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RAPID COLORIMETRIC DETERMINATION OF REDUCED AND OXIDIZED GLUTATHIONE USING AN END-POINT COUPLED ENZYMATIC ASSAY*

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Abstract A simple and rapid colorimetric coupled enzymatic assay for the determination of glutathione is described. The proposed method is based on the specific reaction catalyzed by γ-glutamyltransferase, which transfers the γ-glutamyl moiety from glutathione to an acceptor, with the formation of the γ-glutamyl derivative of the acceptor and cysteinylglycine. The latter dipeptide is a substrate of leucyl aminopeptidase, which hydrolyzes cysteinylglycine to glycine and cysteine that can be easily measured spectrophotometrically. The proposed method was used to measure the content of glutathione in acid extracts of bovine lens, to follow the NADPH dependent reduction of GSSG to GSH catalyzed by the enzyme glutathione reductase and to determine the glutathione content in human astrocytoma ADF cells subjected to oxidative stress. The results obtained showed that the method can be suitably used for the determination of GSH and GSSG in different biological samples and to monitor tissue or cell redox status under different conditions. It is also applicable for following reactions involving GSH and/or GSSG.

Keywords: Glutathione, gamma-glutamyltransferase, lens, glutathione reductase, astrocytoma, oxidative stress

INTRODUCTION

Glutathione (L-gamma-glutamyl-L-cysteinylglycine) is a ubiquitous low molecular weight thiol present in high (mM range) concentrations in cells [1]. The presence of the sulfhydryl group makes glutathione a potent antioxidant, representing the main cellular defence against oxidative stress. Glutathione exerts its antioxidant action both by directly scavenging radicals and by acting as a substrate of antioxidant enzymes, such as glutathione peroxidase or glutathione S-transferases [1-2]. In addition, glutathione plays an important role in the detoxification of drugs, xenobiotics and their metabolites [3] and modulates important cell processes such as DNA and protein synthesis, amino acid transport and apoptosis [4]. Glutathione is present in cells in its reduced form (GSH) and possibly in different oxidized forms namely glutathione disulfide (GSSG) and glutathione mixed disulfides, both with low molecular weight thiols (i.e. Cys or Cys-Gly) and with proteins. In fact, the reversible S-glutathionylation of proteins under oxidative stress conditions has been proposed as a mechanism that allows the protection of proteins from irreversible oxidation [5]. Under physiological conditions, the concentration of GSH is about two orders of magnitude higher than the concentration of glutathione disulfide (GSSG). A decrease in GSH levels or an imbalance in the GSH:GSSG ratio have been found in several pathophysiological conditions, including Parkinson’s disease and other neurological disorders, diabetes and immunodeficiencies [6-8].

Several methods have been developed for the measurement of GSH and GSSG in biological samples. Separative methods, based on the use of HPLC [9-15], gas chromatography [16-17] or capillary electrophoresis [18-19], are sensitive and allow the simultaneous separation and quantification of GSH and GSSG in one single step. However, to increase the sensitivity, some of the separative methods require derivatization [9, 12, 15] or the use of electrochemical [10, 11, 14] or mass spectrometer detectors [13]. Besides requiring specific equipment, these
methods are generally time consuming and inadequate when many samples need to be analyzed. Non-separative methods, generally based on spectrophotometric or fluorimetric measurements of products formed in the reaction of GSH with thiol-reacting agents [20-22], are characterized by a good sensitivity, low cost and simplicity; however, they lack specificity since thiols other than GSH may react with thiol reagents.

Taking advantage of a method for the measurement of γ-glutamyltransferase activity using glutathione as substrate [23], in this paper we propose a new enzyme-based end-point colorimetric method for the specific determination of GSH and oxidized glutathione. Evidence is given for the suitability of the approach to specifically measure the glutathione content in biological samples.

MATERIALS AND METHODS

Materials

Reduced glutathione, glutathione disulfide, dithiothreitol (DTT), Gly-Gly, bovine serum albumin and glutathione reductase (EC 1.8.1.7; GSR) were purchased from Sigma Chemical Co. (St. Louis, MO). Centricon 10 microconcentrators were from Millipore (Billerica, MA). DEAE cellulose (DE-52) was obtained from Whatman (Maidstone, UK). Ninhydrin came from Merck (Darmstadt, Germany). All inorganic chemicals were of reagent grade from BDH (Poole, UK).

