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Simple high-performance liquid chromatographic method for assaying cysteinesulfinic acid decarboxylase activity in rat tissue

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Abstract

A simple method is described for determining cysteinesulfinic acid decarboxylase activity in rat tissue. Enzyme preparations from the liver, kidney and brain were incubated with cysteinesulfinic acid substrate in the presence of pyridoxal 5-phosphate. The enzyme product, hypotaurine, was derivatized with *o*-phthalaldehyde and separated by reversed-phase high-performance liquid chromatography (Capsel Pack AG 120A C_{18} column) using a mobile phase of acetonitrile–water (20:80, v/v) containing 50 m*M* sodium phosphate buffer (pH 6.0) and detected using a fluorometer (excitation at 360 nm and emission at 455 nm). The method described is reproducible and sensitive enough to determine the activity of cysteinesulfinic acid decarboxylase activity in the liver, kidney and brain. This assay was subsequently used to evaluate the effect of dietary proteins whose sulfur amino acid contents differ. Consistent with reported data, compared to casein and whole egg protein, a dietary protein low in sulfur amino acid (soybean protein) increased cysteinesulfinic acid decarboxylase activity in the liver and kidney. This method is therefore applicable to studies on the dietary regulation of cysteinesulfinic acid decarboxylase in rat tissue. © 1997 Elsevier Science B.V.

Keywords: Cysteinesulfinic acid decarboxylase; Enzymes; Taurine; Hypotaurine

1. Introduction

Cysteinesulfinic acid decarboxylase is an enzyme involved in the pathway for synthesizing taurine from cysteine. Cysteine is converted to cysteinesulfinic acid by a reaction catalyzed by cysteine dioxygenase. The reaction catalyzed by cysteinesulfinic acid decarboxylase in turn converts cysteinesulfinic acid to hypotaurine. The hypotaurine thus formed is oxidized to taurine, presumably by a non-enzymatic process [1]. In rodents, cysteinesulfinic acid decarboxylase activity is highest in the liver. Moderate activity is found in kidney, and there is less activity in the brain. No activity has been detected in other tissues [1–6]. Hepatic and renal activity is easily modified by the nutritional and hormonal condition of rats, indicating that this enzyme plays a significant role in regulating taurine biosynthesis. Even though higher levels of dietary protein and sulfur amino acids are associated with higher levels of tissue taurine and its urinary excretion, this nutritional condition lowered cysteinesulfinic acid decarboxylase activity in the liver and kidney [7–11]. Adrenalectomy depressed this activity in the rat liver [9]. Hyperthyroidism, induced in rats by injecting triiodothyronine, depressed hepatic activity while increasing renal activity [12].

Several methods have been proposed for measur-

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ing cysteinesulfinic acid decarboxylase activity in tissue. Earlier studies [13–15] employed a manometric method using a Warburg apparatus. Thus, the evolution of CO_2 following the addition of cysteinesulfinic acid substrate was measured to determine enzyme activity. This method is, however, less sensitive than desired and is inconvenient for handling large numbers of samples, so alternative methods using radioactive substrates have been developed. Enzyme activity is determined by measuring the formation of $^{14}CO_2$ from DL-[1-^{14}C]-cysteinesulfinic acid [2–5,7–12]. It is even more convenient to incubate [^{35}S]cysteinesulfinic acid with an enzyme preparation and to separate out the [^{35}S]hypotaurine thus formed by using Dowex 1-X8 column chromatography and quantifying the result [6]. This last method, however, requires the chemical synthesis of [^{35}S]cysteinesulfinic acid, which is not commercially available [16]. Methods using radioactive substrates are thus sensitive enough to measure cysteinesulfinic acid decarboxylase activity in tissue, but also are comparatively expensive and require special facilities and equipment for handling radioactive compounds. This paper reports a simple high-performance liquid chromatography (HPLC) method to detect cysteinesulfinic acid decarboxylase activity in tissue. It is sensitive enough to detect enzyme activity in the liver, kidney and brain and was applied to a nutritional study examining the effect of dietary protein differing in sulfur amino acid content on enzyme activity in the liver and kidney.

2. Experimental

2.1. Materials

Taurine, cysteinesulfinic acid and *o*-phthalaldehyde (OPA) were purchased from Nacarai Tesque (Kyoto, Japan). Hypotaurine was produced by Sigma (St. Louis, MO, USA). The OPA reagent was prepared by dissolving 20 mg of OPA in 0.4 ml of ethanol and adding 20 μl of 2-mercaptoethanol and 10 ml of borate buffer (0.5 *M*, pH 10.3).

