J. Biochem. Mol. Biol. Vol. 28, No. 2 pp. 170~176 March 31, 1995

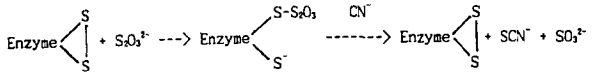
Purification and Characterization of Mouse Liver Rhodanese

Chul Young Lee, Jae Hoon Hwang, Young Seek Lee and Key Seung Cho*

Department of Biochemistry, College of Sciences, Hanyang University, Ansan 425-791, Korea
(Received November 2, 1994)

Abstract: Rhodanese from mouse liver was purified to near homogeneity by ammonium sulfate precipitation, CM-Sephadex ion exchange, hydroxyapatite and Sephacryl S-200-HR gel filtration chromatographies with a purification of 776 folds. The molecular weight was determined by Sephadex G-150 gel filtration and found to be 34.8 KDa. SDS-PAGE showed molecular weight 34 KDa and two identical subunits splitting by aging for 3 weeks at −70℃ the molecular weight of which was 17 KDa. The optimal pH of enzyme activity was 9.4 and the pI value of the enzyme was 6.6. Rhodanese showed the optimal reaction temperature of 25℃ and near linear increasing pattern until 10 min. incubation. K_M values of rhodanese for KCN and $Na_2S_2O_3$ as substrates were 12.5 mM and 8.3 mM, respectively. Rhodanese activity was inhibited by more than 70% at a concentration of 100 μM of Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Cu^{2+}. Other metal ions, such as Mn^{2+}, Mg^{2+}, Ca^{2+}, and Fe^{2+} showed no effect on rhodanese activity.
Key words: aging, enzyme activity, mouse liver, rhodanese.

Since Lang (1933) reported the demonstration of a biological catalyst, rhodanese (EC 2.8.1.1, thiosulfate sulfurtransferase) that converted the cyanide ion into the less toxic thiocyanate, Sörbo (1953a) first crystallized and partially characterized the rhodanese from bovine liver, and subsequent studies with bovine liver and kidney enzymes by a number of investigators have provided information with regard to the mechanism of reaction, and the chemical and physical nature of the enzyme (Westley, 1959; Westley and Green, 1959; Leininger and Westley, 1968; Aird and Horowitz 19 88). This enzyme catalyzes the transfer of the outer sulfate of thiosulfate to cyanide forming the products thiocyanate and sulfide (Sörbo, 1975).



The reaction proceeds by way of a double-displacement mechanism in which a covalent enzyme-sulfur intermediate is formed (Mintel and Westley, 1966). The sulfur atom transferred during the catalysis is bound in persulfide linkage to Cys-247, and substrate CN^- binding seems to involve Arg-186 and Lys-249 (Ploegman *et al.*, 1978; Weng *et al.*, 1978).

In heart and liver tissues, rhodanese was known to localize in the mitochondrial fraction (Westley, 1973) and 30% of its activity existed in membrane-bound form (Ogata and Volini, 1986). The molecular weight and number of polypeptides in rhodanese have been subjects of controversy. Since Sörbo (1953a) initially reported a molecular weight of 37 KDa for rhodanese, numerous investigators suggested that this enzyme was Mr. 33~37 KDa and a dimer composed of two identical subunits, Mr 18~19 KDa (Blumenthal and Heinrikson, 1971; Volini *et al.*, 1978). The dimer theory was later found to be incompatible from the results of three-dimensional structure analysis, and the well-characterized rhodanese from bovine liver mitochondria is a single polypeptide chain of molecular weight, 32,900 daltons composed of 293 amino acids (Russell *et al.*, 1978; Ploegman *et al.*, 1978).

The present paper first describes the isolation and purification of rhodanese from mouse liver. The physical properties and characteristics of enzyme were compared with other rhodanese from various sources.

