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Short communication

Specific determination of threonine in biological samples by gas chromatography with electron capture detection

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Abstract

Threonine was oxidized into acetaldehyde at 0 °C for 30 min with periodic acid. The acetaldehyde formed was converted to a hydrazone with 2,4-dinitrophenyhydrazine. The hydrazone was extracted with *n*-heptane and quantified by gas liquid chromatography with electron capture detection. An internal standard, 2-amino-3-hydroxyhexanoic acid, was used. The calibration curve of threonine was linear up to 200 nmol in 200 μl sample solution and the determination limit of threonine was 1 nmol in 200 μl sample solution. The recoveries were 100.0, 94.0 and 100.0% from homogenates of octopus tentacles and blood plasma and rat livers, respectively. This method was applied to the determination of threonine in tissues of rats given threonine and starved octopuses. This threonine determination method has been used for studies on the metabolism of D-lactate.

Keywords: Threonine; Gas chromatography; Determination

1. Introduction

In the past, we have studied D-lactate metabolism. During the course of these studies, we found that the best precursor for D-lactate formation in cell-free homogenates of *Octopus vulgaris* tentacle tissue was threonine [1]. Subsequently, we studied threonine metabolism in Japanese quail liver and reported that threonine is metabolized as a ketogenic amino acid in the quail liver, whereas it is glucogenic in rat liver [2]. Recently, we reported that in octopus and especially in starved octopus, D-lactate is produced actively from methylglyoxal, which, in turn, is formed via aminoacetone from threonine [3]. For these studies, the threonine concentration in animal tissues had to be measured. Therefore, we established a method for the specific determination of threonine. The method is the subject of this report. At this time, there is only one principal reaction for the specific determination of threonine. This reaction is the oxidative degradation of threonine to acetaldehyde by periodate solution. In a report by Winnick, acetaldehyde was trapped by sodium bisulfite, the excess bisulfite oxidized with iodine, the bisulfite addition product dissociated to acetaldehyde and bisulfite, and, finally, the bisulfite titrated with standard iodine solution [4]. Through this method micromole levels of threonine could be determined. In another procedure, acetaldehyde is treated with aldehyde dehydrogenase. The NADH formed was determined fluorometrically. Threonine could be determined at the nanomole levels using this method [5]. This threonine determination method was applied to only rat blood plasma. In the method presented here, acetaldehyde is converted to 2,4-dinitrophenylhydrazone, which is determined by gas chromatography with electron capture detection. The sensitivity of method was 1 nmol and was applied to some biological experiments.

2. Experimental

2.1. Materials

Periodic acid solution (37%), butyraldehyde, glycine, sodium bisulfite and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Threonine was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). A modification of the method described by Okawa and Sato [6] was used to prepare 2-amino-3-

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hydroxyhexanoic acid. Okawa and Sato synthesized threonine from glycine and acetaldehyde on the basis of a condensation reaction. Cupric glycinate (9 g), sodium carbonate (2.3 g), butyraldehyde (20 ml) and 23 ml water were placed in a 125 ml flask and heated at 50 °C for 1 h with stirring. After the reaction mixture was acidified with 2 M hydrochloric acid, it was filtered. The filtrate was applied to a column (5 cm × 20 cm) of Dowex 50W-X2 (H^+) (100–200 mesh). After washing with 500 ml of water, the column was eluted with 500 ml of 2 M NH_3. The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in about 5 ml water and evaporated to dryness; this procedure was repeated two times, in order to convert the ammonium form to the free form. The residue was dissolved in a minimal amount of water and alcohol was added to the solution. This recrystallization procedure was repeated three times. The yield was about 5 g. The R_f value was 0.71 on silica gel TLC in *n*-butanol/acetic acid/water (4:1:1, v/v/v). In order to confirm the purity and to approximately determine the yield of the product, glycine was also subjected to TLC. The R_f value of glycine was 0.45 in the same system.

