Lipomyces starkeyiの酵素活性測定のための反応液組成 (2)

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資　料

Lipomyces starkeyi の酵素活性測定のための反応液組成（その２）

――トリカルボン酸回路とその周辺の酵素――

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Practical Assay Procedures of Enzymes on Tricarboxylic Acid Cycle and Related Pathways in *Lipomyces starkeyi*

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生化学的見地から微生物の代謝活性を調べる場合，酵素活性を測定する方法が用いられることが多い．しかし，一つの代謝経路上の幾つかの酵素の活性測定に必要な反応液組成を知るだけでも，多くの文献調査が要求される．これらの煩わしさを解決するには，各種酵素の反応液組成をまとめた資料があることが望ましい．このような観点から，我々は前報[1]においてGlycolytic pathway, Pentosephosphate cycle, Glycerol 3-phosphate pathway上の13種類の酵素の反応液組成を示した．

本報では，引き続いてTricarboxylic acid cycle, Ammonium assimilation pathway, Anaplerotic reaction pathway[2], Fatty-acid biosynthesis pathway上の17種類の酵素について記載した．ただし，前報[1]においても述べたが，ここに記述した反応液組成は，各種文献に記載されている組成を基に油脂酵母*Lipomyces starkeyi* IAM4753の粗酵素溶液用に改変したものである．従って，研究対象となる微生物の種類が異なる場合には，試薬や粗酵素液の濃度，pH，活性化剤等について再検討を行う必要があると考えられる．本報で使用した細胞から酵素を取り出す方法[3]，活性測定の条件，試薬（アルドリッチ　ファイン　ケミアルの製品を追加）等は前報[1]に準じた．なお，前報[1]には記述しなかったが，反応液に用いる試薬溶液の多くは，pHを緩衝液のpH付近に調整するとか，使用直前まで冷却あるいは調製後短時間内に使用するなどの注意が心要である。

Table 1. Composition of assay mixtures for enzymes on the tricarboxylic acid cycle.
Common abbreviations used in all tables are as follows : Triehanolamine, triethanolamine hydrochloride-NaOH buffer ; Tris, Tris(hydroxymethyl) aminomethane hydrochloride-HCl buffer ; R., reference ; B., blank ; S., sample.

1-1. Citrate synthase (EC 4.1.3.7)[4]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M Triethanolamine(pH8.2)	2.1	2.1	2.1
5 mM Oxalacetic acid[a]			0.3
1 mM Acetyl coenzyme A		0.3	0.3
2 mM DTNB[b]	0.3	0.3	0.3
Distilled water	0.6	0.3	
1/4 Crude extract	0.01	0.01	0.01
Final volume	3.01	3.01	3.01

Wavelength, 412nm ; $\varepsilon_{412}=13.60$ [$cm^2/\mu mole$]
[a]Solution prepared should be used soon.
[b]5,5'-Dithiobis-(2-nitrobenzoic acid)

1-2. Aconitate hydratase (EC 4.2.1.3)[5]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M Tris(pH8.2)	2.7		2.7
50 mM DL-isocitric acid, trisodium salt			0.3
Distilled water	0.3		
Crude extract	0.05		0.05
Final volume	3.05		3.05

Wavelength, 240nm ; $\varepsilon_{240}=4.88$ [$cm^2/\mu mole$]

1-3. Isocitrate dehydrogenase(NAD) (EC 1.1.1.41)[6]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M Tris(pH7.6)	2.5	2.5	2.5
50 mM DL-isocitric acid, trisodium salt			0.06
0.2 M $MgCl_2 \cdot 6H_2O$	0.1	0.1	0.1
0.2 M Citric acid, monohydrate	0.03	0.03	0.03
10 mM AMP[a]	0.3	0.3	0.3
10 mM NAD[b]		0.05	0.05
Distilled water	0.11	0.06	
Crude extract	0.05	0.05	0.05
Final volume	3.09	3.09	3.09

Wavelength, 340nm ; $\varepsilon_{340}=6.22$ [$cm^2/\mu mole$]
[a]Adenosine-5'-monophosphate, disodium salt
[b]β-nicotinamide-adenine dinucleotide, oxidized form

