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Note

Separation and characterization of ribonuclease A–glutathione mixed disulphide using chromatofocusing and isoelectric focusing

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There have been many reports on the occurrence in biological materials of mixed disulphides of proteins and low-molecular-weight sulphydryl compounds such as glutathione and cysteine[1]. The modification of a number of enzymes by S-thiolation with glutathione has also been reported and its possibility for metabolic regulation discussed[2]. During the course of our study on the identification and biosynthesis of low-molecular-weight mixed disulphides containing cysteine and glutathione in mammalian tissues[3–5], protein–glutathione mixed disulphides attracted our attention. In the present study, we prepared bovine pancreatic ribonuclease A–glutathione mixed disulphide (RNase-SG) as a model for the study of protein–glutathione mixed disulphides. We also report the separation by chromatofocusing and isoelectric focusing of RNase-SG species differing in their glutathione contents, and the values of their isoelectric points, p*I*.

EXPERIMENTAL

Materials

Bovine pancreatic ribonuclease A (E.C. 3.1.27.5) (RNase), dithiothreitol (DTT), oxidized and reduced glutathione (GSSG and GSH, respectively) were obtained from Böhringer Mannheim (Mannheim, F.R.G.). Pharmalyte (pH 3–10), Polybuffer exchanger 94 and Polybuffer 74 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Acrylamide, N,N′-methylenebisacrylamide and N,N,N′,N′-tetramethylethylenediamine were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Guanidine hydrochloride and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Wako (Osaka, Japan). Glutathione reductase [NAD(P)H] (E.C. 1.6.4.2) from yeast and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan).

Preparation of RNase-SG

RNase (140 mg, 10 μmol) dissolved in 20 ml of 6 *M* guanidine hydrochloride containing 2 m*M* EDTA and 0.5 *M* Tris–HCl (pH 8.1) was reduced with 800 μmol of DTT according to the method of Konigsberg[6]. The reduced RNase solution was added to 20 ml of water containing 12 mmol of GSSG, with stirring under an atmosphere of nitrogen. After all the GSSG had dissolved, the solution was adjusted

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to pH 6.5 with 2 *M* sodium hydroxide. The mixture was stirred under a nitrogen atmosphere at 25°C for 3 h. Then the solution was thoroughly dialyzed against 1 m*M* acetic acid at 4°C and lyophilized.

Chromatofocusing

Chromatofocusing was performed at 4°C. A column of Polybuffer exchanger 94 (550 mm × 10 mm) was equilibrated with 25 m*M* imidazole hydrochloride, pH 7.0. RNase-SG (about 30 mg of protein) dissolved in 10 ml of an eluent, Polybuffer 74 hydrochloride (pH 4.0) diluted eight-fold, was applied and the elution was performed at a flow-rate of 34 cm/h.

Isoelectric focusing

Isoelectric focusing was performed at 10°C in a horizontal slab gel of 5% polyacrylamide (11 cm × 10 cm, 0.8 mm thick) containing 2% Pharmalyte and 5% glycerol. Pre-focusing was performed with a constant power of 4.0 W and a maximum voltage of 1000 V for 45 min. The anolyte solution was 1 *M* phosphoric acid and the catholyte was 1 *M* sodium hydroxide. Then 5 μl of a sample were applied in a well 10 mm from the anode and electrofocused for 90 min. After fixation with 10% trichloroacetic acid and 5% sulphosalicylic acid, the protein bands were stained with 0.02% Coomassie brilliant blue R-250.

Determination of pI values

Immediately after the isoelectric focusing, the slab gel was divided into two parts. One part was cut into sections of width 5 mm. Each section was extracted with 5 ml of water and the pH of the extract was determined with a glass electrode at 10°C. Another part was stained as above. The p*I* value of each band was determined by comparison with the pH gradient of the gel.

Identification and determination of GSH bound to RNase

GSH bound to RNase was identified and determined by several methods: (a) analysis of GSH with an amino acid analyzer[7] after the reduction of RNase-SG with DTT; (b) determination of GSH with glutathione reductase and DTNB[8] after the reduction with DTT; (c) acid hydrolysis of RNase-SG and determination of amino acids with an amino acid analyzer[7].