Calf eyes were obtained from freshly slaughtered animals at the local slaughterhouse and the lenses were kept frozen until used. Human astrocytoma (ADF) cells were kindly provided by Dr. W. Malorni, Istituto Superiore di Sanità, Rome, Italy.

Methods

Determination of reduced and oxidized glutathione.

The method is based on the specific reaction catalyzed by γ-glutamyltransferase (E.C. 2.3.2.2; γ-GT), which transfers the γ-glutamyl moiety from glutathione to an
acceptor, with the formation of the γ-glutamyl derivative of the acceptor and Cys-Gly. This dipeptide is readily hydrolized by leucyl-aminopeptidase (E.C. 3.4.11.1; LAP) [24] to form cysteine, which can then be easily measured using a colorimetric assay [25] (Scheme 1). Details on the purification of ancillary enzymes are reported in the Electronic Supplementary Material.

The standard incubation mixture (250 µL final volume) contains 8 mM MgCl₂, 0.2 mM MnCl₂, 40 mM Gly-Gly, 50 mU/mL γ-GT and 50 mU/mL LAP in 32 mM Tris HCl pH 8.5. The reaction is started by the addition of samples. After a 30-min incubation at 37°C, the reaction is stopped with the addition of 12.5 µL of 100% (w/v) trichloroacetic acid (TCA). The incubation mixture is then centrifuged at 12,000 xg for 1 min in a Beckman Microfuge E. The cysteine formed is evaluated spectrophotometrically. Specifically, an aliquot of 200 µL of the supernatant is added to 200 µL of glacial acetic acid and 200 µL of a reagent, prepared by dissolving 250 mg of ninhydrin in 10 mL of glacial acetic acid/4 M HCl (3:2). The mixture is placed in a boiling bath for 4 min. Under these conditions, cysteine, not other aminoacids, specifically reacts with ninhydrin to give a pink colored complex. After cooling on ice, 300 µL of the mixture are diluted with 300 µL of 95% ethanol and the absorbance at 560 nm measured. A blank obtained by adding TCA before the addition of the sample (zero time) is subtracted. In addition, a number of controls can be performed in order to take into account the presence of Cys and Cys-Gly in the samples to be analyzed. Any cysteine present in the samples can be accounted for by performing the spectrophotometric measurement without the addition of the enzymatic ancillary system. Similarly, the presence of Cys-Gly in the sample can be taken into account, once Cys is determined, by preincubating the sample in the presence of LAP alone, stopping the reaction by TCA, and then determining the produced Cys. It is worth noting however, that Cys and Cys-Gly are generally present at very low concentrations in the cell, compared with GSH and GSSG, thus in most cases they are negligible [26].

To measure the concentration of total glutathione equivalents (GSH plus GSSG plus glutathionyl-mixed disulfides), the reducing agent DTT is added to the
standard assay mixture at a final concentration of 2 mM, before performing the GSH assay described above. The concentration of oxidized glutathione in the presence of GSH can be evaluated by the difference between total glutathione and GSH. Oxidized glutathione can be more directly determined following the masking of GSH by preincubating the sample in the presence of 1 mM iodoacetamide at 45°C for 60 min. After incubation, the sample is added to the standard incubation mixture supplemented with 2 mM DTT for the reduction of oxidized glutathione, and the GSH level is then measured as above.

Other methods

γ-GT activity was measured as described [27]. LAP activity was measured as described [24]. One unit of enzyme activity is defined as the amount of enzyme able to generate one µmol of the product per min under the adopted assay conditions.

Protein content in the samples was measured according to Bradford [28], using bovine serum albumin as the standard reference protein.

Processing of biological samples to determine the glutathione content

Bovine lenses were suspended in approximately 5 vol of 200 mM KCl in 100 mM HCl and homogenized at 4°C in a Potter Elvehjem homogenizer. The homogenate was then centrifuged at 10,000 xg for 30 min, ultra filtered on Centricon 10, and the protein free ultra filtrate (lens acid extract) was used for the determination of glutathione. Storage of the lens acid extracts at -20°C for at least one week did not alter the concentration of glutathione.

