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닭의 간 유래의 5,10-Methenyltetrahydrofolate Synthetase의 정제 및 특성

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Purification and Characterization of 5,10-Methenyltetrahydrofolate Synthetase from Chicken Liver

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요약. 닭의 간으로부터 5,10-methenyltetrahydrofolate synthetase를 30-70% 황산암모늄 분획, Q Sepharose Fast Flow anion exchange and Source 15Phe hydrophobic interaction chromatography을 이용하여 정제하였다. 세포 추출물, 황산암모늄 분획, Q Sepharose Fast Flow와 Source 15Phe 단계에서의 비활성은 각각0.0085, 0.031, 0.80 및 1.27 U/mg 이었다. 세포 추출물, 황산암모늄 분획, Q Sepharose Fast Flow와 Source 15Phe 단계에서의 정제도는 각각 1, 3.7, 94.1 및 149.4 이었다. HPLC gel permeation chromatography와 SDS-polyacrylamide electrophoresis 실험으로부터 5,10-methenyltetrahydrofolate synthetase는 분자량이 22.8 kDa인 단량체임을 알 수 있었다. 5-methyl THF과 Mg-ATP의 Km은 각각 7.1 μM 및 63 μM 이었다. 최적온도와 최적 pH는 각각 30 ^{o}C 및 6.0 이었다. 금속이온에 대한 특이성과 스토키오메트리 실험으로부터 최고속도가 Mg^{2+}과 1:1일 때 얻어진다는 것을 알 수 있었다. ATP와 Km은 MgATP, MgCTP, MgUTP 및 MgGTP의 순서로 증가하였으며 최고 역가는 같은 순으로 감소하였는데, 이는 MgATP 가 가장 효과적인 기질임을 증명한다. 이 효소는 tetranitrometane 및 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide에 의해서만 수식되었는데, 이는 tyrosine and carboxylate 잔기가 효소의 활성부위에 존재함을 나타낸다.

주제어: 5,10-Methenyltetrahydrofolate synthetase, 닭의 간, 엽산 대사, 테트라니트로메탄, 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide

ABSTRACT. 5,10-Methenyltetrahydrofolate synthetase from chicken liver was purified through 30-70% ammonium sulfate fractionation, Q Sepharose Fast Flow anion exchange and Source 15Phe hydrophobic interaction chromatography. Specific activities of cell extract, ammonium sulfate, Q Sepharose Fast Flow and Source 15Phe were 0.0085, 0.031, 0.80 and 1.27 U/mg, respectively. Purification fold activities of cell extract, ammonium sulfate, Q Sepharose Fast Flow and Source 15Phe were 1, 3.7, 94.1 and 149.4, respectively. HPLC gel permeation chromatography and SDS-polyacrylamide electrophoresis experiments indicated that the enzyme is a monomeric protein with a molecular weight of 22.8 kDa. Km for 5-methyl THF and Mg-ATP were 7.1 μM and 63 μM, respectively. Optimum temperature and pH were 30 ^{o}C and 6.0, respectively. The data for metal ion specificity and stoichiometry showed that the maximum activity was obtained with a 1:l. ratio of Mg^{2+}. The ATP and Km values increased in the order of MgATP, MgCTP, MgUTP and MgGTP, and the maximum activities also decreased in the same order, indicating MgATP as the most efficient substrate. The enzyme was chemically modified only by tetranitrometane and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide, indicating that tyrosine and carboxylate are present in the active site.

Keywords: 5,10-Methenyltetrahydrofolate synthetase, Chicken liver, Folate metabolism, Tetranitrometane, 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide

INTRODUCTION

5,10-Methenyltetrahydrofolate synthetase (EC 6.3.3.2) catalyzes the conversion of 5-formyltetrahydrofolate (5-formylTHF) into 5,10-methenyltetrahydrofolate (5,10-methenylTHF) along with the hydrolysis of ATP [Holmes & Appling 2002].

