

# Complete Sequence of Glycolytic Enzymes in the Mycorrhizal Basidiomycete, *Suillus bovinus*

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Axenic cultures of *Suillus bovinus* were cultivated in inorganic liquid medium with glucose as a carbon source at 25 °C and continuous supply of oxygen by aeration with compressed air in the dark. Exogenous fructose as sole carbon source yielded about 50% less increase in dry weight than glucose. This resulted from different uptake velocities. Sucrose as sole exogenous carbon source yielded no measurable increase in dry weight.

In glucose cultures, activities of all glycolytic enzymes were found. Maximum specific activities varied largely (from about 60 [fructose 6-phosphate kinase] to about 20 000 [triosephosphate isomerase] nmoles · mg protein<sup>-1</sup> · min<sup>-1</sup>). Apparent  $K_m$ -values also varied over more than two orders of magnitude (0.035 mM [pyruvate kinase] to 6.16 mM [triosephosphate isomerase]). Fructose 6-phosphate kinase proved to be the fructose 2,6-bisphosphate-regulated type, aldolase the divalent cation-dependent (class II) type and glyceralatephosphate mutase the glycerate 2,3-phosphate-independent type of the respective enzymes. Eight of the 10 enzymes exhibited pH-optima between 7.5–8.0. Triosephosphate isomerase and pyruvate kinase showed highest activities at pH 6.5. Regulatory sites within the glycolytic pathway of *Suillus bovinus* are discussed; fructose 6-phosphate kinase appears to be its main bottle neck.

## Introduction

Mycorrhiza, a symbiotic interaction between plant roots and mycelia of higher fungi is widespread in nature (e. g. Trappe, 1962; Harley and Smith, 1983). The special type, ectomycorrhiza, is characterized by a hyphal mantle around young lateral roots and by penetration of hyphae into the intercellular spaces of the root cortex. This tight

vicinity allows delivery of autotrophically produced carbohydrates from plant to fungus and – in turn – supply of minerals and water by fungus to the roots (Melin and Nilsson, 1957; Lewis and Harley, 1965c). Ectomycorrhiza is common in forest trees, and therefore besides of ecological also of economical interest.

In plants, carbohydrates are generally transported as sucrose (Ziegler, 1975). When applied as sole carbon source, this disaccharide has repeatedly been found not to allow growth of isolated mycelia (Lewis and Harley, 1965a; 1965b; Palmer and Hacskeylo, 1970; Chen and Hampp, 1993; see also: Hacskeylo, 1973; Harley and Smith, 1983; Jakobsen, 1991). Rather recently, in elegant experiments with cell cultures of *Picea abies*, Salzer and Hager (1991) solved this contradiction by discovering cell wall-bound sucrase activity for the root cells, but not for hyphae of *Amanita muscaria* and *Hebeloma crustuliniforme*. Thus, the tree supplied glucose and fructose to the fungal cells for uptake. Both these monosaccharides have been found to sustain growth of isolated mycelia. However, glucose yielded greater increase in mass than fructose

**Abbreviations:** ADP/ATP, adenosinediphosphate/adenosinetriphosphate; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid;  $K_m$ , Michaelis constant;  $S_{(0.5)}$ , half saturating substrate concentration for enzymes without Michaelis Menten kinetics; NAD(P)<sup>+</sup>/NAD(P)H, nicotinamide-adenine-dinucleotide (phosphate)oxidized/reduced; TEAE, triethanolaminehydrochloride.

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(Lewis and Harley, 1965a; 1965b; Palmer and Hacksaylo, 1970; Salzer and Hager, 1991) This resulted from a more rapid uptake of glucose, convincingly shown by tracer studies with isolated protoplasts of *Amanita* by Chen and Hampp (1993). The fate of fructose from sucrose splitting is not absolutely clear, yet.

Investigations into enzymes of glucose breakdown in fungal cells have been performed by several authors (e. g. Hacksaylo, 1973; Martin *et al.*, 1987; Jakobsen, 1991; Griffin, 1994; Hampp and Schaeffer, 1995). In the course of these studies, enzymes of the glycolytic pathway, of the oxydative pentosephosphate pathway and of the tricarboxylic acid cycle have been reported to occur in various mycorrhizal fungi, although – as far as we know – there is no complete sequence known in detail, at present. Conclusions on regulation of the respective pathway are therefore not possible, yet, let alone considerations on its alterations by mycorrhization. In this context, most recently Schaeffer *et al.* (1996) reported on different kinetic properties of phosphofructokinase (= fructose 6-phosphate kinase) of *Amanita muscaria* grown isolated or being in contact with roots of *Picea abies*. In our attempt to get more information on metabolic alterations as consequences of mycorrhization, we decided to examine the complete enzymatic sequence from glucose to CO<sub>2</sub> via glycolysis and the tricarboxylic acid cycle and the accompanied nitrogen incorporating enzymes. Objects of investigation were *Pinus sylvestris* and the basidiomycete *Suillus bovinus*, both frequent partners in mycorrhiza and abundant in the northern hemisphere.

