NADP-GDH in 0 1 M $NaHCO_3$, pH 9 6 The colored reaction product was measured in a Titertek Multiskan MCC/340 (Flow Laboratories, McLean, Virginia, USA) at 450 nm The titer was estimated by the effective dose method [28] Pre-immune serum was used as a reference Anticatalytic

immunoprecipitation tests were performed as described by Son et al. [26]. Briefly, cell-free extract (0.08 units of NADP-GDH activity) was mixed with 1–5 µl of antiserum in a total volume of 0.5 ml. After 1 h of incubation at room temperature, the immunoprecipitate was removed by centrifugation at 15,000 *g* for 10 min. The supernatant was used to determine the remaining NADP-GDH activity.

Immunoblotting. After separation by native PAGE, the proteins were transferred to a PVDF membrane (BioRad) with the Phast-System™ blotting unit. Blotting was performed in 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the membranes were blocked for 3 h with 3% (wt/vol) BSA in 10 mM Tris-HCl pH 7.0, 350 mM NaCl. Detection of NADP-GDH on the membranes was achieved in a way similar to the ELISA technique described above. After the blocking step, the membranes were rinsed with washing solution and reacted with immune serum diluted 2000-fold in a washing solution for 18 h. After being rinsed in the washing solution, the membranes were probed for 1 h with a 1000-fold dilution of purified goat-antirabbit IgG-peroxidase conjugate (GARPO, Sigma). After washing, the membranes were incubated in the dark for 10–20 min at room temperature in 0.06% (wt/vol) diaminobenzidine and 0.012% (vol/vol) H_2O_2 in 0.25 M Na_2HPO_4-citrate buffer (pH 6.5).

Results

Fruit bodies were used in the purification procedure because of their high specific activity of NADP-GDH [4]. The use of fruit bodies for the purification of NADP-GDH was merely practical. No differences were observed between the NADP-GDH from mycelium and fruit bodies (data not shown). For purification of the enzyme, the dye Procion Green HE-4BD was selected from a large number of dyes. An amount of 3 g dye/25 g Sepharose CL-6B gave optimal results with regard to recovery of enzyme activity. Binding of the enzyme to the dye matrix was markedly influenced by temperature. Optimum results were achieved at 22°C in buffer A. The enzyme could be eluted from the column biospecifically with 72.3% recovery of activity, by use of 1 mM NADPH. With 1 mM concentrations of NADH or NADP, only 11% and 2% of the activity could be eluted from the column. High concentrations of salt or raising the pH of the buffer to pH 8.0 were also effective in eluting the enzyme. For practical and economical reasons, elution with high salt concentrations was chosen. After optimalization of the method, the column was used in the purification scheme as summarized in Table 1. The enzyme eluted from the Procion Green matrix at 100 mM KCl with 68% recovery. Active fractions were pooled, concentrated, brought into buffer B, and applied on a Protein Pak 5 PW anion-exchange column. The enzyme eluted from this column at 85 mM of potassium phosphate and was purified 126-fold. Electrophoresis of the final preparation on native PAGE revealed a single protein band, as shown in Fig. 1A. The identity of the purified enzyme could be established on gel by activity staining (Fig. 1B). The location of the NADP-GDH activity from cell-free extract, separated on native PAGE, corresponded with an activity staining of the purified enzyme on the same gel. From Ferguson plot analysis of the electrophoresis pattern of purified NADP-GDH on homogeneous 7.5%, 12.5%, and 20% slab-gels, a molecular weight of 330 kDa was estimated. From the elution pattern of NADP-GDH on a Sephacryl S-300 column, a molecular weight of 331 kDa was estimated. After SDS-PAGE, a single band with a molecular weight of 48 kDa was found. On the basis of comparison with marker proteins, its isoelectric point at 15°C was estimated to be 4.83 ± 0.09

Table 1. Purification of the NADP-dependent GDH from *A garicus bisporus*

	Protein (mg)	Total activity[a] (U)	Specific activity ($U \cdot mg^{-1}$)	Recovery (%)
Cell-free extract	237	15.4	0.07	100
Dialysis	103	12.4	0.12	80
Dye-affinity	4.5	8.4	1.85	54
Centriprep	4.5	4.9	1.09	32
Protein Pak DEAE	0.5	4.1	8.20	27

[a] Assayed as the reductive amination reaction.

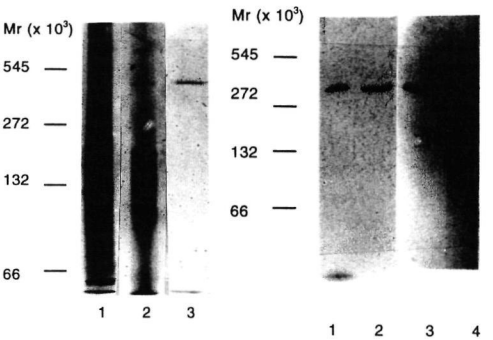

Fig. 1. (**A**) Native PAGE of the purified NADP-GDH on a 10–15% gradient gel. Protein from different purification steps was analyzed by silver staining. Lane 1, cell-free extract (1.0 µg); lane 2, dye affinity chromatography (0.6 µg); lane 3, Protein Pak DEAE chromatography (0.1 µg). (**B**) Native PAGE of NADP-GDH on an 8–25% gradient gel. Cell-free extract and purified NADP-GDH were analyzed by activity staining (lanes 1 and 2) and by immunoblot with anti-GDH (lanes 3 and 4). Lanes 1 and 3, cell-free extract (1.0 µg); lanes 2 and 4, purified enzyme (0.1 µg).

Table 2. K_m values for the substrates of the NADP-dependent GDH from *A garicus bisporus*

Substrate	K_m values (mM)[a]
NH_4Cl	2.1
2-Oxoglutarate	3.2
NADPH	0.074
L-Glutamate	27.0
NADP	0.117

[a] The values are the means of two independent experiments.

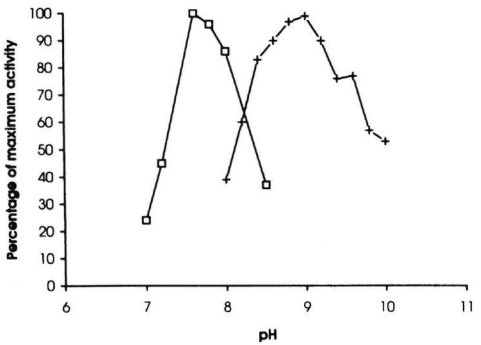

Fig. 2. Effect of pH on the NADP-dependent glutamate dehydrogenase activity. Activities for both amination (□) and deamination reactions (+) at their respective pH optima were set at 100%. Amination and deamination reactions were measured at room temperature in 50 mM Tris/HCl and 100 mM glycine/NaOH buffer, respectively.

(n = 3). For determination of the N-terminal sequence of the protein, purified NADP-GDH was subjected to SDS-PAGE and transferred to a PVDF membrane, according to the methods described by Ploug et al. [23]. Our attempts to sequence, however, were unsuccessful. The N-terminal appeared to be blocked.

Apparent K_m-values for substrates and coenzymes were estimated from Lineweaver-Burke plots (Table 2). The NADP-GDH proved to be strictly specific for NADP(H); no activity was found when NAD(H) was used instead of NADP(H).

The influence of pH on the aminating and deaminating reactions is shown in Fig. 2. The pH optimum for the reductive amination was 7.6, whereas a pH optimum of 9.0 was found for the oxidative deamination. The specific activity of the amination reaction was about 6 times higher (7.8 versus 1.3 U/mg) than that of the deamination reaction at their respective pH optima.

The effect of temperature on the activities of NADP-GDH is shown in Fig. 3. Optimal activity for the aminating reaction was found at 34°C. At elevated temperatures the enzyme proved to be unstable. Incubation of the enzyme at 0°C for 1 h gave no loss of activity. Pre-incubation for 1 h at 25°, 37°, or 55°C, however, gave a loss of activity of 12, 72, and 100%, respectively.

Incubation of the enzyme at 0°C for 1 h with 0.1 M concentrations of urea, KCl, or NaCl gave almost no loss of activity. The same treatment with 2.0 M urea gave a loss of about 65%.

Addition of 3% (vol/vol) of the nonionic detergent Tween 80 to cell-free extract had no effect on the activity. At the same concentration, addition of Nonidet NP 40 or Triton X-100 resulted in about 35% loss of activity. Addition of SDS resulted in complete loss of activity at a concentration of 0.006% (wt/vol).

A number of metabolites were tested for their effect on NADP-GDH activity. Nucleotides like AMP, cAMP, ADP, and ATP were added to the reaction mixture up to concentrations of 4 mM. No effect could be measured on either the aminating or deaminating reaction. At a concentration of 4 mM ADP or ATP, however, a slight inhibitory effect (about 35% inhibition) was observed on the aminating reaction only.

The carboxylic acids citrate, malate, phosphoenolpyruvate, or succinate at concentrations up to 20 mM had no effect on enzyme activity. Pyruvate (20 mM) and oxaloacetate (10 mM) showed about 25% inhibition of both the aminating and deaminating reactions.

Divalent cations like Mg^{++} and Ca^{++} (both 1 mM) had a slight stimulating effect (about 20%) on the aminating activity. The deaminating activity was not influenced. In contrast, the deaminating activity was stimulated about 35% by the addition of 10 mM EDTA.

To test the effects on the equilibrium of the reaction, we added the reaction products to the assay mix. The aminating reaction was not influenced markedly by its reaction product, glutamate. L-glutamate, added in a 12.5 mM concentration, did not decrease the rate of the aminating reaction, while 50 and 100 mM concentrations of L-glutamate decreased the aminating reaction rate only 30% and 60% respectively.

The deaminating reaction was influenced much more by its reaction products. The deaminating reaction rate decreased about 25% on addition of 10 mM 2-oxoglutarate to the assay mix. The presence of NH_4^+ also had marked effects on the deaminating reaction. At concentrations of 0.5 and 5 mM NH_4Cl, the reaction rate was inhibited 40% and 95% respec-

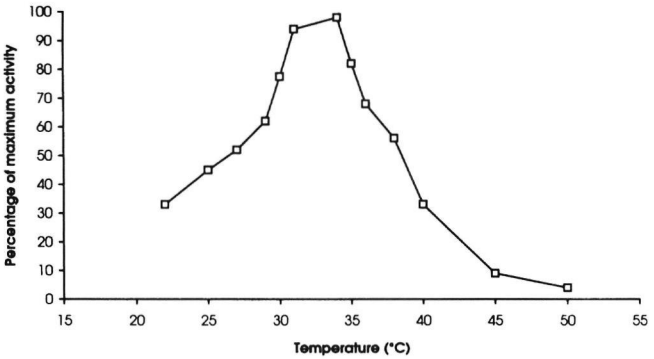

Fig. 3. Effect of temperature on the rate of the reductive amination reaction.

tively. The effect of addition of cofactors on the initial reaction rate was not tested.

The immunization procedure of the New Zealand White rabbit resulted in an NADP-GDH specific antiserum. After the primary injection, the antibody titer of the serum was only 117. After the primary and secondary booster injection, however, the titer was raised to 12,400 and 14,300, respectively. As is shown in Fig. 4, the antiserum was able to precipitate NADP-GDH activity from a cell-free extract in an anticatalytic immunoprecipitation test. Furthermore, specificity of the antiserum was tested on an immuno-blot (Fig. 1B, lanes 3 and 4). Cell-free extract and purified NADP-GDH were subjected to native PAGE on an 8–25% gradient gel and blotted onto a PVDF-membrane. Probing the immuno-blot with the antiserum revealed one strong band for both samples. The location of the bands from the different samples coincided with the activity staining (Fig. 1B, lanes 1 and 2), thus confirming the bands to be NADP-GDH.

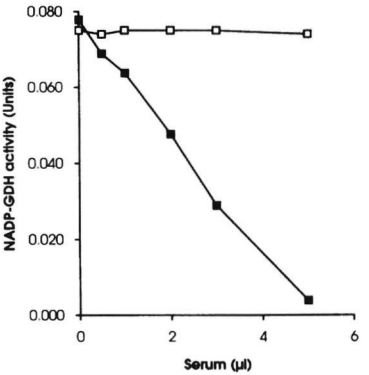

Fig. 4. Anticatalytic immunoprecipitation of NADP-GDH activity from cell-free extract. Either anti-GDH serum (■) or pre-immune serum (□) was mixed with cell-free extract (0.08 U of NADP-GDH activity) in a total volume of 0.5 ml. After 1 h of incubation at room temperature, the mixture was centrifuged at 15,000 *g* for 10 min. Subsequently the NADP-GDH activity in the supernatant was measured.

