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S-(1,2-Dicarboxyethyl)glutathione and S-(1,2-Dicarboxyethyl)L-cysteine in Lens

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Summary: S-(1,2-Dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine were determined by high performance liquid chromatography after reaction with 2,4-dinitrofluorobenzene. By this method the former could be determined in the range 4.05 $\mu\text{mol/l}$ –815 $\mu\text{mol/l}$, and the latter in the range 1.45 $\mu\text{mol/l}$ –1.45 mmol/l. The recovery from cattle lens homogenate was $90.0 \pm 3.2\%$ for S-(1,2-dicarboxyethyl)glutathione and $95.3 \pm 3.1\%$ for S-(1,2-dicarboxyethyl)L-cysteine. Using this method S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine were determined in lenses of several vertebrates and in rat lens during cataract formation by galactose.

S-(1,2-Dicarboxyethyl)glutathion und S-(1,2-Dicarboxyethyl)L-cystein in der Linse des Auges

Zusammenfassung: S-(1,2-Dicarboxyethyl)glutathion und S-(1,2-Dicarboxyethyl)L-cystein wurden mit Hochleistungsflüssigkeitschromatographie nach Reaktion mit 2,4-Dinitrofluorobenzol bestimmt. Mit diesem Verfahren konnte das Erste schon ab 4,05 $\mu\text{mol/l}$ bis zu 815 $\mu\text{mol/l}$, und das Letztere ab 1,45 $\mu\text{mol/l}$ bis zu 1,45 mmol/l bestimmt werden. Die Wiederfindung aus dem Homogenat von Rinderlinsen betrug $90,0 \pm 3,2\%$ für S-(1,2-Dicarboxyethyl)glutathion und $95,3 \pm 3,1\%$ für S-(1,2-Dicarboxyethyl)L-cystein. Unter Verwendung dieses Verfahrens wurden S-(1,2-Dicarboxyethyl)glutathion und S-(1,2-Dicarboxyethyl)L-cystein in den Linsen mehrerer Wirbeltiere und in Rattenlinsen während der Katarakt-Bildung mit Galaktose bestimmt.

Introduction

In 1963 S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine were isolated from calf lens and their chemical structures were determined by comparison of synthesized compounds (1).

S-(1,2-Dicarboxyethyl)glutathione

S-(1,2-Dicarboxyethyl)L-cysteine

Since that time, S-(1,2-dicarboxyethyl)L-cysteine has been reported in the urine and kidney of guinea pig (2), in ox liver and kidney (3), and in ox brain and spinal cord (4). A sensitive determination method for these compounds has, however, not been re-

ported, and their detailed biochemical study is still lacking. We are interested in the physiological significance of these compounds, especially the correlation between their concentration and cataract formation.

Materials and Methods

Materials

2,4-Dinitrofluorobenzene was purchased from Katayama Chemical Industries Co. (Osaka). Acetonitrile and methanol for high performance liquid chromatography (HPLC) were obtained from Wako Pure Chemical Industries (Osaka). S-(1,2-Dicarboxyethyl)glutathione was kindly supplied from Dr. K. Ogata of the Senju Pharmaceutical Co. (Osaka).

Methods

Synthesis of S-(1,2-dicarboxyethyl)L-cysteine

L-Bromosuccinic acid was synthesized from L-aspartate according to the method of Holmberg (5). The hydrobromic acid (5.91 g) and L-cysteine (3.63 g) were dissolved in 40 ml water containing 6 g NaOH, and allowed to stand at 20 °C for 24 h. After the reaction the mixture was acidified with 2 mol/l acetic acid and applied to a Diaion SK (strong acidic, H⁺ form) column (18 × 4.8 cm). The column was washed with 1 l of water and eluted with 1 l of 2 mol/l ammonia. The eluate was concentrated to 300 ml under reduced pressure, aerated at room temperature for 1 h to oxidize excess cysteine, then filtered to remove cystine. The filtrate was applied to an Amberlite CG-50 (acetate form) column (12 × 2.8 cm). The column was washed successively with 500 ml of water, 200 ml of 0.5 mol/l acetic acid and 700 ml of 1 mol/l acetic acid, then eluted with 1750 ml of 2 mol/l acetic acid. The eluate was evaporated under reduced pressure and white crystals were obtained (dry weight 4.2 g).