All data were to be determined for isolated mycelia, for fungus-free tree roots and, finally, for the mycorrhized system produced under controlled conditions.

In this paper, we present basic data for all glycolytic enzymes of the fungus. It was grown in axenic cultures on glucose as the sole carbon source and ammoniumphosphate as the only nitrogen source.

## Material and Methods

Axenic cultures of *Suillus bovinus* (L. ex Fr.) O. KUNTZE, Boletaceae were used. Dicaryotic mycelia were isolated from fruiting bodies collected at Sennefriedhof Bielefeld by U. Röder, Department of Ecology, University Bielefeld.

## Growth conditions

Mycelia were usually grown in suspension cultures in a liquid medium after Kottke *et al.* (1987) containing NH<sub>4</sub><sup>+</sup>-ions as nitrogen and 10 g/l glucose as carbon sources. pH was set to 5.8. The autoclaved (5 min at 1.2 bar) medium was inoculated with suspended hyphae (Gelaire, Laminar Air Flow Class 100, Flow Laboratories, Meckenheim, Germany) and the resulting suspension filled in sterilized (4 h at 160 °C) culture tubes with gas inlet at the bottom (length 45 cm, Ø 4 cm). These were placed in a water bath of 25 °C in the dark.

## Analytical methods

Assay for glucose, fructose, and sucrose in media.

The amounts of glucose, fructose and sucrose in the growth medium were determined enzymatically (Boehringer Test Nr. 716260) and colorimetrically (anthrone reaction, Roe, 1955).

## Determination of dry weight

Hyphae were harvested by filtration of the cell suspension through a Buchner funnel. They were washed with water 3 times, transferred into aluminum vessels, dried at 104 °C for 10 h, cooled to room temperature in a dessicator and weighed on a semimicro-balance.

## Enzyme assays

For determination of enzyme activities mycelia were harvested 5–6 days after inoculation on filter paper in a Buchner funnel. The resulting pellet was washed with water 3 times and suspended in 0.1 M phosphate buffer pH 7.5 plus 0.6 mM DTT (1/5 w/v). Cells were broken by grinding with sea sand in a mortar under cooling with ice. After separation from sand and large cell fragments by filtration through 4 layers of cheese cloth, the resulting homogenates were centrifuged for 20 min at 20 000×g and 4 °C (Sorvall RC-5 Superspeed Refrigerated Centrifuge). The resulting supernatants were used as crude extracts.

Based on prescriptions of Bergmeyer (1974), all enzyme activities were determined photometrically (λ 340 nm) using absorbance changes resulting from oxydation or reduction of NAD(P)H/NAD(P)<sup>+</sup>. All assays were optimized for the *Suil-*

*lus* extracts, i.e., optimum concentrations for substrates and cofactors, optimum pH and, in coupled assay systems, non-limiting concentrations of auxiliary enzymes and pyridine nucleotides had to be determined. This bulk of data will not be presented here in detail. It should, however, be mentioned, that together with the most appropriate method for cell disintegration (cell mill, sonification, grinding) this yielded an improvement for every enzyme ranging from 15% to more than 100%.

*Hexokinase* (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1)

NADP<sup>+</sup> was reduced by oxydation of glucose 6-phosphate produced from glucose and ATP by the enzyme.

Assay: 40 mM TEAE-buffer pH 7.5, 4.0 mM MgCl<sub>2</sub>, 44.4 mM glucose (start), 0.96 mM ATP, 5–10 µg crude extract protein/ml test volume, 0.91 mM NADP<sup>+</sup>, 0.055 units/ml glucose 6-phosphate dehydrogenase.

*Glucose 6-phosphate isomerase* (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)

NADP<sup>+</sup> was reduced by oxydation of glucose 6-phosphate produced from fructose 6-phosphate by the enzyme.

Assay: 85 mM TEAE-buffer pH 7.7, 3.0 mM MgCl<sub>2</sub>, 1.8 mM fructose 6-phosphate (start), 7 µg crude extract protein/ml test volume, 0.30 mM NADP<sup>+</sup>, 0.2 units/ml glucose 6-phosphate dehydrogenase.

*Fructose 6-phosphate kinase* (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11)

NADH was oxydized by the reduction to glycerol 3-phosphate of dihydroxyacetonephosphate resulting from aldolase-catalyzed splitting of fructose 1,6-bisphosphate produced from fructose 6-phosphate and ATP by the enzyme.

