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Note

Gas chromatographic assay for cysteine sulphinate decarboxylase activity in animal tissues

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Cysteine sulphinate decarboxylase (CSD; EC 4.1.1.29) catalyses the decarboxylation of cysteine sulphinic acid (CSA) and cysteic acid to hypotaurine and taurine, respectively [1–3]. CSD has been partially purified from rat liver [3, 5] and dog liver [4], and it requires pyridoxal 5′-phosphate (PLP) and free thiol groups for its maximum activity [1–5]. Although CSD is a key enzyme in taurine biosynthesis, it is absent from mammalian heart containing a high level of taurine [1, 2]. Its activity decreases rapidly in rats fed diets deficient in vitamin B_6 [6] and increases during development [7]. The change of CSD activity is also known to be related to the neuro function [8] and the etiology of essential hypertension [9].

The determination of CSD activity has been carried out by manometric assay [5, 10] of carbon dioxide formed and by radiometric assays [11–15] of [^{14}C]carbon dioxide or [^{35}S]taurine and [^{35}S]hypotaurine. However, the manometric assay is insensitive and non-specific [11]. On the other hand, the radiometric assays are specific and sensitive, but the radioactive substrates are expensive and not easy to prepare.

Recently, we reported a specific and reliable method for the determination of hypotaurine [16] by gas chromatography with hydrogen flame ionization detection (GC–FID) in which hypotaurine was analysed as its volatile derivative, 2-(isobutoxycarbonylamino)-N,N-dibutylethanesulphonamide. In the present paper, we describe a specific assay for CSD activity based on the use of the GC method. By using the new assay method, the distribution of CSD activity in various animal tissues was also studied.

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EXPERIMENTAL

Chemicals

Hypotaurine, CSA and 3-aminopropanesulphonic acid (APS) as an internal standard (I.S.) were purchased from Sigma (St. Louis, MO, U.S.A.). These compounds were dissolved in distilled water at an adequate concentration and stored at 4°C. PLP was obtained from Nakarai Chemicals (Kyoto, Japan). 2-Mercaptoethanol and isobutyl chloroformate (isoBCF) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Cetyldimethylbenzylammonium chloride (CDMBA-Cl, Tokyo Kasei Kogyo) was dissolved in methanol at a concentration of 10%. Methylene blue was purchased from Wako (Osaka, Japan). All other chemicals were of analytical grade.

Preparation of enzyme source

The tissues of oxen (2)*, pigs (3)* and chickens (5)* were kindly supplied from local slaughterhouses. The tissues of the following animals were obtained by dissecting these animals in this laboratory: dogs (2)*, rabbits (2)*, Wistar rats (4)*, ICR mice (8)*, mackerels (5)*, octopuses (10)* and cuttlefishes (3)*. Each tissue was homogenized in 5 volumes of ice-cold 0.25 *M* sucrose for 10 min in a Waring blender, and then the homogenate was centrifuged at 12 000 *g* for 30 min at 4°C. The supernatant (0.4 ml) was applied to a Sephadex G-25 column (5.0 cm×0.9 cm I.D.), and the column was eluted with 10 m*M* phosphate buffer (pH 6.8) at a flow-rate of 20 ml/h. The initial 1.6 ml of the eluate was discarded, and the following 2.4 ml of the eluate were collected as the CSD fraction. Aliquots of this fraction were used for enzymic analysis. The protein concentration was measured by the method of Lowry et al. [17], as modified by Miller [18], with bovine serum albumin as the standard.

Assay of CSD

The reaction mixture contained 0.5 μmol of CSA, 0.2 μmol of PLP, 4 μmol of 2-mercaptoethanol, 100 μmol of phosphate buffer (pH 7.2) and 0.1 μmol of APS (I.S.) in a total volume of 0.8 ml. After preincubation of this mixture at 37°C for 5 min, the assay was started with the addition of the enzyme solution (CSD fraction, containing 0–2 mg of protein). After incubation at 37°C for 30–90 min, the reaction was terminated with 0.4 ml of 0.15 *M* sulphuric acid, and then the acidified mixture was deproteinized by heating in a boiling water-bath for 3 min. After centrifugation at 2000 *g* for 5 min, the protein-free supernatant was used for derivatization of hypotaurine, and the concentration of the products was determined by the GC method described below. A tube that was not incubated served as the blank, and the blank value was substracted from the value of the incubated sample.

