ANALYTICAL SCIENCES AUGUST 2016, VOL. 32

Colorimetric Coupled Enzyme Assay for Cystathionine β-Synthase

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A colorimetric coupled enzyme assay for the determination of cystathionine β-synthase activity is described. The method exploits cystathionine γ-lyase as an ancillary enzyme capable of transforming cystathionine, produced by cystathionine β-synthase, into cysteine. The cysteine is then spectrophotometrically detected at 560 nm, after its specific complexation with ninhydrin. This method was used to detect cystathionine β-synthase in crude extracts, and for the kinetic characterization of the enzyme partially purified from bovine kidney. A rapid two-step protocol is described for the partial purification of cystathionine γ-lyase from bovine kidney, aimed at a suitable and stable ancillary enzyme preparation.

Keywords Cystathionine β-synthase, cystathionine γ-lyase

(Received April 7, 2016; Accepted May 9, 2016; Published August 10, 2016)

Introduction

Cystathionine β-synthase (E.C. 4.2.1.22) (CBS)[1] catalyzes the condensation of L-serine and L-homocysteine (HCys) to generate L,L-cystathionine (Cyt) in the first reaction of the transsulfuration pathway, which is an important route in sulfur metabolism. This pathway is completed by a second reaction, catalyzed by cystathionine γ-lyase (E.C. 4.4.1.1)[2] (CGL), in which cystathionine is transformed into L-cysteine (Cys). The transsulfuration pathway plays an important role in antioxidant cell defence, by providing Cys for glutathione synthesis. In mammalian liver, approximately 50% of the cysteine present in the glutathione pool is derived from HCys through transsulfuration pathway reactions.[3,4] Indeed, transgenic mice knock out for CBS show a significant decrease in the glutathione levels in the brain and liver, with an impairment of the antioxidant ability of these organs.[5] Decreased levels of CBS activity, due to mutations in the CBS gene, are also considered to be the main cause of homocystinuria, an inherited disease characterized by increased concentrations of HCys in plasma and urine.[6] Patients with severe CBS deficiency suffer from lens and skeleton abnormalities, mental disability and vascular dysfunctions. Individuals who are heterozygous for CBS deficiency may only display increased HCys plasmatic levels which, however, are considered to be a risk factor for cardiovascular diseases.[7-10] CBS activity also contributes to the genesis of hydrogen sulfide, a signaling molecule involved in the regulation of several processes, including inflammation, vasodilatation, and apoptosis.[11]

Mammalian CBS is unique in possessing both heme and pyridoxal phosphate (PLP).[12] The heme is located in a N-terminal domain, which, together with a C-terminal regulatory domain interacting with the allosteric effector S-adenosyl methionine (SAM), delimits a catalytic core containing PLP.[13] It has been postulated that the redox status of the heme cofactor may act as a sensor able to link the activity of the transsulfuration pathway with the glutathione levels.[14]

CBS has been most frequently assayed using radioactively labeled serine and measuring the produced labeled Cyt after the separation of products and reagents by different chromatographic techniques, including ion exchange, thin-layer and paper chromatography.[15-19] An assay based on the measurements of labeled water released from tritiated serine has been also proposed.[20] However, despite the sensitivity of these methods, assays using radiolabeled reagents are expensive and laborious. Comparable sensitivity is offered by methods that rely on the determination of Cyt by various mass-spectrometry approaches,[21-23] or by an RP-HPLC technique.[24] More friendly, though less sensitive, spectrophotometric methods have also been proposed, based on either time-point[25,26] or continuous determination of the reaction products. In the latter case, ancillary systems composed of a different number of enzymes have been proposed, which all end with an NADH-dependent reaction.[27,28] Although these latter methods have been adopted for characterization studies on purified CBS, no information is available on their suitability for the detection of CBS activity in crude extracts. Possible limitations in their use may derive from interferences in monitoring the NADH-dependent reaction at 340 nm. Moreover, the possible presence of CGL in the extracts strictly requires one to inhibit this activity in order to preserve Cyt produced by CBS during the assay. In any case, evaluations of CBS activity levels in different tissues and organs have usually been performed with radio isotopic assays.

Here, a new spectrophotometric assay for CBS is proposed, for detecting the enzyme in crude extracts. The assay is based on the colorimetric determination of cysteine produced by the use of CGL as an ancillary enzyme.