After 4 times recrystallization from water and ethanol, needle crystals were obtained. S-(1,2-Dicarboxyethyl)L-cysteine was also synthesized from fumaric acid or maleic acid as follows: the acid (1.16 g) and L-cysteine (1.21 g) were dissolved in 20 ml of 100 g/l sodium carbonate and the mixture was allowed to stand for 48 h. The reaction mixture was purified using a Diaion SK column (20 × 2.8 cm) and an Amberlite CG-45 column (13 × 2.6 cm) as described above.

The physical properties of the products were as follows.

Properties of the S-(1,2-dicarboxyethyl)L-cysteine			
Precursor acid	decomp.	[α] _D ²⁰ in water	yield
Bromosuccinic acid	168 °C	+71.9 (4.18%)	59%
Fumaric acid	119 °C	-20.3 (4.6%)	100%
Maleic acid	135 °C	-13.1 (13.1%)	100%

Each sample of S-(1,2-dicarboxyethyl)L-cysteine was analytically pure and had a R_f value of 0.24 (R_f value of aspartate = 0.21) on paper chromatography (n-butanol : acetic acid : water = 4 + 1 + 1 by vol), and a mobility of 1.9 relative to aspartate on high voltage paper electrophoresis (acetic acid : pyridine : water = 300 + 15 + 2685 by vol, pH 3.1).

HPLC

A Shimadzu liquid chromatography Model 3A (Kyoto) equipped with a Shimadzu variable-wavelength detector and a Shimadzu SGR-1A step gradient apparatus was used. Analysis of S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine

were performed on a 15 × 0.46 cm column of Zorbax ODS, which was obtained from Dupont. Aliquots of 20 to 100 µl of sample solutions were injected into the column, on top of which a 2 µm millipore filter was placed. Acetate buffer (0.01 mol/l, pH 3.65)/acetonitrile (96 + 4 by vol) and the buffer/acetonitrile (94 + 6 by vol) were used as eluent for S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine, respectively. The flow rate was 1.0 ml/min. After the analysis the column was washed by methanol/water (70 + 30 by vol) for 5 min and equilibrated with the elution buffer for 20 min. All runs were performed at 44 °C and the eluate was monitored at 375 nm.

Lens preparation

Lenses were extracted from the eyes of animals immediately after the death. Lens (80 mg) was homogenized in 0.3 ml of water at 0 °C for 1 min. The homogenate was mixed with 2 ml of methanol using a homogenizer with a loosely fitted glass pestle and centrifuged at 1000 g for 15 min. The supernatant was transferred to a 7.5 × 1.0 cm test tube and the precipitate was homogenized with 1.0 ml of methanol/water (70 + 30 by vol.) and centrifuged as mentioned above. This operation was repeated. The methanol extracts were combined and kept in a freezer (-20 °C) over night, then centrifuged as mentioned above to remove precipitate. The supernatant was transferred to a 7.5 × 1.0 cm test tube and evaporated using a Savant Speed Vac Concentrator (model SVC-100H, New York) at room temperature.

The reaction of S-(1,2-dicarboxyethyl)glutathione with 2,4-dinitrofluorobenzene

After evaporation of the methanol extract, the residue was dissolved in 2.0 ml of 0.04 mol/l acetate buffer (pH 5.0). 1 ml of this solution was placed on a DEAE-Sephadex A-25 column (2 ml) previously equilibrated with 0.04 mol/l acetate buffer (pH 5.0). The column was washed successively with 5 ml of water and 5 ml of 1.74 mol/l acetic acid, then eluted with 5 ml of 1 mol/l HCl. The eluate was placed in a 5 ml vial and evaporated using the Speed Vac Concentrator. To this vial 200 µl of 100 g/l sodium carbonate solution and 10 µl of 2,4-dinitrofluorobenzene were added. The vial was tightly capped and shaken mechanically at room temperature for 1 h. After the reaction, 80 µl of 6 mol/l HCl were added, and the reaction mixture was extracted for 30 s with 1.0 ml of benzene using a Vortex-mixer. The vial was centrifuged, and an aliquot of the water layer (20 to 100 µl) was injected for HPLC. Figure 1 (a) and 1 (b) depict the chromatograms of authentic and lens S-(1,2-dicarboxyethyl)glutathione, respectively.

