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# Sensitive determination of cystathionine and assays for cystathionine β- and γ-lyase, as well as cystathionine β-synthase, using high-performance liquid chromatography

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ABSTRACT

Cystathionine was cleaved into 2-ketobutyric acid, cysteine and ammonia by cystathionase. 2-Ketobutyric acid was converted into 3-ethyl-2-hydroxy-6,7-dimethoxyquinoxaline (EHDQ) by reaction with 1,2-diamino-4,5-dimethoxybenzene. When EHDQ was measured in a mobile phase of pH 2.1 using high-performance liquid chromatography with ultraviolet detection, 250 pmol of L-cystathionine in 250 μl of the reaction mixture could be determined. Because EHDQ has a strong fluorescence in a mobile phase of pH 6.5 at 447 nm, on excitation at 365 nm, as little as 2.5 pmol of cystathionine in 250 μl of the reaction mixture could be determined by high-performance liquid chromatography with fluorimetric detection. Cystathionase activity was assayed on the basis of the same principle by determining cystathionine in as little as 63 ng of rat liver by fluorimetric detection. Cystathionine β-synthase activity was measured by the same method by determining cystathionine formed in only 113 ng of wet weight of rat liver. Using these methods, both cystathionine β- and γ-lyase activities in *Saccharomyces cerevisiae* were determined, because quinoxaline derivatives from pyruvate and 2-ketobutyrate could be measured simultaneously by high-performance liquid chromatography.

## INTRODUCTION

Cystathionase catalyses the cleavage of cystathionine, homoserine [1], 2,3-diaminopropionic acid [2], lanthionine, cysteine [3], djenkolic acid [4], L-methionine sulphoximine, L-homocysteine and L-methionine sulphone [6]. L-Cystathionine, which is the best substrate for rat liver cystathionase, is cleaved to 2-ketobutyric acid, cysteine and ammonia [6]. In order to determine levels of cystathionine by measuring cystathionase activity, 2-ketobutyric acid is the preferred product for measurement. The formation of cysteine can also be followed, but it has proved difficult to measure. If the method of Gaitonde [7] is used to determine cysteine, cystathionine and its cleavage product, cysteine produced a similar colour development in the acid ninhydrin reaction.

2-Ketobutyrate has been determined by gas chromatography as a pentafluorophenylhydrazone methyl ester [6], and by high-performance liquid chromatography (HPLC) as a 2,4-dinitrophenylhydrazone [8].

We tried to achieve a more sensitive determination of 2-ketobutyrate as a quinoxaline derivative, which is strongly fluorescent.

Cystathionine β-synthase activity has been measured by either a radioisotopic method which uses $^{14}$C-labelled serine as substrate [9] or a colorimetric method based on a positive ninhydrin reaction with cystathionine formed [10].

The former necessitates the separation of $^{14}$C-labelled amino acids on a Dowex-50 column, and the latter lacks sensitivity although it is rapid.

β-Synthase can also be assayed by determining 2-ketobutyrate from cystathionine in the pres-

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ence of cystathionase, which can be easily prepared from rat liver. Furthermore, cystathonine β-lyase activity can be assayed in the same run by determining pyruvate as 2-hydroxy-6,7-dimethoxy-3-methylquinoxaline. β-Lyase cleaves L-cystathionine into pyruvate, L-homocysteine and ammonia, and is present in microorganisms and plants. These ideas were demonstrated to be applicable to rat liver and *Saccharomyces cerevisiae*.

## EXPERIMENTAL

### *Chemicals*

2-Mercaptoethanol, 2-ketobutyric acid and acetonitrile were purchased from Wako (Osaka, Japan). EDTA disodium and tetrasodium salts were obtained from Dojindo Labs. (Kumamoto, Japan). L-Serine, glucose and chloroform were from Katayama Chemical Industries (Osaka, Japan). Tris was purchased from Sigma (St. Louis, MO, USA). L-Cystathionine was isolated and purified at this University from the urine of a cystathioninuric patient. 1,2-Diamino-4,5-dimethoxybenzene (DDB), 3-ethyl-2-hydroxy-6,7-dimethoxyquinoxaline (EHDQ) and L-homocysteine were synthesized by us [11,12]. Pyridoxal phosphate was kindly supplied by Nisshin Flour Milling (Tokyo, Japan). Bacto-peptone and yeast extract were purchased from Difco Labs. (Detroit, MI, USA).