2.2. Animals and diets

Male Sprague-Dawley rats (four weeks of age) were obtained from Charles River Japan (Kanagawa, Japan). Animals were housed individually in stainless steel mesh cages in a room with controlled temperature (20–22°C), humidity (55–65%) and lighting (lights on from 0700 to 1900 h). They were fed a commercial non-purified diet (Type NMF; Oriental Yeast, Tokyo, Japan). Methods for their care complied with our institute's guidelines for laboratory animal care and use. After five days of acclimatization to the housing conditions, rats were randomly divided into three groups (seven animals each) and fed purified experimental diets containing 20% of dietary proteins differing in sulfur amino acid content (soybean protein, casein and whole egg protein). The basal composition of the experimental diet was (in weight %): protein source, 20; corn oil, 5; corn starch, 15; sucrose, 53.7; cellulose, 2; mineral mixture [17], 3.5; vitamin mixture [17], 1.0 and choline bitartrate, 0.2. The sulfur amino acid content of the soybean protein diet was calculated to be 0.36%, of the casein diet, 0.54% and of the whole egg protein diet, 1.06% [18]. On day 21 of the feeding period, animals were transferred to metabolic cages and urine was collected for two days to measure taurine excretion. Animals were then returned to the usual stainless mesh cages for the remainder of the experimental period. Rats were fed experimental diets for 27 days.

2.3. Assay for cysteinesulfinic acid decarboxylase

At the end of the experimental period, rats were bled from the abdominal aorta, and the liver, kidney and brain were excised. Tissue was homogenized in 0.25 *M* sucrose (7, 5 and 3 ml of the sucrose solution were used for each g of liver, kidney and brain, respectively). Homogenates were centrifuged at 9000 *g* for 10 min and the supernatants were used as enzyme sources in assaying cysteinesulfinic acid decarboxylase activity. The procedures used were essentially the same as developed by Griffith [6], except that the enzyme product, hypotaurine, was determined using HPLC rather than a radiochemical assay. An enzyme source (10 to 50 μl) was added to a small spit tube (4 ml capacity), made up to 50 μl with 0.25 *M* sucrose, and then 100 μl of 120 m*M* potassium phosphate buffer (pH 7.4) containing 5 m*M* dithiothreitol and 0.2 m*M* pyridoxal 5-phosphate were added. The mixture was incubated at 37°C for 15 min and the enzyme reaction was triggered by

adding 50 μl of 20 m*M* cysteinesulfinic acid (neutralized to pH 7.0 with 2 *M* NaOH). The enzyme reaction was continued at 37°C for 10 to 120 min and stopped by placing the tubes in a boiling water bath for 2 min. The mixture was then cooled by placing the tubes in an ice bath for at least 15 min, after which time, they were centrifuged at 2500 *g* for 10 min. A portion of the supernatant (150 μl) was diluted to 5 ml with water and stored at −20°C until analysis. The mixture to which cysteinesulfinic acid solution was added after the boiling step was used as the control.

2.4. HPLC quantification of enzyme products

The HPLC system consisted of the following instruments: A chromato-integrator C-R7A plus; a fluorometer RF-10A; a pump LC-10AD; a column oven CTO-10A; a system controller SCL-10A and a sample injector 7725i. The sample injector was from Rheodyne (Cotati, CA, USA). Other instruments were from Shimadzu (Kyoto, Japan). A 250×4.6 mm Capsel Pack AG 120A C_{18} column (Shiseido, Tokyo, Japan) was used to analyze the enzyme products. Fluorescent adducts were prepared by combining equal parts of the OPA reagent (diluted 1:1 with water) with either a standard solution containing 5 μg/ml each of hypotaurine (36.7 nmol/ml) and taurine (39.9 nmol/ml) or aliquots of the enzyme reaction mixture prepared as described above. After rapid mixing, a 10-μl volume was injected into the HPLC and analyzed at 40°C with a mobile phase of acetonitrile–water (20:80, v/v) containing 50 m*M* sodium phosphate buffer (pH 6.0) at a flow-rate of 1 ml/min for 12 min, and then the flow-rate was increased to 2 ml/min and analysis continued for a total of 20 min. OPA-reactive substances were detected by a fluorometer in excitation mode at 360 nm and emission mode at 455 nm. The chemical structures of cysteinesulfinic acid, hypotaurine and taurine and of their OPA derivatives are shown in Fig. 1.