ADF cells were cultured on plates of 10 cm in diameter with 10 mL of RPMI medium, supplemented with 10% fetal bovine serum (v/v), 2 mM glutamine and 50 mU/mL penicillin/streptomycin. They were then grown at 37 °C in a humidified atmosphere in the presence of 5% CO₂. Before the experiment, approximately 9 million ADF cells (125000/cm²) were transferred to Hank's balanced salt solution (HBSS) and subjected to oxidative
stress through three subsequent additions every 30 minutes of 200 µM H₂O₂.
After 4 h (at 37°C) from the last addition of H₂O₂, cells were withdrawn and lysed
by the freeze/thaw procedure. The suspension was centrifuged at 10,000 xg at 4°C
for 30 min and the supernatant was referred to as cell lysate.

RESULTS

Optimization of the glutathione assay conditions

In order to determine the incubation time and the concentrations of γ-GT and LAP
to be used in the assay, the time-dependent formation of cysteine from
 glutathione, catalyzed by the ancillary enzymes, was followed. This was done by
incubating GSH at four different concentrations (from 50 to 200 µM) in the
standard assay mixture in the presence of 4 mU/mL of both γ-GT and LAP. At
different times, aliquots were withdrawn and assayed for cysteine concentrations
(Fig. 1). After 60 min of incubation a complete transformation of GSH by the
ancillary system was observed for 50, 75 and 100 µM GSH, while at 200 µM the
trasformation was 90% complete. In order to reduce the risk of interference by
samples on the ancillary system, a 12-fold increase in the concentration of both
γ-GT and LAP (50 mU/mL) was adopted as standard assay conditions with 20
min of incubation. Under these conditions, a complete conversion of the standard
solutions of GSH, such as those reported in Fig. 1, was observed (data not shown).
To optimize the direct detection of oxidized glutathione in the presence of GSH,
the reduced sulfhydryls required masking before the reduction of disulfides. Both
iodoacetamide (IAM) and iodoacetic acetic (IAA), two carboxymethylating agents
of thiols, were confirmed not to have interfered with the enzyme ancillary system
and thus could be used to mask GSH. The complete removal of GSH was
observed after incubation in the presence of 1mM IAM at 45°C for 60 min. Under
the same conditions, only 80-90 % GSH masking was observed with IAA (data
not shown). As it had apparently proved to be the most efficient, IAM was
adopted as a blocking agent in this study.
When a mixture of GSH and GSSG both at 50 µM was assayed for GSSG concentration after GSH masking with IAM, GSSG was completely recovered. The GSSG concentration measured by three independent assays was 50.5 µM, coefficient of variation (CV) 4.8%. Note that an excess of IAM, which is left over after the GSH masking step, does not affect the subsequent determination of oxidized glutathione since it is rapidly removed by the excess of DTT present in the incubation mixture.

**Reference standard curves and assay validation**

For the construction of the reference standard curve for GSH measurements, standard GSH solutions (range 20-200 µM in MilliQ grade water) were assayed in triplicate using the procedure described above. Similarly, the reference standard curve for GSSG was obtained with GSSG standard solutions (20-160 µM in MilliQ grade water) analyzed in triplicate using the above procedure. The absorbance at 560 nm was plotted against the standard concentrations and the parameters of the standard curves were determined by linear regression analysis. A linear relationship between absorbance at 560 nm and GSH concentrations was obtained (Fig. 2, top). A typical calibration curve for GSH with 20 µM as the lower limit of quantitation (LLOQ) was defined by $y = 0.0025x$ ($r^2 = 0.996$). A similar linear relationship was observed for the standard curve obtained with GSSG solutions at different concentrations (Fig. 2, bottom). A typical calibration curve for GSSG with 20 µM as LLOQ was defined by $y = 0.0052x$ ($r^2 = 0.994$).

Calibration curves for GSH and GSSG were validated by determining the accuracy (% error) and precision (% CV) at three different concentrations (lowest, middle and highest) of the standard curves. The validation data are reported in Table 1. Both the accuracy and precision of the assays for the measurement of GSH and GSSG are well within the accepted range, which requires a CV and error <15% except for the LLOQ, where both CV and error should not exceed 20% [29].
Applications of the assay

The suitability of the proposed glutathione assay method was verified following a GSH/GSSG inter-conversion reaction and by measuring GSH and oxidized glutathione equivalents in two different biological samples.