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Experimental

Materials

D,L- and L-HCys, Cys, L-serine, Cyt, bovine serum albumin, PLP and D,L-dithiothreitol (DTT) were supplied by Sigma-Aldrich. DEAE 23 SS was from Servacell. Butyl Sepharose 4 Fast Flow was from GE Healthcare. All inorganic chemicals were of reagent grade from BDH Chemicals. Human astrocytoma (ADF) cells were kindly provided by Dr. W. Malorni, Istituto Superiore di Sanità, Rome, Italy. Bovine kidneys were removed from freshly slaughtered animals and stored at –80°C until used. Wistar male rat organs were kindly provided by Dr. M. Cammalleri, Department of Biology, University of Pisa. The organs were removed immediately after the animals had been sacrificed. The organs were then stored at –80°C until use.

Cysteine determination

Cysteine determination was performed essentially according to Gaitonde.[29] Solutions (100 µL) containing Cys were added to 100 µL of glacial acetic acid and 100 µL of a reagent prepared by dissolving 25 mg/mL of solid ninhydrin in a solution of glacial acetic acid and 38% (w/w) HCl (3:2). The mixture was placed in a boiling bath for 4 min. After cooling on ice for 1 min, the mixture was diluted 1:1 with cold 95% ethanol and the absorbance at 560 nm was read within 5 min on a Beckman DU-7 spectrophotometer against a sample (blank) without Cys. For the complex cysteine-ninhydrin, an extinction coefficient at 560 nm of 28.74 ± 0.21 mM^{-1} cm^{-1} was used. This value was derived from a calibration curve obtained using standard solutions of Cys (each assayed in quadruplicate) in the concentrations range 10 - 160 µM, in the presence of all the components of the CBS assay (see below). A typical calibration curve with 10 µM as the lower limit of quantitation (LLOQ) was defined by $y = 0.0052x$ ($r^2 = 0.9987$). The calibration curve was validated by determining the accuracy (% error) and precision (% CV) at three different concentrations (10, 80 and 160 µM) of the standard curve. The accuracy and precision were 10.2 and 0.9% at the lowest Cys concentration, 6.5 and 1.5% at the middle concentration and 0.5 and 1.3% at the highest concentration. Both the accuracy and precision of the assay are well within the accepted range, which requires a CV and error of <15%, except for the LLOQ, where both the CV and error should not exceed 20%.[30] The concentration of Cys in the reference solution was estimated by the Ellman assay;[31] the obtained value was consistent with the nominal value obtained by weight.

Determination of CGL activity

The CGL activity was determined using Cyt as a substrate according to Heinonen[32] with minor modifications, by measuring Cys, as described above. The assay mixture contained 3 mM Cyt, 0.05 mM PLP, 1 mM DTT and 50 mM sodium phosphate buffer pH 8.0 (S-buffer). The mixture was incubated at 37°C. At different times (from 0 to 60 min) aliquots were withdrawn and the reaction was stopped by adding 5% trichloro acetic acid (TCA) followed by centrifugation at 8000*g* for 5 min. Aliquots of the supernatant (100 µL) were used to detect Cys. The absorbance at 560 nm of a sample (blank) obtained from an assay mixture that was quenched immediately after the addition of proteins was subtracted. One unit of CGL activity is the amount of enzyme that catalyzes the production of 1 µmol of Cys per min under the above-mentioned assay conditions.

Preparation of bovine kidney crude extract

Bovine kidney was suspended (0.5 g/mL) in 10 mM potassium phosphate pH 7.0 containing 20 µM PLP and 10 mM EDTA (extraction buffer) and homogenized at 4°C with a blender. The suspension was then centrifuged at 4°C at 10000*g* for 30 min, and the pellet was discarded. This operation was repeated five times in order to completely remove any insoluble material. After filtration through a plug of glass wool and an overnight dialysis against 10 mM sodium phosphate pH 8.0 containing 1 mM EDTA and 5 µM PLP (dialysis buffer), the obtained supernatant was referred to as the "kidney crude extract".