5-formylTHF + MgATP →
5,10-methenylTHF + MgADP + Pi

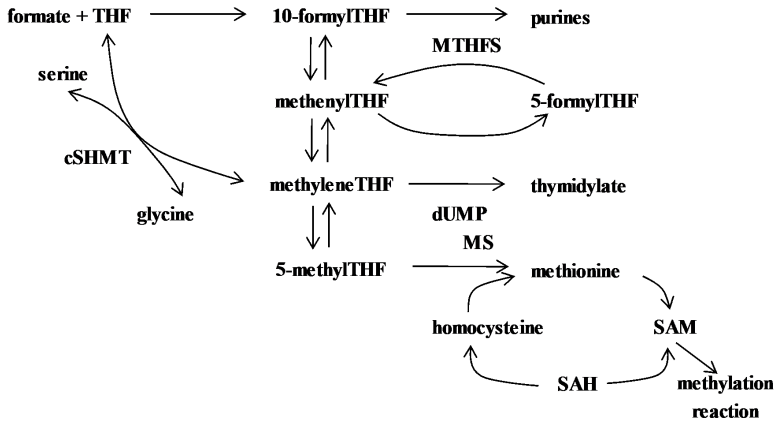

***Fig.* 1.** The folate-dependent one-carbon metabolic pathway in the cytoplasm. THF, MTHFS, cSHMT, SAM and SAH represent tetrahydrofolate, 5,10-methenyltetrahydrofolate synthetase, cytoplasmic serine hydroxymethyltransferase, S-adenosylmethionine and S-adenosylhomocysteine, respectively.

The product of 5,10-methenyltetrahydrofolate synthetase reaction, 5,10-methenylTHF, is then interconverted into reduced one-carbon substituted tetrahydrofolates, as shown in *Fig.* 1.[1] While 5-formylTHF is not directly involved in the metabolic process as a cofactor, 5,10-methenyltetrahydrofolate synthetase-catalyzed reaction and subsequent reactions convert it into cofactors essential to many cellular processes, pathways involving DNA and ATP synthesis, DNA repair, and protein synthesis. 5,10-Methenyltetrahydrofolate synthetase has been used as a target enzyme in developing antiproliferative agents, since this affects the concentrations of 5-formylTHF.[2] Concentrations of 5-formylTHF *in vivo* are regulated by a futile cycle, and 5-formylTHF is synthesized from methenylTHF in a second reaction catalyzed by serine hydroxymethyltransferase as shown in *Fig.* 1. Human 5,10-methenyltetrahydrofolate synthetase with a molecular weight of 23 kDa is the key enzyme in the treatment of several human cancers. 5-FormylTHF is administered either in association with the 5-fluorouracil, antineoplastic pyrimidine analog, to elevate its cytotoxic effects,[3] or to rescue normal cells from the toxic effects of anti-folate methotrexate in high dose levels.[4] Field *et al*. reported that 5,10-methenyltetrahydrofolate synthetase is subject to product inhibition by methenylTHF, which is in equilibrium with 10-formylTHF that in turn tightly binds to 5,10-methenyltetrahydrofolate synthetase.[5] 5,10-Methenyltetrahydrofolate synthetase activity has been purified from pig liver,[6] mouse liver,[7] *Saccharomyces cerevisiae*,[8] sheep liver,[9] *Lactobacillus casei*,[10] rabbit liver[11] and human liver.[12] No studies on chicken liver has yet to be carried out. This report describes the purification to apparent homogeneity of 5,10-methenyltetrahydrofolate synthetase from chicken liver, via 30 - 70% ammonium sulfate fractionation, Q Sepharose Fast Flow anion exchange and Source 15 Phe hydrophobic interaction chromatography and its biochemical characterization with respect to catalytic aspects.

EXPERIMENTAL

Materials.

Chicken liver was obtained from a local meat market and stored at -20 °C. Acetate, Mes[2.5-4; N,N'-bis (2-hydroxyethyl) piperazine], Pipes [(piperazine-N,N'-bis(2-ethanesulfonic acid))], Taps [(3-{[tris-(hydroxymethyl)methyl] amino}propanesulfonic acid)], tetranitromethane and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC) and (6*R,6S*)-5-formylTHF monoglutamate were from Sigma. ATP was purchased from Merck. Q Sepharose fast flow and source 15phe resins were purchased from GE Healthcare. Zorbax G-250 column (4.6 × 250 mm) was from Agilent. All other chemicals were of analytical grade and purchased from commercial suppliers.

Assay of methenyltetrahydrofolate synthetase.