1-4. Isocitrate dehydrogenase (NADP) (EC 1.1.1.42)[7]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M Tris(pH8.0)	2.7	2.7	2.7
50 mM DL-isocitric acid, trisodium salt			0.02
0.2 M $MgCl_2 \cdot 6H_2O$	0.1	0.1	0.1
10 mM NADP[a]		0.05	0.05
Distilled water	0.07	0.02	
Crude extract	0.02	0.02	0.02
Final volume	2.89	2.89	2.89

Wavelength, 340nm ; $\varepsilon_{340}=6.22$ [$cm^2/\mu mole$]
[a]Nicotinamide-adenine dinucleotide phosphate, oxidized form

1-5. 2-Oxoglutarate dehydrogenase comlex[8,9]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.5 M Tris(pH7.6)	1.2	1.2	1.2
2 mM Coenzyme A			0.3
40 mM 2-Ketoglutaric acid	0.3		0.3

32 mM $MgSO_4 \cdot 7H_2O$	0.15	0.15	0.15
78 mM L-cysteine[a]	0.1	0.1	0.13
2 mM TPP[b]	0.15	0.15	0.15
30 mM NAD[c]		0.3	0.3
Distilled water	1.35	0.75	0.45
Crude extract	0.05	0.05	0.05
Final volume	3.0	3.0	3.0

Wavelength, 340nm ; $\varepsilon_{340}=6.22$ [$cm^2/\mu mole$]
[a]L-cysteine monohydrochloride, momohydrate
[b]5, 10, 15, 20-Tetraphenyl-21*H*, 23*H*-porphine
[c]β-nicotinamide -adenine dinucleotide, oxidized form

1-6, Succinyl-CoA hydrolase (EC 3. 1. 2. 3)[10]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.2 M Bicine-KOH buffer(pH8.0)	1.2	1.2	1.2
3 mM Succinyl coenzyme A		1.0	1.0
2 mM DTNB[a]	0.3	0.3	0.3
Distilled water	1.48	0.5	0.48
Crude extract	0.02		0.02
Final volume	3.0	3.0	3.0

Wavelength, 412nm ; $\varepsilon_{412}=13.60$ [$cm^2/\mu mole$]
[a]5,5'-Dithiobis -(2-nitrobenzoic acid)

1-7. Succinate dehydrogenase (EC 1. 3. 99. 1)[11]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.25 M Tris(pH8.0)	1.2	1.2	1.2
20 mM Succinic acid, sodium salt			0.3
2.5 mM MTT[a]	0.3	0.3	0.3
Distilled water	1.45	1.15	0.85
Crude extract	0.05	0.05	0.05
40 mM Phenazine methosulfate[b]		0.3	0.3
Final volume	3.0	3.0	3.0

Wavelength, 578nm;
ε_{578} =approx. 13.0 [$cm^2/\mu mole$]
[a]3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide
[b]Reaction start by addition of the reagent.

1-8. Fumarate hydratase (EC 4. 2. 1. 2)[12]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.5 M Tris(pH8.2)	1.2		1.2
0.5 M L-malic acid, sodium salt			0.3
Distilled water	1.75		1.45
Crude extract	0.05		0.05
Final volume	3.0		3.0

Wavelength, 240nm ; $\varepsilon_{240}=2.44$ [$cm^2/\mu mole$]

1-9. Malate dehydrogenase(NAD) (EC 1.1.1.37)[13,14]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M triethanolamine(pH7.6)	2.8		2.8
5 mM Oxalacetic acid[a]			0.3
10 mM NADH[b]	0.05		0.05
Distilled water	0.33		
Crude extract	0.01		0.01
Final volume	3.16		3.16

Wavelength, 340nm ; $\varepsilon_{340}=6.22$ [$cm^2/\mu mole$]
[a]Solution prepared should be used soon.
[b]β-nicotinamide-adenine dinucleotide, reduced form

Table 2. Composition of assay mixtures for enzymes on the ammonium assimilation pathway.
Common abbreviations used in all tables are as follows : Imidazole, imidazole-HCl buffer ; other abbreviations are the same as table 1.

