A Sensitive Gas Chromatographic Assay for Cystathionase and Cystathionine: Application of This Assay to Biological Samples and Kinetic Studies. Gas Chromatographic Assay for Cystathionase and Cystathionine

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α-Ketobutyric acid is one of the reaction products formed from cystathionine or homoserine by cystathionase. α-Ketobutyric acid was reacted with pentafluorophenyl-hydrazine, and the hydrazone was extracted with ethyl ether, reacted with diazomethane, and subjected to gas–liquid chromatography with an electron capture detector. This method provides a specific and sensitive assay for cystathionase and cystathionine in biological samples.

Keywords——cystathionase; cystathionine; α-ketobutyric acid; pentafluorophenyl-hydrazone; ECD-gas chromatography

Introduction

Cystathionase (EC 4.4.1.1; cystathionine γ-lyase) catalyzes the cleavage of cystathionine, homoserine, α,β-diaminopropionic acid, lanthionine, djenkolic acid and carboxymethylthio-cysteine,[1] and the synthesis of cystathionine [1b,2] and homolanthionine.[3]

Many investigators have isolated cystathionase from various sources and characterized it.[1a,3,4] α-Ketobutyric acid (KBA) is formed by the cleavage of cystathionine and homoserine. Cystathionase activity was determined by measuring KBA formed in a coupled assay with lactate dehydrogenase,[4a] by an isotopic method[6] and from the formation rate of thiol amino acid.[4a] Recently the authors reported a sensitive determination of α-keto acids by GLC.[7]

In this communication, we describe a sensitive assay for cystathionase, based on the determination of KBA, and a sensitive and specific assay for cystathionine in biological samples using cystathionase.

Materials and Methods

Materials

L-Cystathionine was isolated and purified from urine of a cystathionuric patient by Mizuhara and his coworkers at this university. Pentafluorophenylhydrazine (PFPH) was purchased from Aldrich Chemical Co. Pyridoxal phosphate (PALP) was kindly supplied by Nisshin Chemical Co. Ltd. (Tokyo). L-Methionine sulfone, L-homocysteic acid, L-homocysteinesulfinic acid, S-(2-(+)-carboxy-2-hydroxyethyl)-L-homocysteine (L-CHEH) and S-(2-(−)-carboxy-2-hydroxyethyl)-D-homocysteine (D-CHEH) were prepared in our laboratory.[8] KBA and other reagents (all of analytical grade) were purchased from Wako Pure Chemicals Industries Ltd. (Osaka).

Gas chromatographic analysis was carried out as described previously.[7]

Methods

Preparation of Enzyme Source——Animals were decapitated and tissues were excised. The tissues (lung, heart, muscle and intestine) were homogenized in 2 volumes of 1.2% KCl (containing 5×10^{-4} M EDTA and 10^{-4} M PALP) using a Waring blender. Brain, liver, kidney and lens were homogenized in a Teflon-pestled glass homogenizer. The homogenate was centrifuged at $8000 \times g$ for 30 min and aliquots of the supernatant solution were used for enzymatic analysis.

Cystathionase used for kinetic studies and the determination of cystathionine was partially purified from rat liver by the procedure of Kato et al.[9] This preparation, which had an activity of 3.4 U/ml, was stabilized by adding 80% ammonium sulfate and could be stored at 0°C for one year without any significant loss of activity.
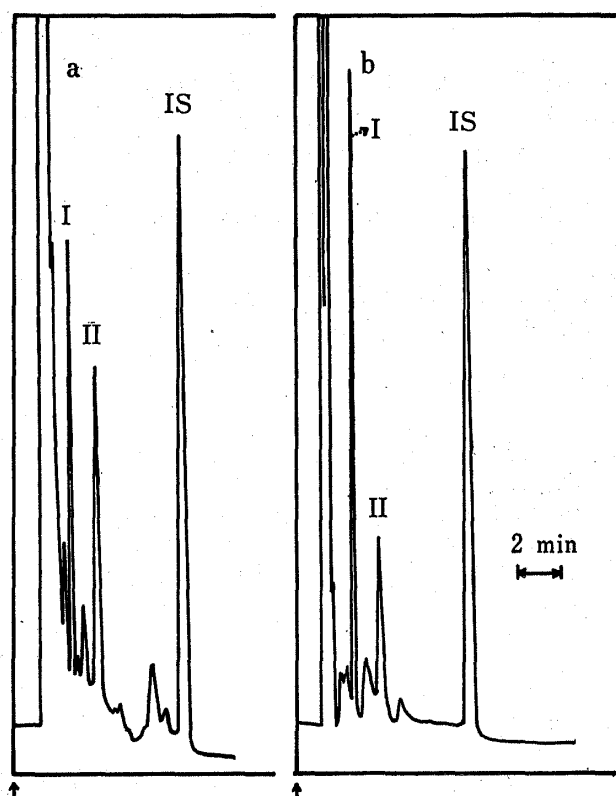
Fig. 1. A Typical Gas Chromatogram of the Pent-
afluorophenylhydrazones of α-Ketobutyric Acid
Methyl Ester