3. Results and discussion

3.1. Analysis of cysteinesulfinic acid decarboxylase activity in rat tissue

Fig. 2 shows typical chromatograms obtained by HPLC of a standard sample containing 5 μg/ml each of hypotaurine (36.7 nmol/ml) and taurine (39.9 nmol/ml), a hepatic enzyme reaction mixture and a control sample. Control samples represent the mixture prepared by adding cysteinesulfinic acid substrate to the enzyme preparation that had been boiled. Hypotaurine and taurine in the enzyme reaction mixture were well separated from the cysteinesulfinic acid and other OPA-reactive substances. Retention times were 2.78±0.00 min for cysteinesulfinic acid, 8.08±0.02 min for hypotaurine and 9.62±0.03 min for taurine (mean±SE of 32 chromatograms). Relative retention times were 2.90±0.01 for hypotaurine and 3.46±0.01 for

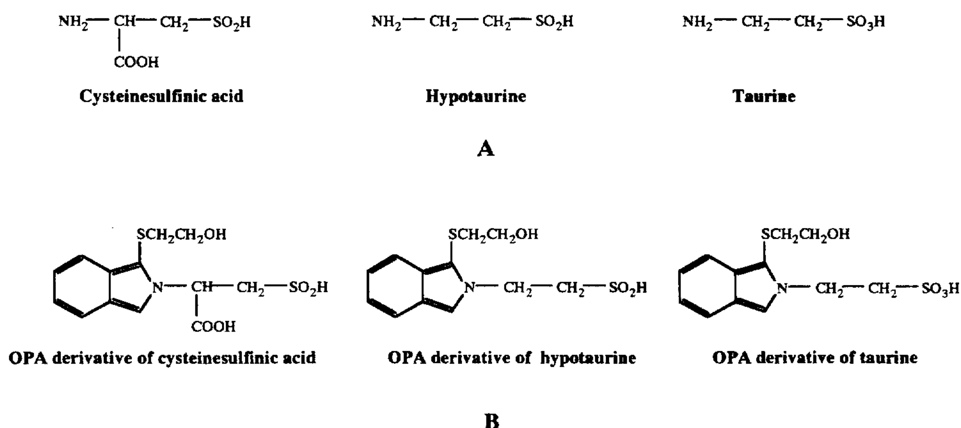

Fig. 1. Chemical structures of (A) cysteinesulfinic acid, hypotaurine and taurine and (B) their *o*-phthalaldehyde (OPA) derivatives.