2.2. Administration of threonine to rats

Male Wistar strain rats, aged 4 weeks, were divided into two groups. One group was given a single dose of threonine (1 g/kg body weight) orally. The second group was the control. The animals were decapitated 1 h later and the organs were removed immediately and used for the determination of threonine.

2.3. Starved octopus

Octopus ocellatus Gray were collected near the Ushimado Marine Laboratory located on the coast of the Inland Sea of Japan and maintained in the laboratory aquarium at 9 ± 1 °C. For a starvation experiment, 16 octopuses were divided into four groups. One group was starved for 17 days and a second group was starved for 32 days. The remaining two groups served as controls. The control groups were fed with *Charybdis japonica* for 17 and 32 days. These experiments were carried out for the purpose of studying threonine metabolism [3].

2.4. Gas chromatography

All samples were analyzed on a Shimadzu GC14A gas chromatograph (Kyoto, Japan) equipped with a Ni-63 electron capture detector. A Hi-Cap CBPI-M25-025 capillary column from Shimadzu (25 m × 0.25 mm i.d.; film thickness, 0.25 μm) was used. The electron capture detector was maintained at 280 °C. The column temperature was raised automatically from 100 to 200 °C at a rate of 20 °C/min, the temperature was then maintained at 200 °C for 5 min, after which the column temperature was raised to 220 °C at a rate of 5 °C/min, and finally increased to 265 °C at a rate of 30 °C/min. The injector block temperature was adjusted to 270 °C. The flow rate of carrier gas (nitrogen) was approximately 1.5 ml/min and the pressure was 1 kg/cm^2. The injection technique was splitless.

2.5. Calibration curve of threonine

Various amounts of threonine (5, 10, 15 and 20 μl 1 mM threonine), 20 μl 1 mM 2-amino-3-hydroxyhexanoic acid as an internal standard, 100 μl 0.5 M disodium hydrogen phosphate, 100 μl 0.05 M periodic acid and 200 μl water were pipetted into a 5 ml vial. The reaction mixture was allowed to stand for 30 min on ice. After the oxidation reaction, 100 μl 0.3 M $NaHSO_3$ was added and then 0.5 ml of a solution consisting of 7 M DNPH and 6 M HCl was added to the reaction mixture. The mixture was reacted at 40 °C for 60 min and extracted twice with 2 ml *n*-heptane for 30 min using a shaker. The heptane layer (3.5 ml) was transferred to a 5 ml vial and evaporated using a Savant Vac concentrator (Model SVS-100H, New York, NY, USA) at room temperature for 20 min. The residue was dissolved in 1 ml *n*-hexane. The hexane layer was diluted with *n*-hexane before injection into gas chromatography if necessary.

2.6. Recovery test

For the purpose of applying this procedure to biological samples, recovery tests were carried out. Various amounts (5, 10, 15 and 20 nmol) of threonine were added to 6000 × *g* octopus tentacle homogenates, rat blood plasma and 6000 × *g* rat liver homogenates from normal rats. The amount of threonine contained in these samples was determined by method A as described below.

2.7. Analytical procedure for the determination of threonine in biological samples

Method A: Fresh octopus tentacles and rat livers were homogenized. Three milliliters methanol was added to 1 ml of the supernatant. After standing the mixture at −20 °C for 30 min, the mixture was centrifuged at 1700 × *g* for 10 min. The precipitate was washed with 4 ml of a methanol/water mixture (70:30, v/v). The methanol and wash supernatants were allowed to stand at −20 °C for 2 h and subsequently centrifuged at 1500 × *g* for 10 min. The extracts were applied onto a Dowex 50W-X2 (H^+, 100–200 mesh) column (5.0 cm × 0.9 cm i.d.), which was washed with 20 ml water and eluted with 20 ml 2 M NH_3. The eluate was concentrated to dryness under reduced pressure using a rotary evaporator. The residue was dissolved in 1 ml water. A plasma sample (0.2 ml) from heparinized rat blood was deproteinized with 2 ml methanol and centrifuged at 1700 × *g* for 15 min. The precipitate was suspended in 1.5 ml 70% methanol (methanol/water, 70:30, v/v) and centrifuged as above. The combined supernatants were treated as described in Section 2.5.