*The figures in parentheses show the number of animals used.

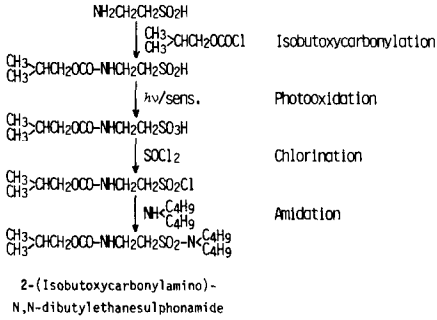

Fig. 1. Derivatization process of hypotaurine.

Derivatization procedure

Hypotaurine was converted into its volatile derivative, 2-(isobutoxycarbonylamino)-N,N-dibutylethanesulphonamide, by the following procedure (see Fig. 1), which is a modification of the previously reported method [16]. An aliquot of the sample solution containing 5–500 nmol of hypotaurine was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap, and the solution was adjusted to pH 10 with 2 *M* sodium hydroxide. Immediately after addition of 0.1 ml of isoBCF, the mixture was shaken with a shaker set at 300 rpm (up and down) for 5 min at room temperature. After washing with 3 ml of diethyl ether, the reaction mixture was adjusted to pH 1–2 with 2 *M* hydrochloric acid, and then 0.5 ml of 0.5 m*M* methylene blue were added. The mixture was irradiated with a 300-W tungsten lamp (Toshiba, Tokyo, Japan) for 5 min from a distance of 20 cm. The reaction mixture was washed twice with 3 ml of diethyl ether. Subsequently, 0.05 ml of 10% CDMBA-Cl and 2 ml of methylene chloride were added to the aqueous layer, and the tube was shaken for 3 min at room temperature. After centrifugation at 2000 *g* for 1 min, the organic layer was transferred to another tube and the solvent was evaporated to dryness at 60 °C under a stream of dry air in a fume-hood. To the residue were added 0.2 ml of thionyl chloride, and the tube was heated at 80 °C for 15 min. The excess thionyl chloride was removed at 80 °C under a stream of dry air in a fume-hood. To the residue were added 0.2 ml of 2 *M* di-*n*-butylamine in acetonitrile, and the mixture was allowed to stand for 5 min at room temperature. The reaction mixture was acidified with 1 ml of 20% phosphoric acid and then extracted twice with 3 ml of *n*-pentane. The solvent was evaporated to dryness, and the residue was dissolved in 0.1 ml of ethyl acetate. Then 2–4 μl of this solution were injected into the gas chromatograph.

Gas chromatography

A Shimadzu 4CM gas chromatograph equipped with a hydrogen flame ionization detector was used. The column packing, 5% SE-54 on 100–120 mesh Uniport HP (Gasukuro Kogyo, Tokyo, Japan), was prepared using toluene as coating solvent according to the solution coating technique [19], and was poured into a silanized glass column (1.0 m×3 mm I.D.). The operating conditions were as follows: oven temperature, 235 °C; injection and detector temperature, 285 °C; nitrogen flow-rate, 50 ml/min. The peak heights of hypotaurine and the I.S. were