Partial purification of CGL from bovine kidney

All operations were performed at 4°C and all centrifugation steps were performed at 10000*g* for 30 min. Solid ammonium sulfate was added to the kidney crude extract under continuous stirring until 15% saturation was reached. The suspension was allowed to equilibrate for 30 min, centrifuged and the pellet discarded. The resulting supernatant was brought to 45% saturation with additional ammonium sulfate, allowed to equilibrate for 30 min and then centrifuged. The 45% saturation pellet was used for the purification of CBS activity (see below). The 45% saturation supernatant was brought to 65% saturation, stirred for 30 min and then centrifuged. The precipitate, containing CGL activity, was dissolved in a minimum volume of extraction buffer and dialyzed overnight against the dialysis buffer. The sample was then added to 30 g of DEAE 23 SS resin, previously equilibrated with 10 mM potassium phosphate pH 7.7 (elution buffer). The suspension was gently stirred for 1 h. The resin was then transferred into a chromatographic column (30 × 3.5 cm), and after packing the resin was eluted at a flow rate of 45 mL/h using the elution buffer supplemented with 30 mM NaCl, until the A_{280} dropped to the baseline. The enzyme was then eluted by applying the elution buffer containing 100 mM NaCl. The fractions containing CGL activity were pooled, dialyzed against the dialysis buffer and concentrated to 7 mg protein/mL. The CGL preparation (20 U/mg) was stable for at least one year at –80°C. No changes in the enzyme activity were observed in the same protein sample after six freezing-thawing cycles.

Determination of CBS activity

The standard mixture for CBS assay contained, in the S-buffer, 2 mM D,L-HCys, 10 mM L-serine, 0.38 mM SAM, 0.25 mM PLP, 1 mM DTT and 18 mU/mL of partially purified CGL from bovine kidney. See Results and Discussion for details on how these conditions were defined.

Partial purification of CBS from bovine kidney

A 45% ammonium sulfate saturation pellet (see above), in which almost no CGL activity was detectable, was dialyzed overnight against the dialysis buffer. The solution was supplemented with 0.6 M ammonium sulfate and then applied to a Butyl Sepharose 4 Fast Flow column (1.5 × 9 cm), previously equilibrated with an elution buffer containing 0.6 M ammonium sulfate. The column was eluted at 45 mL/h with the same buffer until the A_{280} reached the baseline. The elution buffer supplemented with 0.4 M ammonium sulfate was then applied. When the A_{280} reached the baseline, the column was washed with the elution buffer in order to elute CBS. The fractions containing CBS activity were pooled, dialyzed against the dialysis buffer, concentrated to 5 mg/mL and stored at –80°C.

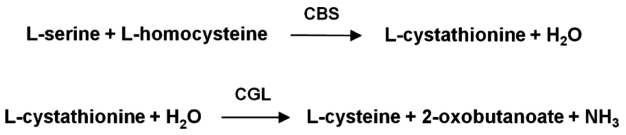

Fig. 1 Principle of the proposed assay.

Other methods

The protein concentration was determined according to Bradford,[33] using bovine serum albumin as a standard. ADF cells were cultured as previously described.[34] Cell lysates were obtained through a freezing and thawing protocol, followed by a 10000*g* centrifugation at 4°C for 30 min. The supernatant, after overnight dialysis against the dialysis buffer, was referred to as the ADF crude extract (2 mg protein/mL). Rat testis and lung were homogenized in sodium phosphate 10 mM pH 7.0 containing 2 mM DTT by a Potter-Elvehjem homogenizer, and the obtained suspensions were centrifuged at 10000*g* at 4°C for 30 min. After overnight dialysis against the dialysis buffer, the supernatants were referred to as testis and lung crude extracts (2 mg protein/mL each). Kinetic parameters were obtained from non-linear regression analysis of kinetic measurements using Graph Pad software.

