Acta Chimica Slovaca, Vol.1, No. 1, 2008, 134 – 142

Intracellular Changes of GABA metabolism in *Trichoderma viride* and other fungi with age and carbon source

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Abstract

The concentration of γ-aminobutyric acid (GABA) in extracts of *Trichoderma viride* grown on sucrose decreased in an age-dependent manner. GABA also disappeared from mycelium when fungus was transferred to the medium with non-saccharide substrate (citrate). Comparative studies with mycelia of *Penicillium simplicissimum* and *Aspergillus nidulans* demonstrated similar development of GABA levels during the cultivation. However, in yeast *Saccharomyces cerevisiae* the amount of GABA was undetectable under aerobic conditions. Results show that GABA shunt is not only active in filamentous fungi but that it seems to be developmentally regulated.

Keywords: filamentous fungi, metabolites, GABA, development, aging

Introduction

γ-Aminobutyric acid (GABA) is mostly known as a neurotransmitter in mammalian brain where it serves as an inhibitory neurotransmitter. Although the metabolic role of GABA in nervous system has been recognized for decades, it is out of focus of neuroscientists, in contrast to non-excitable tissues (organisms) and/or microorganisms.

GABA metabolism is involved in various aspects of both prokaryotic and eukaryotic metabolism. In bacteria, several lines of evidence suggest that GABA metabolism is involved in the intracellular pH regulation in acidic environment (Castanie-Cornet et al., 1999; Ma et al., 2002), which may be mediated by the efflux of GABA (De Biase et al., 1999). GABA shunt is also involved in the degradation of polyamines, e.g. putrescine (Shaibe et al., 1985, Schneider et al., 2002). GABA metabolism in plants covers several metabolic areas. Plants

respond by changes in GABA metabolism upon mechanical and cold stresses (Reddy, 2001) and during the fungal infection (Solomon and Oliver, 2002). It is also involved in processes of plant development and/or differentiation (Gallego et al., 1995, Chen et al., 1994) and reproduction (Ma, 2003; Yang, 2003).

The role of GABA in filamentous fungi is far from being understood and available data are dispersed among several fungal species. In *Neurospora crassa*, concentration of GABA in dry harvested conidia is low but conidia germination was accompanied with GABA formation (Schmit and Brody, 1975) and that glutamic acid decarboxylase (GAD) activity, present mainly in conidia, decreased during germination by an order of magnitude (Christensen and Schmit, 1980). GAD has been purified (Hao and Schmit, 1991) and its expression demonstrated in conidiating cultures (Hao and Schmit, 1993). The fact that GAD appears predominantly in conidia is of interest for the mechanism of conidiation. Kumar et al. (2000) found that GABA shunt is operating in *Aspergillus niger* mycelia. In *Trichoderma viride*, we found that GAD is developmentally regulated and its activity is absent in the non-conidiating mutant (Strigáčová et al., 2001). GABA could be utilized by *Hypocrea jecorina* (*Trichoderma reesei*) similarly as other amino acids (Druzhinina et al., 2006). Metabolic and metabolomic studies of *Fusarium oxysporum* indicate that GABA concentration is elevated in anaerobiosis and increases when glutamate is accumulated (Panagiotou et al., 2005). A heterologous expression and deletion of GAD in *Saccharomyces cerevisiae* showed that the enzyme producing GABA is important for preventing the oxidative stress in these cells (Coleman et al., 2001). The above data show that there are multiple areas of metabolism which involve the metabolism of GABA in fungi. In this work we corroborated our previous data concerning developmental changes of GAD activity by measurements of GABA levels and studied their changes during the adaptation on non-saccharidic substrates.

Experimental

Strains

Trichoderma viride F-534 from the Czechoslovak Collection of Microorganisms, *Aspergillus nidulans* CCM F-266 from Czech Collection of Microorganism, *Penicillium simplicissimum* from prof. W. Burgstaller, University of Innsbruck, Austria, wild type *Saccharomyces cerevisiae* from the collection of Department of Biochemistry and Microbiology, Slovak University of Technology, Bratislava.

