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Partial Purification and Malic Enzyme Studies from *Mucor circinelloides* and *Mortierella alpina*

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Abstract: Malic enzyme (ME, EC 1.1.1.40) from *M. circinelloides* and *M. alpina* were partially purified from crude extract, prepared from mycelia of both fungi after 16 h of growth, by ammonium sulphate precipitation and affinity column (Mimetic Green) to 24 and 22 fold purification, respectively. The enzyme from both fungi showed similar K_M values for its cofactor,NADP, being 0.032 mM and 0.038 mM, respectively. A higher K_m value for malate was observed for malic enzyme from *M. alpina* (1 mM) compared to that of *M. circinelloides* (0.4 mM). Inhibition studies were also carried out using tartronic acid, oxaloacetate, palmitoyl-CoA and oleoyl-CoA. At a concentration of up to 10 mM, tartronic acid caused a 40% inhibition of the activity of this enzyme for both fungi while oxaloacetate showed a higher degree of inhibition towards ME isolated from *M. circinelloides* (70%) compared to that of *M. alpina* (45%). Both oleoyl-CoA and palmitoyl-CoA exhibits a potent inhibitory effect (more than 90% inhibition) towards malic enzyme from both fungi but only at a level above the critical micellar concentration (3μm and 4 μm, respectively).

Key words: Malic enzyme, purification, *Mucor circinelloides* and *Mortierella alpina*

Introduction

Malic enzyme [malate dehydrogenase (decarboxylating) ($NADP^+$)(EC 1.1.1.40)] catalyzes the following reaction.

L-malate + $NADP^+$ → pyruvate + CO_2 + NADPH

This enzyme has been reported to occur in various filamentous fungi such *as Mortierella elongata*, *Aspergillus nidulans*, *Phythium ultimum*, *Entomophthora exitalis* and *Conidiobolus nanodes* (Savitha *et al.*, 1997) where its role in the pyruvate metabolisme has been implicated (Zink, 1972; Zink and Katz, 1973; McCullough and Roberts, 1974). Currently, this enzyme has been suggested to play a vital role in regulating the extent of lipid accumulation in both fungi via direct channeling of NADPH to fatty acid synthase (Wynn *et al.*, 1999). In *Aspergillus nidulans*, a similar observation has also been reported where the inhibition of its activity led to decreased lipid accumulation from 25% (wt/wt) to 5% (wt/wt). It has also been shown to play an important role in fatty acid desaturation as a NADPH provider in *M. circinelloides* (Kendrick and Ratledge, 1992) where a membrane bound malic enzyme was involved. In this study, ME from two oleaginous fungi, *M. circinelloides* and *M. alpina* have been partially purified to determine the catalytic characteristic differences. The effects of four potential inhibitors ie. malate analogs (tartronic acid and oxaloacetate) and fatty-acyl-CoA-esters(palmitoyl-CoA and oleoyl-CoA) towards the enzyme from the two fungi were also investigated.

Materials and Methods

Fungal Cultivation: *M. circinelloides* (CBS 108.16) and *M. alpina peyron* were cultivated in 5 L fermenters containing 4 L of nitrogen-limiting medium (Kendrick and Ratledge, 1992) at 30°C. The medium constituents are as stated below (g/l):
Glucose, 30, ammonium tartrate, 2, KH_2PO_4, 7, Na_2HPO_4, 2, $MgSO_4.7H_2O$, 1.5, yeast extract, 1.5, $CaCl_2$, 0.1, $FeCl_3.6H_2O$, 0.008, $ZnSO_4.7H_2O$, 0.0001, $CuSO_4.5H_2O$, 0.0001, $Co(NO_3)_2.6H_2O$, 0.0001, $MnSO_4.5H_2O$, 0.0001. The fermenters were agitated at 800 rev/min. Air was supplied at 0.5 v/v/min and the pH was maintained at 6.0 by automatic addition of 2 M NaOH and 2 M KOH. The medium was inoculated by the addition of 200 ml culture of each fungi grown in shake flasks for 16 h.

Production of cell free extracts: Both fungi were harvested after 24 h incubation and the mycelium was washed with 500 ml of distilled H_20. It was then resuspended in an extraction buffer to give approximately 20% (wet weight/v) mycelial suspensions. Extraction buffer used was as stated below:
10 mM KH_2PO_4/KOH pH 7.5 containing 20% glycerol, 1 mM each of benzamidine, mercaptoethanol, protease inhibitor cocktail (Sigma), phenil-methyl-sulfonil-fluoride (PMSF) and EDTA.
The mycelial suspensions were then passed twice through a French pressure cell at a pressure of 35 Mpa. French pressed extracts were centrifuged at 16 000 x g for 20 min at 4°C. The supernatant was then obtained and recentrifuged at 100 000 x g for 1 h. The resulting supernatant was referred as cell free extract for further use.