### *Preparation of enzyme source*

Cystathionase and cystationine synthase were purified from rat liver by the procedures of Kato *et al.* [13] and Kashiwamata and Greenberg [10], respectively. The activities of all enzymes are given in units defined as the formation of 1 μmol of product per minute at 37°C.

### *High-performance liquid chromatography*

A Shimadzu LC 3A liquid chromatograph equipped with a variable-wavelength detector (Shimadzu SPD-2A) was used. Quinoxalinol formed from 2-ketobutyric acid was analysed on a 150 mm × 4.6 mm I.D. Chemcopak column (Nucleosil $5C_{18}$, obtained from Chemco Takatsuki, Japan) at 362 nm. Elution was isocratic at a flow-rate of 1.0 ml/min at 40°C, and the mobile phase was a 70:30 (v/v) mixture of 10 m*M* $KH_2PO_4$ (adjusted with phosphoric acid to pH 2.1) and acetonitrile.

To assay the enzyme activities and determine cystathionine more sensitively, a Shimadzu liquid chromatograph LC-6A with a Shimadzu RF-535 fluorescence spectromonitor was used. The excitation and emission wavelengths were 365 and 447 nm, respectively. A 150 mm × 4.6 mm I.D. column of Cosmosil 5C18-AR (Nacalai Tesque, Kyoto, Japan) was used at 40°C with a flow-rate of 1.0 ml/min. The mobile phase was a 70:30 (v/v) mixture of 10 m*M* $K_2HPO_4$ (adjusted with phosphoric acid to pH 6.5) and acetonitrile.

### *Determination of cystathionine and assay of cystathionase*

The principle of the determination of cystathionine and assay of cystathionase is depicted in Fig. 1. To determine cystationine, the sample so-

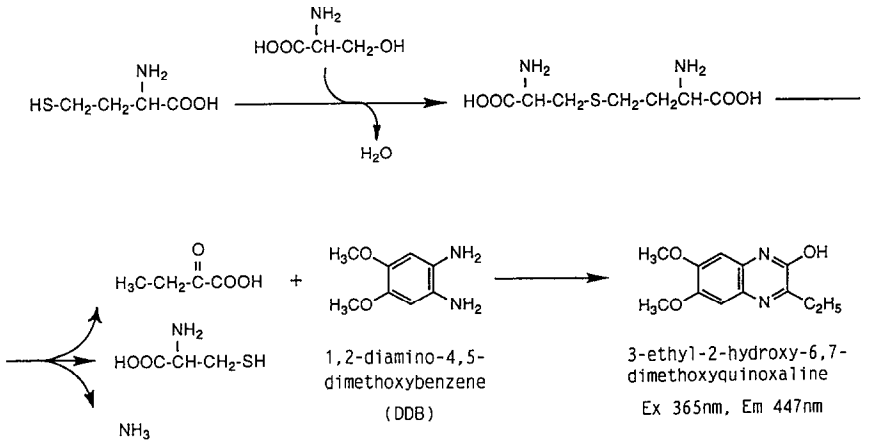


Fig. 1. Reaction scheme for the determination of cystathionine and the assay of cystathionine β-synthase and cystathionine γ-lyase.