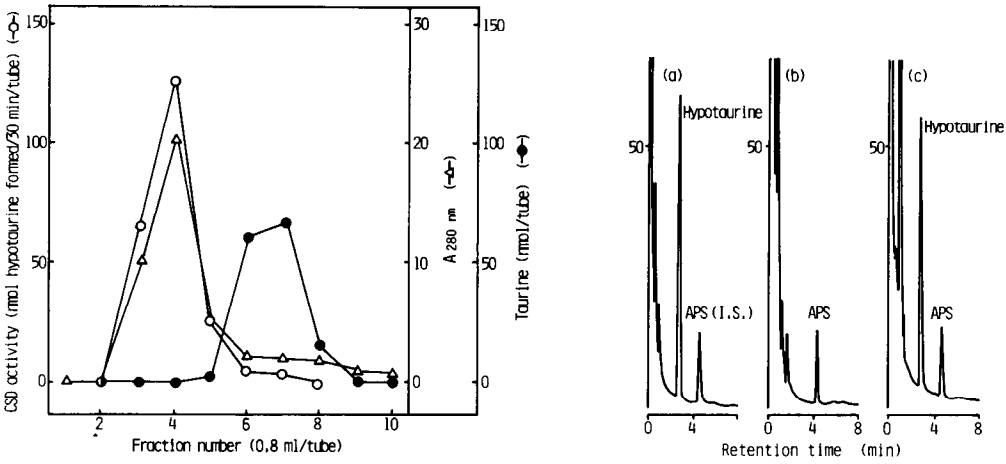

Fig. 2. Column chromatography of rat liver extract in Sephadex G-25; 0.4 ml of the supernatant of tissue homogenate were applied to the column (5.0 cm×0.9 cm I.D.), and elution was performed with 10 m*M* phosphate buffer (pH 6.8) at a flow-rate of 20 ml/h.

Fig. 3. Gas chromatograms obtained from (a) a standard solution containing 200 nmol of hypotaurine, and by the assay of CSD in rat liver incubated at (b) zero time and (c) 30 min. GC conditions are given in Experimental.

measured and the peak-height ratios were calculated. The hypotaurine was quantified by comparison with the calibration curve.

RESULTS AND DISCUSSION

Tissue extracts were prepared by the procedure of Jacobsen et al. [1] using 0.25 *M* sucrose. It was found that the crude extract contained appreciable amounts of taurine, which rendered it difficult to perform accurate assay of low levels of enzyme activity by the present method. However, this problem was solved by the introduction of a gel permeation treatment. As shown in Fig. 2, the endogenous taurine was almost completely removed from enzyme protein fraction (fraction Nos. 3–5). The process was complete within 15 min, and no substantial loss of enzyme activity due to this treatment was observed. Recovery of CSD activity in this fraction was above 97%. The enzyme solution eluted from the gel column was stable for at least one week with little loss of activity when stored at −20°C.

Typical gas chromatograms obtained from standard solution and by the assay of CSD in rat liver are shown in Fig. 3. Each derivative of hypotaurine and APS (I.S.) was eluted as a single and symmetrical peak, and no interfering peak was observed. The recoveries of hypotaurine added to the assay mixture at 10–100 nmol were 92.9–103.7%, and the corresponding relative standard deviations (R.S.D.) were 1.0–4.7% ($n=3$). The detection limit of this method was 5 nmol of hypotaurine formed.

Using rat liver enzyme, the effect of pH, incubation time and of the amount of enzyme on the hypotaurine formation were studied, and the results are summa-

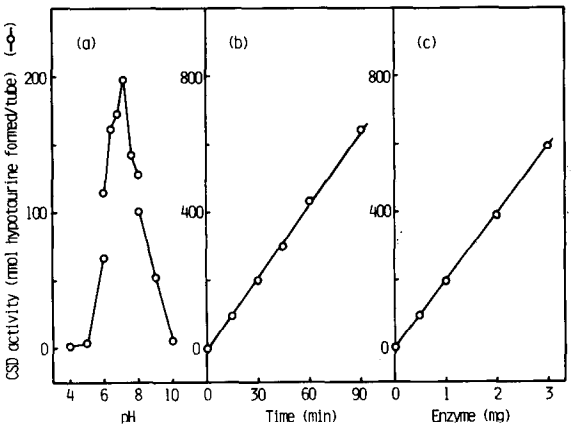

Fig. 4. Effects of (a) reaction pH, (b) incubation time and (c) amounts of enzyme on the reaction rate of CSD. The rat liver extract obtained by gel permeation was used as the enzyme source, and all experiments were carried out as described under Experimental except for the conditions examined.