Partial purification of malic enzyme (ME) from *M. circinelloides* and *M. alpina*: The partial purification of ME was carried out in 2 stages: 1) $(NH_4)_2SO_4$ precipitation between 50 and 60% saturation 2)affinity chromatography using a Mimetic Green 1A6XL (2 x 10 cm, Affinity Chromatography Ltd. Ballasalla, Isle of Man, UK), ME was eluted using a 0 to 250 mM NaCl gradient in the same extraction buffer. Fractions with ME activity and lacking the activities of fatty acid synthase (FAS), malate dehydrogenase (MD), ATP: citrate lyase (ACL), glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH) and NADP: isocitrate dehydrogenase (NADP:ICDH) were pooled and stored at 0°C prior to further analysis.

Kinetic studies: The apparent K_m values of the enzyme from both fungi for its cofactor, NADP and its substrate, malate, were determined by measuring the specific activity of the enzyme at various concentrations of each of the substrates, where the other was kept at a saturating concentration in the reaction mixture. Through Lineweaver-Burk plots, the K_m values for each substrates were determined.

The effect of malate analogues and fatty acyl-CoA esters (FACEs) on malic enzyme activity: The effect of malate analogues (tartronic acid and oxaloacetate) and FACEs (palmitoyl-CoA and oleoyl-CoA) on the activity of ME were determined by measuring the activity of the enzyme performed with the inclusion of each of the compounds at various concentrations compared to the activity performed without the inclusion of any of the inhibitors. Stock solutions of the inhibitors were prepared by dissolving each of the compounds in distilled water.

Enzyme assays: Malic enzyme (EC 1.1.1.40), fatty acid synthase (EC 2.3.1.86), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) ATP:citrate lyase (EC 4.1.3.8), malate dehydrogenase (EC 1.1.1.37) and NADP:isocitrate dehydrogenase (EC 1.1.1.42) were assayed as previously described (Wynn and Ratledge, 1997).

Results and Discussion

Purification of malic enzyme: ME from *M. circinelloides* and *M. alpina* was purified 24 and 22 fold, respectively (Table 1a and b). The purified enzyme lacked the activity of NADP:ICDH, G-6-PDH, 6-PGDH, ACL, FAS and MD which were present at a high activity in the crude extract. However, the recovery of the enzyme was low (2%). Although buffer used included all necessary components for stabilizaing the enzyme, such as glycerol, PMSF, mercaptoethanol and benzamidine, the attempts to stabilize the enzyme were unsuccessful. It is known that addition of PMSF (McElhaney-Feser, 1994), a thiol group reagent (Boulton and Ratledge, 1983) and glycerol were prerequisites in obtaining maximal recoveries in various enzyme purification procedures. Previous reports on the purification of the same enzyme from *M. circinelloides* also showed similar low recovery where only 2% of the enzyme was obtained at the end of the process.

Kinetic studies: The K_m values of malic enzyme from *M. circinelloides* and *M. alpina* for its cofactor, NADP, were similar, being 0.032mM and 0.038 mM, respectively. These values were similar to those reported of ME isolated from stems and roots of wheat (Casati *et al.*, 1997). However, the K_m value for malate of ME from *M. circinelloides* was lower (0.4 mM) compared to that of *M. alpina* (1 mM) denoting a higher specificity for substrates by the enzyme from *M. circinelloides*..

Inhibition studies: Each compound used showed various degrees of inhibition on the activity of ME Table 2.
Tartronic acid at a final concentration of up to10 mM in the reaction mixture showed similar degree of inhibition, approximately between 35 to 40% for both fungi. However, at a lower concentration (2.5 mM), an insignificant inhibition of ME from *M. circinelloides* was observed (5%) while a 100% activity of ME from *M. alpina* was detected. A similar effect was observed when assays of ME from both fungi were carried out with the addition of oxaloacetate. The compound showed a marked inhibitory effect at a final concentration of 2.5 and 10 mM on the activity of ME obtained from *M. circinelloides* (20 and70% inhibition, respectively) compared to that of *M. alpina* (0 and 45% inhibition, respectively). This could be caused by the properties of ME from *M. circinelloides*, probably having a greater affinity towards the compound due to its lower K_m value for malate (0.4 mM) compared to *M. alpina* (1 mM).
Both oleoyl-CoA and palmitoyl-CoA showed a complete inhibition of ME activity from *M. circinelloides* at a final concentration of 1 mM but an insignificant inhibitory effect was observed when concentrations less than 0.1 mM were applied (data not included). Similar results were obtained with ME from *M. alpina* where approximately 90% inhibition was achieved when up to 1 mM oleoyl-CoA and palmitoyl-CoA were added into the reaction mixture. As the concentrations of oleoyl-CoA and palmitoyl-CoA used were far greater than their critical micellar concentrations which are 3μM and 4 μM (Hsu and Powell, 1975) respectively, the inhibition could be due to the compounds acting as detergents. It is known that fatty-acyl-CoA-esters are powerful detergents and could cause irreversible denaturation of the quartenary structure of enzymes (Bloch and Vance, 1977).
FACEs have been shown to play an important role in the

