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Note

Determination of a Small Quantity of Cystine in the Presence of a Large Amount of Cysteine

Shuzo YAMAGATA† and Tomonori IWAMA

Department of Biotechnology, Faculty of Agriculture, Gifu University, Gifu 501-1193, Japan

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A procedure is described to precisely determine a very small amount of cystine in the presence of a large amount of cysteine. After completely modifying cysteine with *N*-ethylmaleimide, the remaining reagent was reacted with DL-homocysteine. Cystine was determined, after being reduced with dithiothreitol, by the reaction with ninhydrin carried out under acidic conditions. The procedure makes it possible to precisely determine the amount of cystine present with cysteine in a concentration ratio of 1:2,000. By employing this procedure, auto-oxidation of cysteine to cystine in a mixture for the L-cysteine α,β-elimination reaction was investigated.

Key words: cystine determination; cysteine; cystathionine; ninhydrin

Various methods have been reported to determine thiols,[1,2] since they play an essential role in organisms. Among them, the spectrophotometric assay can most conveniently be employed. Cysteine can be specifically determined only by the colorimetric method of Gaitonde, using ninhydrin under strongly acidic conditions,[3] even when it exists with other thiols such as homocysteine and glutathione. Cystine can also be determined by applying the same method,[3] after reducing it with a suitable reagent such as dithiothreitol (DTT).[4] The amount of cystine present in biological materials, which usually contain cysteine as well, can be calculated from the difference between the total amount of cysteine plus cystine and that of cysteine.[3] However, this procedure can only be safely applied to exactly determine the concentration of cystine when the concentration is sufficiently high compared with that of cysteine.

When we need to precisely determine a very small amount of cystine present together with a large amount of cysteine, it is theoretically correct to determine cystine by the method of Gaitonde[3] as already mentioned, after completely modifying co-existing cysteine with a proper sulfhydryl reagent. However, the sulfhydryl reagent employed in excess also has to be modified before reducing cystine to avoid its reaction with the cysteine formed. For this purpose, a proper sulfhydryl compound which does not interfere with the method of Gaitonde[3] must be chosen.

This paper describes the conditions for a convenient procedure to determine a very small amount of cystine present in a large amount of cysteine. Results of an investigation on the auto-oxidation of L-cysteine during the incubation procedure for the cysteine α,β-elimination reaction catalyzed by *Saccharomyces cerevisiae* cystathionine γ-lyase (EC 4.4.1.1)[5] and the effect of DTT on protecting it from this oxidation will also be described.

L-Cysteine-hydrochloride was a product of Kishida Chemicals Co. (Osaka, Japan). L-Cystine, pyridoxal 5′-phosphate, DL-homocysteine, *N*-ethylmaleimide (NEM), dithiothreitol (DTT), and ninhydrin were from nacalai tesque (Kyoto, Japan). Glutathione was from Kohjin (Tokyo, Japan). All other reagents were obtained commercially in the highest grade available.

Determination of cystine in the presence of a large amount of cysteine

(*Step 1*) *Modification of cysteine with NEM.* L-Cystine solutions of various concentrations (0.05–1.0 mM) were prepared, all containing 10 mM L-cysteine-hydrochloride, 0.1 M potassium phosphate (pH 7.0), and 1 mM EDTA. NEM was employed to modify all the cysteine molecules, because of its quantitative and rapid reactivity to sulfhydryl groups in addition to its stability at neutral pH.[6] To 0.5 ml each of a cysteine-cystine mixture, the same volume of 16 mM NEM dissolved in 0.2 M potassium phosphate (pH 7.0) was added, and the mixture incubated at 30°C for 5 min (total volume, 1 ml; final concentrations of cysteine and NEM, 5 mM and 8 mM, respectively).

(*Step 2*) *Reaction of NEM remaining with DL-homocysteine.* To completely inactivate excess NEM which would react with cysteine formed from cystine in the subsequent step, DL-homocysteine was chosen as the sulfhydryl compound, since it has been reported not to affect Gaitonde's ninhydrin method.[3]

Portions (0.5 ml each) of the NEM-treated cysteine-cystine mixtures were mixed with the same volume of 20 mM DL-homocysteine and incubated under the same conditions as those already given. Glutathione was also employed at the same concentration in place of DL-homocysteine for the remaining portions (0.5 ml each) of the mixtures.

(*Step 3*) *Reduction of cystine with DTT.* After the incubation, 0.005 ml of 1 M DTT was added to each solution (final concentration, approximately 5 mM), and the mixture incubated at 30°C for 5 min to reduce cystine.