Cultivation

T. viride and *P. simplicissimum* were cultured as described previously (Šimkovič et al., 2002; Strigáčová et al., 2001). *A. nidulans* was cultured on a malt-extract or minimal medium containing per 1 L (pH=6.5): 10 g glucose, 6 g $NaNO_3$, 0.52 g KCl, 0.52 g $MgSO_4.7H_2O$, 1.52 g KH_2PO_4 supplemented with 2 ml of Hunters trace elements. When indicated, sucrose was substituted for an organic acid, namely citric acid. *S. cerevisiae* was cultured in the standard YPD medium or in the sporulation medium containing 1 % potassium acetate, all under similar conditions as above. Solid media were solidified with 15 $g.L^{-1}$ agar.

Methods

Extraction and deproteinization of washed mycelium was performed in 10 % perchloric acid for 24 h at 4 °C. The debris was removed by centrifugation and the perchloric acid was neutralized with 4 M KOH, and pH was adjusted to approximately 5. After removing precipitate by centrifugation the extract was frozen in liquid nitrogen and freeze-dried. In some experiments (indicated in legends to Figures), conidia were removed from mycelia by washing on the nylon mesh prior to the deproteinization and lyophilization. The lyophilized sample was dissolved in appropriate amount of water. Alternatively deuterated water with perdeuterated 3-(trimethylsilyl) propionic acid (TSP) was used to dissolve approx. 20 mg of lyophilized sample for NMR studies. ^{1}H-NMR experiments were carried out in standard 5 mm NMR tubes in 600 MHz Varian Inova NMR spectrometer using standard pulse sequences with water suppression. The spectra were processed and evaluated with VnmrJ (Varian, Inc., USA), Mestre-C (Spain) or Chenomx (Chenomx, Inc., Canada) software. The peak heights were normalized to TSP signal. Thin layer chromatography (TLC) was done on Merck Kieselgel 60 plates using butanol: acetic acid: water mixture (4:1:1). The plates were developed up to the edge, let to dry and developed for the second time. The detection was carried out by spraying the plate with 1 % ninhydrin in ethanol. R_f of spots were compared to those of standard compounds. For the determination of enzyme activities the washed mycelium was resuspended in homogenization buffer (0.5 $mol.L^{-1}$ mannitol; 25 $mmol.L^{-1}$ Tris-HCl (pH 7.4)) with glass beads in the ratio 1:1. The homogenization was performed for 3 x 1 min in an ice bath with a Polytron type homogenizer. Cell debris was removed by centrifugation and the supernatant was used for measurements of enzyme activities. Protein content was assayed with the Bradford method with bovine serum albumin as standard. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed with a kit from Lachema, Czech Republic. Isocitrate dehydrogenase (ICDH) acitvity and 2-

oxoglutarate dehydrogenase (KGDH) were assayed using standard procedures (Ochoa, 1955; Kaufman, 1955) using $NADP^+$ as a co-substrate for KGDH and both NAD^+ and $NADP^+$ for ICDH. The glutamate decarboxylase (GAD) activity was assayed according to Strigáčová *et al.* (2001).

Chemicals

NAD^+ and $NADP^+$ and CoA were purchased from Boehringer Mannheim, Germany, GABA from Sigma, St. Louis, U.S.A., other amino acids from Reachim, Moscow, Russia, α-ketoglutaric from Loba Chemie, Fischamend, Austria, isocitric acids from Koch-Light Laboratories, Colnbrook Bucks, U.K., deuterium oxide (D2O) was purchased from Merck, Darmstadt, Germany, 3-(trimethylsilyl)-propionic acid-d_4, sodium salt (TSP) from Sigma-Aldrich, Steinheim, Germany. Other chemicals were purchased from Lachema, Brno, Czech Republic.

Results

GABA in filamentous fungi and yeasts

The presence of GABA in *Trichoderma viride* mycelia could be demonstrated using ^1H NMR spectroscopy and TLC. The assignment of chemical shifts and multiplicities of major signals observed in NMR spectra were done according to Fan (1996) and with the use of Chenomx NMR Suite. However, GABA almost disappears upon prolonged cultivation of the fungus (Fig. 1A). Besides GABA, the presence of mannitol, betain, glutamate/glutamine, alanine, aspartate, asparagines, leucine, valine, citrate, succinate, acetate, ethanol and lactate could be assigned in the spectra. Similar spectra were found in extracts of mycelia of *A. nidulans* and *P. simplicissimum*. The level of GABA was dependent on the age of mycelium and decreased with its age (Fig. 1A).