2-1. Glutamate dehydrogenase(NAD, NADP) (EC 1.4.1.2, 1.4.1.4)[15, 16]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M Tris(pH8.0)	2.4	2.4	2.4
0.1 M 2-Ketoglutaric acid			0.4
3.2 M NH_4Cl	0.2	0.2	0.2
10 mM NADH[a](or NADPH[b])		0.03	0.03
Distilled water	0.43	0.4	
Crude extract	0.02	0.02	0.02
Final volume	3.05	3.05	3.05

Wavelength, 340nm ; $\varepsilon_{340}=6.22$ [$cm^2/\mu mole$]

[a]β-nicotinamide-adenine dinucleotide, reduced form

[b]Nicotinamide -adenine dinucleotide, phosphate, reduced form

2-2. Glutamate synthase(NADP, NADP) (EC 1.4.1.13, 1.4.1.14)[17, 18, 19]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.1 M Tris(pH8.0)	2.57	2.57	2.57
0.1 M 2-Ketoglutaric acid			0.15
0.17 M L-glutamine	0.2	0.2	0.2
10 mM NADH[a](or NADPH[b])		0.03	0.03
Distilled water	0.18	0.15	
Crude extract	0.05	0.05	0.05
Final volume	3.0	3.0	3.0

Wavelength, 340nm ; $\varepsilon_{340}=6.22$ [$cm^2/\mu mole$]

[a]β-nicotinamide-adenine dinucleotide, reduced

[b]Nicotinamide-adenine dinucleotide phosphate, reduced form

2-3. Glutamine synthetase (EC 6.3.1.2)[20] (The enzyme is assayed with two-steps reaction)

I) 1st reaction

	Pipette into cuvette (ml)		
	R.	B.	S.
0.5 M Imidazole(pH7.2)		0.5	0.5
0.5 M L-glutamic acid			0.5
1 M $MgSO_4 \cdot 7H_2O$		0.1	0.1
1 M L-cysteine[a]		0.1	0.1
50 mM ATP[b]		0.5	0.5
1 M Hydroxylamine hydrochloride[c]		0.1	0.1
Distilled water	2.25	0.9	0.4
Crude extract		0.05	0.05
Final volume	2.25	2.25	2.25

[a]L-cysteine monohydrochloride, momohydrate

[b]Adenosine-5'-triphosphate, disodium salt

[c]5 ml of a stock solution of 2 M hydroxylamine hydrochloride is neutralized to pH 7.2 with 2N NaOH and the volume adjusted to 10ml with distilled water. Neutral solution should be prepared freshly before use.

II) 2nd reaction

After 1st reaction for 20 min, 0.75 ml of following $FeCl_3$ solution is added into 1st reaction mixture.

$FeCl_3$ solution :

10% $FeCl_3 \cdot 6H_2O$ in 0.2 N HCl	0.25ml
24% Trichloroacetic acid	0.25ml
50% (v/v) HCl	0.25ml

After mixing 1st reaction mixture and $FeCl_3$ solution, it is refrigerated immediately and subsequently is centrifuged at 2,500g for 5min. Enzyme activity is determined with adsorbance at 540 nm. L-glutamic acid γ-monohydroxamate (0−2 μmole) is used for the calibration curve. One unit of enzyme is defined as that amount which produces 1.15 micromoles of L-glutamic acid γ-monohydroxamate[20].

Table 3. Composition of assay mixtures for enzymes on the anaplerotic reaction pathway.
Common abbreviations are the same as table 1 and 2.

3-1. Pyruvate carboxylase (EC 6.4.1.1)[4,21]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.25 M Imidazole(pH7.2)	1.2	1.2	1.2
0.3 M Pyruvic acid, sodium salt			0.2
0.3 M $NaHCO_3$	0.16	0.16	0.16
0.1 M $MgSO_4 \cdot 7H_2O$	0.15	0.15	0.15
50 mM ATP[a]	0.06	0.06	0.06
2.2 IU/ml Citrate synthase	0.03	0.03	0.03
1 mM Acetyl coenzyme A		0.3	0.3
2 mM DTNB[b]	0.3	0.3	0.3
Distilled water	1.07	0.77	0.57
Crude extract	0.03	0.03	0.03
Final volume	3.0	3.0	3.0

Wavelength, 412nm ; $\varepsilon_{412} = 13.60$ [$cm^2/\mu mole$]
[a]Adenosine-5'-triphosphate, disodium salt
[b]5,5'-Dithiobis-(2-nitrobenzoic acid)