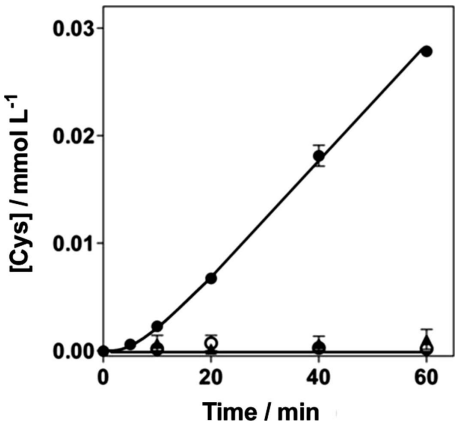

Fig. 2 Time course of cysteine formation from cystathionine through the sequential action of CBS and CGL. The partial purified bovine kidney CBS preparation (2.98 ± 0.11 mU/mg) devoid of CGL activity was incubated (0.17 mg/mL) under the standard assay conditions, and the formed Cys was measured at different times. Open circles and closed triangles refer to an incubation performed in the absence of either CBS or CGL, respectively. Error bars (when not visible are within the symbol size) represent the standard deviation from three independent measurements.

Results and Discussion

Conditions for the assay of CBS

The proposed method for measuring the activity of CBS acting on its physiological substrates HCys and serine, is based on the use of CGL as ancillary enzyme. Cystathionine, the CBS reaction product, was in fact transformed by CGL into Cys, which was then spectrophotometrically detected (see Fig. 1).

The standard mixture for CBS assay contained, in S-buffer, 2 mM D,L-HCys, 10 mM L-serine, 0.38 mM SAM, 0.25 mM PLP, 1 mM DTT and 18 mU/mL of partially purified CGL from bovine kidney. The presence of DTT allowed us to protect Cys from oxidation for at least 1 h at 37°C. After 3 min of pre-incubation at 37°C, the reaction was started by the simultaneous addition of the sample and of the ancillary enzyme. The mixture was then incubated at 37°C. At different times (from zero to 60 min) 120 µL aliquots were withdrawn, the reaction was quenched by adding 5% TCA, and Cys was measured in the supernatant obtained after centrifugation at 8000*g* for 5 min, as described in the Experimental section. Under these conditions (standard assay conditions) one Unit of CBS is defined as the amount of enzyme that catalyzes the production of 1 µmol of Cys per min.

The formation of Cys generated in the coupled assay (Fig. 2) appeared to be linear, after an initial lag phase, from 10 to at least 60 min of incubation. It is worth noting that no Cys formation was observed in the absence of the CBS sample, thus confirming the adequacy of the purification steps for CGL in providing a well performing ancillary system.

Being the lag phase conceivably associated to the attainment of the overall steady state condition in the coupled enzyme system, 20 min of incubation was adopted as a safe starting reference time for the assay, and all the measurements were conducted in the time range of 20 - 60 min.

Care was taken in defining the concentration of the ancillary enzyme suitable for an accurate determination of CBS activity. Different amounts of the partially purified CBS preparation and different amounts of the ancillary enzyme were used to define the optimal assay conditions. The CBS activity detectable using different levels of ancillary enzymatic activity (from zero to 65 mU/mL of CGL in the assay mixture) is reported in Fig. 3A. No differences in CBS activity were observed when CGL activity was higher than 18 mU/mL. This enzymatic activity, combined with the starting reference time of the assay of 20 min was adopted in order to define the standard assay conditions. The linear relationship between the CBS activity, ranging from 0.15 to 1.45 mU/mL, and the amount of CBS sample in the assay (Fig. 3B) confirms the adequacy of the adopted conditions (*i.e.* interval time and ancillary enzyme level) to perform the assay.

It has been reported that the human recombinant CGL is able to catalyze the transformation of HCys through either α,γ-elimination or γ-replacements reactions.[35] The occurrence of these reactions in our CBS assay mixture would lead to a consumption of HCys, thus affecting the measurement of CBS activity. This interference would be especially critical when the assay is used for the kinetic characterization of CBS. However, when the assay mixture containing 18 mU/mL of the ancillary CGL preparation here described was pre-incubated at 37°C for up to 60 min with either 5 mM or 0.18 mM L-HCys, before the addition of CBS, no differences were observed compared to an assay performed without any pre-incubation.

Application of the method

The proposed method was applied to evaluate CBS activity in a bovine kidney extract. An enzyme content of 0.65 ± 0.02 mU/mg of protein (mean ± SD from three independent measurements) was obtained. The intraday variability of the method was estimated by assaying the same bovine kidney extract 20 times using the same volume of extract (60 µL) in a final assay volume of 375 µL. The mean + SD of CBS activity and the CV value were 0.63 ± 0.02 mU/mg of protein and 3.2%, respectively. For evaluating the interday variability of this method, different aliquots of the same bovine kidney extract frozen at –20°C were thawed and assayed for 20 consecutive days.