Method B: Fresh octopus tentacles (2.5 g) were amputated and cut into small pieces with a scissors. The minced tissue was homogenized in 12.5 ml water with a Potter-Elvehjiem homogenizer at 0 °C and 10,000 rpm for 5 min and then centrifuged at 4 °C and 6000 × *g* for 15 min, 1 ml 1.2 M perchloric acid was added to 1 ml of the supernatant. The mixture was allowed to stand for 30 min in ice and centrifuged at 4 °C and 1500 × *g* for 15 min. The supernatant (1 ml) was mixed with 700 μl 2 M dipotassium hydrogen phosphate. After standing at

0 °C for 10 min, the precipitate was removed by centrifugation at 1500 × *g*. The supernatant (200 μl) was treated as described in Section 2.5.

3. Results and discussion

3.1. Reaction conditions for oxidation

Threonine (36 nmol) was dissolved in a mixture of 100 μl 0.5 M Na_2HPO_4 and 100 μl 0.05 M HIO_4 and reacted at 40 °C for different time intervals up to 120 min. Afterwards, 100 μl 3% $NaHSO_3$ was added to the reaction mixture. Acetaldehyde was converted to the hydrazone and determined by gas chromatography. The amount of acetaldehyde available for conversion to hydrazone decreased with increasing reaction time (87, 79 and 69% at 15, 30 and 60 min, respectively) and, after 120 min, was about half of what was at the starting point. To determine the optimum reaction temperature and time, the above experimental procedure was carried out at 0, 20, 30 and 40 °C for 10 and 30 min. From these experimental results, it was decided that the oxidation should be performed at 0 °C for 30 min.

3.2. Calibration curves

Various amounts of threonine (20, 40, 60 and 80 nmol) were oxidized with periodic acid, derivatized to the hydrazone, and then measured by gas chromatography. The peak area ratio (*y*) of the hydrazone compared to the peak area of the butyraldehyde from the internal standard was exactly proportional to the threonine concentration (*x*, nmol) up to at least 200 nmol in 200 μl of the sample solution: $y = 0.00898x$ ($r^2 = 0.992$). The lower determination limit was 1 nmol in 200 μl of sample solution. Since an aliquot (1 μl) from 1 ml of the hexane solution containing the sample was injected to the gas chromatograph, the absolute limit corresponds to 1 pmol. Generally speaking, gas chromatographic determination is highly sensitive, real sensitivity is not so high, because injection amount into gas chromatography is limited. Winnick reported a determination method for threonine. The method was based on oxidation of threonine with periodate and microdiffusion of the acetaldehyde formed. The determination limit of this method was at the micromole level [4]. Nishida et al. reported a specific method for the determination of threonine after an oxidative reaction to acetaldehyde. Acetaldehyde was then oxidized to acetate with aldehyde dehydrogenase, and the NADH formed in the reaction was determined fluorometrically. By this method, 10 nmol in 0.5 ml aqueous solution could be quantified [5]. They estimated the threonine content only in rat blood plasma. Fig. 1 shows the gas chromatograms of authentic and biological samples obtained from the present method. As described in the previous paper, the hydrazones of aldehyde appeared as two peaks in the chromatogram [7]. The peak area was automatically calculated.

3.3. Recovery test and comparison of results obtained by the present method to those obtained by amino acid analysis

Recoveries of 100.0 ± 7.19%, 94.0 ± 3.30% and 100.0 ± 6.57% were obtained for octopus tentacle homogenates, rat blood plasma and rat liver homogenates using method A, respectively. All the experiments were carried out with three replications of each assay. Detailed results are shown in Tables 1–3. When octopus tentacle muscle was prepared using method B, the recovery was 84.8%.