rized in Fig. 4. The optimal pH for the assay of CSD was found to be 7.2 (Fig. 4a). The rate of hypotaurine formation was linear up to 90 min (Fig. 4b). Therefore, we chose an incubation time of 30–90 min for the assay. Hypotaurine formation remained linear as a function of protein concentration between 0 and 3 mg of protein (Fig. 4c). The minimum amount of rat liver extract required for CSD assay was less than 50 μg of protein. In order to determine the saturation conditions of the substrate, the assay was performed in the presence of different concentrations of CSA. The apparent Michaelis constant (K_M) was calculated to be 0.15 m*M* from double reciprocal plots, a value in agreement with that reported previously [1]. The maximum velocity (V_{max}) was calculated as 564 nmol per 30 min. When the CSD reaction was carried out without addition of PLP, the reaction rate dropped to 63% of that obtained under the standard assay conditions. The optimal concentration of PLP was found to be 0.1–0.6 m*M*. The CSD activity was not detected without 2-mercaptoethanol. Similar results were reported by Jacobsen et al. [1]. The optimum conditions as described under Experimental were the same when other animal tissue extacts were used as enzyme source for CSD. The precision of the method was established with respect to repeatability. The R.S.D. was 4.5% ($n=9$) for a mean activity of 199 nmol per 30 min per mg protein.

The method described was used for the assay of CSD activity in various animal tissues. CSD activity was widely found in mammalian tissues, but it was very low or not detectable in non-mammalian tissues (Table I). Liver and kidney obtained from rat, dog and mouse contained high activity, but heart, in which the concentration of taurine is high [2, 20], contained a very low or no activity. These results support the hypothesis that the accumulation of taurine in heart depends on the uptake from plasma [21] or biosynthesis by pathways other than through the CSD reaction [22].

In conclusion, these experiments demonstrate that CSD activity in animal tissues can be successfully measured by the proposed method. This method is specific and sufficiently sensitive, and does not rely on the use of radiolabelled

TABLE I

CSD ACTIVITY IN VARIOUS ANIMAL TISSUES

N.D. = not detectable.

Tissue	CSD activity ($\times 10^{-4}$ μmol/min per mg protein)*									
	Ox	Pig	Dog	Rabbit	Rat	Mouse	Chicken	Mackerel	Octopus	Cuttlefish
Brain	N.D.	6.8	1.4	4.5	2.9	7.2	1.6	N.D.	-	-
Heart	N.D.	1.9	N.D.	2.6	1.1	N.D.	N.D.	N.D.	N.D.	N.D.
Lung	N.D.	2.2	N.D.	6.3	3.5	7.0	N.D.	-	-	-
Gill	-	-	-	-	-	-	-	N.D.	N.D.	N.D.
Liver	N.D.	10.1	36.0	0.9	154.9	29.0	1.8	N.D.	N.D.	N.D.
Spleen	N.D.	N.D.	N.D.	5.1	8.3	1.3	N.D.	N.D.	-	-
Pancreas	N.D.	N.D.	N.D.	N.D.	1.7	9.9	N.D.	-	-	N.D.
Kidney	1.8	1.5	0.8	46.1	13.5	42.4	-	N.D.	N.D.	N.D.
Stomach	N.D.	3.5	N.D.	7.6	3.8	9.1	N.D.	N.D.	N.D.	N.D.
Intestine	-	-	-	-	N.D.	1.9	-	-	-	-
Ovary	-	-	-	-	-	-	-	N.D.	N.D.	-
Testis	-	-	-	-	2.1	-	-	-	N.D.	-
Muscle	N.D.	5.9	-	N.D.	4.2	1.0	0.8	N.D.	N.D.	N.D.

*Each value represents the average obtained from three separate experiments.

materials. A complete assay takes ca. 2 h, and multiple samples can be treated simultaneously, except in the GC separation step. We believe that this method provides a useful tool for biochemical and biomedical research requiring CSD assay.

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