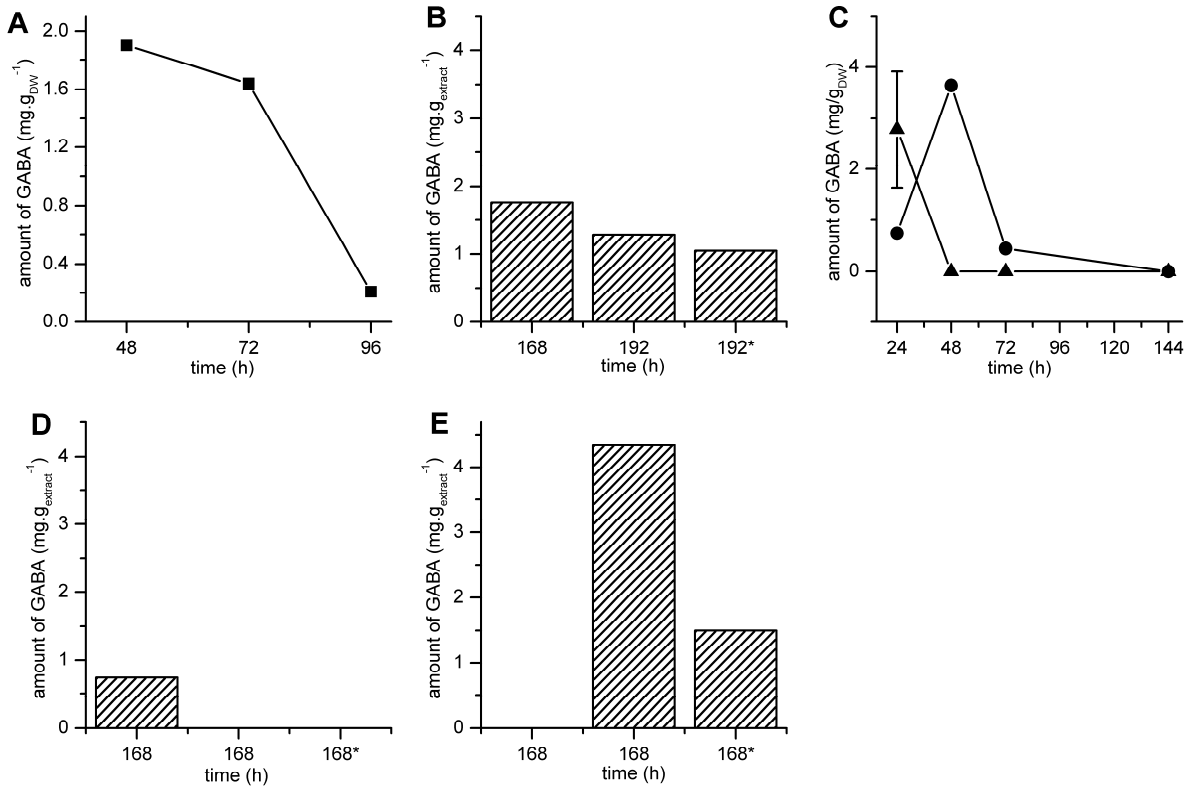

Fig. 1 Time course of intracellular GABA concentrations. GABA levels in *T. viride* (■) grown in liquid Czapek-Dox medium (A) and on Czapek-Dox agar (B), in *P. simplicissimum* (▲) and *A. nidulans* (●) during submerged cultivation in their respective liquid minimal media (C), in *A. nidulans* (D) and *P. simplicissimum* (E) grown on malt-extract agar (first column in D, E) and respective minimal agar (second and third column in D, E). Asterisk denotes extraction of intracellular metabolites without removal of conidia as described in Methods and Results.