a : An aqueous solution (0.25 ml) containing 4 nmol of KBA was
treated as described in Materials and Methods. After dilution
6 times with γ-BHC–benzene, a 1 μl aliquot was injected into
the gas chromatograph.

b : Cystathionine (0.4 mM) was incubated with 0.3 μl of partially
purified cystathionase (1 mU), then the reaction mixture was
treated as described in Materials and Methods, and the final
benzene solution containing 100 ppb γ-BHC (1.0 ml) was dil-
uted 50 times with γ-BHC–benzene. Peaks I and II represent
the PFPH derivative of KBA, and IS is the internal standard
γ-BHC.

Assay Procedure——The reaction mixture
containedP ALP $(5 \times 10^{-5}$ M), m2-ercaptoetha-
nol $(7.5 \times 10^{-3}$ M), EDTA $(7 \times 10^{-3}$ M), Tris-
HCl buffer, pH 8.0 (0.1 M), L-homoserine $(2 \times 10^{-3}$ M) (or L-cystathionine, 4×10^{-4} M), and
enzyme in a total volume of 0.25 ml. The
assay was started with the addition of the
enzyme. After incubation at 37°C for 30 min,
the reactions were terminated with 0.2 ml
of 30% metaphosphoric acid. The amounts
of KBA were measured using a procedure
similar to one described previously.[7] The
acidified reaction mixture was treated with
0.2 ml of 95 mM PFPH in 0.36 N sulfuric acid
(PFPH was dissolved with the aid of heat)
and reacted at 37°C for 2 h. The reaction
mixture was washed with 2 ml and 0.5 ml of
ether after the addition of 0.2 ml of 6 N NaOH.
The hydrazone was extracted with 3 ml of
ether after acidification with 0.2 ml of 6 N HCl.
The ether layer was dried over anhydrous
sodium sulfate and reacted with diazomethane
according to the method of Schlenk and Gell-
erman.[10] After evaporation of the ether
under a gentle stream of nitrogen gas, the
residue was dissolved in 1.0 ml of benzene
containing 100 ppb 1,2,3,4,5,6-hexachlorocy-
clohexane (γ form) (γ-BHC). The benzene
solution was further diluted with the same
benzene, if necessary, and a 1 μl aliquot of it
was injected into the gas chromatograph.
Fig. 1 shows a typical gas chromatogram.
As described in a previous paper,[7] two peaks
due to *syn* and *anti* forms of the hydrazone
appeared. The sum of the two peaks gave
satisfactory results for quantitation, although
the ratio was different in each run.[7]

Assay of Cystathionine and Recovery Test
——Brain tissue, obtained from Japanese
white rabbits, was homogenized in 1.2% KCl
solution as described above, and centrifuged
at 8000 × *g* for 30 min. Various amounts of
cystathionine up to 40 mmol and 1 μl of cystathionase were added to 200 μl of the supernatant.
KBA formation was determined as described in the previous section. No cystathionase activity was found
in 200 μl of the brain supernatant.

Protein Determination——Protein concentrations were determined by means of the biuret reaction.[11]

Results

Characterization of Rat Liver Cystathionase by the Present Method

Kinetic studies of cystathionase activity were performed using the described method.
When 0.2 mM L-cystathionine was used as the substrate, the formation of KBA was proportional
to the enzyme concentration up to 1.4 mU, and in the case of L-homoserine, up to 3.4 mU
(see Fig. 2).