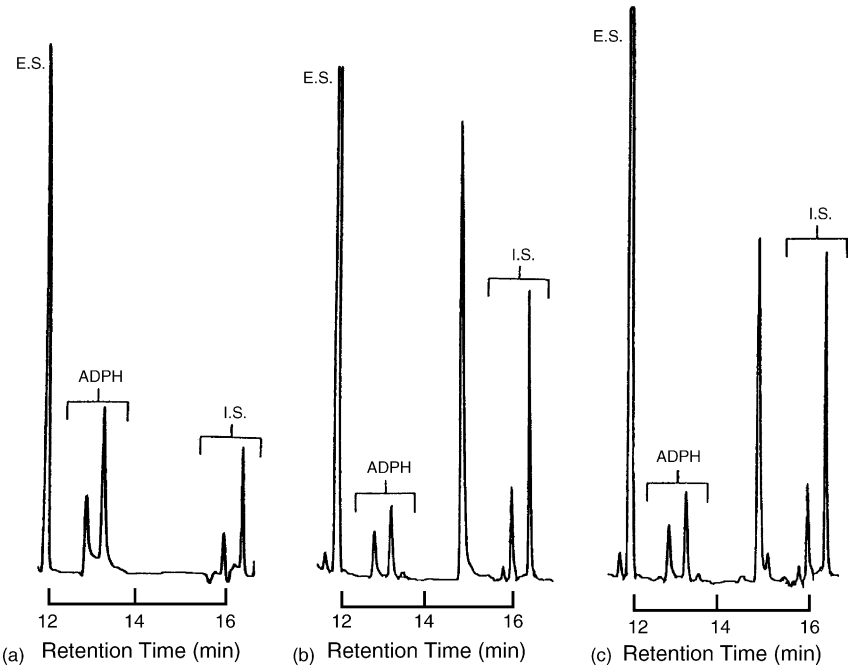

Fig. 1. Gas chromatograms of hydrazones of aldehydes (ADPH). (a) ADPH (authentic): 1 μl of a 1 nmol/ml standard solution of ADPH was injected. (b) ADPH (derived from threonine in the plasma of normal rat): Deprotenized blood plasma of rat (1 ml) was treated as described in Section 2.4. (c) ADPH (derived from threonine in the liver homogenate of normal rat): The liver (2 g) was treated as described in Section 2.4.

Table 1
Recovery of threonine from 6000 × *g* supernatant of octopus foot homogenate

Threonine		Recovery (%)
Added (nmol)	Found (nmol)	
0	1.4	
5	6.5	102.5
10	10.4	90.4
15	15.9	97.1
20	23.4	110.0

Average ± S.D.: 100.0 ± 7.19 (n = 3).

Table 2
Recovery of threonine from rat plasma

Threonine		Recovery (%)
Added (nmol)	Found (nmol)	
0	9.1	
5	14.0	98.3
10	18.6	94.8
15	23.1	93.5
20	26.9	89.2

Average ± S.D.: 94.0 ± 3.30 (n = 3).

Table 3
Recovery of threonine from 6000 × *g* supernatant of rat liver homogenate

Threonine		Recovery (%)
Added (nmol)	Found (nmol)	
0	3.8	
5	9.2	109.6
10	14.0	102.3
15	18.1	95.5
20	22.3	92.6

Average ± S.D.: 100.0 ± 6.57 (n = 3).

Threonine in various rat tissues was determined by the present method and by amino acid analysis. The results are shown in Table 4. These results demonstrate that the present method yielded essentially the same values as those obtained by amino acid analysis.