On transferring the 48 h old *T. viride* mycelia into fresh medium containing citric acid as a sole carbon source the GABA levels dropped, too (Fig. 2).

During the surface cultivation on agar conidia were formed which would interfere with the determination of GABA inside the mycelium. In order to eliminate the effect of conidia formation on GABA levels, one parallel preparation was washed to remove conidia and mycelia were separated by filtration. The washed samples contained more GABA per gram of dry weight (Fig. 1B) suggesting that GABA is concentrated in mycelia rather than in conidia in fungal species involved in this study. Indeed, in conidia only traces of GABA were found (not shown). Even under these conditions, we observed a decrease in intracellular GABA with ageing in *T. viride* (Fig. 1B).

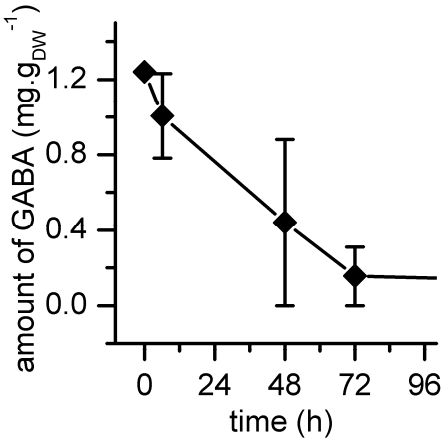

Fig. 2 Time course of intracellular GABA concentration in *T. viride* during adaptation to citrate. Mycelia were grown in liquid Czapek-Dox medium for 48 h and were transferred to the same medium containing 100 mmol.l^{-1} citrate instead of sucrose. Aliquots of mycelia were filtered and deproteinized at time indicated using procedure described in Materials and Methods. Mycelial extracts were analyzed for the presence of GABA by measuring ^{1}H NMR spectra (n = 2).

Similar results were obtained by analysis of extracts of *A. nidulans* and *P. simplicissimum* by ^{1}H NMR (Fig. 1C). At the same time, there were significant differences in GABA content in surface-cultured fungi on media with different composition (Fig. 1D-E).

Yeasts are known to metabolize GABA and posses all necessary genes for both GABA transport and metabolism (Ramos et al., 1985). Surprisingly, we were unable to find detectable amounts of GABA in the extracts of *S. cerevisiae* cultivated either in standard YPD media or in sporulation medium (data not shown). Neither were we able to find GAD activity in yeast after 48 h of cultivation, condition under which *T. viride* had the activity 8.8 nmol.(h.mg$_{prot}$)$^{-1}$. Thus, GABA metabolism does not seem to participate in growth nor in the process of spore formation of *S. cerevisiae*. In these experiments TLC analysis was chosen to supplement NMR measurements. The experiments show that the GABA level in *S. cerevisiae* must be at least one order of magnitude less than those found in *T. viride (*18.4 nmol.mg$_{drw}^{-1}$), *A. nidulans* 35.4 nmol.mg$_{drw}^{-1}$ or in *P. simplicissimum* 27 nmol.mg$_{drw}^{-1}$.

Enzyme activities associated with GABA metabolism

In order to explain differences in GABA levels between *T. viride* and *S. cerevisiae* we measured such enzyme activities in these microorganisms that represent the metabolic environment of the GABA shunt. We have found that specific activities of α-KGD and ICDH were not different between these species, whilst those of AST and ALT were significantly

lower in *S. cerevisiae* (Fig. 3). All activities decreased with increasing cultivation time in both *T. viride* and *S. cerevisae* (Fig. 3A-C).