As shown in Fig. 3-a, the optimum pH for cystathionine was 8, and the buffer effect was
not obvious. Matsuo and Greenberg reported the optimal pH to be 8 for rat liver cystathionase
using homoserine and cystathionine,[1a] and Bikel *et al.* reported it to be 8.1 in Tris buffer for
mouse liver cystathionase with cystathionine.[4b] The pH optimum for γ-cystathionase of
Neurospora was 7.3.[12]

Fig. 3-b shows that the optimal temperature for cystathionine cleavage is 55°C. Mushawar

and Koeppe reported the optimal temperature for cleavage of homoserine to be 45°C.[1d]

The rate of cystathionine cleavage under the standard conditions was linear up to 1 h. K_m values for L-cystathionine and L-homoserine were calculated to be 3.3 mM and 13 mM, respectively, from the double reciprocal plots. The values are in agreement with those reported previously.[1a-d,9] V_{max} values were calculated as 222 nmol/30 min for L-cystathionine
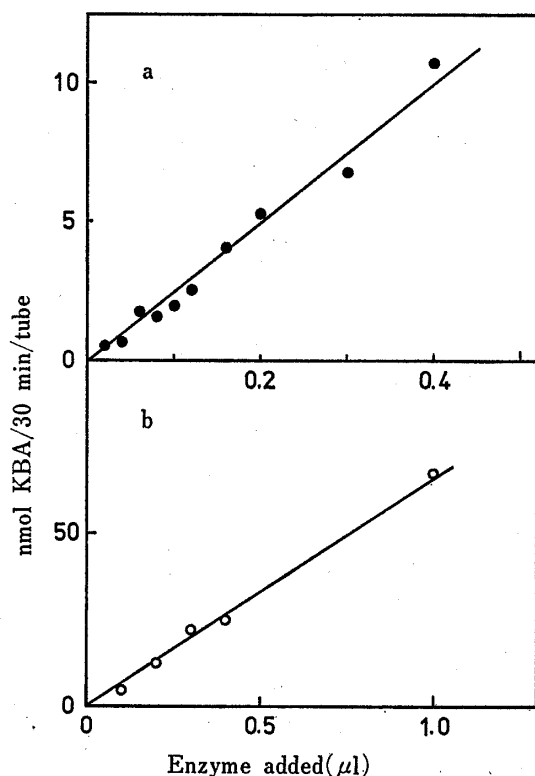

Fig. 2. Effect of Enzyme Concentration on the Formation of KBA by Partially Purified Rat Liver Cystathionase

a : L-Cystathionine (0.2 mM) was incubated with 0.02 to 0.4 μl of partially purified rat liver cystathionase, and the production of KBA was monitored according to the procedure in Materials and methods.
b : L-Homoserine (2 mM) was incubated with 0.1 to 1 μl of partially purified rat liver cystathionase.
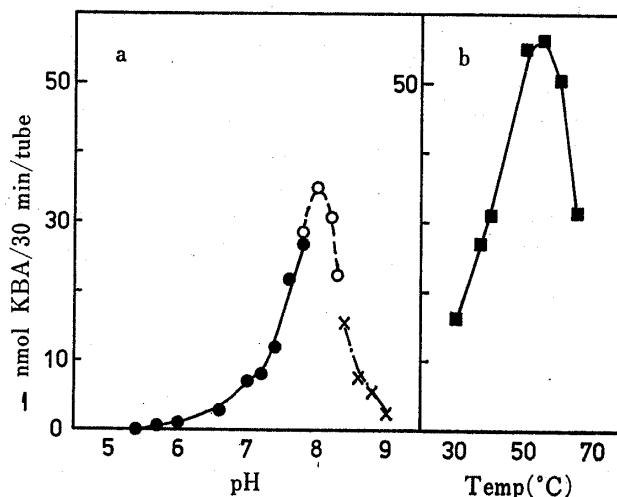

Fig .3. Rate of Cystathionine Cleavage by Partially Purified Rat Liver Cystathionase as a Function of pH and Temperature

L-Cystathionine (0.4 mM) was incubated with 0.3 μl of partially purified rat liver cystathionase in 0.1 M potassium phosphate (●), Tris-HCl (○), or sodium borate buffer (×). The optimum temperature was examined at pH 8 (Tris-HCl buffer) with L-cystathionine (0.4 mM) and paritially purified rat liver cystathionase (0.3 μl) under the standard assay conditions.