The reaction of S-(1,2-dicarboxyethyl)L-cysteine with 2,4-dinitrofluorobenzene

After application of the remaining 1 ml of the sample solution to the DEAE-Sephadex A-25 column, the column was washed with 5 ml of water followed by 5 ml of 0.44 mol/l acetic acid, then eluted with 5 ml of a mixture of 11.4 mol/l HCl and 1.74 mol/l acetic acid (1 + 50 by vol). The eluate was processed in the same manner as for S-(1,2-dicarboxyethyl)glutathione. Representatives of chromatograms of standard and lens sample are shown in figure 2 (a) and 2 (b), respectively.

Cataract formation (6, 7)

Female Wister strain albino rats aged 5 weeks were used after starvation overnight.

Galactose solution (350 g/l galactose; 1.0 ml) was administered orally to each rat in the group on the first day. Thereafter they received solid food (Oriental Yeast Co. Ltd., Tokyo) containing 350 g galactose per kg and water ad libitum for 10 days. Rats of the control group received normal solid food after oral administration of 1.0 ml of physiological saline on the first day.

Fig. 1. High performance liquid chromatograms of authentic S-(1,2-dicarboxyethyl)glutathione (a) and that obtained from bovine lens (b).

(a): An aliquot (10 μ l) of a S-(1,2-dicarboxyethyl)glutathione aqueous solution (2.36 mmol/l) was placed into a 5 ml vial, followed by evaporation under a nitrogen stream. S-(1,2-Dicarboxyethyl)glutathione in the dried residue was reacted with 2,4-dinitrofluorobenzene, and subjected to HPLC as described in "Methods".

(b): Bovine lens (80 mg) was treated with methanol and 2,4-dinitrofluorobenzene as described in "Methods". In both cases, a 20- μ l aliquot of the water layer (290 μ l) was injected for HPLC.

Fig. 2. High performance liquid chromatograms of authentic S-(1,2-dicarboxyethyl)L-cysteine (a) and that obtained from bovine lens (b).

(a): An aliquot (10 μ l) of a S-(1,2-dicarboxyethyl)L-cysteine aqueous solution (4.22 mmol/l) was treated in the same manner as described under figure 1 (a).

(b): Bovine lens (80 mg) was treated in the same manner as described in "Methods".

Identification of the peaks of the 2,4-dinitrophenol derivative of S-(1,2-dicarboxyethyl)glutathione from bovine lens on the high performance liquid chromatogram

Five fresh bovine lenses were homogenized and deproteinized in the manner described above. The extract was applied to the DEAE-Sephadex column and the S-(1,2-dicarboxyethyl)glutathione fraction was reacted with 2,4-dinitrofluorobenzene as described above. An aliquot (200 μ l) of the reaction mixture was injected for HPLC, and two peaks corresponding to 2,4-dinitrophenol derivative of S-(1,2-dicarboxyethyl)glutathione were collected from the end of the column. This fractionation procedure

was repeated 10 times. After evaporation of the combined fraction, the residue was dissolved in 0.5 ml of the mobile phase and further purified by HPLC.

The purified peak fraction was evaporated under reduced pressure and the residue was dissolved in 2 ml of 5.7 mol/l HCl. The solution was transferred to a vial with a tight cap and hydrolysed at 105 $^{\circ}$ C for 9 h after introducing nitrogen gas. The hydrolysate was dried in vacuo, then reacted again with 2,4-dinitrofluorobenzene as described above.

After removal of 2,4-dinitrophenol with benzene, an aliquot of the water layer was analysed by HPLC.