5,10-Methenyltetrahydrofolate synthetase activity was determined by the production of 5,10-CH^+-THF at 360 nm ($\varepsilon_{360} = 25.1 \times 10^3\ M^{-1}\ cm^{-1}$). The standard reaction mixture contained 1 mM MgATP, 500 μM (6*R*,6*S*)-5-formylTHF, 0.5% Triton-X-100, 14 mM 2-mercaptoethanol and 25 mM Mes (pH 6.0), in a final volume of 1 mL. Reactions were performed at 25 °C and monitored with a Perkin Elmer Bio 40 spectrophotometer. One unit of activity represents 1 μmol

of 5,10-CH^+-THF formed per min. The concentration of (6*R*,6*S*)-5-formylTHF monoglutamate was varied from 1 to 500 μM at saturating MgATP concentration (1.0 mM). Similarly, the concentration of MgATP was varied from 0.025 to 5 mM at saturating (6*R*,6*S*)-5-formylTHF concentration (500 μM). Both substrate series were performed in triplicate. Substrate-velocity data were plotted and plugged into the Michaelis-Menten equation using nonlinear regression with "Enzyme Kinetics Module" on Sigma Plot.

Purification of enzyme.

Preparation of cell extracts: The frozen chicken liver (65 g) was thawed and ground in Waring blender using 0.5 ℓ of 25 mM Mes (pH 6.0). The suspension was set at 4 °C overnight and centrifuged for 1 h at 10,000x g using Beckman Centrifuge.

Ammonium sulfate fractionation: Crude protein was obtained by fractionation of 30% to 70% ammonium sulfate saturation. The precipitate, obtained by centrifugation, was dialyzed against 25 mM Mes (pH 6.0) three times for 12 h at 4 °C.

Anion exchange chromatography: The dialyzed enzyme solution was loaded onto a column (2 × 15 cm) of Q Sepharose Fast Flow anion exchange resin equilibrated with the 25 mM Mes (pH 6.0). The column was washed until the A_{280} was < 0.1, and the enzyme was eluted with a 25 - 100 mM linear gradient of Mes (pH 6.0). The fractions containing activity were pooled and solid $(NH_4)_2SO_4$ was added to bring the final salt concentration to 1.0 M.

Hydrophobic interaction chromatography: The protein was then loaded onto a column (2 × 15 cm) of Source 15 Phe resin equilibrated with 1.0 M $(NH_4)_2SO_4$ in 25 mM Mes, pH 6.0, and the enzyme was eluted with a decreasing linear salt gradient [1.0 - 0 M $(NH_4)_2SO_4$)].

SDS-polyacrylamide electrophoresis: Polyacrylamide electrophoresis was done in the presence of sodium dodesyl sulfate (SDS) by the method of Laemmli[13] using 10% polyacrylamide gel and 4% stacking gel. Bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen A (25,700), *L.* casei dihydrofolate reductase (18,000), and lysozyme (14,300) were used as standards.

Molecular weight determination: Molecular weight was estimated by a Zorbax G-250 column (4.6 × 250 mm) preequilibrated with 25 mM Mes (pH 6.0) using Agilent HPLC system.

Protein determination: Protein concentration was determined using the method of Bradford[14] with bovine serum albumin as a standard.

Optimum temperature and stability.

Optimum temperature was obtained by varying the temperature of the reaction mixture. Temperature stability was obtained by preincubating the enzyme at different temperature buffers for 10 min.

Optimum pH and stability.

Optimum pH was obtained by varying the pH values of the reaction mixture. pH stability was obtained by preincubating the enzyme in different pH buffers for 10 min. Buffers at 25 mM final concentration were used in the following pH ranges: acetate, 2.5-4; N,N'-bis(2-hydroxyethyl) piperazine, 4-5; Mes, 5-6.5; Pipes (piperazine-N,N'-bis(2-ethanesulfonic acid)), 6.5-7.5; Taps (3-{[tris-(hydroxymethyl)methyl]amino} propanesulfonic acid), 7.5-9.0. In all cases, overlaps were obtained when buffers were altered so as to make corrections for any spurious buffer effects.

Metal ion specificity and stoichiometry.

Metal ion specificity and stoichiometry were determined according to the procedures developed by Hopkins and Schirch.[11] To determine the metal ion stoichiometry, 0.2 μM of 5,10-methenyltetrahydrofolate synthetase was incubated with 0.2 mM 5-formylTHF and 0.1% β-mercaptoethanol in 1 mL of 25 mM MES (pH 6.0, 30 °C). Varying concentrations of $MgSO_4$ (0~20mM) were added into the reaction mixture and the reaction was started by adding 5 μmoles of NaATP. Relative activities for each $MgSO_4$:ATP ratio were obtained by comparing the experimental rate with the rate of reaction for 1μg of 5,10-methenyltetrahydrofolate synthetase in the standard assay. Experiments for metal ion specificity were conducted in the reaction mixture containing 0.036 μM 5,10-methenyltetrahydrofolate synthetase, 0.2 mM 5-formylTHF 0.1% β-mercaptoethanol and 5 mM tested metal ions in 1 mL of 25 mM MES (pH 6.0, 30 °C). The reaction was started by adding 5 μmoles of NaATP. Relative activities were obtained by comparing the experimental activities with those for 0.036 μM of 5,10-methenyltetrahydrofolate synthetase in the standard assay.