TABLE I. Activity of Cystathionase on Various Sulfur-Containing Amino Acids

Substrate	Concentration	KBA formed
	mM	nmol/30 min/tube[a]
L-Cystathionine	0.8	40.1
DL-Cystathionine	0.8	15.2
L-Homoserine	0.8	19.2
DL-Homoserine	0.8	8.2
L-Homocysteine	2.0	0.6
		nmol/30 min/tube[b]
L-Methionine sulfoximine	20	658
L-Methionine sulfone	20	239
DL-Methionine sulfoxide	20	145

a) Substrates were incubated with 0.3 μl of partially purified rat liver cystathionase under the standard assay conditions.
b) Substrates were incubated at 37°C for 20 h with 2 μl of the enzyme in the standard assay mixture.

and 250 nmol/30 min for L-homoserine.

When L-homoserine was incubated without PALP, the reaction rate dropped to 17.9% of that obtained under the standard assay conditions, while the addition of EDTA and 2-mercaptoethanol had no significant effect on the reaction rate. Similar results were reported by Mud et al.[6] and Matsuo and Greenberg.[13]

The relative activity of the enzyme on a variety of sulfur-containing amino acids is summarized in Table I. Since the enzyme activity towards these amino acids was determined by measuring the formation of KBA, amino acids such as djenkolic acid and carboxymethylthiocysteine were not examined in this paper. The following sulfur-containing amino acids which have a homocysteine moiety, however, were not cleaved to KBA at 2 mM substrate in the standard assay: L-methionine, L-methionine sulfone, DL-methionine sulfoxide, L-homocysteic acid, L-homocysteine sulfinic acid, L-methionine sulfoximine, L-CHEH and D-CHEH. L-Homocysteic acid, L-homocysteine sulfinic acid and L-CHEH were isolated from the urine of homocystinuric and cystathionuric patients,[8] and showed inhibitory effects on the electrical activity of an identified neuron.[14]

Cooper et al. reported the cysthionase catalyzes relatively slow γ-elimination reactions of L-methionine sulfoximate, L-methionine sulfoxide and L-methionine sulfone.[15] When these amino acids are reacted at 20 mM with larger quantities of the enzyme for 20 h, they are cleaved to a lesser extent (Table I). Yao reported that L-CHEH competitively inhibited the cleavage of L-cystathionine and L-homoserine.[16] The Ki value was calculated by the present method

TABLE II.   Distribution of Cystathionase in the Rat

Organ	KBA (nmol)/30 min/mg protein
Liver	378
Kidney	164
Lung	1.9
Intestine	1.1
Heart	n.d.[a]
Muscle	n.d.
Spleen	n.d.
Brain	n.d.
Lens	n.d.

L-Homoserine (2 mM) was incubated with enzyme source (5 μl for liver and kidney and 25 μl for other organs) under the standard assay conditions.
a) not detectable.

TABLE III.   Cystathionase Activities in the Liver of Several Vertebrates

Species	KBA (nmol)/30 min/mg protein
Cyprinus carpio (common carp)	30[a]
Rana catesbiana (bullfrog)	20
Amyda japonica (soft-shelled turtle)	25
Eumeces latiscutatus latiscutatus (lizard)	59
Gallus gallus (chicken)	4.2 (6.9)[b]
Gallus gallus (baby chick)	1.3 (2.4)
Coturnix coturnix japonica (quail)	n.d.[c] (0.9)
Unolonicha striata domestica (bengalee)	n.d.
rabbit	4.5
rat	379

a) L-Homoserine (2 mM) was incubated with enzyme source (5 μl for carp, bullfrog, turtle, lizard and rat, and 25 μl for others) under the standard assay conditions.
b) L-Cystathionine (0.4 mM) was incubated as described above.
c) Not detectable.

to be 100 μM for cystathionine using the Dixon plot.   Other amino acids in the table, which were not utilized as substrates, did not inhibit the cleavage reaction.

Application of the Assay Procedure to the Determination of Cystathionase Activity in Various Tissues

The method described was also used for the assay of cystathionase activity in various tissues.