Materials and Methods

Materials

The standard proteins for molecular weight estimation, acrylamide, bisacrylamide, Tris, CM-Sephadex C 50-120, Sephacryl S-200-HR were purchased from Sigma Chem. Co. (St. Louis, USA). Potassium cyanide, sodium thiosulfate, DTT, PMSF, ammonium persulfate,

*To whom correspondence should be addressed.
Tel: 82-345-400-5511, Fax: 82-345-419-1760.

ammonium sulfate, formaldehyde, HCl, HNO_3, and other inorganic chemicals were purchased from Merck (Frankfurte, Germany). Reagents for protein determination and Affi-gel blue were obtained from BioRad (Richmond, USA). Hydroxyapatite and Sephadex G-150 were purchased from Calbiochem (Lajolla, USA) and Pharmacia (Uppsala, Sweden), respectively.ej

Purification of rhodanese

The homogenation and fractionation of mouse liver followed the method of Volini *et al.* (1967) with slight modification. Rhodanese was purified by ordinary chromatography according to the procedure of Blumenthal and Heinrikson (1971) and Horowitz (1978). Eighty mixed-sex mice, weighing about 30± 3 g, were killed by decapitation from which 120± 15 g of liver tissue was collected for purification of rhodanese each time.

Liver tissue was immediately removed and rinsed several times with cold saline (pH 7.4) to remove blood and impurities. The tissue was finely minced with scissors, suspended in 240 ml of 0.1 M acetate glycine buffer (pH 7.8) containing 1 mM PMSF and 10 mM $Na_2S_2O_3$ (buffer A) and homogenized with teflon homogenizer for 10 min in an ice-bath. The liver homogenate was centrifuged for 30 min. at 3,000×*g* in a refrigerated high-speed centrifuge (Hanil, Model H50A-8). The supernatant was collected and remaining precipitates were rehomogenized with buffer A and centrifuged under the same conditions as above. The combined supernatant was saturated with 35% ammonium sulfate and stirred for 3 h at 4℃ with a magnetic stirrer. The supernatant collected after centrifugation for 30 min at 10,000×g was resaturated with 50% ammonium sulfate and stirred for 3 h at 4℃. The precipitates obtained by centrifugation for 30 min. at 20,000 ×g were dissolved in 10 ml of 50 mM citrate buffer (pH 5.0) containing 10 mM $Na_2S_2O_3$ (buffer B) and dialyzed for 24 h against 3 l of buffer B exchanged 3 times to remove the ammonium sulfate. This dialysate was chromatographed as follows.

CM-Sephadex ion chromatography: The washed CM-Sephadex 50~120 resin mixed with the equilibration buffer (buffer B) was poured into a column (2.5× 50 cm) and equilibrated with a 5 bed volume of buffer B. Twenty milliliters of dialysate were applied and unbound protein was washed out with 500 ml of buffer B. The column was eluted at a flow rate of 1.12 ml/min. with a linear gradient from 0 to 500 mM KCl buffer containing 50 mM citrate, pH 5.0, 10 mM $Na_2S_2O_3$ and 1 mM PMSF (buffer C). Each fraction of 6.3 ml was collected. The activity peak, fractions 124~136, were combined and precipitated with 70% ammonium sulfate. The precipitate obtained was dissolved in 8 ml of 10 mM phosphate buffer (pH 7.0) containing 10 mM $Na_2S_2O_3$ (buffer D) and dialyzed for 12 h. against 3 l of the same buffer and used for the next step.

Affi-gel blue column chromatography: A total volume of 8 ml of rhodanese solution was applied to a Affi-Gel blue column (mesh 100~200; size 1.6×15 cm) equilibrated with a 5 bed volume of buffer D. The column was eluted at a flow rate of 0.45 ml/min. with a linear gradient from 0 to 500 mM KCl buffer D. Each fraction of 3.2 ml was collected. The active fractions, 64~78 were combined and precipitated with 70 % ammonium sulfate saturation. The precipitate dissolved in 5 ml of buffer D was dialyzed for 12 h against 3 l of the same buffer.