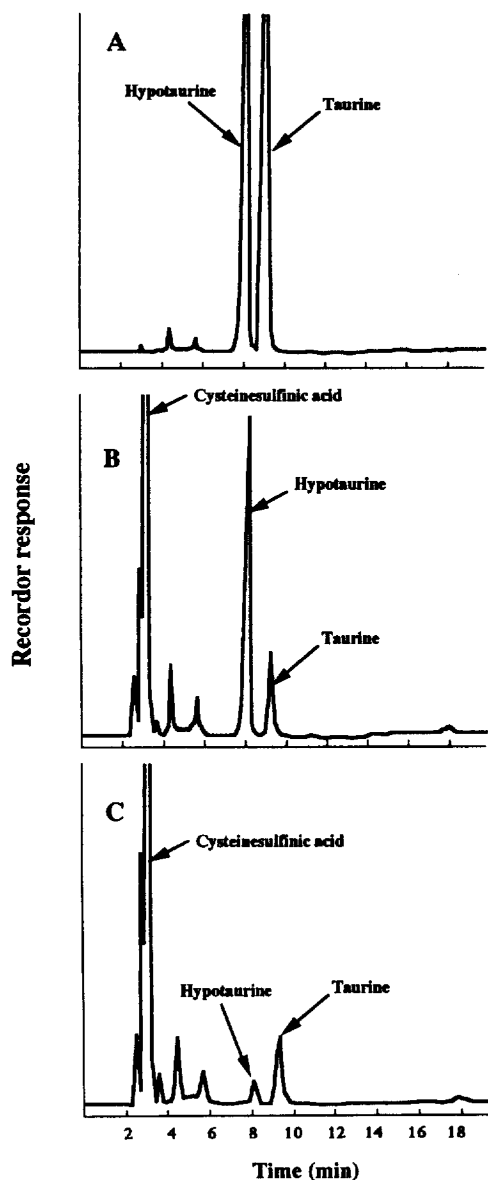

Fig. 2. Chromatograms of samples of (A) authentic hypotaurine and taurine (5 μg/ml each; injected dose 25 ng each), (B) hepatic enzyme reaction mixture and (C) control. The 9000 *g* supernatant fraction (450 μg of protein) of liver homogenates from rats fed laboratory chow was incubated with cysteinesulfinic acid substrate for 30 min and processed for determination of hypotaurine formation by HPLC as detailed in Section 2. Control sample represented the mixture prepared by adding cysteinesulfinic acid substrate after boiling the enzyme preparation.

taurine when the retention time of cysteinesulfinic acid was assigned a value of 1.00. A small but detectable peak, corresponding to the retention time of hypotaurine, was detected in the control sample. When the hepatic enzyme preparation was incubated with the cysteinesulfinic acid substrate for 30 min, the hypotaurine peak showed a marked increase. Compared to the control sample, a small but detectable increase also occurred in the taurine peak in the hepatic enzyme reaction mixture. This suggests that the hypotaurine formed by the enzyme reaction was further oxidized to taurine, presumably by a non-enzymatic process during incubation. The increase in the taurine content in the reaction mixture was thus added to that of hypotaurine to calculate the cysteinesulfinic acid decarboxylase activity, i.e., the rate of hypotaurine formation. A flow-rate of 1 ml/min was used to analyze hypotaurine and taurine in the enzyme reaction mixture. After taurine was eluted, the flow-rate was increased to 2 ml/min, 12 min after the start of the assay, to wash out any OPA-reactive substances. Under these conditions, a small peak was detected at about 18 min, but no peak was observed thereafter. Therefore, 20 min is long enough to wait before injecting the next sample.

When all of the cysteinesulfinic acid substrate (1 μmol) is converted to hypotaurine, the enzyme reaction mixture, prepared as described in Section 2, should give a hypotaurine concentration of 150 nmol/ml. Therefore, standard curves for hypotaurine and taurine were constructed in the range of 3 to 150 nmol/ml (injected amounts were 15 to 750 pmol) (Fig. 3). The data obtained demonstrated a linear relationship between the integrated peak area and the concentration in the range analyzed. Linear regression for each compound was: hypotaurine; $y=85994x+25307$ ($r=1.000$): taurine; $y=96136x+14924$ ($r=0.999$) (y=integrated peak area in arbitrary units, x=concentration, nmol/ml).

In a recovery study (Table 1), we added 50 μl of a solution containing varying amounts of both hypotaurine and taurine to the control samples containing hepatic 9000 *g* supernatants (320–350 μg protein) to which 1 μmol of cysteinesulfinic acid substrate was added after the boiling step (see Section 2.3). The amounts of hypotaurine added to the samples correspond to cysteinesulfinic acid decarboxylase activities of 3.0 to 52.1 nmol/min per mg of protein

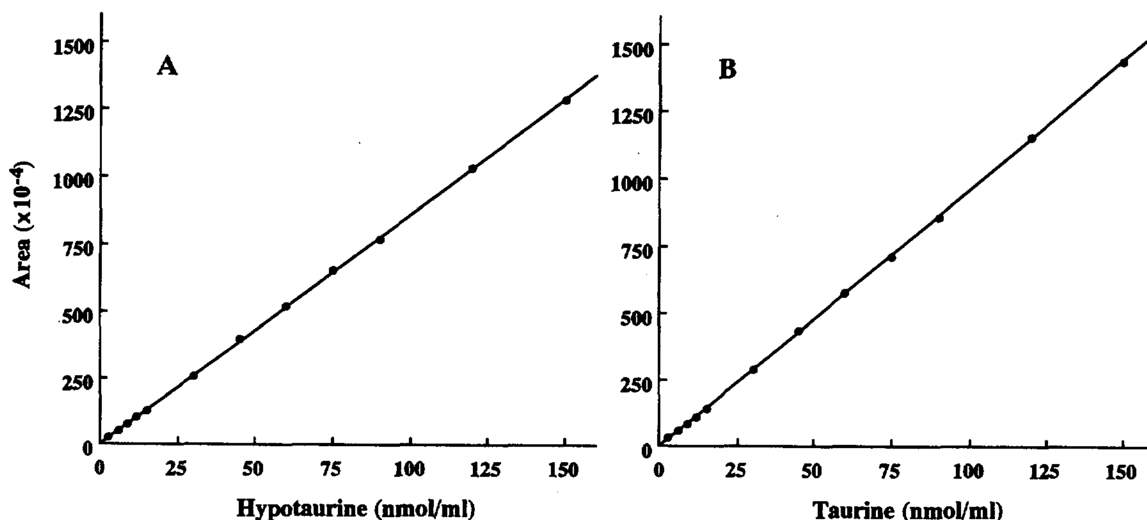

Fig. 3. Standard curves for (A) hypotaurine and (B) taurine.