Assay: 52.1 mM glycylglycine-buffer pH 7.6, 5.2 mM EDTA, 10.9 mM MgCl<sub>2</sub>, 15.2 mM fructose 6-phosphate, 2.4 mM ATP (start), 10 µM fructose 2,6-bisphosphate, 40 µg crude extract protein/ml test volume, 1 unit/ml aldolase, 1 unit/ml triosephosphate isomerase, 0.123 mM NADH, 14 units/ml glycerol 3-phosphate dehydrogenase.

*Aldolase* (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13)

NADH was oxydized by the glycerol 3-phosphate dehydrogenase-catalyzed reduction to glycerol 3-phosphate of dihydroxyacetonephosphate produced from fructose 1,6-bisphosphate by the enzyme.

Assay: 94 mM HEPES-buffer pH 8.0, 1.1 mM fructose 1,6-bisphosphate (start), 3 µg crude extract protein/ml test volume, 1.1 units/ml triosephosphate isomerase, 0.2 mM NADH, 1.2 units/ml glycerol 3-phosphate dehydrogenase.

*Triosephosphate isomerase* (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1)

NADH was oxydized by the glycerol 3-phosphate dehydrogenase-catalyzed reduction to glycerol 3-phosphate of dihydroxyacetonephosphate produced from glyceraldehyde 3-phosphate by the enzyme.

Assay: 243 mM TEAE-buffer pH 6.75 (adjusted with TRIS-buffer), 10.45 mM glyceraldehyde 3-phosphate (start), 0.05–0.1 µg crude extract protein/ml test volume, 0.2 mM NADH, 1.3 units/ml glycerol 3-phosphate dehydrogenase.

*Glyceraldehyde 3-phosphate dehydrogenase* (D-glyceraldehyde-3-phosphate: NAD oxidoreductase, EC 1.2.1.12)

NADH was oxydized by reduction to glyceraldehyde 3-phosphate of glycerate 1,3-bisphosphate by the enzyme. Glycerate 1,3-bisphosphate was produced by glycerate 3-phosphate kinase from glycerate 3-phosphate and ATP.

Assay: 76.6 mM TEAE-buffer pH 8.0, 0.9 mM EDTA, 0.3 mM MgSO<sub>4</sub>, 9.2 mM glycerate 3-phosphate (start), 2.2 mM ATP, 5–10 µg crude extract protein/ml test volume, 0.3 mM NADH, 7.4 units/ml glycerate 3-phosphate kinase.

*Glycerate 3-phosphate kinase* (ATP : 3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3)

NADH was oxydized by glyceraldehyde 3-phosphate dehydrogenase-catalyzed reduction to glyceraldehyde 3-phosphate of glycerate 1,3-bisphosphate produced from glycerate 3-phosphate and ATP by the enzyme.

Assay: 70 mM TEAE-buffer pH 7.0, 0.9 mM EDTA, 3.2 mM  $\text{MgSO}_4$ , 11.9 mM glycerate 3-phosphate (start), 1.3 mM ATP, 6–7  $\mu\text{g}$  crude extract protein/ml test volume, 0.2 mM NADH, 2.5 units/ml glyceraldehyde 3-phosphate dehydrogenase.

*Glyceratephosphate mutase* (Phosphoglycerate phosphomutase, glycerate-2,3-phosphate independent, EC 5.4.2.1)

NADH was oxydized by lactate dehydrogenase-catalyzed reduction to lactate of pyruvate resulting – via phosphoenolpyruvate – from glycerate 2-phosphate produced from glycerate 3-phosphate by the enzyme.

Assay: 90.1 mM TEAE-buffer pH 7.5, 0.33 mM  $\text{MgSO}_4$ , 5.01 mM glycerate 3-phosphate (start), 6–7  $\mu\text{g}$  crude extract protein/ml test volume, 0.17 mM ADP, 0.12 mM NADH, 1.4 units/ml enolase, 1.4 units/ml pyruvate kinase, 9.4 units/ml lactate dehydrogenase.

*Enolase* (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11)

NADH was oxydized by lactate dehydrogenase-catalyzed reduction to lactate of pyruvate produced – via phosphoenolpyruvate – from glycerate 2-phosphate by the enzyme.

Assay: 82 mM TEAE-buffer pH 8.0, 4.1 mM  $\text{MgSO}_4$ , 1.46 mM glycerate 2-phosphate (start), 0.56 mM ADP, 10–15  $\mu\text{g}$  crude extract protein/ml test volume, 0.08 mM NADH, 2.75 units/ml pyruvate kinase, 9.44 units/ml lactate dehydrogenase.