Discussion

Dye-affinity chromatography is routinely used for the purification of dehydrogenases [13, 31], and very specific elution can be achieved with cofactors or nucleotides. The dye Procion Green HE 4BD showed the best performance in the purification of the NADP-GDH of *A. bisporus*. By combination of dye-affinity chromatography and ion-exchange chromatography, the NADP-GDH was purified to apparent homogeneity. Ferguson plot analysis of the electrophoresis pattern on native PAGE and gel permeation chromatography showed its molecular weight to be about 330 kDa. Analysis on SDS-PAGE showed the enzyme to be composed of identical subunits with a molecular weight of 48 kDa. Based on the experimental values, an octameric or a hexameric structure is possible. In general, NADP-GDH of microorganisms is a hexamer composed of identical subunits with a molecular weight between 270 and 320 kDa [6, 7, 20, 25]. Benachenhou-Lahfa et al. [5] compared the structure of NADP-GDH from a wide range of different species and found it to be very conserved. This would mean that NADP-GDH of *A. bisporus* is most likely a hexamer.

The temperature optimum, pH optima, and K_m-values of *A. bisporus* NADP-GDH for 2-oxoglutarate, L-glutamate, and ammonia are similar to those of other fungal glutamate dehydrogenases [2, 7, 8, 20, 27]. Like *Laccaria laccata* NADP-GDH [8], *A. bisporus* NADP-GDH did not exhibit biphasic kinetics for

ammonia as shown by the NADP-GDHs from *Coprinus lagopus* [2], *Sphaerostilbe repens* [7], *Neurospora crassa* [32], and *Cenococcum graniforme* [20]. NADP-GDH from *A. bisporus* proved to be strictly specific for NADPH; no activity was observed with NADH.

The deaminating reaction was shown to be inhibited strongly by the presence of ammonia; at a 5 mM concentration, ammonia decreased the deaminating reaction rate about 95%. The effect of L-glutamate on the aminating reaction was much less pronounced; a 100 mM concentration of L-glutamate was needed to decrease the aminating reaction rate about 60%. The inhibiting effect of ammonia on the deaminating reaction has also been reported for other fungi [2, 7, 8]. The inhibiting effect of ammonia on the deaminating reaction and the difference in affinity of *A bisporus* NADP-GDH for ammonia and glutamate are in favor of the hypothesis that in vivo this enzyme operates mainly in the direction of glutamate formation.

NADP-GDH activity was not much affected by the metabolites tested. Slight inhibitory effects were noted with 4 mM concentrations of ADP and ATP, 10 mM pyruvate, and 20 mM oxaloacetate. Several authors [11, 25, 27] have reviewed the effects of metabolites on the GDHs of fungi. Although the activity of GDHs of lower fungi is affected by the presence of nucleotides, such effects have not been reported on the NADP-GDHs of higher fungi. Divalent cations at a 1 mM concentration had a slight stimulating effect on the aminating activity. Wootton [32] studied the effect of divalent cations on NADP-GDH activity of *N. crassa* by adding their Cl^- salts to assays of purified *N. crassa* NADP-GDH. He reported nonspecific inhibitory effects at concentrations greater than 50 mM, probably owing to the Cl^--anions.

According to Stewart et al. [27], the lower fungi have only one GDH enzyme, which is responsible for both ammonia assimilation and deamination of glutamate. In those organisms there will be a necessity for the enzyme to evolve complex regulatory properties. However, possession of both a biosynthetic NADP-linked GDH and a catabolic NAD-linked enzyme by higher fungi renders it unnecessary for these enzymes to have complex regulatory properties Furthermore, in view of the moderate effects on NADP-GDH activity achieved by relatively high concentrations of the metabolites, an in vivo regulatory role of these substances appears to be unlikely.

Our immunological experiments showed that we successfully raised an antiserum against the purified NADP-GDH. Using this antiserum, we were able to precipitate NADP-GDH activity in a cell-free extract. Furthermore, Western-blot analysis of the cell-free extract revealed only one band, thus demonstrating the specificity of the antiserum.

In summary, the enzyme resembles NADP-GDH from other fungal sources with respect to its molecular and kinetic properties. With the aid of the NADP-GDH-specific antiserum, further study will focus on the regulation and location of NADP-GDH in *A. bisporus*.

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Nucleotide sequence and expression of the gene encoding NADP-dependent glutamate dehydrogenase (*gdhA*) from *Agaricus bisporus*.

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Abstract

The gene encoding NADP-dependent glutamate dehydrogenase (*gdh*A) was isolated from an *Agaricus bisporus* recombinant phage λ library The deduced *A. bisporus* NADP-GDH amino acid sequence represents a 457 amino acid protein and is highly homologous to the amino acid sequences derived from previously isolated and characterized genes coding for microbial NADP-GDH The open reading frame is interrupted by six introns The position of none of the introns is conserved with respect to the conserved positions of the two introns located in the open reading frame of the ascomycete fungi *Aspergillus nidulans* and *Neurospora crassa* Northern analysis suggests that the *A. bisporus gdh*A gene is transcriptionally regulated and that, unlike in ascomycetes, transcription of this gene is repressed upon the addition of ammonium to the culture

Key words *Agaricus bisporus*, NADP-dependent glutamate dehydrogenase, molecular cloning, gene structure, mushroom

Introduction

The common white mushroom *Agaricus bisporus* (Lange) Imbach is grown in large amounts for commercial purposes During commercial fruiting substancial amounts of organic nitrogen compounds are added to increase the yield, however little knowledge has been gained about the biochemistry, genetics and physiology of its nitrogen metabolism This is in part due to the secondarily homothallic life cycle which complicates classical genetic analysis The basidiospores are predominantly heterokaryotic and fertile and identification of the rare homokaryons among the offspring by classical means is laborious and time consuming (Raper et al 1972) The information derived from such knowledge however, could be helpful in developing new commercial strains and in developing more sophisticated cultivation systems

A. bisporus can utilize a variety of nitrogen sources including ammonium as inorganic nitrogen source Ammonium plays a central role in the nitrogen metabolism of many yeasts and fungi (Jennings 1988, Ahmad and Hellebust 1991) Utilization of ammonium is catalyzed either by a NADP-dependent glutamate dehydrogenase (NADP-GDH, EC 1 4 1 4) or by glutamine synthetase (GS, EC 6 3 1 2) (Pateman 1969) NADP-GDH is also supposed to occupy a pivotal role in general metabolism, since it provides a link between amino acid metabolism and the Krebs cycle The metabolic role of NADP-GDH in the assimilation of ammonium in the ascomycete fungi *Neurospora crassa* and *Aspergillus nidulans* was elucidated through studying a number of mutant strains Mutations in the structural gene for NADP-GDH relieve repression by ammonium in *A. nidulans* (Kinghorn and Pateman 1973) and *N. crassa* (Dunn-Colemann et al 1979) Such mutants are hampered in the production of glutamate and therefore glutamine which is produced by amidation of glutamate Tracer studies using $^{15}NH_4^+$ confirmed the role of NADP-GDH in the assimilation of ammonium in *A. nidulans* (Kusnan et al 1989) and extended this role to the ectomycorrhizal ascomycete *Cenococcum geophilum* (Martin et al 1988) and the basidiomycete *Stropharia semiglobata* (Schwartz et al 1991) Evidence for transcription regulation of the *A nidulans* NADP-GDH gene was obtained by a study on the steady state transcript levels of the gene on different nitrogen sources (Hawkins et al 1989)

The NADP-GDH of the basidiomycete *A. bisporus* is expressed both in mushrooms and vegetatively growing mycelium (Baars et al 1994) Unlike the situation in *A. nidulans* and *N*

crassa the expression of the enzyme is repressed in the presence of ammonium and derepressed if glutamate is the sole nitrogen source (Schaap et al 1995a) The primary structure of the gene encoding NADP-GDH is highly conserved throughout nature and this allowed us to clone and to study the expression of the corresponding *A. bisporus* gene

Materials and methods

Agaricus strains and culture conditions, bacterial strains, DNA techniques and chemicals

Agaricus bisporus Horst®U1 and its homokaryotic constituents, strains 39 and 97, were used from the collection of the Mushroom Experimental Station (Horst, The Netherlands) The strains were cultured on DT80-glucose medium according to Sonnenberg et al (1988)
Escherichia coli LE392 (Promega, Madison, USA) was used for phage amplification and λ DNA isolation *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, MD, USA (BRL)) was used for plasmid transformation and propagation
Plasmid pUC19 (Yannisch-Perron et al 1985) was used as vector for (sub) cloning and amplification of obtained DNA fragments Standard DNA manipulations were carried out essentially as described in Sambrook et al (1989) Restriction enzymes and other enzymes used for DNA manipulations were purchased from BRL and used according to the suppliers instructions DNA sequences were determined by the method of Sanger *et al.* (1977) using a T7 sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) Herring sperm DNA was obtained from Boehringer Mannheim GmbH (Mannheim, Germany) Synthetic deoxyoligonucleotides used for sequencing were purchased from Pharmacia Biotech

Cloning of the *A. bisporus* gene encoding NADP-GDH

Two degenerate oligonucleotide primers were used in a polymerase chain reaction (PCR) using strain 39 genomic DNA as template
Primer 1 (sequence 5'-CT(A/G/C/T)AA(A/G)TT(T/C)(C/T)TIGGITT(T/C)GA(A/G)C-3') encodes *N. crassa* NADP-GDH amino acids 91-97 and primer 2 (sequence 5'-CC(A/G)TA(A/G/T/C)CCIGTIGC(T/C)TCIGG-3') encodes the antisense codons for *N. crassa* NADP-GDH amino acids 193-199 (Kinnaird and Fincham, 1983) The initial denaturation step of 3 min at 95°C was followed by 30 cycles of 1 min at 95°C, 1 min at 48°C and 1 min at 72°C and a final cycle of 5 min at 72°C using a Sensa DNA processor (Sensa, Amersfoort, The Netherlands) The amplified 430 bp product was labelled with [α-^{32}P] dATP using the method of Feinberg and Vogelstein (1983) and was used as a probe for a standard screening of 15000 plaques corresponding to 5 genome equivalents of an *A. bisporus* λ EMBL4 genomic library of strain 39 Hybridization was carried out overnight at 65°C in standard hybrization buffer (SHB, 6x SSC, 5x Denhardts solution, 0 5% SDS and 100 µg/ml denatured herring sperm DNA) The filters were washed at 65°C using stringent conditions, dried and exposed on Kodak X-omat AR films (Eastman Kodak Co , Rochester, New York, USA) at -70°C using intensifying screens Plaques giving a positive duplo hybridization signal were purified λ DNA was isolated using standard techniques and analyzed by Southern hybridization using the PCR product as probe Hybridizing fragments were cloned in pUC19 and analyzed by sequence analysis The

corresponding cDNA was isolated from a mixed primordia, small fruit body cDNA library (De Groot et al 1995) using hybridization conditions described above and a genomic 1 9 kb *Bgl*II fragment comprising the complete coding region of *gdh*A as a probe (Fig 1, probe P1)
The transcription initiation site was deduced by primer extension mapping according to Calzone et al (1987)

Isolation of total genomic DNA and Southern blot analysis of cultured *A. bisporus* strains

Total DNA from the strains was isolated according to the method of De Graaff et al (1988), digested with various restriction enzymes, separated on 0 8% agarose gels and transferred onto Hybond-N hybridization transfer membranes (Amersham International plc , Amersham, UK) Chromosomal DNA preparation and clamped homogeneous electric field (CHEF) electrophoresis conditions were performed as described by Sonnenberg et al (1991) using a Biorad CHEF DrII system (Biorad Laboratories Inc , Richmond, California, USA) After separation chromosomes were transferred onto a Hybond-N membrane The membranes were hybridized overnight at 65°C in SHB using the genomic 1 9 kb *Bgl*II fragment of *gdh*A as a probe, washed at 65°C using stringent conditions and exposed to X-ray films as described above The CHEF blot was reprobed with a mixture of 200 random cDNA clones using the same conditions to make the chromosomes visible

Northern analysis, preparation of cell-free extracts and assay of enzyme activities