(*Step 4*) *Determination of cysteine formed.* Cysteine

† To whom correspondence should be addressed. Tel (Fax): +81-58-293-2933; E-mail: yamagata@cc.gifu-u.ac.jp

Abbreviations: DTT, dithiothreitol; NEM, *N*-ethylmaleimide

produced was determined by the method of Gaitonde.[3] Samples (0.3 ml) were mixed with the same volume of acetic acid and then with the same volume of Gaitonde's ninhydrin solution,[3] this being followed by heating in boiling water for 10 min. After cooling with tap water, the colored solutions were mixed with 2.1 ml of ethanol, and the absorbance of each at 560 nm was determined. A 0.2 mM L-cysteine-hydrochloride solution containing 5 mM DTT was used as a standard. The solutions containing 10 mM DL-homocysteine (or glutathione) and 5 mM DTT were also subjected to the same determination to ascertain that these reagents would not interfere with the determination under the conditions employed.

(*Step 5*) *Calculation of cystine concentration.* As cystine gives two molecules of cysteine after its reduction and the original cystine solutions were finally diluted 4.02 fold, the cystine concentration of each was calculated from the cysteine concentration finally obtained by multiplying by 2.01.

The results are summarized in Fig. 1(A) which shows a proportional relationship between the cystine concentration determined and the concentration of cystine dissolved in the original solution (0.05-1.0 mM). Both DL-homocysteine and glutathione were good modifiers of remaining NEM, giving straight lines. The same experiment was carried out in a narrower cystine-concentration range, 0.005–0.1 mM, and the result is shown in Fig. 1(B). Determinations in both the experiments were reproducible. The combined results indicate that we could precisely assay L-cystine in a concentration range of 0.005–1.0 mM in the presence of 10 mM L-cysteine (a ratio range of 1/2,000–1/10), without being interferred with by the latter. The recovery of cystine dissolved in the original solutions together with 10 mM cysteine was approximately 85%. Approximately the same recovery was obtained in other experiments in which cysteine was dissolved at concentrations of 1 mM and 5 mM. Employment of an increased concentration of DTT (10 mM) also did not essentially change the value. The lower recovery is considered to have been due to insufficient color development, being affected by various substances present in the solutions (*Step 3*). This was confirmed by the subsequent experiment, in which the same procedure was performed without adding either cystine or cysteine in *Step 1*. Cysteine and DTT were dissolved in the solution obtained after *Step 2* at concentrations of 0.2 mM and 5 mM, respectively. After incubating at 30°C for 5 min, this solution was subjected to the ninhydrin reaction as already described, and the coloring was compared with that obtained for the standard solution containing 0.2 mM cysteine and 5 mM DTT. The former solution showed an absorbance of approximately 80% that of the latter. However, the lower recovery could be corrected by carrying out a standard experiment.

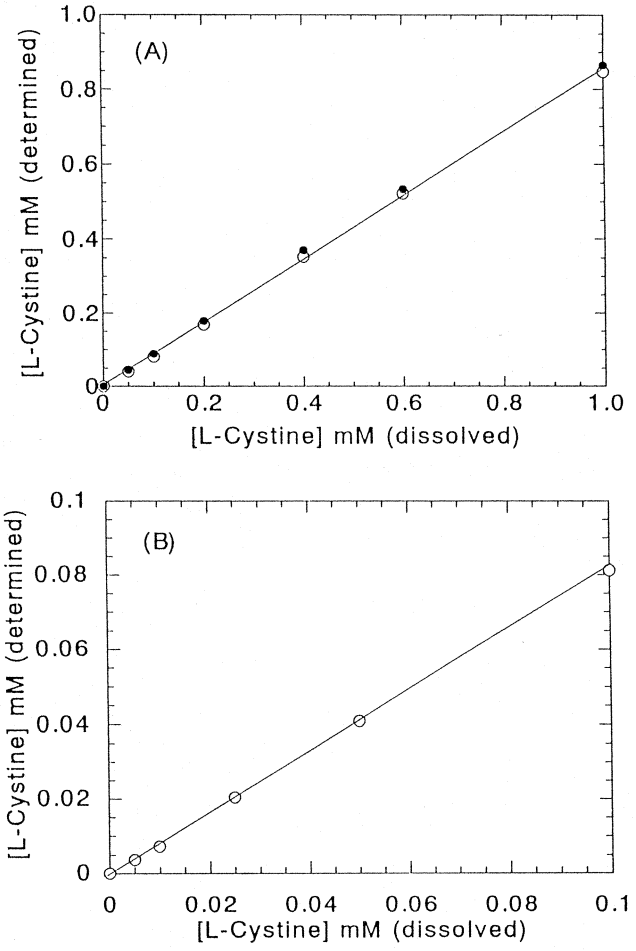

Fig. 1. Determination of Cystine at Low Concentrations in the Presence of 10 mM L-Cysteine.