*Pyruvate kinase* (ATP : pyruvate 2-O-phosphotransferase, EC 2.7.1.40)

NADH was oxydized by lactate dehydrogenase-catalyzed reduction to lactate of pyruvate produced from phosphoenolpyruvate and ADP by the enzyme.

Assay: 166 mM HEPES-buffer pH 6.5, 15 mM KCl, 3.75 mM  $\text{MgSO}_4$ , 0.81 mM phosphoenolpyruvate, 2.35 mM ADP (start), 6–7  $\mu\text{g}$  crude extract protein/ml test volume, 0.2 mM NADH, 6.4 units/ml lactate dehydrogenase.

### Soluble protein

Soluble protein was determined according to Lowry *et al.* (1951) with bovine serum albumine as reference.

## Results

### Growth of mycelia

For experiments, isolated axenic mycelia of *Suillus bovinus* were cultivated in inorganic liquid medium with an organic carbon source in culture tubes in the dark. Growth was improved by ample supply of oxygen and by preventing settling down of the cells. Both was accomplished by continuous aeration with compressed air from the bottom of the culture tube. Largest increase in dry weight was obtained at 25 °C. It was lower by about 20% at 20 °C or 30 °C, respectively. Mainly because of uptake of ammonium ions, the pH of the medium dropped from 5.8 in the beginning to about 4.0 at harvest (after 5–6 days). These changes were accepted because the mycelia exhibited a pronounced tolerance for acidic environment: Production of dry weight was found best between pH 5.0 and 3.0 (Fig. 1). It should be noted that under the extreme acidic and under the extreme alkaline conditions tested there was a decrease in dry weight, indicating limited survival of the mycelia. Under the culture conditions applied, the initially inoculated small flakes of hyphae developed into more or less solid „balls“ of about 1 cm Ø within 5–6 days. Increase in dry weight was about 3 times larger than that in Erlenmeyer flasks or on agar plates containing the same nutrients.

Among the common carbon sources tested, such as glucose, fructose and sucrose, glucose yielded

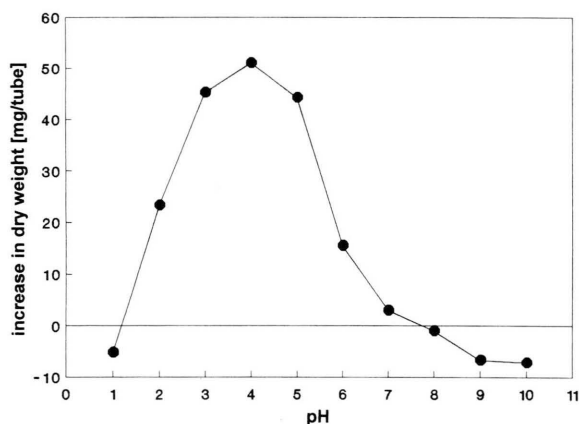


Fig. 1. Influence of pH on growth of mycelia of *Suillus bovinus*.

Growth conditions: liquid inorganic media of different pH with glucose as sole carbon source, continuous aeration with compressed air, 25 °C, darkness.



about twice as much dry matter as fructose. Sucrose did not lead to any measurable increase in dry weight. This is in accordance with literature data for other mycorrhizal fungi (see Introduction). Different growth seems to be largely due to different uptake velocities for the sugar molecules. Uptake studies with samples of the same dense suspension of hyphae in phosphate buffer revealed an about two times faster disappearance of glucose from the medium than of fructose. They yielded no significant change in the amount of sucrose applied (Fig. 2). This also corresponds to the already mentioned results of tracer studies with isolated protoplasts of *Amanita muscaria* by Chen and Hampp (1993).

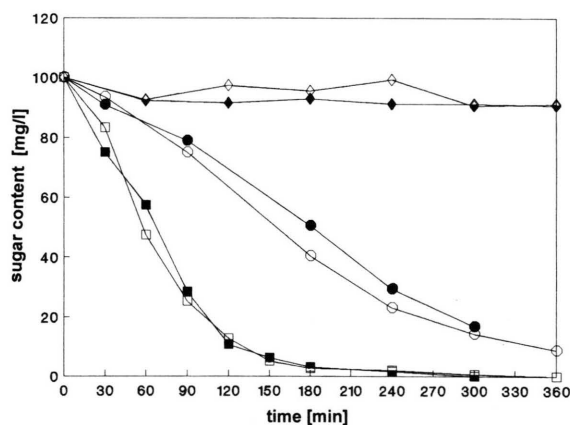


Fig. 2. Decrease of glucose (—□—), fructose (—○—) or sucrose (—◇—) in media of isolated axenic mycelia of *Suillus bovinus*.