RESULTS

Preparation of RNase-SG and separation by isoelectric focusing

Fig. 1 shows a typical isoelectric focusing of RNase-SG prepared by the present method. Eight bands were clearly separated. They were numbered from the side of the cathode as shown for lane 6. The preparations shown in Fig. 1 were prepared by treating the reduced RNase with different amounts of GSSG. The molar ratios of GSSG to DTT used in the reduction of RNase were 3, 5, 10 and 15 for the RNase-SG shown in lanes 3, 4, 5 and 6, respectively. The contribution from each band was estimated by scanning densitometry. When the molar ratio was 3, the most abundant species was band 1 (27% of the total), followed by band 2 (19%) and RNase without GSH constituted 17%. At a molar ratio of 15, all the RNase was converted into

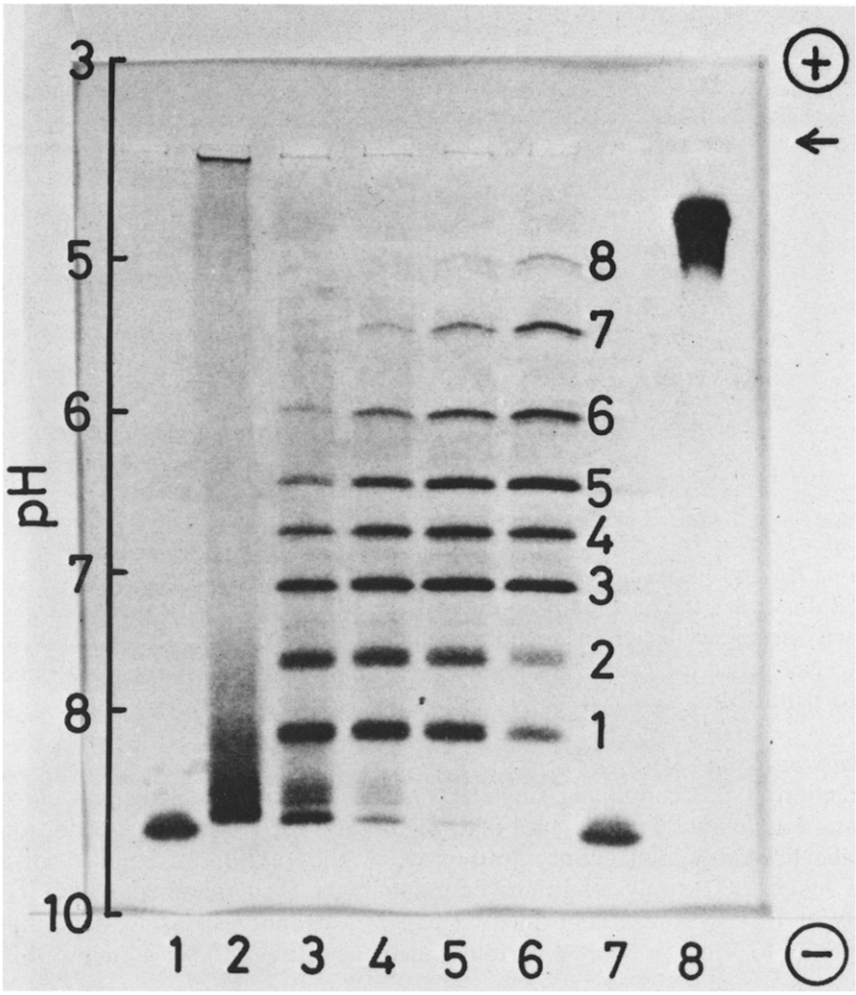

Fig. 1. Isoelectric focusing of RNase-SG in a slab polyacrylamide gel containing 2% Pharmalyte (pH 3–10). Proteins were stained with Coomassie brilliant blue R-250. Lanes 1 and 7, native RNase; 2, reduced RNase; 3–6, RNase-SG prepared by treating reduced RNase with GSSG at GSSG to DTT ratios of 3, 5, 10 and 15; 8, bovine serum albumin. Conditions as in Experimental. The arrow indicates the position of sample application.

RNase-SG. Thus, the higher the GSSG concentration, the greater were the amounts of RNase-SG species with higher band numbers.

The effect of pH on the formation of RNase-SG was examined between pH 5.8 and 7.8. The amounts of bands with higher band numbers were slightly greater than those of the other bands at pH 6–7 under the present preparative conditions. Therefore, the preparation of RNase-SG was performed at pH 6.5.

The pI values of RNase-SG

From the results obtained in six separate experiments, the p*I* value of each band at 10°C was as shown in Table I.