RESULTS AND DISCUSSION

Purification of methenyltetrahydrofolate synthetase.

***Table* 1.** Purification of chicken liver 5,10-methenyltetrahydrofolate synthetase

Step	Protein (mg)	Units (U)	Specific Activity (U/mg)	Yield (%)
Cell extract	1321.2	11.2	0.0085	100
Ammonium Sulfate	276.3	8.5	0.031	75.9
Q Sepharose Fast Flow	6.4	5.1	0.80	45.5
Source 15 Phe	3.3	4.2	1.27	37.5

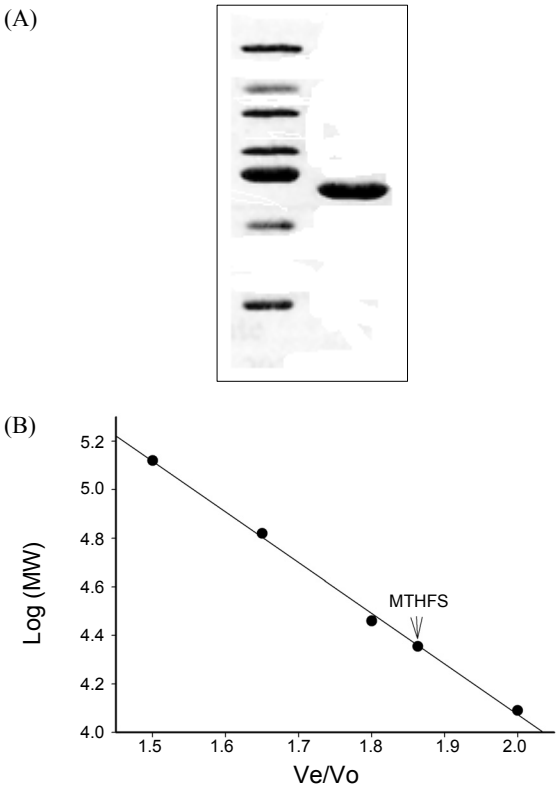

***Fig*. 2.** Molecular weight of the purified enzyme determined by SDS-polyacrylamide gel electrophoresis (A) and HPLC (B). In (A), standard proteins were bovine serum albumin (68 kDa), ovalbumin (43 kDa), glyceraldehydes 3-phosphate dehydrogenase (36 kDa), carbonic dehydrogenase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactalbumim (14 kDa). Left and right lanes stand for standard markers and purified enzyme, respectively. In (B), molecular weight markers were bovine serum albumin (dimmer, 132 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

The purification of 5,10- methenyltetrahydrofolate synthetase from a homogenate of chiken liver is summarized in *Table* 1. Specific activities of cell extract, ammonium sulfate, Q Sepharose Fast Flow and Source 15 Phe were 0.0085, 0.031, 0.80 and 1.27 U/mg, respectively. Purification fold activities of cell extract, ammonium sulfate, Q Sepharose Fast Flow and Source 15 Phe were 1, 3.7, 94.1 and 149.4, respectively. As shown in *Fig*. 2A, the analysis of enzyme preparation via SDS-PAGE in the final stage of the isolation procedure showed the enzyme to be homogeneous with a subunit molecular weight of 22.8 kDa.

Molecular weight.

Standard proteins and purified enzyme were passed through gel permeation column (Zorbax G-250, 4.6 × 250 mm) of Agilent HPLC system. The relationship of molecular weight and Ve/Vo in *Fig*. 2B shows that the molecular weight of the enzyme is 22.8 kDa. Combined with SDS-polyacrylamide gel electrophoresis data, the enzyme was determined as a monomeric protein. The data obtained from human liver,[4] human cytosolic[12] and human mitochondrial 5,10-methenyltetrahydrofolate synthetase[15] showed that human enzymes are identical and momomeric proteins with a molecular weight of 23 kDa. Anguera *et al*. reported that mouse 5,10-methenyltetrahydrofolate synthetase has a molecular weight of 23 kDa and is 84% identical in amino acid sequence to the human enzyme and the protein sequence of mouse enzyme is 84%, 76% and 28% identical with those of human, rabbit and *S. cerevisiae*, respectively.[7] On the other hand, the molecular weights of *S. cerevisiae*,[8] *Lactobacillus casei*,[10] rabbit[11] and pig[6] 5,10-methenyltetrahydrofolate synthetases were reported to be 28 kDa, 23 kDa, 28 kDa and 23 kDa in the monomeric form, respectively. Jolivet *et al*. reported that human 5,10-methenyltetrahydrofolate synthetase has a 77% amino acid homology with rat liver enzyme, but 22% with bacterial enzyme.[4] These indicate that 5,10-methenyltetrahydrofolate synthetase is simple hyperbolic and non-regulatory enzyme, unlike other multimeric enzymes in vivo.