Growth conditions: liquid media pH 4.5, aeration with compressed air, 25 °C, darkness. open symbols = colorimetric determinations, closed symbols = enzymatic determinations.

In addition, the catabolism of fructose is likely to be slower. The phosphorylating enzyme, hexokinase, necessary to introduce the sugar molecule into degrading pathways, exhibited a much lower affinity towards fructose than towards glucose. The apparent  $K_m$ -values differed by one order of magnitude ( $K_m$  (fructose) = 4.49 mM,  $K_m$  (glucose) = 0.37 mM).

#### Carbohydrate degrading enzymes

There is clear evidence for glucose and fructose degradation via the glycolytic pathway in *Suillus bovinus*. In crude extracts of mycelia from the above glucose-cultures, activities of all enzymes in-

volved could be detected. Maximum specific activities measurable with optimized assays (= *in vitro* enzyme capacities) varied largely. They ranged from about 60 to more than 20000 nmoles · mg protein<sup>-1</sup> · min<sup>-1</sup> (Table I). Lowest capacity was found for fructose 6-phosphate kinase ( $58 \pm 3$  nmoles · mg protein<sup>-1</sup> · min<sup>-1</sup>), highest for triosephosphate isomerase ( $20960 \pm 799$  nmoles · mg protein<sup>-1</sup> · min<sup>-1</sup>). Fructose 6-phosphate kinase activity was still much lower, sometimes not measurable at all, when analyzed without addition of fructose 2,6-bisphosphate. Remarkably low was also the capacity of aldolase ( $157 \pm 6$  nmol · mg protein<sup>-1</sup> · min<sup>-1</sup>). This enzyme proved severely

Table I. Maximum activities measurable (=capacities) of glycolytic enzymes in crude extracts of mycelia of *Suillus bovinus*.

Growth conditions: liquid inorganic medium with glucose as sole carbon source pH 5.8, continuous aeration with compressed air, 25 °C, darkness. For preparation of crude cell extracts and enzyme assays see Material and Methods.

Enzyme	Capacity [nmol · mg protein <sup>-1</sup> · min <sup>-1</sup> ]
Hexokinase	351 ± 11
Glucose 6-phosphate isomerase	1818 ± 204
Fructose 6-phosphate kinase	58 ± 3
Aldolase	157 ± 6
Triosephosphate isomerase	20960 ± 799
Glyceraldehyde 3-phosphate dehydrogenase	2951 ± 220
Glycerate 3-phosphate kinase	1593 ± 135
Glyceratephosphate mutase	989 ± 42
Enolase	616 ± 40
Pyruvate kinase	598 ± 30

dependent on divalent cations. Their removal by EDTA led to an almost complete loss in activity (Table IIA) which could be re-established by addition of several heavy metal ions (Table IIB). Among the elements tested, cobalt turned out to be most effective. It was followed by iron, manganese, nickel and zinc. Copper ions proved to be ineffective. From these data, the enzyme belongs to the class II aldolases (Rutter, 1964). Addition to the untreated enzyme of the heavy metal ions tested, never resulted in significant increases in activity. On the contrary, in some cases it led to slight ( $Zn^{2+}$ ) or even severe ( $Cu^{2+}$ ) inhibitions. Application of  $K^+$  ions, which improved enzyme activity *in vitro*, as it is known from literature (Rutter *et al.*, 1966), did not result in restauration of the EDTA-reduced activity. Glyceratephosphate mu-

Table II. Inhibition by EDTA (A) and subsequent re-stauration by heavy metal ions (B) of aldolase activity in crude extracts of mycelia of *Suillus bovinus*.

Growth conditions: liquid inorganic medium with glucose as sole carbon source pH 5.8, continuous aeration with compressed air, 25 °C, darkness. For preparation of crude cell extracts and enzyme assays see Material and Methods. (100 = 168 nmol · mg protein<sup>-1</sup> · min<sup>-1</sup>).

A						
Exposure time						
EDTA [mM]	5 min	10 min	15 min	20 min	25 min	
0.0	100	100	100	100	100	
0.5	82	51	32	22	12	
1.0	67	28	15	5	5	
2.0	54	17	6	6	4	
4.0	40	8	4	3	4	
8.0	33	3	3	3	2	

B						
0.5 mM EDTA exposure time 25 min						
Heavy metal [mM]	Co <sup>2+</sup>	Fe <sup>2+</sup>	Mn <sup>2+</sup>	Ni <sup>2+</sup>	Zn <sup>2+</sup>	Cu <sup>2+</sup>
0.0	8	8	8	8	8	8
0.5	7	30				
1.0	97	74	52	23	13	6

tase exhibited no demand for glycerate 2,3-bisphosphate *in vitro*. It, therefore, is considered independent of this compound.