Mycelium of strain Horst®U1 was grown on agar plates containing DT80 glucose medium covered with a cellophane sheet After 14 days of incubation at 24°C, 80 colonies were collected, fragmented in a Waring blender and subsequently used to inoculate fifteen, 250 ml Erlenmeyer flasks each containing 50 ml of DT80 glucose medium except that either 20 mM glutamate or 20 mM proline was used as sole nitrogen source The cultures were allowed to grow without shaking for 15 days (glutamate) or 20 days (proline) at 24°C after which a solution of $(NH_4)_2HPO_4$ (pH 6 8) was added to a final ammonium concentration of 20 mM At given time intervals three individual cultures were pooled The mycelium was harvested by filtration over nylon gauze, immediately frozen in liquid nitrogen and stored at -70°C In one control experiment NaCl was added instead to a final concentration of 20 mM Other control cultures contained DT80 glucose with 10 mM $(NH_4)_2HPO_4$ or a mixture of 10 mM glutamate and 5 mM $(NH_4)_2HPO_4$ as sole nitrogen sources In a separate experiment mycelium was harvested seven hours after the addition of 5 mM dl-phosphinotricin (PPT) to a culture grown on DT80 glucose and 20 mM glutamate Total RNA was isolated from the samples using TRIzol™ Reagent (BRL) The concentration of the RNA was determined spectrophotometrically and equal amounts of RNA was denatured in 10xSSC and 6 15 M formaldehyde and spotted on Hybond-N membrane, or denatured using glyoxal and DMSO by standard techniques, separated on a 1 6% agarose gel and transferred to Hybond-N membrane Sizes of transcripts were determined by co-electrophoresis of RNA molecular weight markers (BRL) which were stained separately after transfer with methylene blue (Sambrook et al 1989) The membranes were hybridized overnight at 42°C in SHB and 50% formamide using the 1 9 kb *Bgl*II fragment of *gdh*A or a 1 2 kb *Sal*I-*Sph*I fragment of *pki*A, the gene encoding pyruvate kinase (Schaap et al 1995a) or a *Kpn*I-*Xba*I fragment comprising the complete cDNA of the gene encoding ribosomal protein S15a as probes (Schaap et al 1995b) and washed at 65°C using stringent conditions All membranes were stripped according to the

suppliers instructions and rehybridized with a 900 bp *Eco*RI fragment encoding the by sequence analysis identified 3' end of 28S ribosomal RNA (EMBL accession no. X91812) using conditions described above to provide for a loading control.

The same mycelium samples were used for determination of enzyme activities. Preparation of cell-free extracts of the samples and assay of enzyme activities were performed as described by Baars et al. (1995a). Total protein concentrations of the extracts were determined using the Biorad protein assay kit and bovine γ-globulin as a standard.

Results

Cloning of the *A. bisporus gdh*A gene

Two degenerate oligonucleotide primers were designed on the basis of two highly conserved regions in the primary structure of the $NADP^+$-GDH sequences of *A. nidulans* (Hawkins et al. 1989), *N. crassa* (Kinnaird and Fincham 1983) and *Saccharomyces cerevisiae* (Nagasu and Hall 1985; Scott Moye et al. 1985). The primers were used in a PCR using the homokaryotic *A. bisporus* strain 39 genomic DNA as template. The 430 bp PCR product obtained was used as a probe for the screening of the λEMBL4 genomic library. A 1.6 kb *Sst*I fragment which hybridized with the PCR product, was subcloned in pUC19 and subjected to sequence analysis. Comparison of the inferred amino acid sequences with published $NADP^+$-GDH sequences confirmed that this fragment contained a part of the *A. bisporus gdh*A gene. The 1.6 kb *Sst*I fragment was subsequently used in a Southern analysis of genomic DNA of *A. bisporus* strains 39 and 97, the two infertile homokaryotic constituents of the cultivated strain

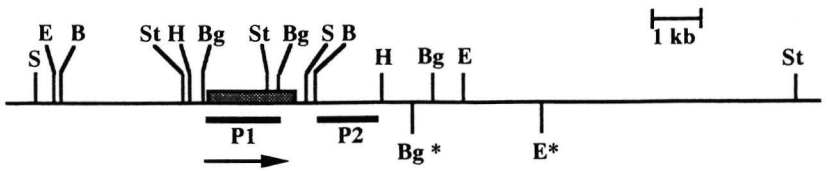

Fig. 1. Genomic map of the gene encoding NADP-GDH of strain 39. Only restriction enzymes used in the cloning procedure are shown and abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*DIII; S, *Sal*I; St, *Sst*I. The shaded box represents the *gdh*A gene. The arrow indicates direction of transcription. Thick bars (P1 and P2) indicate the *Bgl*II and the *Sal*I-*Hin*DIII genomic fragments which were used as probes for RFLP and CHEF analysis. The B^* and E^* sites indicated below the horizontal line are the alternative location of the *Bgl*II and *Eco*RI sites encountered in strain 97 using P1 or P2 as probe.

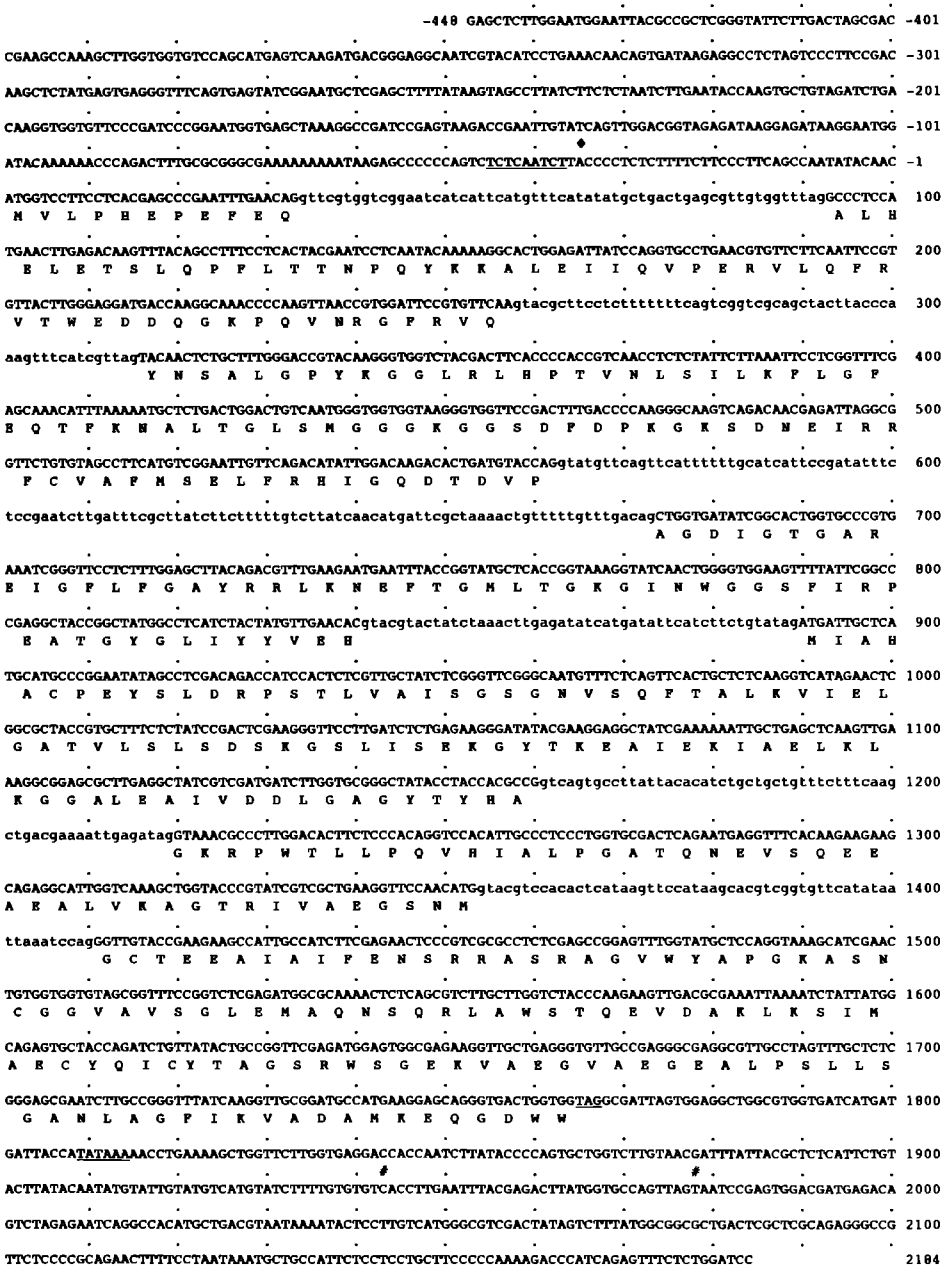
-448 GAGCTCTTGGAATGGAATTACGCCGCTCGGGTATTCTTGACTAGCGAC -401
CGAAGCCAAAGCTTGGTGGTGTCCAGCATGAGTCAAGATGACGGGAGGCAATCGTACATCCTGAAACAACAGTGATAAGAGGCCTCTAGTCCCTTCCGAC -301
AAGCTCTATGAGTGAGGGTTTCAGTGAGTATCGGAATGCTCGAGCTTTTATAAGTAGCCTTATCTTCTCTAATCTTGAATACCAAGTGCTGTAGATCTGA -201
CAAGGTGGTGTTCCCGATCCCGGAATGGTGAGCTAAAGGCCGATCCGAGTAAGACCGAATTGTATCAGTTGGACGGTAGAGATAAGGAGATAAGGAATGG -101
ATACAAAAAACCCAGACTTTGCGCGGGCGAAAAAAAAATAAGAGCCCCCAGTCTCTCAATCTTACCCCTCTCTTTTCTTCCCTTCAGCCAATATACAAC -1
ATGGTCCTTCCTCACGAGCCCGAATTTGAACAGgttcgtggtcggaatcatcattcatgtttcatatatgctgactgagcgttgtggtttagGCCCTCCA 100
M V L P H E P E F E Q A L H
TGAACTTGAGACAAGTTTACAGCCTTTCCTCACTACGAATCCTCAATACAAAAAGGCACTGGAGATTATCCAGGTGCCTGAACGTGTTCTTCAATTCCGT 200
E L E T S L Q P F L T T N P Q Y K K A L E I I Q V P E R V L Q F R
GTTACTTGGGAGGATGACCAAGGCAAACCCCAAGTTAACCGTGGATTCCGTGTTCAAgtacgcttcctctttttttcagtcggtcgcagctacttaccca 300
V T W E D D Q G K P Q V NR G F R V Q
aagtttcatcgttagTACAACTCTGCTTTGGGACCGTACAAGGGTGGTCTACGACTTCACCCCACCGTCAACCTCTCTATTCTTAAATTCCTCGGTTTCG 400
Y N S A L G P Y K G G L R L H P T V N L S I L K F L G F
AGCAAACATTTAAAAATGCTCTGACTGGACTGTCAATGGGTGGTGGTAAGGGTGGTTCCGACTTTGACCCCAAGGGCAAGTCAGACAACGAGATTAGGCG 500
E Q T F K N A L T G L S M G G G K G G S D F D P K G K S D N E I R R
GTTCTGTGTAGCCTTCATGTCGGAATTGTTCAGACATATTGGACAAGACACTGATGTACCAGgtatgttcagttcatttttttgcatcattccgatatttc 600
F C V A F M S E L F R H I G Q D T D V P
tccgaatcttgatttcgcttatcttcttttgtcttatcaacatgattcgctaaaactgtttttgtttgacagCTGGTGATATCGGCACTGGTGCCCGTG 700
A G D I G T G A R
AAATCGGGTTCCTCTTTGGAGCTTACAGACGTTTGAAGAATGAATTTACCGGTATGCTCACCGGTAAAGGTATCAACTGGGGTGGAAGTTTTATTCGGCC 800
E I G F L F G A Y R R L K N E F T G M L T G K G I N W G G S F I R P
CGAGGCTACCGGCTATGGCCTCATCTACTATGTTGAACACgtacgtactatctaaacttgagatatcatgatattcatcttctgtatagATGATTGCTCA 900
E A T G Y G L I Y Y V E H M I A H
TGCATGCCCGGAATATAGCCTCGACAGACCATCCACTCTCGTTGCTATCTCGGGTTCGGGCAATGTTTCTCAGTTCACTGCTCTCAAGGTCATAGAACTC 1000
A C P E Y S L D R P S T L V A I S G S G N V S Q F T A L K V I E L
GGCGCTACCGTGCTTTCTCTATCCGACTCGAAGGGTTCCTTGATCTCTGAGAAGGGATATACGAAGGAGGCTATCGAAAAAATTGCTGAGCTCAAGTTGA 1100
G A T V L S L S D S K G S L I S E K G Y T K E A I E K I A E L K L
AAGGCGGAGCGCTTGAGGCTATCGTCGATGATCTTGGTGCGGGCTATACCTACCACGCCGgtcagtgccttattacacatctgctgctgtttctttcaag 1200
K G G A L E A I V D D L G A G Y T Y H A
ctgacgaaaattgagatagGTAAACGCCCTTGGACACTTCTCCCACAGGTCCACATTGCCCTCCCTGGTGCGACTCAGAATGAGGTTTCACAAGAAGAAG 1300
G K R P W T L L P Q V H I A L P G A T Q N E V S Q E E
CAGAGGCATTGGTCAAAGCTGGTACCCGTATCGTCGCTGAAGGTTCCAACATGgtacgtccacactcataagttccataagcacgtcggtgttcatataa 1400
A E A L V K A G T R I V A E G S N M
ttaaatccagGGTTGTACCGAAGAAGCCATTGCCATCTTCGAGAACTCCCGTCGCGCCTCTCGAGCCGGAGTTTGGTATGCTCCAGGTAAAGCATCGAAC 1500
G C T E E A I A I F E N S R R A S R A G V W Y A P G K A S N
TGTGGTGGTGTAGCGGTTTCCGGTCTCGAGATGGCGCAAAACTCTCAGCGTCTTGCTTGGTCTACCCAAGAAGTTGACGCGAAATTAAAATCTATTATGG 1600
C G G V A V S G L E M A Q N S Q R L A W S T Q E V D A K L K S I M
CAGAGTGCTACCAGATCTGTTATACTGCCGGTTCGAGATGGAGTGGCGAGAAGGTTGCTGAGGGTGTTGCCGAGGGCGAGGCGTTGCCTAGTTTGCTCTC 1700
A E C Y Q I C Y T A G S R W S G E K V A E G V A E G E A L P S L L S
GGGAGCGAATCTTGCCGGGTTTATCAAGGTTGCGGATGCCATGAAGGAGCAGGGTGACTGGTGGTAGGCGATTAGTGGAGGCTGGCGTGGTGATCATGAT 1800
G A N L A G P I K V A D A M K E Q G D W W
GATTACCATATAAAAAACCTGAAAAGCTGGTTCTTGGTGAGGACCACCAATCTTATACCCCAGTGCTGGTCTTGTAACGATTTATTACGCTCTCATTCTGT 1900
ACTTATACAATATGTATTGTATGTCATGTATCTTTTGTGTGTCACCTTGAATTTACGAGACTTATGGTGCCAGTTAGTAATCCGAGTGGACGATGAGACA 2000
GTCTAGAGAATCAGGCCACATGCTGACGTAATAAAATACTCCTTGTCATGGGCGTCGACTATAGTCTTTATGGCGGCGCTGACTCGCTCGCAGAGGGCCG 2100
TTCTCCCCGCAGAACTTTTCCTAATAAATGCTGCCATTCTCCTCCTGCTTCCCCCAAAAGACCCATCAGAGTTTCTCTGGATCC 2184