lution was incubated at 37°C for 30 min with pyridoxal 5-phosphate (PALP) (50 $\mu M$), 2-mercaptoethanol (7.5 m$M$), Tric–HCl, pH 8.0 (0.1 $M$) and 0.2 mU of cystathionase with a final volume of 0.25 ml in a 1.5-ml Eppendorf microtube. After incubation, the reaction was terminated by the addition of 250 $\mu$l of 2 $M$ HCl, and the mixture was centrifuged at 12 000 $g$ for 3 min. The supernatant was transferred to a 5-ml vial, and 500 $\mu$l of a 0.1 $M$ DDB solution in 1 $M$ HCl were added. The mixture was allowed to react at 40°C for 30 min, then 3 ml of chloroform were added and the vial was tightly capped and vigorously shaken. A 2-ml volume of the chloroform layer was pipetted into a 5-ml test-tube and evaporated using a Savant Speed Vac concentrator (Model SVC-100H, Savant Instruments, Hicksville, NY, USA) at room temperature. The residue was dissolved in 200 $\mu$l of the mobile phase, and 20 $\mu$l were injected into HPLC system.

*Assay of cystationine β-synthase activity*

The principle of the assay of $\beta$-synthase shown in Fig. 1 indicates a coupled reaction with cystathionase. L-Homocysteine, which is a substrate for the synthase, is also a substrate for cystathionase. Since the product of the synthase, cystathionine, is 67 times better as a substrate for cystathionase than L-homocysteine, the substrate for $\beta$-synthase [6], the coupled reaction proceeds favourably in the direction of cystathionine degradation. As a result, the assay of synthase activity is feasible.

A sample solution containing synthase was preincubated with 120 $\mu M$ PALP, 7 m$M$ EDTA, 7.5 m$M$ 2-mercaptoethanol, 100 m$M$ Tris–HCl (pH 8.0) and 0.2 mU of cystathionase (in large excess to the $\beta$-synthase) at 37°C for 5 min in a 1.5-ml microtube. Then 11 $\mu$mol of L-serine and 10 $\mu$mol of L-homocysteine were added, and the reaction mixture was brought up to 250 $\mu$l with water and incubated at 37°C for 30 min. At the end of the incubation period, 250 $\mu$l of 2 $M$ HCl were added to terminate the reaction, and the mixture was centrifuged. These reactions were performed in a test-tube. The derivatization was done as described under *Determination of cystathionine and assay of cystathionase.*

*Recovery test of cystathionine*

Various amounts of cystathionine were added to 50 $\mu$l of the supernatant obtained from rat brain homogenate centrifuged at 9000 $g$, in which no cystathionase activity was detected [6], and their recoveries were measured by the procedure described above.

*Yeast cultivation and assay of cystathionine β- and γ-lyase*

A 600-$\mu$l volume of a suspension of *S. cerevisiae* (wild type, IS66-4C) preincubated at 30°C for 11 h in 15 ml of medium (2% glucose, 2% bacto-peptone and 1% yeast extract) were incubated in 60 ml of the medium at 30°C for 8.5 h. The cells were precipitated by centrifugation at 1300 $g$ for 3 min and washed with buffer (20 m$M$ potassium phosphate, pH 7.5, 0.1 m$M$ EDTA and 50 $\mu M$ PALP) three times. The cells were then homogenized with glass beads (0.3 mm) by Vibrogen (Edmund Bahler, Tübingen, Germany) at 4°C for 150 s, followed by centrifugation at 13 000 $g$ for 45 min. The supernatant was dialysed at 4°C for 24 h, and the cystathionine $\beta$-lyase and $\gamma$-lyase activities were assayed. The procedure was carried out under the same conditions as the cystathionase assay described above, except for using 100 m$M$ potassium phosphate (pH 7.5) and 20% acetonitrile in the mobile phase. The reaction conditions for the formation of 2-hydroxy-6,7-dimethoxy-3-methylquinoxaline from pyruvate and its analysis were reported in detail in a previous paper [11].

## RESULTS

Fig. 2a shows the chromatographic trace of authentic EHDQ, and Fig. 2b that of EHDQ formed from 150 pmol of cystathionine by the use of partially purified cystathionase. Fig. 2c shows the cystathionase activity in the supernatant from rat liver (8 $\mu$g protein) centrifuged at 9000 $g$. The supernatant was incubated with 0.1 $\mu$mol of cystathionine, and the separation was monitored fluorimetrically.