Enzyme capacities do not include informations on substrate affinities. These are expressed by apparent  $K_m$ - or  $S_{0.5}$ -values. They were found to differ by more than one order of magnitude (Table III). In most cases low capacities were ac-

Table III. Apparent  $K_m$ - or  $S_{0.5}$ - values and Hill coefficients of glycolytic enzymes of mycelia of *Suillus bovinus*.

Growth conditions: liquid inorganic medium with glucose as sole carbon source pH 5.8, continuous aeration with compressed air, 25 °C, darkness. For preparation of crude cell extracts and enzyme assays see Material and Methods.

Enzyme	$K_m$ ( $S_{0.5}$ ) [mM]	Hill coefficient
Hexokinase	0.37	1.03
Glucose 6-phosphate isomerase	0.35	1.04
Fructose 6-phosphate kinase	0.11	1.93
Aldolase	0.37	1.01
Triosephosphate isomerase	6.16	1.00
Glyceraldehyde 3-phosphate dehydrogenase	1.38	1.04
Glycerate 3-phosphate kinase	1.06	0.99
Glyceratephosphate mutase	0.17	0.99
Enolase	0.25	1.02
Pyruvate kinase	0.035	0.98

companied by low apparent  $K_m$ -values, high capacities by high apparent  $K_m$ -values, i.e., enzymes existing with comparatively small capacities had high, those existing with comparatively large capacities had low substrate affinities.

Only one of the enzymes, fructose 6-phosphate kinase, exhibited a homotropic effect for its substrate: When assayed in presence of fructose 2,6-bisphosphate, its activity did not follow Michaelis Menten kinetics, but showed a Hill coefficient of almost 2. This was different at absence of fructose 2,6-bisphosphate. Under these conditions, the Hill coefficient was 1.00. For all the other enzymes it was also close to unity (Table III).

Determined in artificial buffer systems, pH-dependencies exhibited maxima between pH 7.5 and 8.0 for 8 of the 10 enzymes. Triosephosphate isomerase and pyruvate kinase showed maximum activity around pH 6.5 (Fig. 3). In most cases the maxima were relatively sharp. Deviation from optimum pH by one order resulted in drop of activity of 50% or more.

## Discussion

Our data on glucose, fructose and sucrose as sole carbon sources for growth of isolated mycorrhizal fungi are not new. They extend, however, existing respective knowledge on another mycorrhizal basidiomycete, *Suillus bovinus*, which proves to be a suitable object for further physiological research.

The data on carbohydrate degrading enzymes presented, also confirm existing information on general involvement of the glycolytic pathway from glucose to pyruvate in carbohydrate catabolism of mycorrhizal fungal cells (Martin *et al.*, 1987; Jakobsen, 1991; Griffin, 1994; Hampp and Schaefer, 1995), but they extend respective knowledge by presenting activities and kinetic data of the complete sequence of enzymes in one fungal species. Additionally, they contain further characteristics of some of the enzymes. To our knowledge, such information has not been reported before.

As to enzyme characterization, our data shows, that fructose 6-phosphate kinase of *Suillus bovinus* is the fructose 2,6-bisphosphate-dependent type, that aldolase is the cation-dependent class II type, and that glyceratephosphate mutase is the glycerate 2,3-phosphate-independent type of the respec-