*GSR catalyzed reduction of GSSG.* Fig. 3 reports both the GSSG and GSH concentrations at different times, following the NADPH-dependent reduction of GSSG catalyzed by GSR. In this case, reaction mixture aliquots were assayed for total glutathione and, following the GSH masking by IAM, for GSSG. With this approach it was possible to monitor the reaction by quantifying the substrate and product, thus enabling a comparison with the direct monitoring of NADPH oxidation at 340 nm.

*Glutathione levels in bovine lens extracts.* Four different lens acid extracts were analyzed in triplicate as described in the Methods, by measuring total soluble glutathione and soluble oxidized glutathione after the sulphhydryls had been masked with IAM. The concentrations of GSH (mean ± SD) were 0.77 ± 0.05, 0.75 ± 0.07, 0.78 ± 0.09 mM, 0.88 ± 0.04, respectively, while oxidized glutathione in all the samples was not detectable. These results account for a concentration of GSH (mean ± SEM) in the bovine lens of 7.7 ± 0.70µmol/g wet weight of lens and are consistent with previously reported data [18]. Attempts to reveal the presence in the sample extracts of either Cys or Cys-Gly failed (data not shown), confirming the rather low levels of these thiol compounds in the lens [30], at least within the detectability limits offered by the method.

The recoveries of GSH and GSSG from lens acid extract samples supplemented with either GSH or GSSG are reported in Table 2. After spiking three different lens acid extracts with 0.500 mM GSH, the mean (± SD) concentrations of the added GSH was 0.502 ± 0.020 (4% CV). Similarly, after spiking with 0.100 mM GSSG, the mean (± SD) concentrations of the added GSSG was 0.106 ± 0.002 (2% CV).

The intraday variability of the method was estimated on one lens extract, which was assayed twenty times using the same volume of extract (40 µL) in a final assay volume of 250 µL. The mean ± SD of GSH concentration and CV value
were $0.89 \pm 0.07$ mM ($8.9 \pm 0.75$ µmol/g wet weight of lens tissue) and 8.4%, respectively. Since GSSG was not detectable in the lens acid extract, the intraday variability of the method for GSSG determination was determined in an acid lens extract sample, spiked with 0.500 mM GSSG, which was assayed twenty times using 40 µL of the spiked sample in a final assay volume of 250 µL. The mean ± SD GSSG concentration and CV values were $0.520 \pm 0.047$ mM and 9.0%, respectively.

Glutathione levels in human ADF cell extracts. Finally, the proposed method was applied to determine the content of intracellular glutathione in a human astrocytoma cell line, subjected to an oxidative insult by exposure to H$_2$O$_2$ (see Methods). After H$_2$O$_2$ exposure, a decrease in the intracellular reduced glutathione content was observed in the cells subjected to oxidative stress, compared with cell control incubation (Fig.4, black bars). No appreciable increase in low molecular weight oxidized forms of glutathione was observed, as it appears evident from the comparison between reduced glutathione content (Fig.4, black bar) and the glutathione content measured in the supernatant after removal of proteins (Fig.4, dashed bar). However, following the oxidative treatment, a significant increase in glutathione-mixed disulfides with proteins occurred as it appears evident from the difference between total glutathione (Fig.4, white bars) and the glutathione content measured in the supernatant after removal of proteins (Fig.4, dashed bars).

DISCUSSION

GSH and oxidized glutathione (GSSG plus GS-mixed disulfides) concentrations and their ratios are important indexes of cell redox status. Therefore, methods that can specifically and accurately measure these metabolites in biological samples may be useful in studies in which cells, tissues and organs undergo oxidative stress. Our proposal for a new specific colorimetric method to measure glutathione concentrations in biological samples is aimed at the specific determination of the glutathionyl moiety of glutathione, both in its reduced state and when reversibly linked through a disulfide bond.
When compared to other non-separative methods based on spectrophotometric or fluorimetric measurements of products formed in the reaction of GSH with thiol-reacting agents [20-22], the proposed method is less sensitive but has the advantage of a greater specificity. This is a relevant aspect in the analysis of biological samples (e.g. cell lysates) that possibly contain thiols that can interfere with thiol-reacting agents. The specificity of the two reaction steps catalyzed by the enzymatic ancillary system together with the specificity of the cysteine determination enables both GSH and oxidized glutathione to be measured. The latter is defined as the equivalent of the glutathionyl moiety present in GSSG as well as in mixed disulfides with different low molecular weight thiols (i.e. Cys and Cys-Gly) and with proteins. Clearly, when the presence of Cys and Cys-Gly and their mixed disulfides is negligible, the method is useful for specifically evaluating the redox couple GSH/GSSG. This occurs in several cell systems and conditions [26, 30]. The presence of Cys and/or Cys-Gly, can be in any case tested by this method using appropriate controls performed without the addition of the ancillary system or by adding LAP alone.