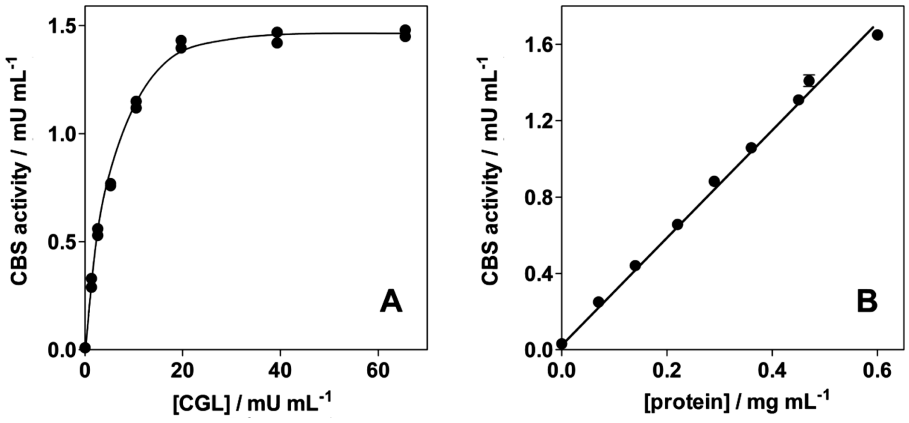

Fig. 3 Optimization of assay conditions. Panel A: The CBS reaction rate was measured using the proposed assay under the standard conditions with different concentrations of the ancillary enzyme. The final protein concentration of the CBS preparation in the assay was 0.5 mg/mL. Panel B: Different amounts of partially purified CBS were assayed in standard conditions. The measured activity is reported as a function of the protein content of the CBS sample. Error bars (when not visible are within the symbol size) represent the standard deviation from three independent measurements.

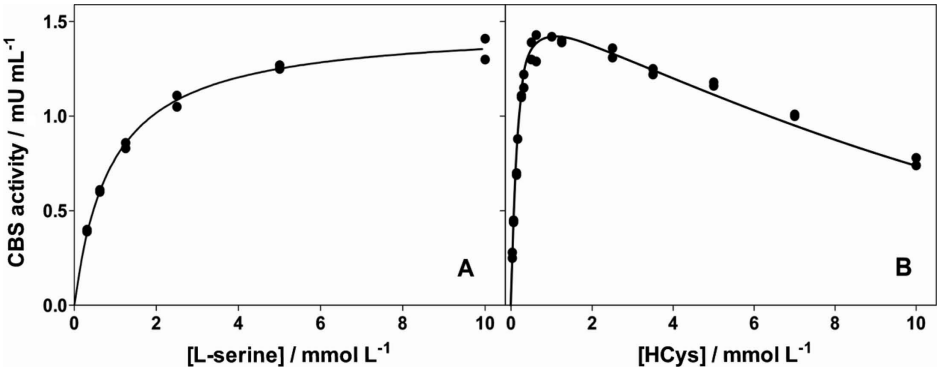

Fig. 4 Dependence of CBS reaction rate on the substrate concentration. The CBS reaction rate was measured using the proposed assay with different concentrations of either L-serine (Panel A) or L-HCys (Panel B). All the assays were performed under standard conditions using the partial purified bovine kidney CBS preparation (0.6 mg/mL protein final concentration in the assay). The data obtained for L-HCys concentrations above 1 mM were not considered for determining the kinetic parameters.

The mean + SD of CBS activity and CV value were 0.59 ± 0.05 mU/mg of protein and 8.5%, respectively. No significant differences were observed between the CBS activity measured on the first day and activity measured after 20 days.

Using the same methodological approach adopted to measure the CBS activity in the bovine kidney extract, other crude extracts from both cultured cells and rat tissues were assayed for CBS. When analyzed for CBS activity, crude extracts from ADF cells, as well as extracts from rat testis and lung revealed a linear progression of the reaction over time, which allowed us to determine a CBS content of 0.56 ± 0.01, 0.40 ± 0.01 and 0.31 ± 0.01 mU/mg of protein, respectively.