Fig. 2. (page 84) Nucleotide sequence and deduced amino acid sequences of the *A. bisporus gdh*A gene Only the sense strand of the gene is shown from 5' to 3' end and is numbered from the first nucleotide of the presumed initiation codon Intervening sequences are in lower case The putative CAAT box is underlined The major transcription startsite (♦) and the location of the polyA tails encountered in the cDNA sequences (#) are indicated above the nt sequence The nt sequence will appear in EMBL databases under accession no X83393

Horst®U1 This allowed us to construct a limited genomic map of the *gdh*A gene and its surroundings (Fig 1) In order to determine the complete sequence of *gdh*A we cloned a 1 9 kb *Bgl*II fragment and an overlapping 4 kb *Hin*DIII fragment from a positive phage and we sequenced both DNA strands of relevant parts using standard subcloning techniques Gaps were filled using sequence specific oligonucleotides as primers

Structure of the *A. bisporus gdh*A gene

Analysis of the DNA sequence indicated that the open reading frame is interrupted by six introns In order to confirm these introns we isolated several independent *gdh*A cDNA clones from a cDNA library which was constructed from polyA rich RNA isolated from primordia and small mushrooms of strain Horst®U1 Two independent cDNA clones were sequenced and the sequences confirmed the presence and location of the predicted six introns in the open reading frame of *gdh*A (Fig 2) The 3' end of the two individual cDNAs were located 175 bp and 209 bp downstream of the TAG stopcodon (Fig 2) The nucleotides surrounding the startcodon fit to the consensus start sequence of highly expressed genes (Kozak, 1989) The major transcription initiation site was identified at position -35 relative to the startcodon and a CAAT motif which matches at 8 out of 9 positions with the conserved motif found in higher eukaryotes is found starting at position -45 (Fig 2) (Nussinov 1990) In the 3' non coding region of the *gdh*A transcript the sequence TATAAA is found starting at position 1809 (Fig 2) This sequence resembles the sequence AATAAA found in higher eukaryotes where it functions in these organisms as a processing and polyadenylation signal (reviewed by Humphrey and Proudfoot 1988)

The codon usage of the *A. bisporus gdh*A gene shows a limited bias towards a thymine in the third position of a codon (33 1%) whereas in 60 8% of the codons a pyrimidine is used in the third position of a codon

The complete amino acid sequences of several microbial NADP-GDHs and the GDH amino acid sequences of two vertebrates have been determined The deduced amino acid sequence of *A. bisporus* NADP-GDH was aligned with the deduced amino acid sequences of these glutamate dehydrogenases The alignment showed a striking similarity between *A. bisporus* NADP-GDH and other fungal NADP-GDHs with tendency for this to occur in discrete highly conserved blocks rather than evenly spread The conserved regions include amino acids proposed to be involved in binding of the cofactor and dicarboxylate substrates and in catalysis (Mattaj et al 1982) The similarity with *E. coli* NADP-GDH was also high, whereas substantial divergence was found with the vertebrate enzymes which have a dual coenzyme specificity (Smith et al 1974)

Genomic organization of the *gdh*A gene

In order to evaluate the use of the *gdh*A gene as a restriction fragment lenght polymorphism (RFLP) marker a detailed genomic restriction map was constructed of strains 39 and 97, the homokaryotic constituents of Horst®U1 Therefore a Southern blot of various genomic DNA digests of strains 39 and 97 was probed with a 1 9 kb *Bgl*II fragment comprizing a major part of the *gdh*A coding region (Fig 1, probe P1) and an adjacent 1 2 kb *Bam*HI-*Hin*DIII fragment located downstream of *gdh*A (Fig 1, probe P2) Different patterns were found in strain 39 and 97 for *Eco*RI and *Bgl*II (Fig 3) In addition probe P1 was used to identify the chromosome on which *gdh*A is located The 13 chromosomes were released from protoplasts of strain 39 and strain 97 and separated by clamped homogenous field electrophoresis (CHEF) using the conditions described by Sonnenberg et al (1991) followed by Southern analysis of the separated chromosomes The location of the chromosomes was made visible by rehybridization of the same membrane with a mixture of 200 randomly selected cDNA clones isolated from the mixed cDNA library of primordia and small mushrooms (Fig 3) The result made it clear that the *gdh*A gene is located on chromosome 10 of both strains

Regulation of expression of *gdh*A

A. bisporus NADP-GDH activity is repressed in the presence of ammonium and derepressed if glutamate is used as the sole nitrogen source The regulation of the gene was studied by Northern analysis Equal amounts of total RNA extracted from mycelium continuously growing on glutamate or proline for 15 or 20 days before and after addition of 20mM ammonium was hybridized with the 1 9 kb genomic *Bgl*II fragment of *gdh*A

The *Bgl*II fragment hybridized with a transcript of approximately 1700 nt Addition of ammonium resulted in a decrease of the steady state level of *gdh*A transcripts by a factor 10 within two hours The reduced transcript levels were maintained throughout the time course of the experiment and were equal to the *gdh*A transcript level observed in mycelium utilizing ammonium as sole nitrogen source (Fig 4) A similar decrease in steady state level of *gdh*A transcripts was observed seven hours after the addition of 5 mM dl-phosphinotricin (PPT) (results not shown) which blocks the enzyme activity of glutamine synthetase (GS) (Lea et al 1984, Baars et al 1995b) No effect was observed on *gdh*A mRNA levels upon addition 20mM Na^+ instead of 20 mM NH_4^+ to the culture (Fig 4, panel A and B, lanes 6) The same membranes were hybridized with the gene encoding the *A. bisporus* ribosomal protein S15a and with the *A. bisporus* pyruvate kinase gene (*pki*A) No effect was observed on the *s15a* mRNA levels (Fig 4, panel C and D) or on the *pki*A mRNA levels (Fig 4 panel E and F) upon addition of ammonium All Northern blots were rehybridized with a DNA probe encoding a part of the *A. bisporus* 28S rRNA to provide for a loading control (Fig 4, panel G and H)

The enzyme activities of NADP-GDH, NAD-GDH (EC 1 4 1 2), GS and glutamate pyruvate transaminase (GPT, EC 2 6 1 2) were measured in extracts of the same mycelium before and after addition of $(NH_4)_2HPO_4$ and seven hours after addition of PPT The NADP-GDH levels observed in mycelium utilizing glutamate or proline as nitrogen source were 10 to 12 times higher in comparison to mycelium utilizing ammonium as nitrogen source After addition of ammonium a slow decrease in NADP-GDH activity was observed The NAD-GDH values were similar reported values (Baars et al 1994, Schaap et al 1995a) and did not change significantly after addition of ammonium The GS values measured in the proline utilizing culture were initially five fold higher

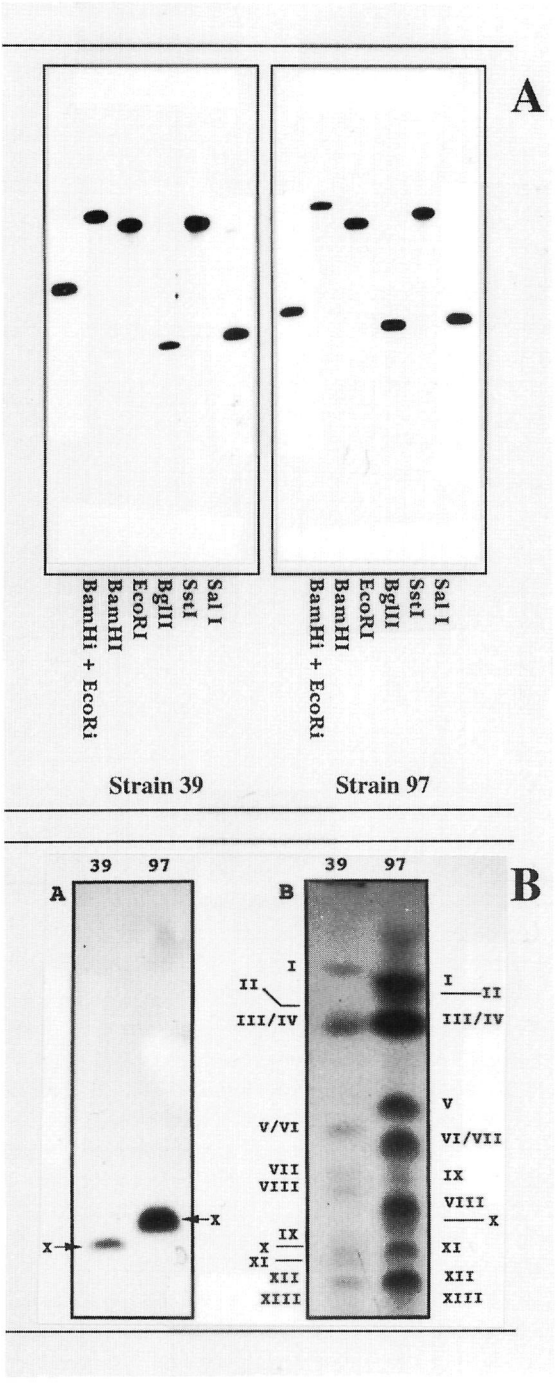

Fig. 3. A: Southern analysis of genomic DNA of strains 39 and 97 using a 1.2 kb *Bam*HI-*Hin*DIII fragment located downstream of *gdh*A as probe. Restriction enzymes used for the detection of polymorphisms are shown below the lanes.
B: Southern analysis of chromosome separation by CHEF. Strain numbers are indicated above the panels. Panel A was hybridized with a 1.9 kb *Bgl*II fragment of *gdh*A and panel B was hybridized with a mixture of 200 cDNA clones randomly isolated from primordia and small fruit bodies. Chromosomes are numbered according to Sonnenberg *et al.* 1991.