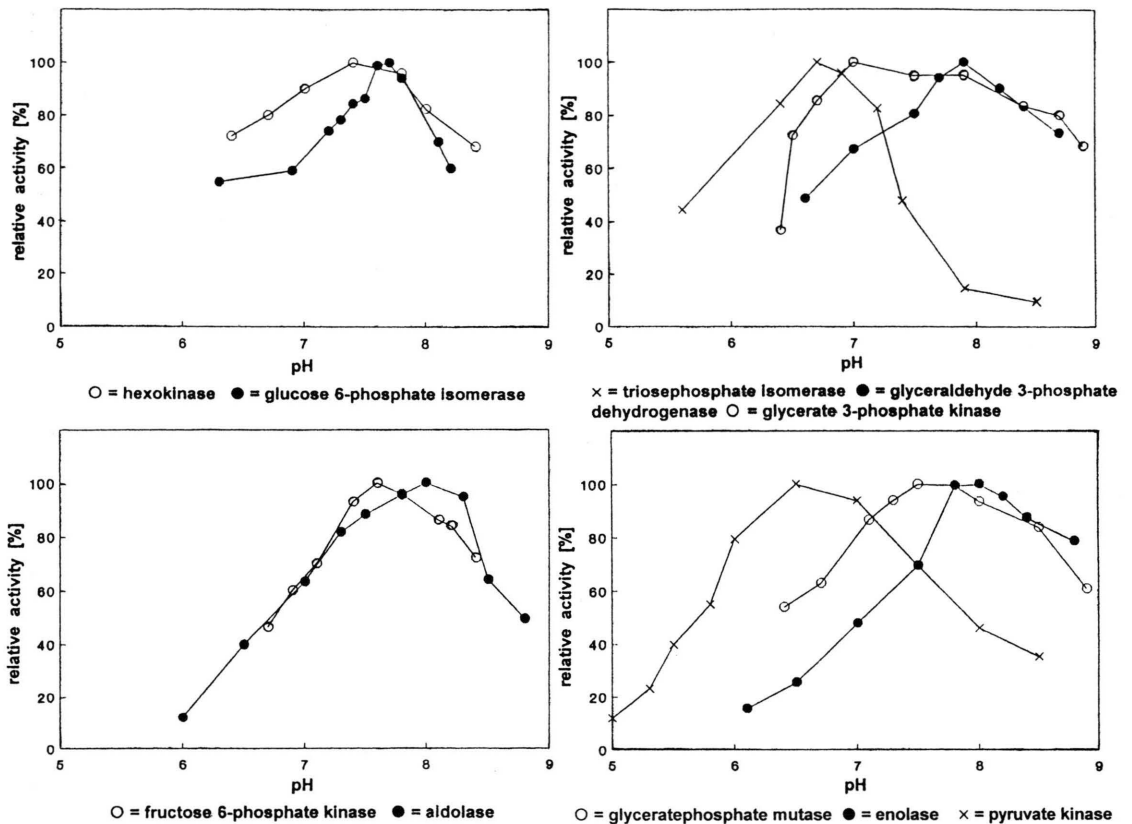


Fig. 3. pH-dependencies of the activities of glycolytic enzymes of *Suillus bovinus*.

Growth of mycelia: liquid inorganic medium with glucose as sole carbon source pH 5.8, continuous aeration with compressed air, 25 °C, darkness. Enzyme assays: see Material and Methods.

Buffers used: HEPES (hexokinase, aldolase, pyruvate kinase)

TEAE (glucose 6-phosphate isomerase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, glyceral 3-phosphate kinase, glyceralphosphate mutase, enolase), Glycylglycine (fructose 6-phosphate kinase).

tive enzyme. This is in good agreement with the known occurrence of these enzymes in various sources of living matter:

Fructose 2,6-bisphosphate-dependent fructose 6-phosphate kinase has been found in various heterotrophic, (Pilkis *et al.*, 1981; Van Schaftingen, 1987), but not in autotrophic organisms in which a  $PP_i$ -dependent fructose 6-phosphate phosphorylating enzyme is activated by this compound (Stitt, 1990). Among the former, there is already another mycorrhizal basidiomycete, *Amanita muscaria* (Schaeffer *et al.*, 1996). When effective, fructose 2,6-bisphosphate exhibits high stimulatory efficiency on fructose 6-phosphate kinase.

Class II aldolases are typical for bacteria, cyanobacteria and fungi (Rutter *et al.*, 1966; Horecker *et*

*al.*, 1972). In photoautotrophic organisms, such as several green, red and brown algae as well as higher plants (Russel and Gibbs, 1967; Willard and Gibbs, 1968), it has been found residing in chloroplasts, while there were class I aldolases in the cytosol of these organisms. The observed properties of the *Suillus* enzyme correspond largely to those of yeast aldolase. Inactivated by EDTA-treatment, the activity of this enzyme can also be restored by  $Co^{2+}$ ,  $Fe^{2+}$ ,  $Mn^{2+}$  and  $Ni^{2+}$ , not by  $Cu^{2+}$  ions. A striking difference, however, is the respective effectiveness of  $Zn^{2+}$ . It is highest for the yeast enzyme (Kobes *et al.*, 1969), but very low for that of *Suillus*. High effectiveness of  $Zn^{2+}$ ,  $Co^{2+}$  and  $Fe^{2+}$  has also been reported for the aldolases of some bacteria (Groves *et al.*, 1966).

Besides the glycerate 2,3-bisphosphate-dependent glyceratephosphate mutase, also the glycerate 2,3-phosphate-independent type of the enzyme seems to be widespread. It has been described for various autotrophic and heterotrophic organisms including some ascomycetes such as *Aspergillus* and *Neurospora* (Grisolia, 1962; Ray and Peck, 1972; Grisolia and Carreras, 1975).