using an incubation period of 30 min. After centrifugation, 200 μl of the supernatant were diluted to 5 ml and analyzed by HPLC and the amounts of hypotaurine and taurine in the mixture were calculated using the standard curves obtained as above. The mean recoveries of 98.8 to 101% for hypotaurine and 96.2 to 109% for taurine were very satisfactory.

Fig. 4 shows hypotaurine formation as a function of the incubation time and the enzyme amount using hepatic 9000 *g* supernatants as an enzyme source from rats fed laboratory chow or purified diets containing soybean protein and whole egg protein. The enzyme reactions were linear up to at least 60 min using 340–380 μg of protein. The reactions were also linear as a function of the enzyme amount employed up to 680 μg of protein in a 30-min incubation. Activity was higher in rats fed soybean protein than in those fed egg protein or laboratory chow. Data (Fig. 4B) suggested that the enzyme reaction is linear at least up to 60% substrate conversion.

In results obtained using renal 9000 *g* supernatants as an enzyme source (460–510 μg of protein) from rats fed purified diets containing soybean protein and whole egg protein (Fig. 5), enzyme reactions were linear up to at least 120 min. Reactions were also linear as a function of the enzyme amount up to 930 μg of protein in a 60-min incubation. Again, activity was higher in rats fed soybean protein than in those fed egg protein.

Cysteinesulfinic acid decarboxylase activity is much lower in the brain than in the liver or kidney. Our HPLC method was found to be sensitive and reliable enough, however, to detect activity in the brain (Fig. 6). Using 9000 *g* supernatants (380–470

Table 1
Recovery of hypotaurine and taurine from an enzyme mixture

Amount added (nmol)		Amount recovered (nmol)		Recovery (%)	
Hypotaurine	Taurine	Hypotaurine	Taurine	Hypotaurine	Taurine
31.25	7.8125	31.1±1.2	8.53±0.82	99.4±3.7	109±10
62.5	15.625	61.7±0.9	15.0±0.4	98.8±1.4	96.2±2.4
125	31.25	125±6	31.7±1.6	100±5	101±5
500	125	507±13	124±5	101±3	98.9±4.1

Each value represents mean±SE (using five different preparations of 9000 *g* supernatants of liver homogenate).

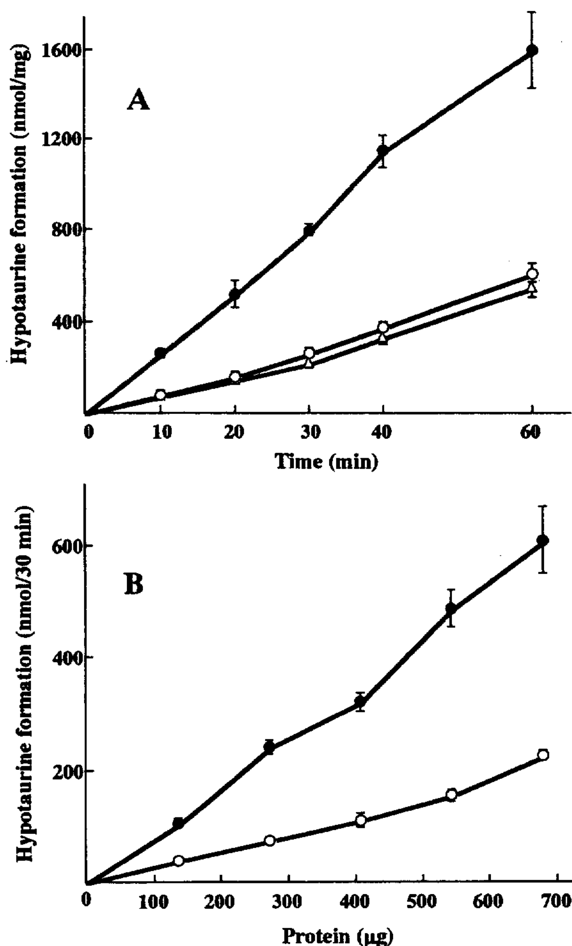

Fig. 4. Hypotaurine formation as a function of (A) incubation time and (B) amount of enzyme using 9000 *g* supernatants of liver homogenates obtained from rats fed (△) laboratory chow and purified diets containing (●) soybean protein and (○) whole egg protein as an enzyme source. Each value represents mean±SE for three rats.