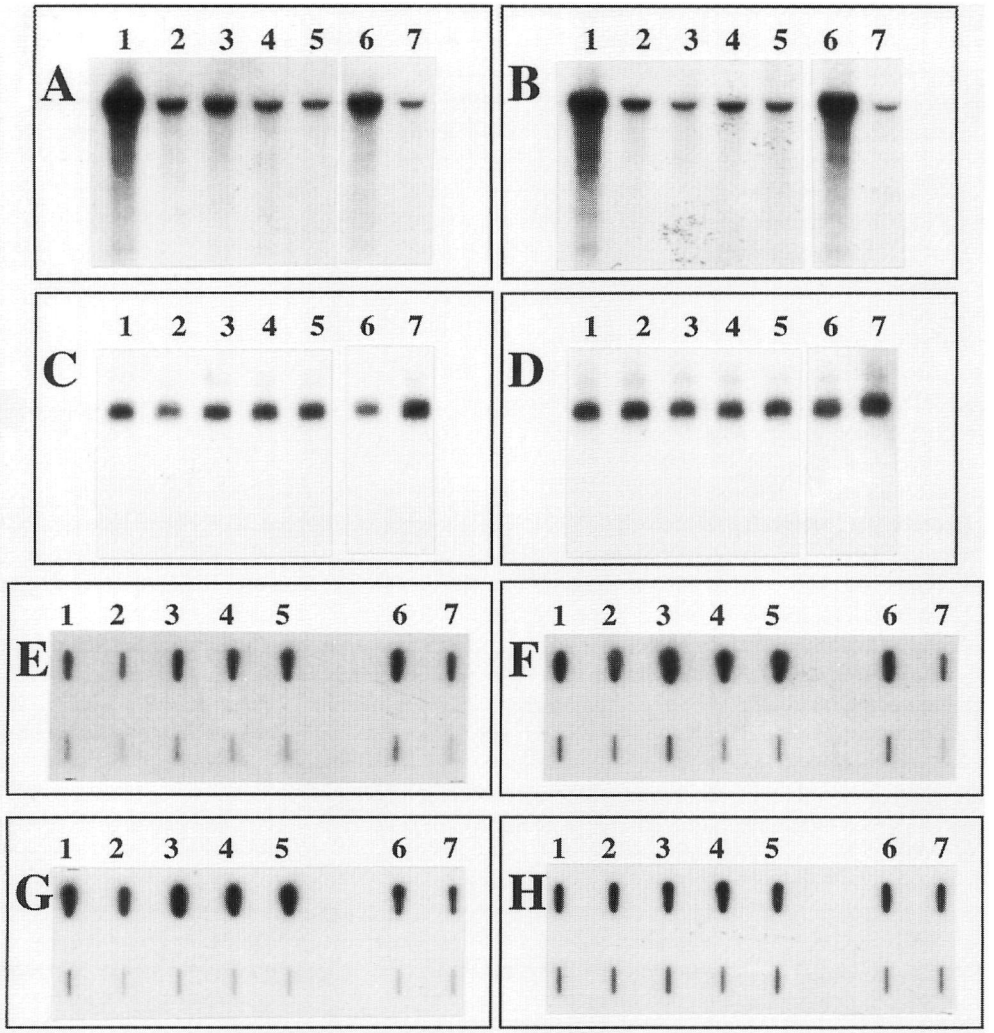

Fig. 4. Northern analysis of total RNA isolated from *A. bisporus* Horst®U1 grown on glutamate (panel A.C.E and G) or proline (panel B, D, F and H) as sole nitrogen source before and after the addition of 20 mM ammonium.

Panel A, B, C and D; 7 µg total RNA was separated on a 1.6% agarose gel, transferred to Hybond-N and hybridized with a genomic 1.9 kb *Bgl*II fragment of *gdh*A (panel A and B) or the complete cDNA of *s15a* (panel C and D). Lanes 1 to 5; total RNA isolated before and 2, 4, 8, and 24 h after addition of 20 mM ammonium. Lane 6; total RNA isolated 8 h after the addition of 20 mM Na^+ to the cultures and lane 7; total RNA isolated from mycelium grown on 20 mM ammonium for 14 days.

Panel E, F, G and H; Northern spotblots of 1µg (upper row) and of 0.1 µg (lower row) of the same RNA samples probed with a 1.2 kb *Sal*I-*Sph*I fragment of *pki*A (panel E and F) or a 900 bp *Eco*RI fragment encoding a part of the 26S rRNA (panel G and H). Slot numbers correspond to lane numbers.

than the glutamate utilizing culture and showed a slow decrease in activity. The initial GPT activity in the proline utilizing culture was also five fold higher than in the glutamate utilizing culture. However, a three fold increase in GPT activity is observed in the glutamate utilizing culture after the addition of ammonium. Finally, addition of PPT to a glutamate utilizing culture did not significantly change the enzyme activities of NADP-GDH, GS and GPT. The results are summarized in Table 1.

Table 1. Enzyme activities in U/mg in *A. bisporus* mycelium grown on 20 mM glutamate or on 20 mM proline before and after the addition of 20 mM ammonium or 5 mM PPT.

Substrate	Incubation time (h)	NADP-GDH	GS	GPT
glutamate + NH_4^+[b]		0.006	0.030	0.170
NH_4^+[b]		0.005	0.019	n.d.[c]
glutamate	0	0.058	0.056	0.073
	8	0.035	0.036	0.100
	24	0.029	0.047	0.227
proline	0	0.071	0.261	0.364
	8	0.079	0.165	0.411
	24	0.060	0.141	0.294
PPT added[a]	8	0.071	0.071	0.097
no PPT added[a]	8	0.037	0.042	0.076

[a] PPT was added only to cultures grown on 20 mM glutamate as nitrogen source.
[b] Control cultures were grown on 10 mM glutamate and 10 mM ammonium or 20 mM ammonium.
[c] n.d.; not determined.

Discussion

There is an increasing amount of literature concerning the physiology and molecular genetics of nitrogen metabolism in the ascomycetes *A. nidulans*, *N. crassa* and *S. cerevisiae*. These fungi can use a wide variety of nitrogen sources for growth. Maximum growth rates are obtained using ammonium, glutamate or glutamine as nitrogen sources and growth on these compounds leads to a strong repression of a whole range of enzyme activities involved in the utilization of more complex nitrogen compounds. Little is known about the nitrogen metabolism of the basidiomycete *A. bisporus*, but it shares with the ascomycetes the ability to use different nitrogen sources for growth.

Glutamate and glutamine play crucial roles in the assimilation of ammonium and bring about nitrogen catabolite control in *A. nidulans*, *N. crassa* and *S. cerevisiae*. It is clear therefore that the

enzymes involved in glutamate and glutamine metabolism must play a key role in nitrogen metabolism GDH catalyses the reversible amination of 2-oxoglutarate to glutamate and is an enzyme involved in primary nitrogen metabolism. Ascomycetes and basidiomycetes have gained two GDH enzymes which use different cofactors (for a review see Jennings, 1988) Pateman (1969) showed that the activity of NADP-GDH in *N. crassa* and in *A. nidulans* is derepressed upon growth on ammonium and repressed when growth took place on glutamate This suggests that in these fungi the NADP-GDH serves to synthesize glutamate In the basidiomycete *Schizophyllum commune* on the other hand, the level of NADP-GDH is approximately tenfold lower during growth on ammonium sulphate compared to growth on glutamate as nitrogen source (Dennen and Niederpruem, 1964) and in the basidiomycete *Coprinus cinereus* NADP-GDH is repressed by urea and ammonium while NAD-GDH is subject to carbon catabolite repression and urea derepression. (Steward and Moore 1974).

NADP-GDH has been purified from *A. bisporus* and was extensively characterized (Baars et al 1995a) The expression of NADP-GDH upon growth on ammonium or combinations of ammonium and glutamate was approximately tenfold lower compared to growth on glutamate as sole nitrogen source This suggested that in *A. bisporus* NADP-GDH is not primarily involved in ammonium assimilation However, elucidation of the metabolic role of NADP-GDH in *A. bisporus* by a classical genetic approach is presently not feasible In order to study the regulation of NADP-GDH in *A. bisporus* using different nitrogen sources, we have therefore cloned and characterized *gdh*A, the gene encoding NADP-GDH and used this gene as a probe in Northern analyses We have cloned this gene by taking advantage of the finding that the protein sequence of NADP-GDH of different species share regions of high homology Two degenerate oligomers were designed on such regions and with PCR a probe was generated which was used to screen a genomic library of *A. bisporus* Comparison of the coding regions of the gene encoding NADP-GDH from *A. bisporus* with NADP-GDHs from fungal origin revealed a high homology Less homology was observed with *E. coli* NADP-GDH (McPherson and Wootton 1983) and the lowest homology was observed with the vertebrate GDHs especially at the carboxyterminal end The vertebrate GDHs can use NADP as cofactor but prefer NAD as cofactor (Smith et al , 1974) and the carboxyterminal end is probably involved in binding of this factor (Wootton, 1974)

Sequence analysis further revealed that the coding region of *gdh*A is interrupted by six introns which were confirmed by comparing the genomic sequence with cDNA sequences The sequences of exon-intron and intron-exon splice boundaries fit to the fungal splice consensus sequences (Unkles, 1992) The location of the two intervening sequences in the *gdh*A genes of *A. nidulans* and *N. crassa* is conserved However, the location of none of the six introns in the *A. bisporus* *gdhA* gene corresponds to these locations This is in agreement with the observation of Harmsen et al (1991) that there is a clear boundery between ascomycetes and basidiomycetes with respect to intron positions

Southern analysis of separated chromosomes showed that *gdh*A is located on chromosome 10 of both homokaryotic constituents of Horst®U1 Comparison of the homokaryotic constituents by Southern analysis of their digested genomic DNAs revealed polymorphic patterns for *Eco*RI and *Bgl*II This polymorphism can be used for discrimination between homokaryons and heterokaryons in the offspring of Horst®U1 and thus this gene can be used as a tool in the development of classical genetics of *A. bisporus*.

The activity of NADP-GDH in mycelium growing on ammonium or combinations of ammonium and glutamate is approximately tenfold lower compared to activity found in mycelium growing on glutamate as sole nitrogen source This suggested that the steady state mRNA level

of *gdh*A is regulated in some way by an extracellular or intracellular ammonium concentration Therefore we designed a medium shift experiment in which ammonium was added to cultures using only glutamate or proline as nitrogen sources We measured the steady state mRNA levels of *gdh*A before and at different time points after the addition of ammonium In addition we measured in the same RNA samples the mRNA levels of *pki*A, and of the gene encoding the ribosomal protein S15a We also measured the NADP-GDH, NAD-GDH, GS and GPT enzyme activities before and after addition of ammonium Northern analysis showed a rapid response of the *gdh*A mRNA level to the addition of ammonium Within two hours after addition the mRNA level was decreased by a factor 10 (Fig 4) The mRNA levels of *pki*A and of the gene encoding ribosomal protein S15a are not noticeable influenced by the addition of ammonium indicating that ribosome biosynthesis and growth continues A similar decrease in *gdh*A mRNA levels was obtained by the addition of PPT to a culture which used glutamate as sole nitrogen source The glutamine analog PPT inhibits the amidation of glutamate by GS and as a result of that the intracellular pool of ammonium accumulates through the continuous deamination of glutamate by NAD-GDH and/or NADP-GDH thereby decreasing the *gdh*A transcript level

We also observed that the NADP-GDH enzyme activity remained high for at least 24 h after addition of ammonium suggesting the regulation of NADP-GDH in vegetatively growing mycelium occurs, at least for a major part, at the level of transcription and that NADP-GDH is a very stable enzyme *in vivo* This also implies that after addition of ammonium there is still a high level of NADP-GDH for a long period and the organism has to respond to that GPT is one of the major transaminase activities found in *A. bisporus* (Baars et al 1994) After addition of ammonium a significant transient increase in GPT activity (Table I) and an accumulation of alanine (results not shown) was observed in the glutamate utilizing culture This can compensate the overactivity of NADP-GDH by recycling the surplus of 2-oxoglutarate which is aminated by NADP-GDH A similar phenomenon is observed in *N. crassa* The pathway for the biosynthesis of glutamine and alanine compete in *N. crassa* with each other for the available glutamate If the GS activity is low the surplus glutamate is converted into alanine (Kanamori et al 1982) The reason for the higher GPT and GS activities observed in the culture grown on proline remains obscure However, our results clearly show that regulation of *A. bisporus* NADP-GDH in response to different nitrogen sources is markedly different from the responses of NADP-GDHs of ascomycete fungi

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Summary

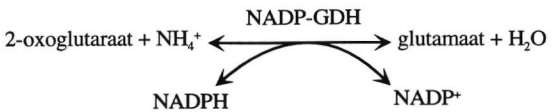
Summary and concluding remarks

Despite its long history of cultivation and the large amounts in which it is grown for human consumption nowadays, knowledge of the basic metabolism of *A. bisporus* is scarce when compared to other industrial crops. This thesis describes the results of a study of the nitrogen metabolism of *A. bisporus*.