Considering regulatory sites within the glycolytic sequence, no unequivocal conclusions can be drawn from the data available. Nevertheless, an attempt shall be made, to get first insight into these processes. Comparison of the determined *in vitro* enzyme capacities alone is not likely to reflect the situation *in vivo*. They are determined at saturating substrate concentrations, and thus depend largely on amounts and on turnover numbers of the enzymes; they do not include substrate affinities. Consideration of the latter appears necessary, however, because presence of saturating substrate concentrations for all enzymes appears unlikely *in vivo*. We do not know the respective substrate concentrations at the site of action, but – as a compromise – we decided to use  $K_m$ -values as indicators for enzyme affinity, being fully aware, of course, that exactly half-saturating substrate concentrations are also unlikely to exist for every enzyme. Thus, the quotient “enzyme capacity ·  $K_m^{-1}$ ” was chosen as a more appropriate means for comparison. Finally, pH-dependencies had to be taken into consideration. This also could not be done precisely because the cytosolic pH of *Suillus* is unknown. Exemplarily we present a calculation for pH 7.1 (Table IV), a value well within the range of 6.8–7.4 usually assumed for cytosol. It can, however be noted, that use of other pH-values of this range do not alter the conclusions drawn in the following essentially. Nevertheless, the striking deviation of the pH-optima of triosephosphate isomerase and pyruvate kinase from the pH-optima of the other enzymes deserves attention. It certainly points to regulatory significance *in vivo* and demands for further clarification.

We feel, that three aspects make the resulting relative numbers appear useful indicators for comparison of *in vivo* efficiencies of the enzymes. First, the isomerases, glucose 6-phosphate isomerase and triosephosphate isomerase, and the mutase, glyceratephosphate mutase turn out to be least

Table IV. Tentative enzyme efficiencies at pH 7.1 of the glycolytic enzymes in crude extracts of mycelia of *Suillus bovinus*.

Relative numbers (rounded),  $F_{pH}$  = capacity at pH 7.1 · maximum capacity<sup>-1</sup>.

Enzyme	Enzyme capacity
	$K_m (S_{0.5}) \cdot F_{pH}$
Hexokinase	900
Glucose 6-phosphate isomerase	3700
Fructose 6-phosphate kinase	400
Aldolase	300
Triosephosphate isomerase	3100
Glyceraldehyde 3-phosphate dehydrogenase	1500
Glycerate 3-phosphate kinase	1500
Glyceratephosphate mutase	5100
Enolase	1300
Pyruvate kinase	15200

limiting. For enzymes catalyzing energy-independent steps within a metabolic sequence, this is to be expected. Second, the closely interdependent enzymes glyceraldehyde 3-phosphate dehydrogenase and glycerate 3-phosphate kinase, and additionally the neighbouring enolase, show comparably high rates. Third, fructose 6-phosphate kinase comes out as a major bottle neck, hexokinase as another minor rate limiting step of the pathway. Regulatory significance of these enzymes has been found for all sorts of living matter. Accepting these results as evidence for reliable use of the data, the two remaining enzymes, aldolase and pyruvate kinase, exhibit unexpected characteristics. Even by taking into account that aldolase is the only enzyme within the glycolytic pathway which produces two interconvertible product molecules, glyceraldehyde 3-phosphate and dihydroxyacetonephosphate, its tentative efficiency still does not meet that of hexokinase. Aldolase, therefore, would be one more enzyme with regulatory significance. This quality is not known for aldolases from other sources. It, therefore, might be a speciality in *Suillus* (and other fungi?) but, of course, we must have in mind that we did not succeed in determination of maximum capacity, i.e., did not find all essential cofactors for the *in vitro* assay. The extraordinary high tentative efficiency of pyruvate kinase is very unusual. Because of it, the enzyme is not likely to have regulatory significance which has been observed for autotrophic or-



ganisms, but seems to be absent in heterotrophic cells (Turner and Turner, 1980; Ruyters, 1982). The comparatively very high substrate affinity of pyruvate kinase of *Suillus* might be caused by competition for its substrate. Other phosphoenolpyruvate consuming enzymes would be carboxylases producing C<sub>4</sub>-dicarbonic acids necessary to fill up the tricarboxylic acid cycle when drained off for production of amino acids such as glutamate and aspartate. Both are often considered to be those

products of nitrogen incorporation in the fungal cell which – in case of mycorrhiza – are delivered to the root (France and Reid, 1983; Martin *et al.*, 1987; Chalot *et al.*, 1991; Griffin, 1994). In mycelia of *Amanita muscaria*, activities of such carboxylases have been found recently by Wingler *et al.* (1996). Of course, irregularities within the tricarboxylic acid cycle cannot be excluded, at present. These questions will be dealt with in a following publication.

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