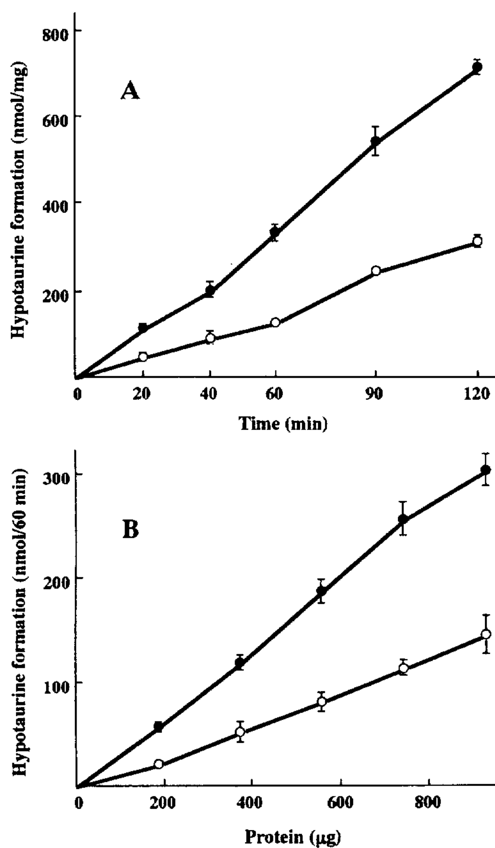

Fig. 5. Hypotaurine formation as a function of (A) incubation time and (B) amount of enzyme using 9000 *g* supernatants of kidney homogenates obtained from rats fed purified diets containing (●) soybean protein and (○) whole egg protein as an enzyme source. Each value represents mean±SE for three rats.

μg of protein) or whole homogenates (1100–1200 μg of protein) as enzyme sources, the rate of hypotaurine formation was linear up to 180 min.

3.2. Hepatic and renal cysteinesulfinic acid decarboxylase activity in rats fed different proteins

Taurine concentrations in the liver and kidney, and serum and urinary taurine excretion increased as the sulfur amino acid content increased in the diet (Table 2). The activity of cysteinesulfinic acid decarboxylase, in contrast, decreased as the sulfur amino acid content increased in the diet. Hepatic activity in rats fed laboratory chow was about the same as in animals fed purified diet containing whole egg protein and the renal activity in these rats was about the same as in rats fed purified diet containing casein. Taurine formation represented 0.3–1.0% of the total cysteinesulfinic acid decarboxylase activity for the liver and 2.1–5.5% of that for the kidney. Enzyme activity in the 9000 *g* supernatant fraction of the brain in rats fed laboratory chow was 1.42±0.15 nmol/min per mg of protein, and taurine formation represented 5.2% of the total activity.

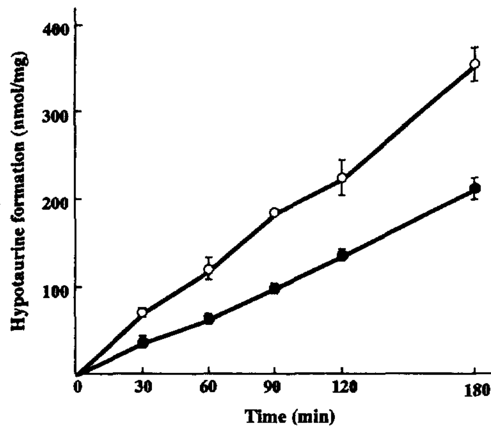

Fig. 6. Hypotaurine formation as a function of incubation time using (●) unfractionated brain homogenates and (○) 9000 *g* supernatants of homogenates obtained from rats fed laboratory chow as the enzyme source. Each value represents mean±SE for five rats.