3-2. Phosphoenolpyruvate carboxylase(EC 4.1.1.31)[22]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.25 M Tris(pH7.4)	1.2	1.2	1.2
0.1 M PEP[a]			0.12
0.3 M $NaHCO^3$	0.1	0.1	0.1
0.1 M $MgSO_4 \cdot 7H_2O$	0.1	0.1	0.1
2.2 IU/ml Citrate synthase	0.03	0.03	0.03
1 mM Acetyl coenzyme A		0.3	0.3
2 mM DTNB[b]	0.3	0.3	0.3
Distilled water	1.24	0.94	0.82
Crude extract	0.03	0.03	0.03
Final volume	3.0	3.0	3.0

Wavelength, 412nm ; $\varepsilon_{412} = 13.60$ [$cm^2/\mu mole$]
[a]Phosphoenolpyruvic acid, monopotassium salt
[b]5,5'-Dithiobis-(2-nitrobenzoic acid)

3-3. Phosphoenolpyruvate carboxykinase(ATP)[a] (EC 4.1.1.49)[23,24]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.25 M Tris(pH7.4)	1.2	1.2	1.2
0.1 M PEP[b]			0.12
0.3 M $NaHCO^3$	0.1	0.1	0.1
0.1 M $MgSO_4 \cdot 7H_2O$	0.1	0.1	0.1
60 mM ADP[c]	0.2	0.2	0.2
2.2 IU/ml Citrate synthase	0.03	0.03	0.03
1 mM Acetyl coenzyme A		0.3	0.3
2 mM DTNB[b]	0.3	0.3	0.3
Distilled water	1.04	0.74	0.62
Crude extract	0.03	0.03	0.03
Final volume	3.0	3.0	3.0

Wavelength, 412nm ; $\varepsilon_{412} = 13.60$ [$cm^2/\mu mole$]
[a]True activity of this enzyme(PEPCK) is calculated by following equation.
True value of PEPCK activity = (PEPCK activity) − activity of phosphoenolpyruvate carboxylase)
[b]Phosphoenolpyruvic acid, monopotassium salt
[c]Adenosine-5'-diphosphate, disodium salt
[d]5,5'-Dithiobis-(2-nitrobenzoic acid)

3-4. Malic enzyme (EC 1.1.1.40)[25]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.4 M Triethanolamine(pH7.6)	1.2	1.2	1.2
60 mM L-malic acid			0.25
0.12 M $MnCl_2 \cdot 6H_2O$	0.1	0.1	0.1
50 mM NADP[a]		0.06	0.06
Distilled water	2.12	2.06	1.82
Crude extract	0.03	0.03	0.03
Final volume	3.0	3.0	3.0

Wavelength, 340nm ; $\varepsilon_{340} = 6.22$ [$cm^2/\mu mole$]
[a]Nicotinamide-adenine dinucleotide phosphate, oxidized form

Table 4. Composition of assay mixture for enzyme on the fatty-acid biosynthesis pathway.
Common abbreviations are the same as table 1 and 2.

4-1. ATP citrate lyase (EC 4.1.3.8)[26]

	Pipette into cuvette (ml)		
	R.	B.	S.
0.5 M Triethanolamine(pH8.2)	1.7	1.7	1.7
0.2 M Citric acid, monohydrate	0.3	0.3	0.3
0.2 M $MnCl_2 \cdot 6H_2O$	0.15	0.15	0.15
0.2 M 2-Mercaptoethanol	0.15	0.15	0.15
50 mM ATP[a]		0.3	0.3
10 mM NADH[b]	0.05	0.05	0.05
20 IU/ml MDH[c]	0.02	0.02	0.02
Distilled water	0.6	0.3	
Crude extract	0.05	0.05	0.05
2 mM Coenzyme[b]			0.3
Final volume	3.02	3.02	3.02

Wavelength, 340nm ; $\varepsilon_{340} = 6.22$ [$cm^2/\mu mole$]
[a]Adenosine-5'-triphosphate, disodium salt
[b]β-nicotinamide-adenine dinucleleotide, reduced form
[c]Malate dehydrogenase(NAD)
[d]Reaction start by addition of the regent.

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