Table 4
Threonine content of various rat tissues after giving the animals threonine p.o. as determined by the present method and amino acid analysis

Tissues	Control group (μmol/g tissues)	Threonine content	
		Present method (μmol/g tissues)	Amino acid analysis (μmol/g tissues)
Liver	0.067 ± 0.04	2.05 ± 0.31	2.04
Muscle	n.d.	1.67 ± 0.06	1.56
Kidney	n.d.	3.94 ± 0.32	n.d.
Brain	n.d.	1.05 ± 0.02	n.d.
Plasma	0.055 ± 0.02*	1.33 ± 0.09*	n.d.

Threonine content was measured using method A (n = 3). * denotes the unit for plasma content: μmol/ml.

Table 5
Threonine content of the tentacles and mantle of fed and starved octopuses

	Days	Fed group (μmol/g.w.w.)	Starved group (μmol/g.w.w.)
Tentacle	17	0.876 ± 0.15	0.385 ± 0.06
Mantle	32	0.582 ± 0.14	0.448 ± 0.04
Mantle	17	0.897 ± 0.11	0.581 ± 0.09
	32	0.803 ± 0.17	0.584 ± 0.02

Threonine content was measured using method B (n = 3).

3.4. Threonine contents in various rat tissues after threonine administration

As shown in Table 4, orally administered threonine was taken up and transported to various tissues in rats within one hour. All the experiments were carried out with three replications of each assay. The amino acid was accumulated at concentrations 30 and 24 times higher than that normally found in the liver and kidney, respectively. In 1989, Semon et al. [8] and Soemitro et al. [9] reported the contents of free amino acids in blood plasma, brain, liver and muscle of Sprague–Dawley albino rats. They reported the relationship between dietary protein intake and amino acid levels in tissues of rats. The concentrations of most amino acids in the tissues were proportional to protein intake. The threonine levels in rat tissues in the present report were similar to those reported by Semon et al. [8] and Soemitro et al. [9].

3.5. Threonine concentration in the tentacles and mantle of octopus after starvation

In a previous paper, we reported that threonine was a ketogenic amino acid in quail liver and a glucogenic amino acid in mammals [2]. More recently, we reported that in octopus, especially in starved octopus, D-lactate was actively produced from methylglyoxal, which is formed via aminoacetone from threonine and glycine [3]. In connection with these experiments, we determined the content of threonine in the tentacles and mantle of octopus. As can be seen from Table 5, when octopuses were starved, the concentration of free threonine decreased in both tissues. This means that threonine was used for energy production during starvation [3]. As can be seen from Table 4, when excess amounts of threonine were administered to rats, it was accumulated in the tissues. This accumulation of excess threonine in rat tissues contrasts with the decrease of the amino acid level in starving octopuses.

In conclusion, data presented here show that threonine levels in several organs were exactly measured with good recovery and sensitivity. The determination method could apply to the biochemical and nutritional research.

References

[1] S. Akagi, S. Ohmori, Amino Acids 26 (2004) 169.
[2] S. Akagi, K. Sato, S. Ohmori, Amino Acids 26 (2004) 235.
[3] T. Fujisawa, S. Akagi, M. Kawase, M. Yamamoato, S. Ohmori, J. Exp. Zool. A Comp. Exp. Biol. 303 (6) (2005) 489.

[4] T. Winnick, J. Biol. Chem. 142 (1942) 461.
[5] T. Nishida, S. Kume, M. Saito, M. Suda, J. Biochem. 81 (1977) 1085.
[6] K. Okawa, M. Sato, Japan Pat. 2964 (1959) (Chem Abs. 54 (1960) 13018).
[7] H. Ohata, M. Otsuka, S. Ohmori, J. Chromatogr. B 693 (1997) 297.
[8] B.A. Semon, P.M.B. Leung, Q.R. Rogers, D.W. Gietzen, J. Nutr. 119 (1989) 166.
[9] S. Soemitro, K.P. Block, P.L. Crowell, A.E. Harper, J. Nutr. 119 (1989) 1203.