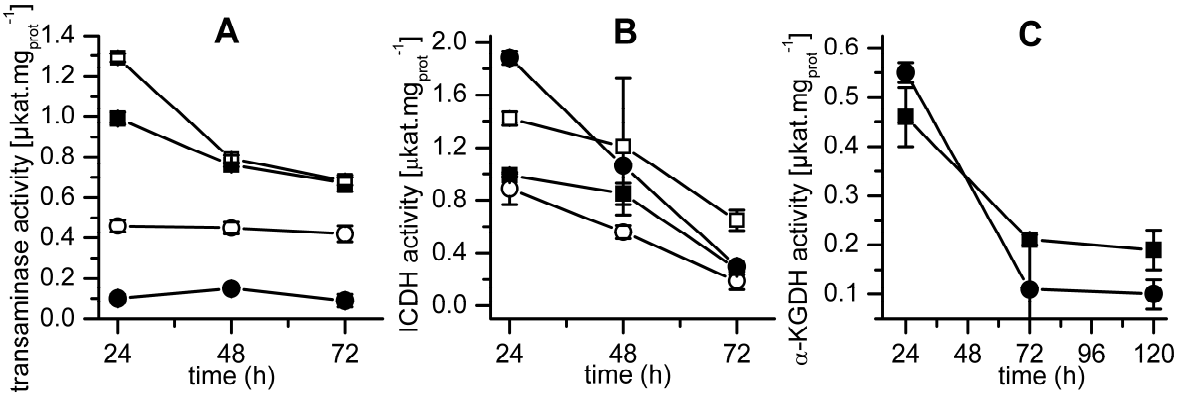

Fig. 3 Time course of (A) alanine aminotransferase (full symbols), aspartate aminotransferase (open symbols), (B) NAD^+-dependent (closed symbols) and $NADP^+$-dependent isocitrate dehydrogenase (open symbols) and (C) 2-oxoglutarate dehydrogenase enzymatic activities in *T. viride* mycelium (squares) and *S. cerevisiae* cells (circles). n = 2

Discussion

Presented results extend reports describing the occurrence of GABA in fungi. Intracellular amounts of GABA have a maximum in all studied fungi at about 24-48 h after which the levels drop with the age of the fungus. These results correlate with data of Strigáčová *et al.* (2001) who measured GAD activity and our other earlier results (Chovanec et al., 2005 and citations therein) where a biphasic time-courses of several metabolic parameters were found. Experiments also demonstrate that the GABA metabolism is active in both submerged and aerial mycelia.

Adaptation of mycelia to citrate was accompanied with the rapid disappearance of GABA (Fig. 2) indicating the absence of operation of GABA shunt under these conditions. One could speculate that GABA shunt is active during glucose catabolism but not under conditions when gluconeogenesis is a predominant metabolic pathway. As nitrogen source was not changed in our experiments, this cannot explain observed differences, thus the participation of GABA shunt in nitrogen metabolism seems improbable.

We have not observed any GABA in yeasts in any of the experiments. These data are striking as GABA was found in brewer's yeast autolysate (Holdsworth and Neville, 1988) and because yeasts possess all the enzymes of GABA shunt (Ramos et al., 1985) including transport systems for GABA uptake (Vissers et al., 1989; Bermudez Moretti et al., 1995).

These enzymes are inductive and could be induced by GABA (Talibi et al., 1995). As yeast extract is a component of YPD cultivation medium (as a source of GABA and glutamate), it could be expected that the GABA shunt will be active under our conditions in *S. cerevisiae*. Our results imply that additional post-transcriptional factor(s) are required to activate GABA shunt in *S. cerevisiae*, which should be investigated in detail in the future. One of the reasons why GABA levels in *S. cerevisiae* are low could be caused by lower activities of transaminases and isocitrate dehydrogenase (Fig. 3). Our results are in agreement with data of Tran-Dinh *et al.* (1996) who found only 2.6 % exchange of glutamate with Krebs cycle in *S. cerevisiae*. Thus, there is a clear-cut difference between *S. cerevisiae* and filamentous fungi in the metabolism of GABA under aerobic conditions. The fact that GABA shunt is absent under anaerobic conditions in *S. cerevisiae* seems to be well documented (Muratsubaki, 1987; Albers et al., 1998), similarly as in *Fusarium oxysporum* (Panagiotou et al., 2005). In *Trichoderma viride*, GABA disappeared from mycelia cultivated under anaerobic conditions (Chovanec et al., 2005). This indicates that GABA metabolism is closely linked to oxidative metabolism in both yeasts and filamentous fungi. The question of whether the GABA shunt is involved in some specific area(s) of metabolism, such as mycelial growth and differentiation in mycelial fungi, remains to be investigated in the future.