As summarized in Table II, crude extracts, from rat liver and kidney showed enzyme activity, the former having the highest activity.   Lung and intestine obtained from rat showed little or no activity.   A comparison of cystathionase activity in the liver of various vertebrates is given in Table III.   This indicates that the activity is highest in rat liver and lowest in the livers of birds.

Application of the Assay Procedure to the Determination of Cystathionine

The calibration curve of cystathionine was linear up to 40 nmol of L-cystathionine. Recoveries of 106.5±4.8% ($n=4$) of L-cystathionine added to rabbit brain supernatant were obtained, and as little as 5 nmol of L-cystathionine in the incubation mixture (0.25 ml) was specifically measurable.

Discussion

Cystathionase activity could be determined by measuring μmol-range KBA liberated by the method of Matsuo and Greenberg.[1a,4b,9]   The procedure we have described is approximately 100 times more sensitive.   Also, the described method is thought to be more specific than the method of Matsuo and Greenberg, since KBA was determined by GLC with an electron capture detector.[7]   By the present method, cystathionase activity in 0.33 mg of fresh rat liver can be measured.   The amount of tissue required for the accurate and reproducible assay of cystathionase has not been reported previously.

It has been reported by many investigators that rat liver posesses the highest activity of cystathionase.[1a,6,17]   In contrast to our results, weak cystathionase activity was found in the brain.[6,17b,18]   This discrepancy may be due to the presence of lactate dehydrogenase and NADH in the 8000×g supernatant of brain homogenate.   In order to apply the present method accurately to tissue extracts containing lactate dehydrogenase, NADH has to be eliminated by dialysis.   The presence of cystathionine in the brain and lens[19] may account for the absence of cystathionase in them.   Cystathionase activity was not found in human fetal liver and brain, though an enzyme system for cystathionine synthesis was present.[20]   Thus, human fetal liver was found to contain considerable amounts of cystathionine.[27]

Cystathionase activity is found at very low levels in bird liver (Table III).   Cysteine may be obtained from serine and hydrogen sulfide by cystathionine β-synthetase (EC 4.2.1.22, serine sulfhydrase).[21]

Cystathionine is widely found in the urine of cystathionuria patients,[22] premature infants,[23] vitamin B_6-deficient animal,[24] and also in brain tissues[25] as well as in plants[26] and microorganisms.[27]   A sensitive and specific assay for cystathionine is needed in the fields of biochemistry and clinical chemistry.   An earlier method for the determination of cystathionine utilized spectrophotometry based on a characteristic ninhydrin reaction,[28] but it is less sensitive (μmol level) and specific that the present method.   A sensitive and specific isotopic method,[29] which used enzyme to catalyze the exchange of radioactive cysteine into cystathionine is available.   This method, however, requires the use of a special enzyme and radioisotope, which is disadvantageous.   Needless to say, an ordinary amino acid analyzer and gas chromatograph with a flame ionization detector can be used for the determination of cystathionine.[30,31] However, these methods were not aimed at the determination of cystathionine alone, and are more time-consuming and less sensitive than our method.   For these reasons, the method pre-

sented here should be useful in various fields as a sensitive and specific assay for cystathionine.