Authentic S-(1,2-dicarboxyethyl)glutathione was treated in the same manner.

Results

Standard curves

Figure 3 illustrates the standard curves for S-(1,2-dicarboxyethyl)glutathione (a) and S-(1,2-dicarboxyethyl)L-cysteine (b). The curves were linear at least up to 815 μ mol/l (a) and 1.45 mmol/l (b). As little as 4.08 μ mol/l of the former and 1.45 μ mol/l of the latter could be detected by the present procedure.

Fig. 3. Calibration curves of S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine.

Varying amounts (10, 30, 50, 100 μ l) of S-(1,2-dicarboxyethyl)glutathione (2.36 mmol/l) and S-(1,2-dicarboxyethyl)L-cysteine (4.22 mmol/l) aqueous solutions were treated as described under figure 1 (a) and figure 2 (a).

Recovery tests

Various amounts of S-(1,2-dicarboxyethyl)glutathione or S-(1,2-dicarboxyethyl)L-cysteine were added to bovine lens homogenate and their recoveries were measured in a separate run by the procedure described above. The results are summarized in table 1, which shows recoveries of $90.0 \pm 3.2\%$ for S-(1,2-dicarboxyethyl)glutathione and $95.3 \pm 3\%$ for S-(1,2-dicarboxyethyl)L-cysteine.

Tab. 1. Recovery of S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine added to cattle lens homogenate.

	S-(1,2-Dicarboxyethyl)-glutathione added		S-(1,2-Dicarboxyethyl)-glutathione determined		Recovery	
	(nmol/80 mg lens)	(nmol/80 mg lens)	%	C.V. (%)	Mean $\pm$ S.D.	
S-(1,2-Dicarboxyethyl)-glutathione	0	9.76			90.0 $\pm$ 3.21	
	7.09	16.3	92.2	2.16		
	17.7	24.9	85.5	1.96		
	28.4	36.0	92.4	1.52		

	S-(1,2-Dicarboxyethyl)-L-cysteine added		S-(1,2-Dicarboxyethyl)-L-cysteine determined		Recovery	
	(nmol/80 mg lens)	(nmol/80 mg lens)	%	C.V. (%)	Mean $\pm$ S.D.	
S-(1,2-Dicarboxyethyl)-L-cysteine	0	3.10			95.3 $\pm$ 3.09	
	6.33	9.21	96.5	1.36		
	19.0	20.4	91.1	1.41		
	25.3	28.0	98.4	0.813		

Various amounts of S-(1,2-dicarboxyethyl)glutathione or S-(1,2-dicarboxyethyl)L-cysteine were added to the lens homogenate, and each was measured by the respective procedure described in the text.

Each value is the mean of duplicate determinations.

The coefficients of variation (C.V. %) are given as C.V. (%) =

$$\frac{\text{S.D.}}{\text{mean}} \times 100.$$

S-(1,2-Dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine in lenses of various animals

The described method was used for the determination of S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine in lenses of various vertebrates. As shown in table 2, S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine were found in all animals examined and the former was found in much higher concentrations than the latter. S-(1,2-Dicarboxyethyl)glutathione was found in especially high concentration in rabbit lens.

Confirmation of identity of the peaks of 2,4-dinitrophenol derivative of S-(1,2-dicarboxyethyl)glutathione from bovine lens on the high performance liquid chromatogram

Identification of peaks corresponding to the 2,4-dinitrophenol derivative of S-(1,2-dicarboxyethyl)glu-

Tab. 2. Distribution of S-(1,2-dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine in lens of various vertebrates.

	S-(1,2-Dicarboxyethyl)-glutathione (nmol/g lens)	S-(1,2-Dicarboxyethyl)-L-cysteine (nmol/g lens)
Cattle	115 $\pm$ 9.66	34.4 $\pm$ 5.83
Rat	119 $\pm$ 10.6	3.68 $\pm$ 0.57
Guinea pig	58.6 $\pm$ 4.38	16.5 $\pm$ 1.76
Rabbit	462 $\pm$ 113	38.5 $\pm$ 16.8
Dog	255 $\pm$ 61.1	19.8 $\pm$ 9.56
Tuna	309 $\pm$ 138	11.5 $\pm$ 1.21

All lenses were treated immediately after death as described in the text.

tathione from bovine lens was confirmed by comparison with the authentic peptide.