In summary, presented data demonstrate the dependence of GABA metabolism as reflected by GABA content on both mycelial age and the kind of carbohydrate catabolised. Also they demonstrate differences in GABA content between yeasts and mycelial fungi, which may be explained by lower activities of transaminase and isocitrate dehydrogenase activities.

Acknowledgement

This work was supported by the Slovak State Programme (Project No. 2003SP200280203) and the Slovak Grant Agency VEGA (grants No. 1/2334/05 and 1/3251/06).

References

Albers E, Gustafsson L, Niklasson C, Lidén G (1998) Microbiol 144: 1683-90

Bermudez Moretti M, Correa Garcia S, Ramos EH, Batlle A (1995) Cell Mol Biol (Noisy-le-grand) 41: 843-849

Castanie-Cornet MP, Penfound TA, Smith D, Elliott JF, Foster JW (1999) J. Bacteriol 181: 3525-35

Chen Y, Baum G, Fromm H (1994) Plant Physiol 106: 1381-1387

Chovanec P, Kaliňák M, Liptaj T, Prónayová N, Jakubík T, Hudecová D, Varečka Ľ (2005) Can J Microbiol 51: 853-62

Christensen RL, Schmit JC (1980) J Bacteriol 144: 983-990

Coleman ST, Fang TK, Rovinsky SA, Turano FJ, Moye-Rowley WS (2001) J Biol Chem 276: 244–250

De Biase D, Tramonti A, Bossa F, Visca P (1999) Mol Microbiol 32: 1198-211

Druzhinina IS, Schmoll M, Seiboth B, Kubicek CP (2006) Appl Environ Microbiol 72: 2126-2133

Fan TWM (1996) Prog nucl magn reson spectrosc 28: 161-219

Gallego PP, Whotton L, Picton S, Grierson D, Gray JE (1995) Plant Mol Biol 27: 1143-51

Hao R, Schmit JC (1991). J Biol Chem 266: 5135-5139

Hao R, Schmit JC (1993) Biochem J 293: 735-738

Holdsworth ES, Neville EE (1988) Biochem Int 17: 1107-1116

Kaufman S (1955) Methods Enzymol 1: 714-722

Kumar S, Punekar NS, SatyaNarayan V, Venkatesh KV (2000) Biotechnol Bioeng 67: 575-84

Ma H (2003) Curr Biol 13: 834-836

Ma Z, Richard H, Tucker DL, Conway T, Foster JW (2002) J Bacteriol 184: 7001–7012

Muratsubaki H (1987) J Biochem (Tokyo) 102: 705-14

Ochoa S (1955) Methods Enzymol 1: 699-704

Panagiotou G, Christakopoulos P, Olsson L (2005) J Biotechnol 118: 304–315

Panagiotou G, Villas-Bôas S G, Christakopoulos P, Nielsen J, Olsson L (2005) J Biotechnol 115: 425–434

Ramos F, el Guezzar M, Grenson M, Wiame JM (1985) Eur J Biochem 1491: 401-404

Reddy ASN (2001) Plant Sci 160: 381–404

Schmit JC, Brody S (1975) J Bacteriol 124: 232-242

Schneider BL, Ruback S, Kiupakis AK, Kasbarian H, Pybus C, Reitzer L (2002) J Bacteriol 184: 6976-6986

Shaibe E, Metzer E, Halpern YS (1985) J Bacteriol 163: 933–937

Šimkovič M, Kaliňák M, Burgstaller W, Varečka Ľ (2002) FEMS Microbiol Lett 213: 21-26

Solomon PS, Oliver RP (2002) Planta 214: 414-20

Strigáčová J, Chovanec P, Liptaj T, Hudecová D, Turský T, Šimkovič M, Varečka Ľ (2001) Arch Microbiol 175: 32-40

Talibi D, Grenson M, Andre B (1995) Nucleic Acids Res 23: 550-557

Tran-Dinh S, Beganton F, Nguyen TT, Bouet F, Herve M (1996) Eur J Biochem 242: 220-227

Vissers S, Andre B, Muyldermans F, Grenson M (1989) Eur J Biochem 181 357-361

Yang Z (2003) Dev Cell 5: 185-6