TABLE I

pI VALUES AND MOLAR RATIOS OF GSH AND RNase OF RNase-SG SPECIES

Values of pI are means of six separate experiments determined by isoelectric focusing in slab polyacrylamide gels containing 2% Pharmalyte (pH 3–10) at 10°C. GSH and RNase were determined by acid hydrolysis and amino acid analysis of single RNase-SG species separated by chromatofocusing. GSH/RNase values are means of two separate experiments.

	Band No.							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
	pI							
	8.2	*7.6*	*7.1*	*6.7*	*6.4*	*6.0*	*5.4*	*4.9*
GSH/RNase	–	–	2.93	4.23	4.98	5.90	7.26	7.74
Nearest integer	–	–	3	4	5	6	7	8

Separation of RNase-SG species by chromatofocusing

Fig. 2 illustrates the chromatofocusing of a RNase-SG preparation. Peaks 3–8 were clearly separated. Fractions exhibiting maximum absorption at 280 nm were subjected to isoelectric focusing. As shown in Fig. 3, peak 8 corresponded to band 8, peak 7 to band 7, etc.

GSH contents in RNase-SG species

A fraction (1 ml) containing single RNase-SG species obtained upon chromatofocusing was treated with 20 μmol of DTT at pH 9 for 1 h. After the addition of sulphosalicylic acid to a final concentration of 3%, the solution was applied to an amino acid analyzer. The only compound liberated by the DTT treatment was GSH. The identity of GSH was also confirmed by paper electrophoresis using Whatman 17 Chr paper at 40 V/cm in 50 m*M* pyridine–acetic acid (pH 6.0) for 40 min. GSH

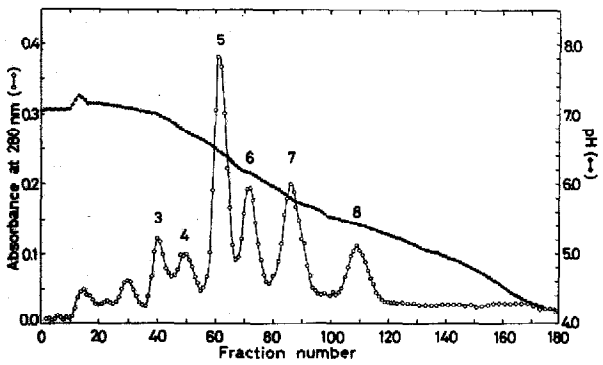

Fig. 2. Chromatofocusing of RNase-SG at 4°C. Column: Polybuffer exchanger 94 (550 mm × 10 mm). Starting buffer: 25 m*M* imidazole hydrochloride, pH 7.0. Eluent: Polybuffer 74 hydrochloride (1:8 dilution), pH 4.0. Flow-rate: 34 cm/h. Fractions of 3 ml were collected.