The results of the method with different biological samples show its suitability in determining GSH and GSSG, and also in revealing different oxidized forms of glutathione (S-glutathionyl modified proteins). When applied to follow the NADPH dependent reduction of GSSG to GSH catalyzed by the enzyme glutathione reductase, the versatility of the method can be seen as it enables the time course of the reaction to be determined by measuring both substrate and product concentrations. This may be a significant feature in cases where the spectrophotometric enzyme assay is impaired by unfavourably high optical density values of cell extracts or by enzyme activities (i.e. NADPH oxidases) that may interfere with the measurement of the oxidation of NADPH at 340 nm.

An assay of GSH and oxidized glutathione in cell-free extracts of both bovine lens and human ADF cells, revealed how in these samples, soluble total glutathione is essentially found in a reduced state (i.e. GSH). Note that, on the basis of the results of spiked sample analysis performed on lens extracts, where added GSSG
was quantitatively recovered, the presence of any soluble oxidized glutathione fraction should be readily detectable.

No GSSG was detected in human ADF cells even following oxidative stress induced by hydrogen peroxide. In this case, however, the observed decrease in GSH values induced by the oxidative insult is paralleled by an easily detectable increase in the equivalent of oxidized glutathione associated with reversibly S-glutathionylated high molecular weight species (i.e. S-glutathionyl- modified proteins) (Fig. 4). As mentioned above, also in oxidatively stressed ADF cells, the levels of both reduced and oxidized Cys or Cys-Gly appear to be below the threshold of detectability using this method. Thus the generation of glutathione-proteins mixed disulfides appears to be the only pathway for this cell system to react to the adopted stress conditions and can be seen as a chance to counteract irreversible cell damage [5].

In conclusion, we have developed a new, simple and fast colorimetric enzyme-based end-point method to determine glutathione. The method is specific and accurate, and can be applied to follow the redox flux of glutathione in various biological samples. Work is in progress to adapt the method in order to devise a microtiter plate assay, which would allow the rapid simultaneous analysis of a large number of different samples.

ACKNOWLEDGEMENTS

We are indebted to Dr. G. Pasqualetti and Dr. R. Di Sacco (veterinary staff of Consorzio Macelli S. Miniato, Pisa) for their valuable co-operation in the bovine lens collection. Thanks are also due to Dr. Mariarosaria Loffreno for her helpful assistance in purifying γ-GT from bovine kidney.

REFERENCES

Table 1. Validation data of the assay for measurement of GSH and GSSG. The accuracy (% error) and precision (% coefficient of variation, CV) at three different concentrations (lowest, middle and highest) of the standard curve were determined.

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<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>Concentration measured (µM)</th>
<th>% error</th>
<th>% CV</th>
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<td>19.8</td>
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<tr>
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<tr>
<td>GSH</td>
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<td>3.8</td>
<td>6.5</td>
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<td>GSSG</td>
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<td>160</td>
<td>138.4</td>
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<td>7.3</td>
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Table 2. Recovery of GSH and GSSG from spiked bovine lens acid extracts.

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<td>GSSG</td>
<td>0.100</td>
<td>0.104</td>
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<tr>
<td>Lens extract 2</td>
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<td>0.493</td>
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<tr>
<td></td>
<td>GSSG</td>
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<td>0.107</td>
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<tr>
<td>Lens extract 3</td>
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<td>0.485</td>
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<tr>
<td></td>
<td>GSSG</td>
<td>0.100</td>
<td>0.106</td>
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FIGURE LEGENDS

Scheme 1 Principle of the method. γ-GT: γ-glutamyltransferase; LAP: leucyl-aminopeptidase

Fig. 1 Time course of cysteine production from GSH. The concentrations of cysteine formed from GSH are reported vs. the time of incubation. Four different concentrations of GSH were used: 50 µM (squares), 75 µM (circles), 100 µM (triangles) and 200 µM (diamonds)