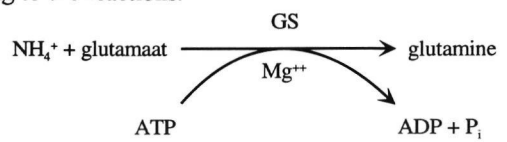
Current knowledge on nitrogen metabolism in fungi has mainly been derived from studies using the yeast *Saccharomyces cerevisiae* and the filamentous ascomycete fungi *Aspergillus nidulans* and *Neurospora crassa*. From these studies considerable similarity in nitrogen metabolism is suggested for the ascomycete fungi. However, since reports on nitrogen metabolism of basidiomycete fungi are relatively scarce, it is not clear whether the details are common to all filamentous fungi.

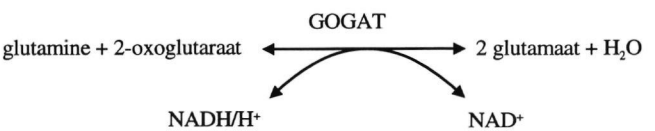
This thesis describes the results of a study on the nitrogen metabolism of the basidiomycete fungus *Agaricus bisporus* strain Horst® U1, known as the commercial white button mushroom. An overview of different aspects of the nitrogen metabolism is described in the introduction (**Chapter 1**). From this overview it is clear that ammonium assimilation plays a pivotal role in the regulation of nitrogen metabolism. An overview of the possible pathways by which ammonium assimilation may proceed in *A. bisporus* was obtained by making an inventory of the main nitrogen assimilating enzymes (**Chapter 2**). Both vegetative mycelium and fruit bodies were included in this survey. It was shown that *A. bisporus* has the enzymic potential to assimilate ammonium by the activities of glutamine synthetase (GS), NADP-dependent glutamate dehydrogenase (NADP-GDH) and NAD-dependent glutamate dehydrogenase (NAD-GDH). Next to this, glutamate synthase (GOGAT) activity and a number of major transaminating activities like glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and alanine-glyoxylate transaminase (AGT), were found.

Based on these enzymes two possible pathways of ammonium assimilation could be postulated. The first possibility is offered by the NADP-GDH pathway according to the following reversible reaction:



A second possibility, the GS/GOGAT pathway, is offered by the sequential action of GS and GOGAT according to the reactions:





It is assumed that primary ammonium assimilation is mediated by either NADP-GDH or GS, while NAD-GDH has a catabolic role in the degradation of glutamate

To obtain some information on the physiological role of the main ammonium assimilating enzymes *A. bisporus* was grown on a number of different defined nitrogen sources Good growth was found in a defined buffered medium on glucose as a carbon source and a number of organic nitrogen compounds or ammonia as a nitrogen source However no growth was observed with nitrate as a nitrogen source *A. bisporus* was not able to use organic nitrogen sources 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 en 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

The effects of growth on ammonium or glutamate as a nitrogen source were studied in more detail and the results are reported in **Chapter 3** Mycelium of *A. bisporus* was grown in batch cultures on different concentrations of ammonium, glutamate and glucose When grown on ammonium, activities of NADP-GDH and GS were repressed NAD-GDH activity was about 10 times higher than the activities of NADP-GDH and GS At concentrations below 8 mM ammonium, NADP-GDH and GS were slightly derepressed

With glutamate as a nitrogen source activities of NADP-GDH and GS were derepressed, compared to growth on ammonium activities of these two enzymes were about 10-fold higher Activities of GDH's showed no variation at different glutamate concentrations Activity of GS was slightly derepressed at low glutamate concentrations

Growing *A. bisporus* on both ammonium and glutamate as nitrogen sources, resulted in enzyme activities comparable to growth on ammonium alone Activities of NADP-GDH, NAD-GDH and GS were not influenced by the concentration of glucose in the medium In mycelium starved for nitrogen, activities of NADP-GDH, NAD-GDH and GS were derepressed while in carbon-starved mycelium the activity of GS and both GDH's was repressed

The concentration of glucose in the cultivation medium did not affect the enzyme activities When compared with the results of studies concerning ammonium assimilation by yeasts and a range of other fungi a number of similarities were observed The derepression of NAD-GDH activity by glutamate is shown by most fungi Also the responses of GS activity towards different concentrations or different types of nitrogen sources show no large differences with the responses observed in most other fungi studied so far

With regard to NADP-GDH the responses can be divided into two groups When grown on ammonium as a nitrogen source high activities of NADP-GDH have been reported for *N. crassa*, *A. niger* and *Fusarium oxysporum* In these organisms NADP-GDH is repressed by glutamate However, in *Schizophyllum commune* and *Hebeloma cylindrosporum*, like in *A. bisporus*, NADP-GDH appears to be repressed by ammonium and derepressed by glutamate as a nitrogen source In *Pleurotus ostreatus* NADP-GDH was found to be absent

These results did not allow us to decide whether ammonium assimilation was mediated by the concurrent action of both NADP-GDH and GS or by the action of either one of these enzymes

To be able to study the early products of ammonium assimilation in *A. bisporus* ^{15}N-labelled

ammonium was fed to actively growing mycelium The mycelium used in these experiments was grown on a defined medium with ammonium as a nitrogen source The results of this study are described in **Chapter 4** The products of ammonium assimilation were analysed with ^{15}N-NMR. Participation of GS, GOGAT and NADP-GDH were determined by inhibiting GS with phosphinothricin and GOGAT with azaserine These inhibitors are unlikely to affect the activity of NADP-GDH *in vivo*

When inhibitors were absent from the incubation media, peaks could be observed at 40 62 ppm and 42 75 ppm in the ^{15}N-NMR spectra, corresponding with incorporation of labeled nitrogen into the α-N position of glutamate or glutamine and the α-N position of alanine, respectively Furthermore a large peak was found at 111 84 ppm, corresponding with the labelling of the γ-N position of glutamine Addition of inhibitors had pronounced effects on the ^{15}N-NMR spectra of extracts from the mycelia Inhibition by azaserine results in the accumulation of labelled glutamine γ-N, at the expense of the amounts of α-N peaks of glutamate/glutamine and alanine Addition of phosphinothricin to the incubation medium resulted in a complete inhibition of the incorporation of ^{15}N-ammonium

The physiological effects of adding these inhibitors were tested by analysing the pools of free amino acids Inhibition of GS by phosphinothricin resulted into a 10-fold decrease of the glutamine pool of the mycelium and an increase of the pools of glutamate and alanine Inhibition of GOGAT by azaserine resulted in a marked accumulation of the pools of glutamine and alanine

These results clearly indicate that, under the conditions used, ammonium assimilation is mainly catalysed by the enzymes of the GS/GOGAT pathway Furthermore it was shown that both transamination of glutamate with pyruvate to yield alanine and the formation of N-acetylglucosamine are major routes in nitrogen metabolism of *A. bisporus*

The purification of GS is described in **Chapter 5** The native enzyme has a molecular weight of 325 kD and consists of eight 46 kD subunits Its pI was found at 4 9 Optimal activity was found at 30°C Stability declined rapidly at temperatures above 20°C Based on the number of subunits and its low thermostability *A. bisporus* GS is likely to be a type II GS
The enzyme exhibits a K_m for glutamate, ammonium and ATP of 22 mM, 0 16 mM and 1 25 mM, respectively, in the biosynthetic reaction, with optimal activity at pH 7 The affinity of *A. bisporus* GS for its substrates is similar to that of GS from other fungal sources
In a number of organisms metabolites like L-alanine, L-histidine, L-tryptophan, anthranilic acid and 5'-AMP have been proposed to act as feedback inhibitors of GS activity However, GS from *A. bisporus* is only slightly inhibited by 10 mM concentrations of these metabolites Therefore a major role of these metabolites in the regulation of *A. bisporus* GS is not very likely Methionine sulfoximine and L-phosphinothricine strongly inhibited GS from *A. bisporus* For the transferase reaction K_i-values were 890 μM and 240 μM for methionine sulfoximine and phosphothricine, respectively For the biosynthetic reaction K_i was 17 μM for both methionine sulfoximine and phosphothricine

The purification of NADP-GDH is described in **Chapter 6** The molecular weight of the native enzyme was 330 kD The enzyme is composed of identical subunits of 48 kD and probably has a hexamer structure The pI of the enzyme was found at pH 4 8 The N-terminus of the purified protein appeared to be blocked so N-terminal sequencing was not possible
The K_m values for ammonium, 2-oxoglutarate, NADPH, L-glutamate and NADP were 2 1, 3 2, 0 074, 27 0 and 0 117, respectively The enzyme was specific for NADP(H)
The pH optima for the amination and deamination reactions were found to be 7 6 and 9 0,

respectively Optimal activity was found at 33°C The molecular characteristics and the kinetic parameters of *A. bisporus* NADP-GDH are very similar to the characteristics of fungal GDH's in general The kinetic parameters suggest a role in the reductive amination of 2-oxoglutarate since the affinity of NADP-GDH for ammonium and 2-oxoglutarate is higher than its affinity for glutamate Furthermore ammonium ions exert a strongly inhibitory effect on the deamination of glutamate No regulation by metabolites could be demonstrated

To facilitate further study, antibodies were raised against the purified enzyme and tested for their specificity using immunoprecipitation tests and immunoblotting Furthermore the gene encoding NADP-GDH (*gdh*A) was isolated from an *A. bisporus* recombinant phage λ library (**Chapter 7**) The *A. bisporus* NADP-GDH amino acid sequence represents a 457 amino acid protein and shows much similarity to the genes coding for microbial NADP-GDH in general With regard to the non-coding regions within the open reading frame some differences were found The open reading frame is interrupted by six introns The position of none of the introns is conserved with respect to the conserved positions of the two introns located in the open reading frame of the ascomycete fungi *Aspergillus nidulans* and *Neurospora crassa* This would suggest that these ascomycetes are only distantly related to *A. bisporus*

To study transcription of *gdhA*, mycelium was grown on a medium capable of inducing high activities of NADP-GDH Subsequently ammonium was added to the cultures and steady state transcript levels of *gdhA* were measured before and at different time points after addition of ammonium Northern analysis showed a rapid response of the *gdhA* transcript levels Within two hours after addition the level of transcription was decreased by a factor 10 Transcript levels of the unrelated genes coding for pyruvate kinase and ribosomal protein S15a were not influenced by the addition of ammonium A similar decrease in *gdhA* transcript levels occurred when ammonium assimilation (catalysed by GS) in glutamate grown mycelium was inhibited by the addition of phosphinothricine As a result of this inhibition ammonium derived from the turn-over of cellular components will accumulate intracellularly So, Northern analysis suggests that the *A. bisporus gdh*A gene is transcriptionally regulated and that, unlike in ascomycetes, transcription of this gene is repressed upon the addition of ammonium to the culture

In summary, the physiological role of GS and GOGAT is clearly demonstrated Both the kinetic properties of the purified enzyme and the analysis of the products of the incorporation of ^{15}N-ammonium strongly suggest that GS is a key enzyme in the primary incorporation of ammonium by *A. bisporus*

In contrast, the physiological roles of NADP-GDH and NAD-GDH are not yet fully understood On the basis of mutant studies of *A. nidulans*, *N. crassa* and *S. cerevisiae* anabolic and catabolic roles were proposed for NADP-GDH and NAD-GDH, respectively Based on its relatively high activity in mycelium grown either glutamate or ammonium, a catabolic role of NAD-GDH could also be possible in *A. bisporus*