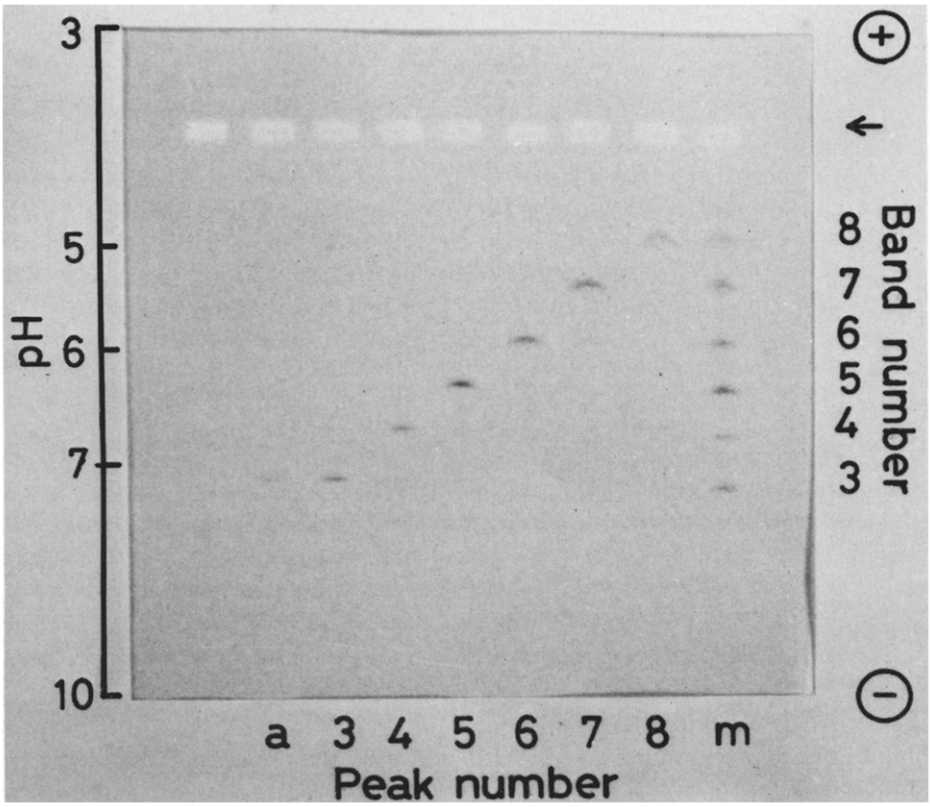

Fig. 3. Isoelectric focusing of the peak fractions shown in Fig. 2. Conditions as in Fig. 1. m = Mixture of RNase-SG species; a = fraction 30.

moved about 4 cm and GSSG about 6 cm toward the anode, and they were well separated from DTT and RNase which remained near the origin. GSH and GSSG were further identified using glutathione reductase and DTNB[8] after extraction with water of the area containing GSH and GSSG.

In order to determine the GSH content, a fraction containing a single RNase-SG species obtained upon chromatofocusing was hydrolyzed in 6 *M* hydrochloric acid at 110°C for 22 h and amino acids were determined by an amino acid analyzer. The GSH content was calculated from the increase in the amount of Glu. The molar ratio of GSH to RNase was determined on the basis of the amino acid residues of RNase[9]. Asp and Val were taken as the references because they are not present in GSH and gave constant values after the acid hydrolysis. As shown in Table I, the ratios of GSH to RNase indicate that band 8 contained 8 mol of GSH per mol of RNase, band 7 contained 7 mol of GSH per mol of RNase, etc. The exact values of the GSH contents in bands 1 and 2 have not been determined because pure preparations of these bands have not yet been obtained. In one experiment, however, the molar ratio of GSH to RNase obtained with a mixture of these bands was 1.76. Therefore, it was assumed that bands 1 and 2 contained 1 and 2 mol of GSH per mol of RNase, respectively.

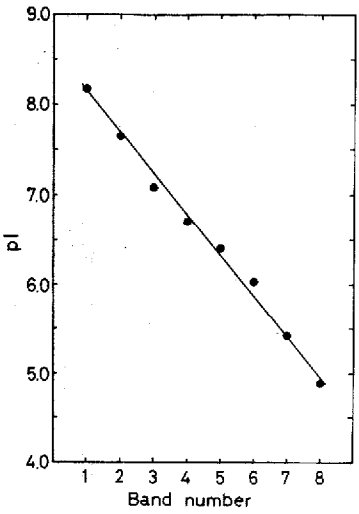

Fig. 4. The relationship between the p*I* value and the GSH content in RNase-SG species. The band number corresponds to the GSH content (mol/mol).

DISCUSSION

The results reported show that reduced RNase was converted into the mixed disulphide of RNase and GSH when treated with GSSG. GSH was liberated from the preparation upon treatment with DTT. However, GSH was not eliminated by chromatofocusing, isoelectric focusing, paper electrophoresis and gel filtration on Sephadex G-25. These results indicate that GSH is bound to RNase by the disulphide bond.

Analyses of the preparations by chromatofocusing and isoelectric focusing have shown that the disulphides comprise eight species differing in their GSH contents. Thus, these disulphides were designated as RNase-SG_1, -SG_2, -SG_3, -SG_4, -SG_5, -SG_6, -SG_7 and -SG_8. The binding of GSH, an acidic tripeptide, to a RNase molecule by the disulphide bond results in a decrease in the p*I* value of RNase, in proportion to the number of bound GSH, as illustrated in Fig. 4. The average decrease was 0.47 per bound GSH. Thus, the RNase-SG species differ considerably in their p*I* values, although their molecular weights are similar. Therefore, chromatofocusing and isoelectric focusing are the most suitable methods for the preparative separation and analyses of RNase-SGs. These methods will also be useful for the study of other protein–glutathione mixed disulphides.

As shown in Fig. 1, bands with lower band number, especially RNase-SG_1 and -SG_2, were broader than the others. This seems to indicate the heterogeneity of the RNase-SG which differs in the site of bound GSH.

In some preliminary experiments, the mixture of RNase-SG species exhibited little RNase activity and also little effect on the activity of native RNase. However, further studies on the activities of the individual RNase-SG species are needed.

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