

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——

長沼孝文・伊藤 功・横山 等・辻井栄作
竹下昌利・武井雅也・兎束保之

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

TAKAFUMI NAGANUMA, ISAO ITO, HITOSHI YOKOYAMA,
EISAKU TSUZII, MASATOSHI TAKESHITA, MASAYA TAKEI
and YASUYUKI UZUKA

*Department of Fermentation Technology, Faculty of Engineering,
Yamanashi University. Kofu 400*

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、引き続き Tricarboxylic acid cycle, Ammonium assimilation pathway, Anapleurotic 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必要である。

Table 1. Composition of assay mixtures for enzymes on the tricarboxylic acid cycle.

Common abbreviations used in all tables are as follows: Triethanolamine, 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 ; $\epsilon_{412}=13.60$ [cm²/μmole]

^aSolution prepared should be used soon.

^b5,5'-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 ; $\epsilon_{240}=4.88$ [cm²/μmole]

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 ·6H ₂ O	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, 340 nm ; $\epsilon_{340}=6.22$ [cm²/μmole]

^aAdenosine-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 ·6H ₂ O	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 ; $\epsilon_{340}=6.22$ [cm²/μmole]

^aNicotinamide-adenine dinucleotide phosphate, oxidized form

1-5. 2-Oxoglutarate dehydrogenase complex^(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 · 7H ₂ O	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 ; $\epsilon_{340} = 6.22$ [cm²/μmole]

^aL-cysteine monohydrochloride, monohydrate

^b5,10,15,20-Tetraphenyl-21*H*, 23*H*-porphine

^cβ-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 ; $\epsilon_{412} = 13.60$ [cm²/μmole]

^a5,5'-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;

$\epsilon_{578} = \text{approx. } 13.0$ [cm²/μmole]

^a3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide

^bReaction 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 ; $\epsilon_{240} = 2.44$ [cm²/μmole]

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 ; $\epsilon_{340} = 6.22$ [cm²/μmole]

^aSolution prepared should be used soon.

^bβ-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 ₄ Cl	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 ; $\epsilon_{340} = 6.22$ [cm²/μmole]^aβ-nicotinamide-adenine dinucleotide, reduced form^bNicotinamide -adenine dinucleotide, phosphate, reduced form2-2. 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 ; $\epsilon_{340} = 6.22$ [cm²/μmole]^aβ-nicotinamide-adenine dinucleotide, reduced^bNicotinamide - adenine dinucleotide phosphate, reduced form2-3. 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 · 7H ₂ O		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

^aL-cysteine monohydrochloride, monohydrate^bAdenosine-5'-triphosphate, disodium salt^c5 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 FeCl₃ solution is added into 1st reaction mixture.FeCl₃ solution :

10% FeCl ₃ · 6H ₂ O in 0.2 N HCl	0.25ml
24% Trichloroacetic acid	0.25ml
50% (v/v) HCl	0.25ml

After mixing 1st reaction mixture and FeCl₃ 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 ·7H ₂ O	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 ; $\epsilon_{412} = 13.60$ [cm²/μmole]^aAdenosine-5'-triphosphate, disodium salt^b5,5'-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 ·7H ₂ O	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 ; $\epsilon_{412} = 13.60$ [cm²/μmole]^aPhosphoenolpyruvic acid, monopotassium salt^b5,5'-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 ·7H ₂ O	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 ; $\epsilon_{412} = 13.60$ [cm²/μmole]^aTrue activity of this enzyme (PEPCK) is calculated by following equation.

True value of PEPCK activity =

(PEPCK activity) - activity of phosphoenolpyruvate carboxylase

^bPhosphoenolpyruvic acid, monopotassium salt^cAdenosine-5'-diphosphate, disodium salt^d5,5'-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 ·6H ₂ O	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 ; $\epsilon_{340} = 6.22$ [cm²/μmole]^aNicotinamide-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 ·6H ₂ O	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 ; $\epsilon_{340} = 6.22$ [cm²/μmole]

^aAdenosine-5'-triphosphate, disodium salt

^bβ-nicotinamide-adenine dinucleotide, reduced form

^cMalate dehydrogenase(NAD)

^dReaction start by addition of the reagent.