With regard to the metabolic role of NADP-GDH, however, no firm conclusions can be drawn Based on the kinetic properties of the purified enzyme a role in the reductive amination of 2-oxoglutarate is suggested However the analysis of the products of the incorporation of ^{15}N-ammonium does not suggest an anabolic role for NADP-GDH Also the results of the Northern analysis, which show that the expression of mRNA transcripts of the NADP-GDH gene is inhibited by ammonium ions, argue against a role in the formation of glutamate

In *A. bisporus*, studies concerning the repression and derepression of enzyme activities when grown on different nitrogen sources, proved to have only very limited value in predicting the metabolic role of the enzymes studied

On the other hand study of mutants of *A. bisporus* defective in either NADP-GDH, NAD-GDH or both would probably be very helpful in depicting the metabolic role of these two enzymes Unfortunaly it is very difficult to obtain mutants of this organism The main problem is the availability of an appropriate unit for treatment, for example uninucleate haploid asexual spores As has been discussed in the introduction to this thesis, *A. bisporus* does not produce such spores The use of basidiospores or hyphal fragments for the production of mutants is complicated by the fact that the majority of these cells are multinucleate Furthermore *A. bisporus* basidiospores germinate poorly This problem however would be overcome by the use of protoplasts instead of basidiospores The conditions for the production of protoplasts of *A. bisporus* are well documented

In conclusion, our results show large similarity in the molecular and kinetic characteristics of the *A. bisporus* enzymes involved in ammonium assimilation when compared to other fungi Despite this similarity in the tools used of ammonium incorporation, physiological studies strongly suggest that the use of these tools is regulated in a different way in *A. bisporus* So, the general model which has been proposed for ascomycete fungi cannot be adopted to describe ammonium assimilation in *A. bisporus.* Especially the role of NADP-GDH, an enzyme with high activity in maturing fruit bodies, is poorly understood

Since changes in nitrogen metabolism affect many aspects of the life cycle of micro-organisms, a relation between nitrogen metabolism and the practice of commercial growing is not purely hypothetical In view of the differences between nitrogen metabolism in *A. bisporus* and nitrogen metabolism in other industrial fungi like *Aspergillus*- and *Neurospora*-species, further study will be indispensable to understand the mechanisms that underly its biology

Samenvatting

Samenvatting

De champignon is bij velen bekend als een smakelijk onderdeel van de maaltijd. Deze populariteit heeft er voor gezorgd dat de teelt van champignons gedurende de laatste tientallen jaren is uitgegroeid tot industriële vormen. Over de omstandigheden waaronder de teelt van champignons optimaal verloopt is veel bekend en er bestaat een speciale beroepsopleiding die tot doel heeft aankomende champignontelers de finesses van de teelt bij te brengen. De kennis die op deze opleidingen wordt overgebracht is vergaard in een lang proces van uitproberen, vallen en opstaan.

Ondanks de grote schaal waarop de champignon geteeld wordt, is echter nog slechts weinig bekend over de fysiologie van deze schimmel. Een grotere bekendheid met de fysiologie van de champignon zou een betere sturing van het onderzoek mogelijk kunnen maken. Om die reden zou meer onderzoek naar de fundamentele biologie van de champignon welkom zijn.

Dit proefschrift beschrijft de resultaten van een studie naar de stikstofhuishouding van de champignon (*Agaricus bisporus*). In **Hoofdstuk 1** wordt de levenscyclus en de teelt van de champignon beschreven. Daarnaast wordt een overzicht gepresenteerd van wat bekend is over de stikstofhuishouding van schimmels in het algemeen en de champignon in het bijzonder.

In de stikstofhuishouding van schimmels zijn drie groepen van biochemische processen te onderscheiden. Een overzicht van deze processen is weergegeven in Fig. 1. De eerste groep omvat de afbraak van stikstofhoudende substraten (katabole processen). Hierbij komt ammonium vrij. De tweede groep processen omvat de inbouw van het gevormde ammonium in de aminozuren glutamaat en glutamine (ammonium assimilatie). De derde groep omvat de anabole processen. Glutamaat en glutamine kunnen als grondstof dienen om alle stikstofhoudende componenten van de cel te kunnen maken. Bijvoorbeeld, eiwitten vormen een belangrijk bestand-

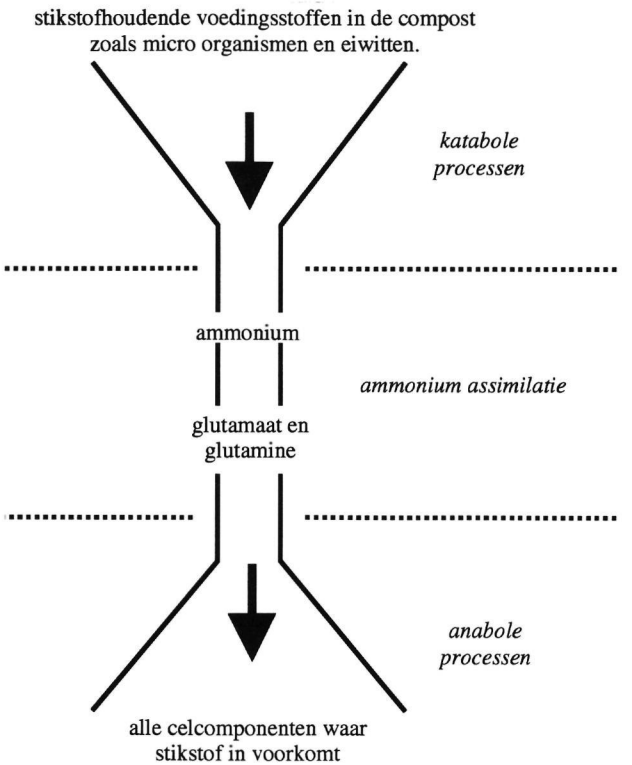


Fig. 1. Globaal overzicht van de stikstofhuishouding

deel van de cel en bestaan uit lange ketens van aminozuren Deze aminozuren kunnen uit glutamaat gemaakt worden Elk van deze groepen biochemische omzettingen wordt mogelijk gemaakt door een aantal enzymen

In dit onderzoek hebben wij ons beperkt tot de bestudering van de ammonium assimilatie De reden daarvoor is dat er reeds relatief veel onderzoek is gedaan aan de enzymen die de champignon ter beschikking heeft om stikstofhoudende substraten af te kunnen breken Echter over hoe de champignon het ammonium dat gevormd wordt uit de afbraak van substraten gebruikt om celmateriaal te maken is nog weinig bekend Toch is deze omzetting erg belangrijk Uit onderzoek aan andere schimmels is bekend dat ammonium en de aminozuren glutamaat en glutamine een grote rol spelen in de regulatie van de enzymen die stikstofhoudende substraten kunnen afbreken Anders gezegd, een teveel of een tekort van deze stoffen vormen voor de cel het signaal om meer of minder substraat-afbrekende enzymen te maken De manier waarop dit gebeurt is nog niet volledig bekend Het is echter wel duidelijk dat de inbouw van ammonium in glutamaat en glutamine het middelpunt van de stikstofhuishouding vormt

Om duidelijkheid te verkrijgen over de manier waarop ammonium assimilatie plaats vindt in de champignon, is gekeken welke enzymen die in staat zijn om ammonium om te zetten in aminozuren ook daadwerkelijk aanwezig zijn in het organisme (**Hoofdstuk 2**) Het blijkt dat de champignon over 3 enzymen beschikt die ammonium kunnen inbouwen in een aminozuur, nl glutamine synthetase (GS), NADP-afhankelijk glutamaatdehydrogenase (NADP-GDH) en NAD-afhankelijk glutamaat dehydrogenase (NAD-GDH) Daarnaast beschikt de champignon over een aantal enzymen die de stikstof die in een aminozuur gebonden is verdelen over andere aminozuren De belangrijkste van deze enzymen zijn het glutamaat synthase (GOGAT) en transaminases zoals glutamaat-pyruvaat transaminase, glutamaat-oxaloacetaat transaminase en alanine glyoxylaat transaminase

Met behulp van deze enzymen kan op twee manieren ammonium omgezet worden in glutamaat en glutamine De reacties die daarbij een rol spelen zijn weergegeven in Fig 2
Het NADP-GDH biedt de mogelijkheid ammonium in te bouwen in glutamaat volgens reactie1 Deze reactie is omkeerbaar Het NAD-GDH is in staat om eenzelfde reactie uit te voeren, maar gebruikt daarbij NADH als cofactor i p v NADPH Een tweede mogelijkheid om glutamaat te maken wordt gevormd door de gezamelijke actie van de enzymen GS en GOGAT, volgens de reacties 2 en 3 Koppeling van deze beide reacties geeft een bijna onomkeerbare reactie in de richting van de vorming van glutamaat

Als er twee manieren zijn om ammonium in te bouwen in aminozuren, dringt zich de vraag op of beide systemen naast elkaar werken of dat slechts één van beide manieren wordt gebruikt? Om deze vraag te beantwoorden zijn een aantal benaderingen van het probleem mogelijk Elk van deze benaderingen kan een gedeelte van de vraag beantwoorden Vaak wordt gebruik gemaakt van mutanten die één van de te bestuderen enzymen mist Uit speciale substraat-behoeften is dan misschien af te leiden welke rol het missende enzym speelt in de stikstofhuishouding Helaas is het maken van mutant van de champignon erg moeilijk Het speciale karakter van de champignon-cel is daar "schuld" aan De champignon-cel bevat meerdere kernen (1 tot 25) met in elke kern de informatie om het te onderzoeken enzym aan te maken Als in één kern deze informatie door mutatie beschadigd wordt, kan de informatie uit een andere kern gebruikt worden om het enzym alsnog te maken Momenteel is er geen techniek voorhanden die in alle kernen dezelfde mutatie teweegbrengt

Ook uit de verandering van hoogte van de enzym-activiteiten in relatie tot de aard en de

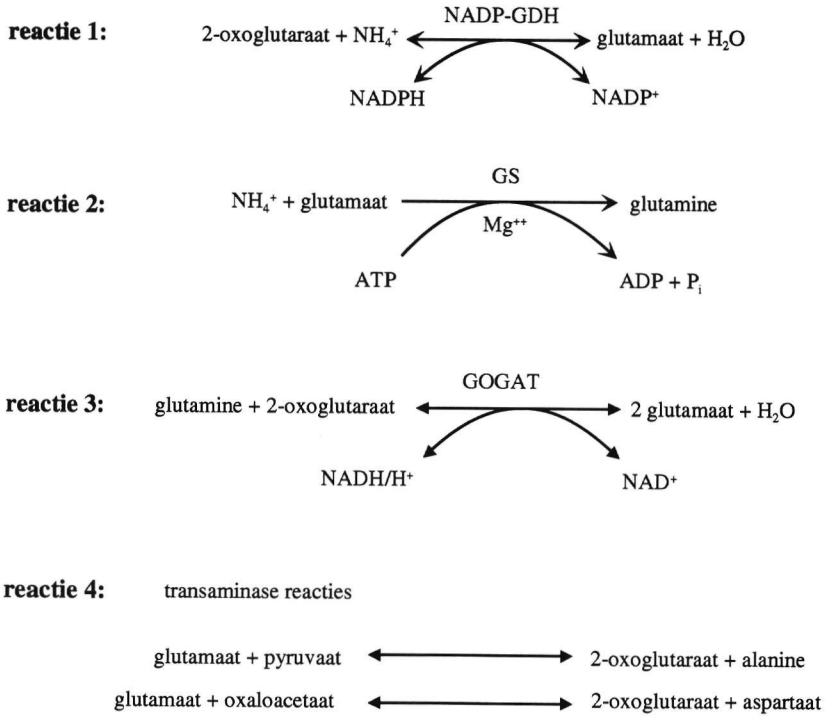


Fig. 2. Schema van reactievergelijkingen die een rol spelen bij de inbouw van ammonium.

hoeveelheid van het stikstofhoudende substraat is informatie over de fysiologische rol van de enzymen af te leiden. Daarom is de champignon gekweekt op een aantal goed bekende stikstofbronnen. Champignon mycelium groeit goed op een combinatie van glucose en ammonium of organische stikstofbronnen (zoals ureum, aminozuren en urinezuur). Het bleek niet mogelijk om champignon mycelium te kweken met nitraat als stikstofbron. Ook kweek op aminozuren als enige koolstof- en stikstofbron bleek niet mogelijk.
De specifieke activiteit van GS, NAD-GDH en NADP-GDH bleek te variëren bij kweek op verschillende stikstofbronnen. De hoogste specifieke activiteit van GS, NAD-GDH en NADP-GDH werd gevonden als glutamaat gebruikt werd als stikstofbron, terwijl de laagste specifieke activiteiten werden gevonden bij kweek op ammonium als stikstofbron.