*Calibration curves and limit of determination for cystathionine*

Various amounts (0.2–200 nmol) of L-cysta-

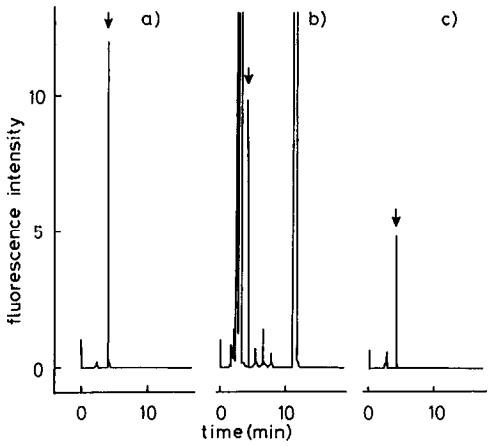


Fig. 2. High-performance liquid chromatograms. The arrow shows the peak of authentic EHDQ or that derived from 2-ketoglutarate. (a) EHDQ (1 nmol) was dissolved in 20 ml of the mobile phase (pH 6.5) and 20 μl were injected. (b) An example of the determination of L-cystathionine by partially purified cystathionase. L-Cystathionine (150 pmol) was incubated with cystathionase at 37°C for 30 min in the presence of PALP, EDTA and mercaptoethanol. After the addition of 250 μl of 2 *M* HCl, the 2-ketobutyrate formed was converted into EHDQ by DDB (50 μmol) in 1 *M* HCl at 40°C for 30 min. EHDQ was extracted with 3 ml of chloroform, and 2 ml of the extract were evaporated to dryness. The residue was dissolved in 200 μl of the mobile phase, and 20 μl were injected. (c) An example of assay of cystathionase activity in rat liver. L-Cystathionine (0.1 μmol) was incubated with the 9000 *g* supernatant of rat liver (8 μg of protein) in the presence of the cofactors described in (b).

thionine were added to 0.25 ml of the assay mixture and treated as described under *Determination of cystathionine and assay of cystathionase.* The absorbance at 362 nm ($y$) was directly proportional to the L-cystathionine concentration ($x$, nmol): $y = 4.97 \cdot 10^{-4}x + 4.44 \cdot 10^{-4}$ ($r = 0.9993$, $n = 12$). The determination limit was 250 pmol in 0.25 ml of the assay mixture. When HPLC with fluorimetric detection was used, a plot of fluorescence intensity ($y$) *versus* the L-cystathionine concentration ($x$, pmol) in the assay mixture gave a straight line from 1 to 300 pmol in 0.25 ml of the assay mixture ($n = 14$, range 32); $y = 7.0 \cdot 10^{-2}x - 4.98 \cdot 10^{-2}$ ($r = 0.9989$). From the curve, the determination limit was 2.5 pmol in 0.25 ml of the assay mixture. When HPLC with fluorometric detection was used, cystathionine could be determined in a very small amount of rat brain (0.3 mg of protein).

Both the cystathionase assay and the cystathionine determination could be performed by HPLC with UV or fluorescence detection. Although the latter is *ca.* 100 times more sensitive, the former is sufficient to determine cystathionine or to assay cystathionase activity in usual biological samples.

*Recovery tests of cystathionine*

Various amounts of cystathionine were added to the supernatant from rat brain homogenate centrifuged at 9000 *g*, and their recoveries were measured by the procedure described above. The results are summarized in Table I, which shows recoveries of 98.3 ± 1.35% ($n = 3$). As shown in Table I, a considerable amount of cystathionine exists in the brain, because of the absence of cystathionase [6]. The value of 0.977 is attributed not to intrinsic 2-ketobutyrate but to cystathionine, because 2-ketobutyrate was not detectable in the rat brain homogenate.

