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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.

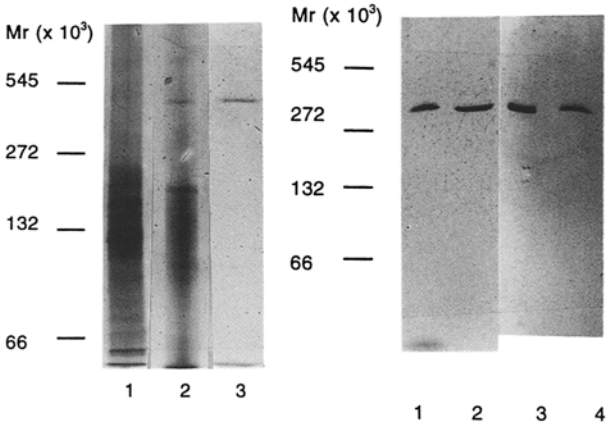

Fig. 1. (**A**) Native PAGE of the purified NADP-GDH on a 10–15% gradient gel. Protein from different purification steps was analyzed by silver staining. Lane 1, cell-free extract (1.0 µg); lane 2, dye affinity chromatography (0.6 µg); lane 3, Protein Pak DEAE chromatography (0.1 µg). (**B**) Native 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.

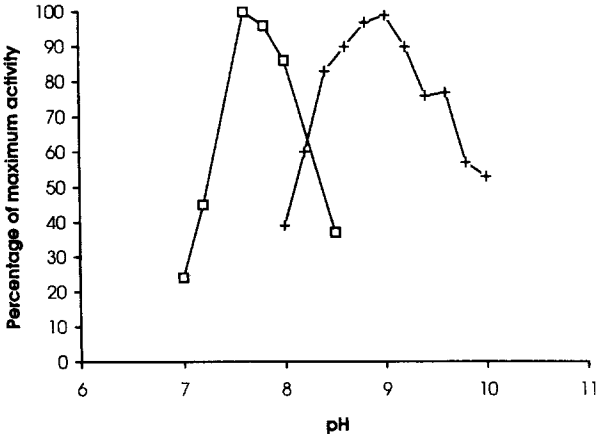

Fig. 2. Effect of pH on the NADP-dependent glutamate dehydrogenase activity. Activities for both amination (□) and deamination reactions (+) at their respective pH optima were set at 100%. Amination and deamination reactions were measured at room temperature in 50 mM Tris/HCl and 100 mM glycine/NaOH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

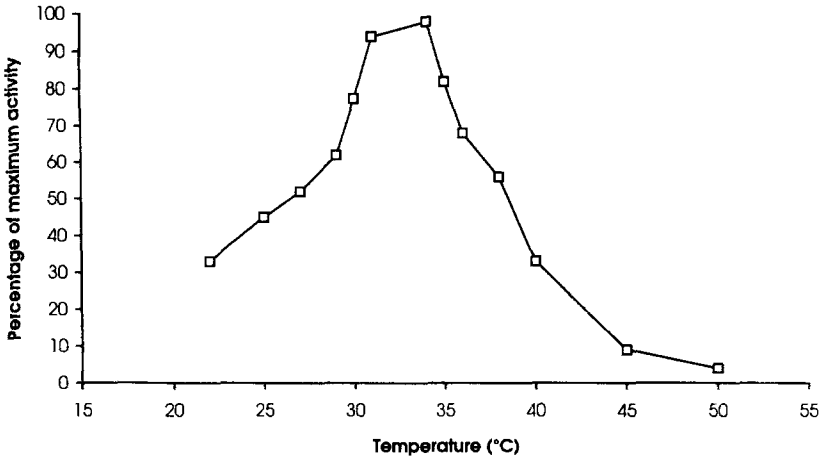

Fig. 3. Effect of temperature on the rate of the reductive amination reaction.

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.

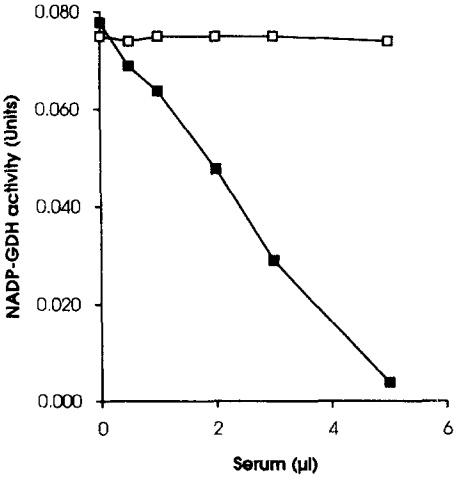

Fig. 4. Anticatalytic immunoprecipitation of NADP-GDH activity from cell-free extract. Either anti-GDH serum (■) or pre-immune serum (□) was mixed with cell-free extract (0.08 U of NADP-GDH activity) in a total volume of 0.5 ml. After 1 h of incubation at room temperature, the mixture was centrifuged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