Om de effecten van groei op ammonium of glutamaat als stikstofbron nader te bestuderen, werd champignon mycelium gekweekt op een reeks verschillende concentraties van ammonium, glutamaat en glucose (**Hoofdstuk 3**). Bij kweek op ammonium werden lage activiteiten van GS en NADP-GDH gevonden. Bij ammoniumconcentraties lager dan 8 mM waren de activteiten iets hoger dan bij ammoniumconcentraties hoger dan 8 mM. De activiteit van NAD-GDH was ongeveer 10 keer hoger dan de activiteit van GS en NADP-GDH.
Bij kweek op glutamaat werden 10 maal hogere activiteiten gevonden van GS en NADP-GDH, vergeleken met de kweek op eenzelfde concentratie ammonium. Variatie in de concentratie glutamaat gaf geen verschillen in activiteit van GS en NADP-GDH te zien. Ook kweek op

verschillende concentraties glucose gaf geen verschillen in activiteit van GS en NADP-GDH te zien
Als een combinatie van glutamaat en ammonium (in een aantal verschillende verhoudingen) als stikstofbron werd aangeboden, werden steeds activiteiten van GS en NADP-GDH gevonden die vergelijkbaar waren met activiteiten zoals die gevonden werden bij kweek op alleen ammonium De aanwezigheid van ammonium als stikstofbron onderdrukt dus hoge enzymactiviteiten In mycelium dat geen stikstof meer tot zijn beschikking had werden hoge activiteiten van GS, NADP-GDH en NAD-GDH gevonden, terwijl in mycelium dat geen koolstof meer ter beschikking had lage activiteiten van deze enzymen gevonden werden

Als deze resultaten worden vergeleken met de resultaten van studies naar de ammonium assimilatie door gisten en een aantal andere schimmels, vinden we een aantal paralellen, met name in de activiteiten van GS en NAD-GDH onder verschillende kweekkondities, en verschillen met name in de respons van de NADP-GDH activiteiten op kweek met ammonium of glutamaat als stikstofbron Op basis van deze resultaten is het echter niet mogelijk om een uitspraak te doen over de rol die GS en NADP-GDH spelen in de inbouw van ammonium

Een andere benadering die gebruikt kan worden om de rol van de enzymen in de inbouw van ammonium te bestuderen maakt gebruik van gelabeld stikstof (^{15}N) Door ammonium met daarin een gelabeld stikstofatoom als stikstofbron aan te bieden aan het mycelium, kan gevolgd worden in welke moleculen het ammonium wordt ingebouwd **(Hoofdstuk 4)**
Als NADP-GDH een belangrijke rol speelt in de inbouw van ammonium, zal het gelabelde stikstof-atoom in glutamaat verschijnen Als GS en GOGAT een belangrijke rol spelen, zal het gelabelde stikstof-atoom eerst in glutamine en met enige vertraging in glutamaat verschijnen Om de interpretatie van de resultaten te vergemakkelijken, kan bovendien gebruik gemaakt worden van speciale remmers De werking van GS en GOGAT kan geremd worden met respectievelijk phosphinothricine en azaserine Het NADP-GDH heeft weinig last van deze twee remstoffen
Indien het experiment werd uitgevoerd zonder remmers toe te voegen kon de aanwezigheid van het gelabeld stikstof worden aangetoond in glutamaat, alanine en glutamine Toevoegen van phosphinothricine (de remmer van GS) had tot gevolg dat geen gelabeld ammonium meer werd ingebouwd Analyse van de aminozuursamenstelling van het mycelium gaf aan dat de concentratie van glutamine een factor 10 lager werd, terwijl de concentraties van alanine en glutamaat toenamen Het toevoegen van azaserine had tot gevolg dat de concentratie van glutamine in het mycelium toenam Dit bleek zowel uit de analyse van de aminozuursamestelling van het mycelium als uit de verhouding tussen het signaal van het gelabeld glutamine en het gelabeld glutamaat
Deze resultaten laten duidelijk zien dat onder de omstandigheden waarin wij het experiment hebben uitgevoerd de enzymen GS en GOGAT het grootste gedeelte van de inbouw van ammonium verzorgen

Aangezien het ook mogelijk is om uit de karakteristieken van de bestudeerde enzymen informatie af te leiden omtrent hun rol in de inbouw van ammonium, zijn zowel GS **(Hoofdstuk 5)** als NADP-GDH **(Hoofdstuk 6)** gezuiverd

GS bleek een eiwit met een molecuulgewicht van 325 kD te zijn dat is opgebouwd uit 8 subeenheden van 46 kD Het iso-electrisch punt was 4 9 De hoogste activiteit werd gemeten bij 30°C en bij temperaturen boven 20°C liep de stabiliteit van de enzymactiviteit sterk terug Op basis van deze karakteristieken is het erg waarschijnlijk dat het GS van de champignon een type II GS is De K_m-waarden voor glutamaat, ammonium, en ATP in de biosynthetische

reactie waren resp. 22 mM, 0.16 mM en 1 25 mM De biosynthetische reactie verliep het best bij pH 7 Deze waarden komen overeen met waarden die voor GS uit andere schimmels zijn gevonden.
In een aantal organismen wordt de activiteit van GS beinvloed door alanine, histidine, tryptofaan, anthranilzuur en 5'-AMP. De activiteit van GS uit de champignon bleek echter niet beinvloed te worden door de aanwezigheid van deze stoffen Methioninesulfoximine en phosphothricine bleken GS uit de champignon zeer sterk te remmen.

Voor het gezuiverde enzym NADP-GDH werd een molekuulgewicht van 330 kD gevonden Het enzym bestaat uit 6 identieke eenheden van 48 kD Het iso-electrisch punt van het enzym was 4.9 De N-terminus van het enzym was geblokkeerd. De K_m-waarden van NADP-GDH voor ammonium, 2-oxoglutaraat, NADPH, glutamaat en NADP waren achtereenvolgens 2.1 mM, 3 2 mM, 0.074 mM, 27 mM en 0 117 mM. Het enzym was specifiek voor NADPH en de maximale activiteit werd gevonden bij 33°C Deze waarden komen sterk overeen met waarden die in het algemeen voor NADP-GDH uit schimmels worden gevonden. Er zijn een aantal stoffen beschreven die in andere micro-organismen de activiteit van NADP-GDH kunnen beinvloeden. Deze stoffen hadden echter geen effect op de activiteit van NADP-GDH uit de champignon De door NADP-GDH bevorderde reactie is reversibel,maar op basis van de gevonden Km-waarden lijkt het waarschijnlijk dat de reactie in de richting van de vorming van glutamaat ligt Ook de sterk remmende werking van ammonium op de afbraak van glutamaat door NADP-GDH maakt dit aannemelijk
Uit een vergelijking van K_m-waarden van GS en NADP-GDH voor ammonium wordt duidelijk dat GS bij lage concentraties ammonium beter kan binden dat NADP-GDH

Om de rol van NADP-GDH in de inbouw van ammonium uitgebreider te kunnen bestuderen, werd het gen dat codeert voor NADP-GDH (*gdhA*) geisoleerd (**Hoofdstuk 7**). Dit gen codeert voor een eiwit van 457 aminozuren en vertoont veel overeenkomst met de genen die in andere micro-organismen voor NADP-GDH coderen Het gen wordt onderbroken door 6 niet-coderende stukjes (intronen) Op basis van de plaats van deze intronen in het gen, lijkt er weinig verwantschap te bestaan tussen de champignon (een basidiomycete schimmel) en de ascomycete schimmels *Neurospora crassa* en *Aspergillus nidulans*
Bij kweek op glutamaat werd een hoge activiteit van NADP-GDH gevonden Daarmee samenhangend werd tevens een grote hoeveelheid mRNA gevonden dat codeert voor dit enzym Indien ammonium werd toegevoegd aan deze kweek bleek dat reeds 2 uur na toediening de hoeveelheid mRNA dat codeert voor NADP-GDH met een factor 10 was afgenomen. De hoeveelheid mRNA die codeert voor de enzymen pyruvaatkinase en ribosomaal eiwit 15A bleek niet te zijn afgenomen, zodat aangenomen kan worden dat dit een specifieke afname van NADP-GDH mRNA is Uit alle resultaten lijkt het aannemelijk dat de activiteit van NADP-GDH in de cel wordt gereguleerd op het niveau van de transcriptie Deze gegevens komen goed overeen met de enzym-activiteiten die gevonden zijn bij kweek van champignon mycelium op ammonium, glutamaat en een combinatie van beiden

Samenvattend kunnen we zeggen dat het aannemelijk is dat GS en GOGAT een prominente rol spelen bij de inbouw van ammonium door de champignon De karakteristieken van het gezuiverde GS en de analyse van de inbouw van gelabeld stikstof bieden voldoende houvast voor deze conclusie.
Over de rol van de beide glutamaatdehydrogenasen (NAD-GDH en NADP-GDH) bestaat nog onduidelijkheid Het lijkt er sterk op dat de regulatie van NADP-GDH in champignon aanzienlijk afwijkt van de regulatie zoals die beschreven is voor NADP-GDH in ascomycete schim-

mels Op basis van de bestudering van mutanten van *N. crassa*, *A nidulans* en *Saccharomyces cerevisiae* is duidelijk geworden dat in deze schimmels en gist NADP-GDH een rol heeft in de aanmaak van glutamaat, terwijl NAD-GDH een rol heeft in de afbraak ervan De relatief geringe invloed van ammonium op de activiteit van NAD-GDH in *A. bisporus* in vergelijking met de veel grotere invloed op de activiteit van NADP-GDH en GS sluit een rol voor NAD-GDH in de afbraak van glutamaat niet uit

De analyse van de inbouw van gelabeld stikstof en de analyse van NADP-GDH specifiek mRNA na toevoeging van ammonium maken een rol van NADP-GDH in de vorming van glutamaat onwaarschijnlijk

Toch zou een beter begrip van de functie van NAD-GDH en NADP-GDH gewenst zijn Analyse van vruchtlichamen van de champignon maakt duidelijk dat deze beide enzymen een hoge activiteit vertonen tijdens de vruchtlichaamvorming Een relatie tussen deze enzymen en het mechanisme van vruchtlichaamvorming is dan ook niet denkbeeldig

Tot slot kan gezegd worden dat de resultaten van deze studie suggereren dat er grote verschillen zijn tussen de manier waarop de inbouw van ammonium wordt gereguleerd in ascomyceten en de manier waarop dit gebeurt in de champignon

Dankwoord

Nu mijn proefschrift zijn voltooiing nadert, is het goed om even stil te staan bij alle mensen die er aan hebben meegewerkt Nu ik er over nadenk zijn er dat nogal wat geweest
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Curriculum vitae

Johannes Jacobus Petrus Baars werd op 21 juli 1961 te Roosendaal geboren Zijn belangstelling voor de "levende natuur" bleek al op jonge leeftijd Op peuter-leeftijd was zijn grote wens om later directeur van de dierentuin te worden
Om aan die prille grote wens te kunnen voldoen heeft hij een aantal schooltypen doorlopen Hij behaalde in 1977 en 1979 achtereenvolgens het MAVO- en het HAVO-diploma en in 1984 werd de tweedegraads onderwijsbevoegdheid voor biologie en scheikunde aan het Mollerinstituut te Tilburg behaald Hij vervolgde zijn studie biologie aan de Katholieke Universiteit Nijmegen In april 1988 legde hij het doctoraal examen af met als hoofdvak Dierfysiologie (Prof Wendelaar Bonga) en als bijvakken Biochemie (Prof Bloemendaal) en Microbiologie (Prof Vogels)
Na ruim een jaar in een leger-groen tenue te hebben vertoefd werd hij als assistent in opleiding (AIO) aangesteld op de afdeling Microbiologie van de Katholieke Universiteit Nijmegen Vanaf november 1989 tot november 1993 werkte hij aan een samenwerkingsprojekt tussen dit laboratorium en het Proefstation voor Champignoncultuur te Horst betreffende het stikstof-metabolisme van de champignon De resultaten van dit onderzoek zijn grotendeels beschreven in dit proefschrift
Vanaf november 1994 is hij als junior onderzoeker in tijdelijke dienst verbonden aan de afdeling Microbiologie van de Katholieke Universiteit Nijmegen en gedetacheerd bij het Proefstation voor Champignoncultuur te Horst

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