Fig. 2 Calibration curves for GSH and GSSG. Calibration curves for GSH (top) and GSSG (bottom) were drawn assaying standard GSH solutions (range 20-200 µM) and standard GSSG solutions (range 20-160 µM) as described in the Materials and Methods. Each point is the average of three measurements. Error bars represent SD; when not visible, SD is within the symbol size

Fig. 3 Time course of the NADPH dependent reduction of GSSG catalyzed by glutathione reductase. GSH (circles) and GSSG (squares) concentrations were determined from the calibration curves of Fig. 2; NADPH concentration (triangles) was determined spectrophotometrically from the absorbance at 340 nm

Fig. 4 Effect of H₂O₂ exposure on the intracellular glutathione content of ADF cells. Human ADF cells were subjected to oxidative stress as described in the Materials and Methods. Control cells were incubated in the same conditions in the absence of H₂O₂. After incubation, cells were lysed and the lysates were divided into three aliquots. The first aliquot was immediately treated with DTT 2 mM for 30 minutes at 37°C prior to protein precipitation with 0.6M perchloric acid (PCA); after centrifugation at 10,000 xg for 5 minutes and neutralization with 0.35 M K₂CO₃, GSH was measured on the supernatant (total glutathione, white bars). The second aliquot was immediately treated with PCA for deproteinization; after centrifugation and neutralization with K₂CO₃, GSH was measured on the supernatant after reduction with DTT (total glutathione minus the glutathione involved in mixed disulfides with protein, dashed bars). The third aliquot was treated in the same manner of the second aliquot except that the reduction with DTT prior of GSH measurement was omitted (reduced glutathione, black bars). Error bars represent SEM (n=3)
\[ \gamma\text{-GT} \]

\[ \text{GSH} + \text{Gly-Gly} \rightarrow \gamma\text{-Glu-Gly-Gly} + \text{Cys-Gly} \]

\[ \text{LAP} \]

\[ \text{Cys-Gly} + \text{H}_2\text{O} \rightarrow \text{Cys} + \text{Gly} \]
Purification of ancillary enzymes

We purified in our lab the ancillary enzymes needed for the assay; the purification of both enzymes was very fast and did not require more than a couple of days.

γ-GT was partially purified from bovine kidney. The kidney was weighed and homogenized in 5 vol (w/v) of 50 mM Tris-HCl buffer (pH 8.0). After stirring in an ice-cold bath for 30 min, the suspension was centrifuged at 3,000 xg for 15 min at 4°C. The supernatant was ultracentrifuged at 100,000 xg for 60 min. The precipitate was collected and resuspended in 50 mM Tris-HCl buffer (pH 8.0) supplemented with 1% Triton X-100. After overnight stirring at 4°C, ammonium sulphate was added to a final 50% of salt saturation. After centrifugation at 10,000 xg for 15 min, the sediment was resuspended in 50 mM Tris-HCl buffer (pH 8.0) and stored at 4°C until use. The γ-GT preparation obtained as described above is devoid of Cys-Gly hydrolase activity and stable for at least two months.

LAP was partially purified from bovine lens. Lenses were weighed and suspended in 5 vol (w/v) of 10 mM sodium phosphate buffer (pH 7.0) supplemented with 5 mM DTT, and stirred in an ice-cold bath for 45 min. The suspension was then centrifuged at 10,000 xg for 40 min at 4°C. The supernatant was added to 0.25 vol of DE-52 equilibrated with 10 mM sodium phosphate buffer pH 7.0 supplemented with 2 mM DTT, and stirred in an ice-cold bath. After 45 min the resin was filtered through a Buchner funnel, and the filtrate containing the Cys-Gly hydrolase activity was used for further purification. An equal volume of a mixture of chloroform/octanol (95:5) was added to the filtrate and the mixture was vigorously shaken at room temperature for 10 min. The emulsion was then centrifuged at 250 xg for 3 min and the supernatant, containing the enzyme activity, was collected by suction. Chloroform/octanol treatment was repeated until a little emulsion had formed. An amount of 2.5 vols of ice cold ethanol were slowly added to the supernatant resulting from the chloroform/octanol treatment. After 30 min stirring and subsequent centrifugation at 17,500 xg for 20 min, the precipitate was resuspended in 50 mM Tris-HCl buffer (pH 8.0) and stored at 4°C.
until use. The LAP preparation obtained as described above is devoid of any γ-glutamyltransferase activity and stable for at least two months.