Metal Ion Specificity and Stoichiometry.

The synthetase reactions require the presence of a divalent metal ion. The relative rates of the reaction with several divalent metal ions are summarized in *Table* 2. The data show that the enzyme has a poor specificity for its divalent metal ion requirement. Titrations with Mg^{2+} and NaATP were carried out in order to determine the function of the metal ion. The maximum activity was obtained with 1:l. ratio of Mg^{2+} and ATP, indicating that the function of the divalent ion is to form a metal-ATP complex and neutralize the negative charge of ATP. Our data are qualitatively consistent

***Table* 2.** Specificity of 5,10-methenyltetrahydrofolate synthetase for metal ion

Metal Ion	Relative Activity (%)
Mg^{2+}	100
Zn^{2+}	61
Cd^{2+}	72
Ca^{2+}	84
Co^{2+}	100
Mn^{2+}	100

***Table* 3.** Specificity of 5,10-methenyltetrahydrofolate synthetase for trinucleotide

Trinucleotide	Km (μM)	Relative Activity (%)
MgATP	63	100
MgCTP	81	91
MgUTP	85	87
MgGTP	96	71

***Table* 4.** Km values of 5,10-methenyltetrahydrofolate synthetase from different species

Species[a]	5-formylTHF (μM)	Mg-ATP (μM)
L. casei [Grimshaw *et al*., 1984]	0.6	1.0
Human [Bertrand *et al*., 1987]	4.4	20
Rabbit [Hopkins & Schirch, 1984]	0.5	300
S. cerevisiae [Holmes & Appling, 2002]	33	43
M. pneumoniae [Chen *et al*., 2005]	165	166
Mouse [Anguera *et al*., 2004]	5.0	769
Pig [Cho, 2008]	6.5	75
Chicken [this study]	7.1	63

[a]Km values were taken from the relevant references.

with the results from Hopkins and Schirch.[11]

Nucleotide Specificity

Experiments for nucleotide specificity were conducted using four different metal-chelated nucleotide as shown in *Table* 3. The data show that Km increases in the order of MgATP, MgCTP, MgUTP and MgGTP, but the maximum velocity decreases in the same order, indicating that MgATP is the best substrate. These are also qualitatively consistent with the results from Hopkins and Schirch.[11]

Kinetic parameters.

The kinetic constants for 5,10-methenyltetrahydrofolate synthetase-catalyzed formation of 5,10-methenylTHF were obtained. As shown in *Table* 4, Km values of the 5-methyl THF and ATP were 7.1 μM and 63 μM, respectively. The Km of 5-formylTHF of the chicken liver 5,10-methenyltetrahydrofolate synthetase is similar to the Km values of the human,[12] mouse[7] and pig[6] enzymes; it is 11.8-fold and 14.2-fold higher than the Km values of the *L. casei*[10] and rabbit[11] enzymes, respectively; 4.7-fold and 23.4-fold lower than the Km values of the *S. cerevisiae*[8] and *Mycoplasma pneumoniae* enzymes,[16] respectively. The Km of Mg-ATP for chicken 5,10-methenyltetrahydrofolate synthetase is lower than the Km values of the rabbit,[8] mouse,[7] pig[6] and *Mycoplasma pneumoniae*[16] enzymes, but higher than the Km values from *L. casei*,[10] human[12] and *S. cerevisiae* enzymes.[8] The data indicate that 5,10-methenyltetrahydrofolate synthetases from rabbit and *L. casei* have the highest affinity for 5-methyl THF and ATP, respectively, as shown in *Table* 4.

