Homogeneous Enzymatic Assay for L-Cysteine with βC-S Lyase

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We have developed a new enzymatic assay for determining L-cysteine concentration. The method involves the use of βC-S lyase from Streptococcus anginosus, which catalyzes the α,β-elimination of L-cysteine to hydrogen sulfide, pyruvate, and ammonia. The production of pyruvate is measured by D-lactate dehydrogenase and NADH. The decrease in NADH was proportional to the L-cysteine concentration up to 1.0 mM. When serum samples were used, within-day and day-to-day coefficient variations were below 4%. This method is simple, and can easily and reliably be used for accurate determination of L-cysteine concentration in serum or other samples.

Key words: L-cysteine; assay method; βC-S lyase; Streptococcus anginosus

In cells, homocysteine arises from the metabolism of methionine, an essential sulfur amino acid, and transforms to cystathionine and then to L-cysteine (L-Cys). In numerous studies, total homocysteine concentrations have been found to increase in patients with arterial sclerosis, renal failure, and other diseases.1) More studies have reported that total serum or plasma homocysteine is an independent risk factor for cardiovascular disease.1) Hence many methods have been developed to measure homocysteine, such as HPLC and enzymatic assays.2–6)

In contrast, analysis of L-Cys in these clinical conditions has been reported only rarely.7,8) Because there is extensive interconversion between these metabolites, analysis of them in biological samples is necessary to examine their role in human disease. Recently, a new enzymatic colorimetric assay for L-Cys has been reported.9) In this assay, L-Cys is eliminated by methionine a,γ-lyase, but the substrate specificity of methionine a,γ-lyase is lower, and especially reacts homocysteine as well as methionine. Hence this assay method must remove the homocysteine of the samples in advance, and therefore it takes a lot of time (total analysis time, 50 min) and effort.

We were interested in L-cysteine desulphhydrase (Lcd) for measurement of L-Cys concentration. Lcd is a pyridoxal 5'-phosphate-dependent enzyme that catalyzes the α,β-elimination of L-Cys to pyruvate, ammonia, and hydrogen sulfide. In this paper, we report a new enzymatic assay method of L-Cys with Lcd from Streptococcus anginosus.9,10) This enzyme was highly specific to L-Cys, and did not react to homocysteine, methionine, or D-cysteine in accordance with previous results.9) The reaction sequence for the determination of L-Cys is summarized below.

Step 1: Lcd

L-Cys + H2O → pyruvate + NH3 + H2S

Step 2: D-lactate dehydrogenase

pyruvate + NADH + H+ → lactate + NAD+

Lcd forms pyruvate from L-Cys, and the decrease in NADH caused by the following reaction of lactate dehydrogenase is spectrophotometrically measured. Under optimal conditions, this assay can easily and reliably be used for the accurate determination of L-Cys in serum and other samples.

S. anginosus Lcd was prepared from recombinant Escherichia coli cells as previously described.9) D-Lactate dehydrogenase (EC 1.1.1.28) was from Toyobo (Osaka, Japan). L-Cys, NADH, and all other reagents of analytical grade were purchased from Nacalai Tesque (Kyoto, Japan). L-Cys solutions were prepared by dissolving L-Cys in distilled water. Sera obtained were stored at −20°C until assayed. Sample absorbance was measured with a Model 7150 automated analyzer (Hitachi, Tokyo).

The reaction reagent consisted of reagents R1 and R2. The final reaction mixture concentrations of reagent R1 were 50 mM potassium phosphate buffer (pH 7.0), 0.12 mM NADH, and 60 U/ml of D-lactate dehydrogenase. Those of reagent R2 were 50 mM potassium phosphate buffer (pH 7.0), 0.06 mM pyridoxal 5'-phosphate, and 0.022 mg/ml of Lcd. The reaction was initiated by mixing 0.25 ml of reagent R1 and 0.010 ml
of sample. After 5 min at 37°C, 0.050 ml of reagent R2 was added, and absorbance at 340 nm was measured at 37°C for 5 min. Other settings used for the automated analyzer were as follows: measurement interval, 12 s; assay mode, end-point assay; subwavelength, 546 nm.

The time courses of the absorbance changes are shown in Fig. 1. When 0.10 and 0.20 mM L-Cys solutions were used as calibrators, absorbance decreased proportionally to the L-Cys concentrations (−21.6 mAbs for 0.10 mM and −35.9 mAbs for 0.20 mM). The reaction for distilled water as a sample blank was slightly detected (−5.2 mAbs). Hence, we thought that an end-point assay could be adopted.

The standard curves for L-Cys standards are presented in Fig. 2. They were linear between 0.10 and 1.0 mM L-Cys of sample. The influence of sample dilution was also examined, using different sera. When L-Cys samples were diluted with sera having L-Cys concentrations of approximately 0.14 and 0.03 mM respectively and assayed, good linearity were found (Fig. 2). These results indicate that our method is suitable for assay of samples. The method is able to detect 0.05 mM L-Cys out of consideration to the quantitation limit of absorbance change.

The specificity of our method was examined, and no assay response was detected for other L- or D-amino acids, including cysteine (data not shown). This result is reasonable because S. anginosus Lcd did not react to any amino acid species except L-Cys, DL-cystathionine, L-cystine, S-(2-aminoethyl)-L-cysteine, 3-chloro-DL-alanine, and S-methyl-L-cysteine.\(^9\) The subsequent reaction of D-lactate dehydrogenase has been already used in a practical assay of serum glutamate-pyruvate transaminase, and substrates or coenzymes of the enzyme also gave no interference.

For precision study, five different serum samples were assayed 20 times. For the day-to-day precision study, two serum samples were assayed once a day for 7 d. The results are summarized in Table 1. Within-day coefficient variations ranged from 1.4 to 3.9%, and day-to-day coefficient variations were 3.7 and 3.8%.

The analytical recovery for L-Cys added to a serum sample was also determined (Table 1). When 0.20 mM of L-Cys was added to a serum sample containing 0.11 mM endogenous L-Cys, the recovery and day-to-day coefficient variation were 100% and 1.2% respectively.

In conclusion, it was found that our assay method can easily and reliably be used in the accurate determination of L-Cys concentration. In particular, this method is useful for routinely assaying serum L-Cys. We have
provided a full-automatic assay system, since with this method it is not necessary to pretreat samples manually to eliminate other sulfur amino acids.

The thiol group of L-Cys allows it to form a disulfide bond with other thiol-containing molecules, such as L-Cys itself, homocysteine, and the cysteine residues of several proteins. If samples are reduced in advance by reducing reagents, such as L-dithiothreitol and /C12-mercaptoethanol, total L-Cys released from disulfide bonds can be determined by our assay method. Investigation of L-Cys concentrations using normal and abnormal patients’ sera is now in progress.

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References