The proposed assay method was used for the kinetic characterization of CBS in the partially purified enzyme preparation of bovine kidney. Figure 4 gives the reaction rates measured at different concentrations of either L-serine or L-HCys. In the case of L-serine (Fig. 4A), a K_M of 0.90 ± 0.04 mM (mean ± SE) was obtained, a value which is comparable to those measured for other mammalian CBS,[19,36,37] and for the yeast recombinant enzyme.[27] In the case of L-HCys, an inhibition by substrate was observed when the level of L-HCys was increased to above 2 mM (Fig. 4B). This effect has already been reported for CBS,[17,27,36] which may in part explain the wide range of K_M values for HCys, comprised between 0.08 and 20 mM, reported in the literature for the mammalian enzyme.[8,14,25,27,38,39] In this regard, also the presence of SAM has been demonstrated to strongly affect the K_M values.[39] Also CGL is inhibited by HCys (60% residual activity with 10 mM L-HCys and 2.8 mU/mL of CGL). However, the observed inhibition is not sufficient to make the adopted ancillary system limiting in the assay. In fact, the dependence of the CBS reaction rate on the CGL concentration observed when the assay was conducted in the presence of 10 mM D,L-HCys could be superimposed over that reported in Fig. 3A, in which D,L-HCys was kept at 2 mM (data not shown).

Two different protein concentrations in the assay mixture, *i.e.* 0.30 (data not shown) and 0.60 mg/mL (Fig. 4B), were used for the kinetic analysis. While a two-fold increase in V_{max} was observed (0.88 ± 0.02 and 1.72 ± 0.05 μmol L^{-1} min^{-1}), the K_M values obtained considering substrate concentrations not higher than 1 mM were, as expected, not significantly different, *i.e.* 0.13 ± 0.01 and 0.16 ± 0.02 mM.

Finally, when D,L-HCys was used as a substrate, while V_{max} (measured using a protein concentration of 0.6 mg/mL) remained unchanged compared to that measured using L-HCys, an approximately two-fold increase in K_M (0.30 ± 0.03 mM) was obtained. This confirms the stereo specificity of the enzyme,[27] which only recognizes the L-enantiomer.

It may be worth comparing the present coupled enzyme assay with a previously published end-point assay method, in which the product of the CBS catalyzed reaction, Cyt, was directly measured at 453 nm after complexation with ninhydrin.[25] The advantage of this method to directly quantitate Cyt is, however, greatly overcome by a lower sensitivity with respect to our proposed method. In fact, the ratio between the extinction of the Cys-ninhydrin complex (ε_{560}) and the extinction coefficient of Cyt-ninhydrin complex (ε_{453}) ranges from approximately 17[25,29] to 24, referring to the ε values coming from our measurements (ε_{560} = 28.74 ± 0.21 mM^{-1} cm^{-1}; ε_{453} = 1.21 ± 0.01 mM^{-1} cm^{-1}). Moreover, the direct evaluation of Cyt as a means to assay CBS in the presence of enzymes such as CGL or Cyt-β-lyase, possibly present in crude extracts preparations,[40] would require us to supplement the assay mixture of enzymes inhibitors. In this regard, propargyl glycine and copper sulfate have been used as inhibitors of CGL; however, while propargyl glycine may interfere, contributing, even though modestly, to the 453 nm absorbance of the Cyt-ninhydrin complex (unpublished results), copper sulfate, more generally, is a source of ionic species certainly non recommendable in enzyme assays, especially when thiol carrying molecules are around.[41,42] Such problems are avoided in the proposed method.

Conclusion

In conclusion, a spectrophotometric coupled enzyme assay for the determination of cystathionine β-synthase activity was optimized, based on the use of cystathionine γ-lyase as an ancillary enzyme. The method appears to be a simple and suitable means both for evaluating the activity of cystathionine β-synthase in crude extracts and for a kinetic characterization of the enzyme.

Acknowledgements

This work was supported in part by the Ministero Istruzione, Università, Ricerca, PRIN Project 2009M8FKBB_003 and in part by Pisa University. We are indebted to Dr. G. Pasqualetti and Dr. R. Di Sacco (veterinary staff of Consorzio Macelli S. Miniato, Pisa) for their valuable operation in the bovine kidney collection.

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