文 献

- 1) 長沼, 兎束, 田中, 植月, 三森, 久保田, 小泉; *J. Inst. Enol. Vitic. Yamanashi Univ.*, **22**, 19 (1987).
- 2) Conn, E. E., Stumpf, P. Y., Bruening, G., Doi, R. H.: 生化学, (田宮, 八木訳), 第5版, p. 357, 東京化学同人, 東京 (1988).
- 3) Naganuma, T., Uzuka, Y., Tanaka, K.: *Anal. Biochem.*, **141**, 74 (1984).
- 4) Parvin, R.: *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p. 16, Academic Press, New York and London (1969).
- 5) Reeves, H. C., Rabin, R., Wegener, W. S., Ajl, S. J.: *Methods in Microbiology*, (Norris, J. R., Ribbons, D. W.), Vol. 6A, p. 433, Academic Press, London and New York (1971).
- 6) Kornberg, A.: *Methods in Enzymology*, (Colowick, S. P., Kaplan, N. O.), Vol. 1, p. 707, Academic Press Inc., Publishers, New York (1955).
- 7) *BIOCHEMICALS*, p.61, Oriental Yeast Co., Ltd., Tokyo (1982).
- 8) 生化学辞典, (今堀, 山川監修), p.215, 東京化学同人, 東京 (1984).
- 9) Sanadi, D. R.: *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p.52, Academic Press, New York and London (1969).
- 10) Gergely, J.: *Methods in Enzymology*, (Colowick, S. P., Kaplan, N. O.), Vol. 1, p.602, Academic Press Inc., Publishers, New York (1955).
- 11) Veeger, C., DerVartanian, D. V., Zeylemaker, W. P.: *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p. 81, Academic Press, New York and London (1969).
- 12) Hill, R. L., Bradshaw, R. A.: *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p.91, Academic Press, New York and London (1969).
- 13) 生化学辞典, (今堀, 山川監修) p.1356, 東京化学同人, 東京 (1984).

- 14) Kawaguchi, A., Bloch, K. : *J. Biol. Chem.*, **251**, 1406 (1976).
- 15) 生化学辞典, (今堀, 山川監修) p.380, 東京化学同人, 東京 (1984).
- 16) *Method Sheets of Enzyme Determination (Biochemica Information)* : p.11,
ペーリンガー・マンハイム山之内株式会社.
- 17) 生化学辞典, (今堀, 山川監修) p.379, 東京化学同人, 東京 (1984).
- 18) Stewart, G. R., Mann, A. F., Fentem, P. A. : *The Biochemistry of Plants*, Vol. 5, p.309, Academic Press, Inc., (1980).
- 19) Tochikura, T., Sung, H-C, Tachiki, T., Kumagai, H. : *Agric. Biol. Chem.*, **48**, 2149 (1984).
- 20) Elliott, W. H. : *Methods in Enzymology*, (Colowick, S. P., Kaplan, N. O.), Vol. 2, p.337, Academic Press Inc., Publishers, New York(1955).
- 21) Young, M. R., Tolbert, B., Utter, M. F. : *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p.250, Academic Press, New York and London (1969).
- 22) 田口, 香月 : 糖代謝とその制御, (千葉, 田中, 宇井企画・編集), (蛋白質 核酸 酵素 臨時増刊), Vol. 22, No.14, p. 260, 共立出版, 東京 (1977).
- 23) Woronick, C. L., Johnson, M. J. : *J. Biol. Chem.*, **235**, 9 (1960).
- 24) 千葉 : 糖代謝とその制御, (千葉, 田中, 宇井企画・編集), 蛋白質 核酸 酵素 臨時増刊), Vol. 22, No.14, p. 249, 共立出版, 東京 (1977).
- 25) Hsu, R. Y., Lardy, H. A. : *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p. 230, Academic Press, New York and London (1969).
- 26) Takeda, Y., Suzuki, F., Inoue, H. : *Methods in Enzymology*, (Lowenstein, J. M.), Vol. 13, p.153, Academic Press, New York and London (1969).