Table 1a: Partial purification of malic enzyme from *M. circinelloides*. ME obtained was free from the activity of FAS, ACL, MD, G-6-PDH, 6-PGDH and NADP:ICDH

	Total activity (nmol/min)	Specific activity (nmol/min/mg protein)	Purification (fold)
Crude Extracts	10271	26	1
$(NH_4)_2SO_4$ precipitation (50-60%)	6163	441	17
Mimetic Green	205	620	24

Table 1b: Partial purification of malic enzyme from *M. alpina*. ME obtained was free from the activity of FAS, ACL, MD, G-6-PDH, 6-PGDH and NADP:ICDH

	Total activity (nmol/min)	Specific activity (nmol/min/mg protein)	Purification (fold)
Crude Extracts	15780	93	1
$(NH_4)_2SO_4$ precipitation (50-60%)	10257	551	6
Mimetic Green	316	2009	22

Table 2: The effect of tartronic acid, oxaloacetate and fatty acyl-CoA esters on malic enzyme activity. An assay of ME without any inhibitors is regarded as 100 % activity

Inhibitors	Relative inhibition of ME (%)		
	Final concentration (mM)	*M. circinelloides*	*M. alpina*
Tartronic acid	2.5	6	0
	5.0	20	15
	10.0	42	40
Oxaloacetate	2.5	20	0
	5.0	40	10
	10.0	70	45
Oleoyl-CoA (18:1)	0.1	65	70
	0.5	75	75
	1.0	100	88
Palmitoyl-CoA (16:0)	0.1	60	60
	0.5	75	75
	1.0	100	85

regulation of lipid biosynthesis of oleaginous yeasts by inhibiting the activity of citrate translocase which facilitates the translocation of citrate from mitochondria to the cytosol for the generation of acetyl-CoA (Evans *et al.*, 1983). In *Lipomyces starkeyi*, both compounds have been shown to exhibit a significant degree of inhibition towards the activity of ATP:citrate lyase at a concentration of 10 μM and below (Boulton and Ratledge, 1983). This provides evidence suggesting the physiological role for the inhibition of ATP: citrate lyase from *L. starkeyi* in the regulation of lipogenesis. However, results presented here indicate that it is unlikely that the control of lipid synthesis in both fungi being achieved by the inhibitory effect of FACEs towards the activity of ME.

References

Bloch, K. and D. Vance, 1977. Control mechanisms in the synthesis of saturated fatty acids. Annu. Rev. Biochem., 46: 263-268

Boulton, C.A. and C. Ratledge, 1983. Partial purification and some properties of ATP:citrate lyase from the oleaginous yeast *Lipomyces starkeyi*. Journal of General Microbiology, 129: 2863-2869

Casati, P., C.P. Spampinato and C.S. Andreo, 1997. Characteristics and physiological function of NADP-Malic enzyme from wheat. Plant and Cell Physiol., 38: 928-934

Evans, C.T., A.H. Scragg and C. Ratledge, 1983. Regulation of citrate efflux from mitochondria of oleaginous and non-oleaginous yeasts by long chain fatty acyl-CoA esters. Eur. J. Biochem., 132: 617-622

Hsu, K.H.L. and B.M. Powell, 1975. Inhibition of citrate synthase by oleoyl-CoA: a regulatory phenomenon. Proc. Natl. Acad. Sci. USA., 72: 4729-4733

Kendrick, A. and C. Ratledge, 1992. Desaturation of polyunsaturated fatty acids in *Mucor circinelloides* and the involvement of the novel membrane-bound malic enzyme. European J. Biochemistry, 209: 667-673.

McCullough, W. and C.F. Roberts, 1974. The role of malic enzyme *in Aspergillus nidulans*. FEBS letters, 41: 238-242.

McElhaney-Feser G.E. and R.L. Cihlar, 1994. Purification and characterization of fatty acid synthase from *Candida albicans* strain 4918 and two derived spontaneous cerulenin-resistant. J. Medical and Veterinary Mycology, 32: 13-20

Savitha, J., J. P. Wynn and C. Ratledge, 1997. Malic enzyme: its purification and characterization from *Mucor circinelloides* and occurrence in other oleaginous fungi. World J. Microbiology and Biotechnology, 13: 7-9.

Wynn, J.P. and C. Ratledge, 1997. Malic enzyme is a major source of NADPH for lipid accumulation by *Aspergillus nidulans*. Microbiology, 143: 253-357.

Wynn, J.P., A.A. Hamid and C. Ratledge, 1999. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. Microbiology, 145: 1911-1917.

Zink, M.W., 1972. Regulation of the two malic enzymes in *Neurospora crassa*. Canadian J.s Microbiology, 18: 611-617

Zink, M.W. and J.S. Katz, 1973. Malic enzyme of *Fusarium oxysporum*. Canadian J. Microbiology, 19: 1187-1196.