This study presented a simple and reliable HPLC method for detecting cysteinesulfinic acid decarboxylase activity in tissue. The detectable hypotaurine peak in control samples limited the sensitivity of our present method, however. Because the hypotaurine peak in control samples proportionally increased as the enzyme amount used in the incubation mixture increased, lengthening the time for the enzyme assay is the conventional way of improving the accuracy of the method. It was also found that control values were affected by the nutritional condition of the animals. Thus, for a 9000 *g* supernatant fraction of liver homogenates as the enzyme source (330–410 μg of protein), control values were 15.4±0.9 nmol for rats fed purified diets containing soybean protein, 17.0±1.6 nmol for those fed casein, 23.5±3.2 nmol for those fed whole egg protein and were 9.2±0.5 nmol for rats fed laboratory chow. Enzyme activity was high and control values were low in rats fed

Table 2
Hepatic and renal cysteinesulfinic acid decarboxylase activity and parameters for taurine metabolism in rats fed proteins differing in sulfur amino acid content

	Diet			
	Purified diets containing different proteins			Chow
	Soybean protein	Casein	Whole egg protein	
Cysteinesulfinic acid decarboxylase (nmol/min per mg)				
Liver				
Hypotaurine	29.6±2.2[a]	19.6±2.4[b]	11.6±1.4[c]	8.85±0.89
Taurine	0.093±0.009[a]	0.101±0.016[a]	0.127±0.022[a]	0.063±0.010
Total	30.1±2.2[a]	20.0±2.4[b]	11.9±2.2[c]	9.21±0.89
Kidney				
Hypotaurine	5.81±0.39[a]	4.33±0.29[b]	3.29±0.32[c]	4.29±0.31
Taurine	0.128±0.013[a]	0.148±0.043[a]	0.197±0.092[a]	0.213±0.090
Total	6.12±0.40[a]	4.63±0.36[b]	3.58±0.32[c]	4.86±0.61
Tissue taurine				
Liver (μmol/g)	0.323±0.029[a]	0.836±0.172[b]	4.22±0.61[c]	ND
Kidney (μmol/g)	3.18±0.26[a]	7.41±0.58[b]	13.4±1.0[c]	ND
Serum (μmol/dl)	8.30±0.83[a]	16.2±1.3[b]	18.6±1.1[b]	ND
Urinary taurine (μmol/d)	0.27±0.02[a]	6.02±3.35[b]	79.7±4.1[c]	ND

Each value represents mean±SE (seven rats per group for those fed purified diets containing different proteins and five rats per group for animals fed laboratory chow). Statistical examinations were conducted among rats fed purified diets.
[abc]Values sharing different superscript letters are significantly different at $P<0.05$.
ND=not determined.

soybean protein, so the control value in these rats represented a mere 5.9±1.5% of the total hypotaurine peak using a 30-min reaction time. The corresponding value was 25.4±1.5% in rats fed whole egg protein where enzyme activity was low and control values were high. These values decreased as the length of time for the enzyme assay increased and were 3.0±0.7% for rats fed soybean protein and 13.1±2.4% for rats fed whole egg protein after a 60-min incubation time.

Although our HPLC and other methods were not compared in the present study, the hepatic enzyme activity in rats fed laboratory chow and measured using HPLC appeared to be comparable to those measured radiochemically. Using a 20 000 *g* supernatant fraction of liver homogenates from rats fed laboratory chow as an enzyme source and using ^{14}C-labeled substrate, it has been reported that enzyme activity was 4.8–8.5 nmol/min per mg of protein [2,5]. Griffith [6] reported that the hepatic enzyme activity in rats fed laboratory chow was 15 nmol/min per mg of protein using 7000 *g* supernatants of tissue homogenates as an enzyme source and using ^{35}S-labeled substrate. It has been demonstrated that higher levels of dietary protein and sulfur amino acids are associated with lower cysteinesulfinic acid decarboxylase activity in the liver and kidney [7–11]. Consistent with these observations, enzyme activity in the liver and kidney in this study deceased as the sulfur amino acid content of the diet increased. Our proposed HPLC method is therefore applicable to studies of the nutritional regulation of cysteinesulfinic acid decarboxylase.

In conclusion, our proposed HPLC method was sensitive enough to detect cysteinesulfinic acid decarboxylase activity in the liver, kidney and brain. This method is not expensive and does not require special facilities or equipment to handle radioactive compounds. This method was also applicable to a study of the effect of dietary protein, differing in sulfur amino acid content, on enzyme activity in the liver and kidney. This method should greatly facilitate studies on the regulation of taurine synthesis in organisms.

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