The 2,4-dinitrophenyl-peptide was synthesized from authentic S-(1,2-dicarboxyethyl)glutathione, hydrolysed by hydrochloric acid, then reacted again with 2,4-dinitrofluorobenzene. Analysis of the 2,4-dinitrophenyl amino acids by HPLC gave four peaks with retention times of 13.6, 14.4, 16 and 17.6 min, which (from previous standardized analysis) are due to the two optical isomers of S-(1,2-dicarboxyethyl)L-cysteine, and to glycine and glutamic acid, respectively. The ratios of peak areas were 1.1 : 1 : 2.03 : 8.7. In comparison, 2,4-dinitrophenyl amino acids from the 2,4-dinitrophenol derivative of S-(1,2-dicarboxyethyl)glutathione from bovine lens gave also four peaks with peak area ratios of 1.2 : 1 : 2 : 8.4.

This evidence shows that peaks corresponding to the 2,4-dinitrophenol derivative of S-(1,2-dicarboxyethyl)glutathione from bovine lens were genuine and had no contamination.

Changes in the level of S-(1,2-dicarboxyethyl)glutathione in rat lens during the galactose cataract formation

Since the rat cataract formation by galactose is triggered in the first week (8), we examined the time course of the level of S-(1,2-dicarboxyethyl)glutathione in rat lens in the early stages of galactose feeding. As shown in figure 4, S-(1,2-dicarboxyethyl)glutathione levels in rat lens of the galactose feeding group decreased to 50% of controls within 48 h, to 30% on 5th day and 25% on 10th day.

Fig. 4. Changes in the level of S-(1,2-dicarboxyethyl)glutathione in rat lens during the galactose cataract formation. Rats were divided into two groups, i.e. the cataract (●) and the normal group (○) ($n = 3$ at the respective time intervals), and were treated as described in "Cataract formation". After 1, 2, 3, 5, 7 and 10 days, they were decapitated, lenses were immediately excised and were treated with methanol and 2,4-dinitrofluorobenzene as described in "Methods". An aliquot (20 μ l) of the water layer (290 μ l) was injected for HPLC.
 | | Represents half of the standard deviation.

Discussion

S-(1,2-Dicarboxyethyl)glutathione and S-(1,2-dicarboxyethyl)L-cysteine were reported by Calam & Waley in 1963, but a sensitive analytical procedure for these compounds has been lacking until now. We first analysed S-(1,2-dicarboxyethyl)L-cysteine by gas-liquid chromatography with an electron capture detector (9). This method was briefly as follows: S-(1,2-dicarboxyethyl)L-cysteine was esterified in methanol containing hydrochloric acid (50 g/kg) in a vial at 100 °C for 30 min and reacted with heptafluorobutyric anhydride at 40 °C for 30 min. This derivative showed a single peak on ECD-gas chromatogram and its calibration curve was linear from 0.063 to 6.33 μ mol/l. S-(1,2-Dicarboxyethyl)glutathione was hydrolysed in 6 mol/l HCl in a vial at 110 °C for 6 h and S-(1,2-dicarboxyethyl)L-cysteine formed was determined. This method was sensitive, but it had the disadvantage that it could not directly determine S-(1,2-dicarboxyethyl)glutathione and was time-consuming.

Consequently, further investigations were undertaken to determine this peptide and amino acid using HPLC. The two substances were measured in separate runs by the present HPLC method, to avoid the problem of overlapping peaks in the simultaneous

determination. Calam & Waley reported that the concentration of S-(1,2-dicarboxyethyl)glutathione was 30 μ mol/100 g of calf lens and that of S-(1,2-dicarboxyethyl)L-cysteine was lower (1). This value of S-(1,2-dicarboxyethyl)glutathione in calf lens was about 3 times as much as that determined by us. As noted in table 2, the S-(1,2-dicarboxyethyl)L-cysteine content in lenses of various animals is one-third to one order of magnitude lower than the content of S-(1,2-dicarboxyethyl)glutathione.