L-Cystine was dissolved at the various concentrations indicated in 0.1 M potassium phosphate (pH 7.0) containing 1 mM EDTA and 10 mM L-cysteine. Immediately after preparing each mixture, the solution was treated with an excess amount of NEM to react with all cysteine molecules, before unreacted NEM was modified with a sufficient amount of DL-homocysteine (○) or glutathione (●). Cystine was then determined by the method of Gaitonde[3] after being reduced with 5 mM DTT, using L-cysteine as a standard. Figures A and B show the relationships between the concentration of cystine dissolved with L-cysteine and that determined in cystine-concentration ranges of 0.05–1.0 mM and 0.005–0.1 mM, respectively. See the text for detailes of the conditions.

Application of the procedure to determine the cystine formed from L-cysteine during incubation

The formation of cystine from cysteine during incubation of the mixture used for the L-cysteine α,β-elimination reaction would interfere with an exact analysis of the enzymatic catalysis, since both amino acids can be substrates of *S. cerevisiae* cystathionine γ-lyase.[5] In order to study how much cystine would be formed and to what extent we could protect cysteine from oxidation under the conditions of the enzymatic reaction, we determined cystine formed from cysteine in the reaction mixture with and without DTT.

L-Cysteine-hydrochloride was dissolved at different concentrations (1, 5, and 10 mM) together with the same components as those contained in the mixture for the cysteine α,β-elimination reaction, except for the enzyme (0.1 M potassium phosphate (pH 7.8)/1 mM EDTA/0.2 mM pyridoxal 5′-phosphate).[5] The solutions (1 ml each in a 1.5-ml Eppendorf tube with a cap) were incubated at 30°C for 30 min with and without DTT added at 1, 5, or 10 mM.

Table 1. Concentration of Cystine Produced by the Auto-oxidation of Cysteine under Various Conditions

L-Cysteine in 1st incubation (mM)	No DTT (mM)	1 mM DTT (mM)	5 mM DTT (mM)	10 mM DTT (mM)
1	0.01	0.006	0.006	0.006
5	0.025	0.009	0.015	0.011
10	0.038	0.026	0.022	0.025

The concentration of cystine was calculated from the concentration of cysteine in the final solution by multiplying by 2.01 (see the text for details).

Portions (0.5 ml each) of the incubated solutions containing cysteine and produced cystine were subjected to the procedure already mentioned. The results are summarized in Table 1.

The results show that L-cysteine dissolved at different concentrations (1, 5, or 10 mM) together with the other compounds just described was oxidized to form cystine (0.01, 0.025, or 0.038 mM, respectively) in the absence of DTT. These concentrations of cystine formed correspond to 1.0, 0.5 and 0.38% of the cysteine concentrations given to the incubation mixture, respectively. This implies that the concentration of oxygen in the mixture was very limited under the conditions employed.

DTT at a concentration of 1 mM exhibited its significant effect to protect cysteine from oxidation; it reduced the production of cystine by approximately 50% when cysteine was incubated at 1 mM and at 5 mM. The effect of DTT (1 mM) was approximately the same as that observed when it was used at higher concentrations (5 mM and 10 mM).

It has been described that cystine is present in tissue cells of normal animals in a proportion of approximately 10% or less the amount of cysteine.[3,7-9] We could employ the procedure just described to precisely determine a small quantity of cystine existing together with large amounts of thiols after a preliminary experiment to check the approximate concentration of cysteine, from which we can estimate the required concentrations of NEM and homocysteine to be used. A specific determination of cystine has been reported, utilizing the specificity of the cystine-binding protein of *Escherichia coli*.[10] This is very powerful for special purposes to determine a very small amount of cystine. However, it is inconvenient for researchers to prepare the protein themselves. In comparison, the present procedure consumes much less time and is less expensive. The procedure could also be useful for diagnostic purposes, particularly in measuring the cystine/cysteine ratio in biological materials from patients with cystinurea and cystinosis.[9,11]

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