TABLE I

RECOVERIES OF CYSTATHIONINE ADDED TO THE SUPERNATANT FROM RAT BRAIN HOMOGENATE

Various amounts of L-cystathionine were added to 50 μl of the supernatant of rat brain homogenate. L-Cystathionine was measured by the method described in the legend of Fig. 2b.

| Concentration (nmol/17 mg wet weight brain) | | Recovery (%) | C.V. (%) |
|---|---|---|---|
| Added | Determined | | |
| 0 | 0.977 | | |
| 1.00 | 1.96 | 98.3 | 1.88 |
| 1.50 | 2.48 | 100 | 2.66 |
| 2.00 | 2.91 | 96.7 | 2.27 |
| Mean ± S.D. | | 98.3 ± 1.35 | |

*Assay of cystathionase activity*

Cystathionase in the supernatant from rat liver homogenate centrifuged at 9000 *g* was assayed by HPLC with UV and fluorimetric detection. The curve obtained with UV detection showed excellent linearity over the range from 0.77 μg to at least 15.5 μg of protein, corresponding to 13.25–265 μg wet weight of rat liver. When rat liver cystathionase activities were assayed by HPLC with fluorimetric detection, the fluorescence intensity $y$ (range 32) plotted against liver protein concentrations was linear from 3.62 to 96.7 ng of protein (corresponding to 62.5 ng to 1.667 μg wet weight of liver; $y = 0.230x - 0.051$ ($r = 0.9976$, $n = 12$).

*Assay of cystathionine β-synthase activity*

After preincubation with cystathionase and various amounts of cystathionine β-synthase, L-serine and L-homocysteine were added and the mixture was incubated for 30 min. The 2-ketobutyrate formed was converted into EHDQ by DDB in 1 *M* HCl and analysed by HPLC. The resulting calibration curve of the synthase activity of rat liver was linear from 1.4 mU to at least 11 mU. When the activity of the synthase in the supernatant from rat liver homogenate centrifuged at 9000 *g* was determined, a linear relationship was observed between 6.5 and 193 ng of protein in 250 μl of the reaction mixture, which corresponds to 0.113–3.33 μg wet weight of rat liver: $y = 0.201x + 0.011$ ($r = 0.998$, $n = 15$), where $y$ is the fluorescence intensity (range 32) and $x$ is the protein concentration (ng).

*Cystathionine β-lyase and γ-lyase in S. cerevisiae*

Cystathionine β-lyase and γ-lyase, which occur in yeast, cleave L-cystathionine to pyruvate and 2-ketobutyrate, respectively. Since 2-ketobutyrate and pyruvate could be easily determined by our HPLC method as the corresponding quinoxaline derivatives in the same run, we applied this method to the assay of both enzyme activities. The peak of the former appeared at 8 min and that of the latter at 4 min under the HPLC conditions described above. The activities of β-lyase and γ-lyase in wild-type yeast (IS66-4C) were 0.3 and 3.4 mU/mg protein.

## DISCUSSION

In 1982 we reported a sensitive and specific determination of cystathionine using GC [6]. In those days HPLC was not as widely used as it is today. In 1986 Bergad and Rathbun [8] reported an HPLC determination of cystathionase activity in human and rabbit lenses [8].

The HPLC method with fluorimetric detection presented here is *ca.* 2000 times more sensitive than the GC method. The method of Bergad and Rathbun [8] analysed 2-ketobutyrate as two peaks (*syn* and *anti*), and was less sensitive than the method described here. Our HPLC method can use either UV or fluorimetric detection. The former can be widely used, because HPLC with UV detection is commonly available. Fluorimetry should be used when only small amounts of sample are available. For example, cystathionase activity in twenty human hair-roots could be assayed by this method. Details of these results will be reported elsewhere.

The fluorescence intensity of EHDQ varies with pH. Since the relative intensities were 1:3.8:4:2.8 at pH 2.1, 6, 6.5 and 7, respectively, a pH 6.5 was chosen for the mobile phase.

The metabolism of sulphur-containing amino acids is not at all clear in all organisms. Ono studied cysteine formation using many kinds yeast mutant [14]. Yeast is an important biotechnological and biochemical tool. At the beginning of a cooperative study with his group, we applied our method to the determination of cystathionine β-lyase and γ-lyase in wild-type yeast. It was found that the activity of the latter was eleven times higher than that of the former, indicating that wild-type yeast produces much more cysteine than homocysteine under these conditions.

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