It was noteworthy that the S-(1,2-dicarboxyethyl)glutathione concentration in lens decreased rapidly during the development of cataract. Reddy reported the level of glutathione in rat lens fell rapidly during cataract formation (8). We also determined the glutathione level of lens during cataract formation by feeding galactose and obtained same results. When S-(1,2-dicarboxyethyl)glutathione and glutathione levels are plotted as a percentage of each control on the Y axis, and dietary exposure (days) is plotted on the X axis, each curve decreases to 50% on the second day, 30% on the third and 25% on the 10th day. This means that rates of synthesis and degradation of glutathione in rat lenses of during galactose cataract may be same as those of S-(1,2-dicarboxyethyl)glutathione. However, glutathione protects strongly against photooxidation in bovine lens homogenate, whereas S-(1,2-dicarboxyethyl)glutathione stimulates photooxidation (10). We also monitored the concentrations of S-(1,2-dicarboxyethyl)L-cysteine in rat lens during cataract formation, but its level was too low to measure after the second day on the diet. In general, free amino acids were found to decrease rapidly during cataract development (11, 12).

Ophthalmic acid and norophthalmic acid, analogues of glutathione, have been also reported to be present in bovine lens. We tried to determine whether their concentrations change during cataract formation in the galactose-fed rat, using the present method, but unfortunately they could not be detected either in the lenses of normal or cataract rats.

As shown in figure 1, S-(1,2-dicarboxyethyl)glutathione synthesized from glutathione and fumarate as well as that occurring in the lens from cattle, rabbit and guinea pig gave two adjacent peaks on the high performance liquid chromatogram. On the other hand, similar analyses of lenses from dog, rat and tuna showed the same two adjacent peaks, but the first of these was higher than the second. On the other hand, S-(1,2-dicarboxyethyl)L-cysteine, which was synthesized from fumarate or occurred in animal lens, also appeared as two peaks on HPLC. Similar-

ly, synthesized and urinary S-(1,2-dicarboxyethyl)L-cysteine gave adjacent two peaks on the amino acid analyser (2), but one peak on the ECD-gas chromatogram (9). This is because diastereoisomers at the dicarboxyethyl group of both compounds are present. But the presence of diastereoisomers of a natural amino acid or peptide is not usual. If glutathione or cysteine in lens reacts nonenzymatically with fumarate, diastereoisomers must be formed. Calam & Waley demonstrated, however, that S-(1,2-dicarboxyethyl)glutathione was not an artifact from glutathione and fumarate, but a genuine constituent of the lens, by removing endogenous glutathione from the homogenate with N-ethylmaleimide. Therefore, we synthesized the optically pure S-(1,2-dicarboxyethyl)L-cysteine and S-(1,2-dicarboxyethyl)glutathione, by reacting L-bromosuccinic acid and L-cysteine or glutathione in sodium hydroxide or sodium carbonate solution. However, the reaction products also gave two adjacent peaks on HPLC, and are identical with the compounds formed by reaction of fumarate or malate with cysteine or glutathione. Although, as described above, S-(1,2-dicarboxy-

ethyl)L-cysteine synthesized from three materials showed slight differences of specific rotation, they gave the same two adjacent peaks. Recently, Nishiyama & Kuninori showed that an addition compound of N-ethylmaleimide and glutathione, S-(N-ethyl-2,5-dioxopyrrolydino-3-yl)glutathione, gave two peaks on the high performance liquid chromatogram and separated and purified each diastereoisomer (13). They reported kinetic studies of racemization of the optically pure compound (14). This evidence suggests that the asymmetric carbon of the dicarboxyethyl group easily undergoes racemization. The reason for the higher first peak in the HPLC of S-(1,2-dicarboxyethyl)glutathione from the lenses of dog, rat and tuna is not elucidated.

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