References and Notes

1) *a*) Y. Matsuo and D.M. Greenberg, *J. Biol. Chem.*, **230**, 545 (1958);   *b*) Y. Matsuo and D.M. Greenberg, *J. Biol. Chem.*, **234**, 516 (1959);   *c*) D.M. Greenberg, P. Mastalerz, and A. Nagabhushanam, *Biochim. Biophys. Acta*, **81**, 158 (1964);   *d*) I.K. Mushahwar and R.E. Koeppe, *J. Biol. Chem.*, **248**, 7407 (1973);   *e*) K. Yao, M. Kinuta, and Y. Watanabe, *Physiol. Chem. Physics*, **10**, 569 (1978).
2) F. Chatagner, M. Tixier, and C. Portemer, *FEBS lett.*, **4**, 231 (1969).
3) H.H. Tallan, T.A. Pascal, K. Schneidman, B.M. Gillam, and G.E. Gaull, *Biochem. Biophys. Res. Commun.*, **43**, 303 (1971).
4) *a*) M. Flavin and C. Slaughter, "Methods in Enzymology," Vol. 17B, ed. by H. Tabor and C.W. Tabor, Academic Press, New York, 1971, pp. 433—439;   *b*) I. Bikel, T.N. Pavlatos, and D.M. Livingston, *Arch. Biochem. Biophys.*, **186**, 168 (1978).
5) D.M. Greenberg, "Methods in Enzymology," Vol. 5, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1962, pp. 936—942.
6) S.H. Mudd, J.D. Finkelstein, F. Irreverre, and L. Laster, *J. Biol. Chem.*, **240**, 4382 (1965).
7) S. Ohmori, Y. Tanaka, M. Ikeda, and K. Hirota, *Anal. Biochem.*, **112**, 204 (1981).
8) *a*) S. Ohmori, H. Kodama, T. Ikegami, S. Mizuhara, T. Oura, G. Isshiki, and I. Uemura, *Physiol. Chem. Physics*, **4**, 286 (1972);   *b*) H. Kodama, S. Ohmori, M. Suzuki, and S. Mizuhara, *Physiol. Chem. Physics*, **2**, 287 (1970).
9) A. Kato, M. Ogura, H. Kimura, T. Kawai, and M. Suda, *J. Biochem.*, **59**, 34 (1966).
10) H. Schlenk and J.L. Gellerman, *Anal. Chem.*, **32**, 1412 (1960).
11) G. Beisenherz, H.J. Boltze, T. Bücher, R. Czod, K.H. Garbade, E. Meyer-Ahrendt, and G. Pfleiderer, *Z. Naturforsch.*, **8b**, 555 (1953).
12) M. Flavin and A. Segal, *J. Biol. Chem.*, **239**, 2220 (1964).
13) Y. Matsuo and D.M. Greenberg, *J. Biol. Chem.*, **234**, 507 (1959).
14) H. Takeuchi, A. Mori, M. Kohsaka, and S. Ohmori, *Brain Res.*, **67**, 342 (1974).
15) A.J.L. Cooper, R.A. Stephani, and A. Meister, *J. Biol. Chem.*, **251**, 6674 (1976).
16) K. Yao, *Physiol. Chem. Physics*, **7**, 401 (1975).
17) *a*) D.K. Rassin and G.E. Gaull, *J. Neurochem.*, **24**, 969 (1975);   *b*) K. Heinonen, *Biochem. J.*, **136**, 1011 (1973).
18) R. Griffiths and N. Tudball, *Life Sci.*, **19**, 1217 (1976).
19) Y. Watanabe, *Jap. J. Clin. Opthal.*, **25**, 585 (1971).
20) G. Gaull, J.A. Sturman, and N.C.R. Räihä, *Pediat. Res.*, **6**, 538 (1972).
21) *a*) J. Brüggemann, K. Schlossmann, M. Merkenschlager, and M. Waldschmidt, *Biochem. Z.*, **335**, 392 (1962);   *b*) J. Brüggemann and M. Waldschmidt, *Biochem. Z.*, **335**, 408 (1962).
22) H. Harris, L.S. Penrose, and D.H. Thomas, *Ann. Human Genet.*, **23**, 442 (1959).
23) H. Przyrembel and H.J. Bremer, *Clin. Chim. Acta*, **41**, 95 (1972).
24) *a*) P. Fourman, J.W. Summerscles, and D.M. Morgan, *Arch. Dis. Child.*, **41**, 273 (1966);   *b*) D.B. Hope, *Biochem. J.*, **66**, 486 (1957).
25) J.J. Volpe and L. Laster, *J. Neurochem.*, **17**, 425 (1970).
26) M.J. Horn and D.B. Jones, *J. Biol. Chem.*, **139**, 649 (1941).
27) *a*) F.M. Harold, *J. Bact.*, **84**, 382 (1962);   *b*) N.H. Horowitz, *J. Biol. Chem.*, **171**, 255 (1947).
28) S. Kashiwamata and D.M. Greenberg, *Biochim. Biophys. Acta*, **212**, 488 (1970).
29) A.H. Datko, S.H. Mudd, and J. Giovanelli, *Anal. Biochem.*, **62**, 531 (1974).
30) M. Friedman, A.T. Noma, and J.R. Wagner, *Anal. Biochem.*, **98**, 293 (1979).
31) J. Desgres, D. Boisson, and P. Padieu, *J. Chromatogr.*, **162**, 133 (1979).