***Table* 5.** Effect of various chemicals on the activity of 5,10-methenyltetrahydrofolate synthetase[a]

Chemicals[b]	Concentration (mM)	Relative Activity (%)
Control	-	100
p-Hydroxylmercuriobenzoate	1	100
	10	100
5,5'-Dithiobis-(2-nitrobenzoic acid)	1	100
	10	100
Diethylpyrocarbonate	1	100
	10	100
Pyridoxal phosphate	1	100
	10	100
Phenylglyoxal	1	100
	10	100
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide	1	15
	10	0
Tetranitromethane	1	38
	10	0
N-bromosuccinimide	1	100
	10	100

[a]Reaction mixture (pH 6.0 and 30 °C) contained 25 mM Mes, 0.01 unit of enzyme, 140 μM 5-formylTHF, 1.5 mM of MgATP and different concentrations of chemicals. The enzyme was incubated with a chemical for 10 min before the reaction started with substrates. [b]No stochiometry was carried out.

Effect of chemicals.

Table 5 shows the effects of chemical modification reagents on the enzyme activity. Among them, only tetranitrometane and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide showed significant inhibition indicating that tyrosine and carboxylate are involved in either the binding of the substrate or the catalytic process. Our result is qualitatively

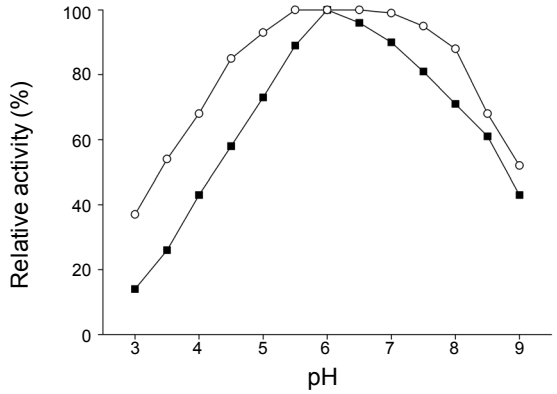

***Fig*. 3.** Effect of temperature on the 5,10-methenyltetrahydrofolate synthetase activity. ■-■ and ○-○ represent optimum temperature and temperature stability, respectively.

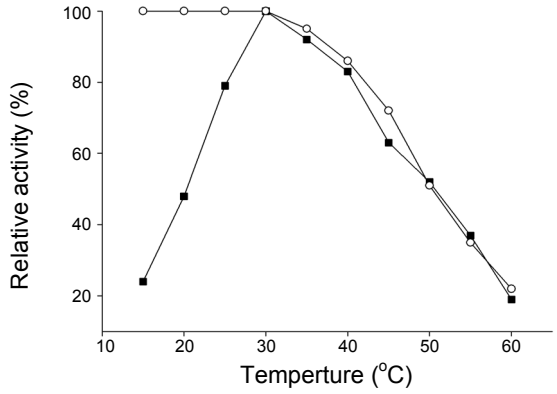

***Fig*. 4.** Effect of pH on 5,10-methenyltetrahydrofolate synthetase activity. ■-■ and ○-○ represent optimum pH and pH stability, respectively.

consistent with the report of Cho[6] that carboxylate and tyrosine were modified by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and tetranitrometane, respectively, and with the report of Wu *et al.* that tyrosine mutation to alanine at 153 and aspartate mutation to alanine from human 5,10-methenyltetrahydrofolate synthetase resulted in a huge decrease in the activity.[17] Further studies, such as those on pH-dependent kinetics would be helpful in explaining the function of tyrosine and carboxylate.

Effect of temperature.

As shown in *Fig*. 3, optimum temperature was obtained at 30 °C and enzyme was stable up to 30 °C. Optimum temperatures of human,[12] pig,[6] rabbit[11] and yeast[8] 5,10-methenyltetrahydrofolate synthetases were reported to be 37 °C, 35 °C, 30 °C and 30 °C, respectively.

Effect of pH.

Optimum pH was 6.0, as shown in *Fig*. 4. Optimum pHs of human,[12] pig,[6] yeast[8] and rabbit[11] 5,10-methenyltetrahydrofolate synthetases were 6.0, 6.5, 7.0 and 6.0, respectively. The data indicate that the protonation states of functional amino acid residues in the active sites of the reported methenyltetrahydrofolate synthetases are well preserved. Enzyme was stable at pH 5.5 ~ 7.0, with a slight inactivation at pH 4.5 ~ 5.5 and 7.0 ~ 8.0. The data are qualitatively consistent with the results of pig liver 5,10-methenyltetrahydrofolate synthetase, showing that enzyme was stable at pH 4.5 ~ 7.0 with a slight inactivation at 7.0 ~ 8.0.[6]

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