Hydroxyapatite column chromatography: Hydroxyapatite resin, 20 g equilibrated with buffer D was poured into a column (2.0×12 cm) and washed with a 5 bed volume of the same buffer. Five ml of dialysate obtained from the previous step was applied to the column and unbounded protein was washed out with 100 ml of buffer D. The elution was started with 100 ml of buffer D and phosphate gradient from 0 to 300 mM and collected 3.0 ml/tube at a flow rate of 0.52 ml/min. The fractions with high activity, 38~48, were combined and concentrated by 70% ammonium sulfate saturation. The precipitate was dissolved in 2.5 ml of 50 mM phosphate buffer (pH 7.0) containing 10 mM $Na_2S_2O_3$ (buffer E) and dialyzed for 12 h to remove salts with the same buffer.

Sephacryl S-200-HR gel filtration: Rhodanese fraction from step 3 was applied to Sephacryl S-200-HR gel column (1.6×125 cm) equilibrated with buffer E. The column was eluted with buffer E at a flow rate of 0.14 ml/min and collected in a 1.4 ml/tube. The active fractions, 134~147, were combined and precipitated with 70% ammonium sulfate saturation. The precipitate was dissolved in 2 ml of 50 mM phosphate buffer (pH 7.0) (buffer F) and dialyzed for 12 h with the same buffer, separated into aliquots and stored at −70℃.

Rhodanese assay

Enzyme activity was determined by a modification of the method of Sörbo (1953a) to detect the concentration of SCN^- formed by the reaction of CN^- to $Na_2S_2O_3$. The reaction mixture was composed of 0.6 ml of 50 mM borate buffer (pH 9.4), 0.2 ml of 0.25 M KCN, 0.2 ml of 0.25 M $Na_2S_2O_3$ and 10 μl of enzyme preparation. The mixtures were incubated for 1 min at 25℃ and stopped by addition of 0.5 ml of 15% formaldehyde. After addition of 1.5 ml of 10% ferric nitrate solution, an absorbance was detected at 460 nm with a spectrophotometer (Beckman, Model DU-50). The standard curve was prepared with potassium thiocyanate by measuring an absorbance at 460 nm.

Molecular weight determination

The molecular weight of rhodanese was estimated by gel filtration chromatography on a calibrated Sephadex G-150 column (1.6×90 cm). Standard proteins were amylase (200 KDa), bovine serum albumin (66 KDa), ovalbumin (43 KDa), carbonic anhydrase (29 KDa), and cytochrome C (12.4 KDa). The molecular weight of enzyme was also determined by SDS-PAGE as described by Laemmli (1970). The gel consisted of 5% acrylamide stacking gel and 15% acrylamide separating gel. After loading the sample, the slab gel (40× 140×1.5 mm) was run for 2 h at 15 mA and then run for 6 h at 30 mA. The standard proteins used were bovine serum albumin (66 KDa), ovalbumin (45 KDa), pepsin (43.7 KDa), trypsinogen (24.4 KDa), β-lactoglobulin (18.4 KDa), and lysozyme (14.3 KDa).

Isoelectric focusing

In order to determine the isoelectric point (pI) of rhodanese, isoelectric focusing was performed in Phast-Gel IEF gel (39×47×0.35 mm, pH range 3 to 9) on a Phast system (Pharmacia). The combination of standard proteins used for isoelectric focusing were amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase (pI 6.55), horse myoglobin (pI 6.85), horse myoglobin (pI 7.35), lentil lectin (pI 8.15), lentil lectin (pI 8.45), lentil lectin (pI 8.65), and trypsinogen (pI 9.3). Proteins in the isoelectric focusing gel were stained with 0.02% PhastGel Blue R solution for 25 min.

Protein determination

Protein concentration was measured as described by Bradford (1976) with a Bio-Rad protein assay reagent, using bovine serum albumin as a standard.

Results and Discussion

Purification of enzyme

In order to purify the rhodanese from the crude cell-free extracts of mouse liver, 120 g of liver was homogenized and fractionated as described in the Material and Methods section, and chromatographed on a CM-

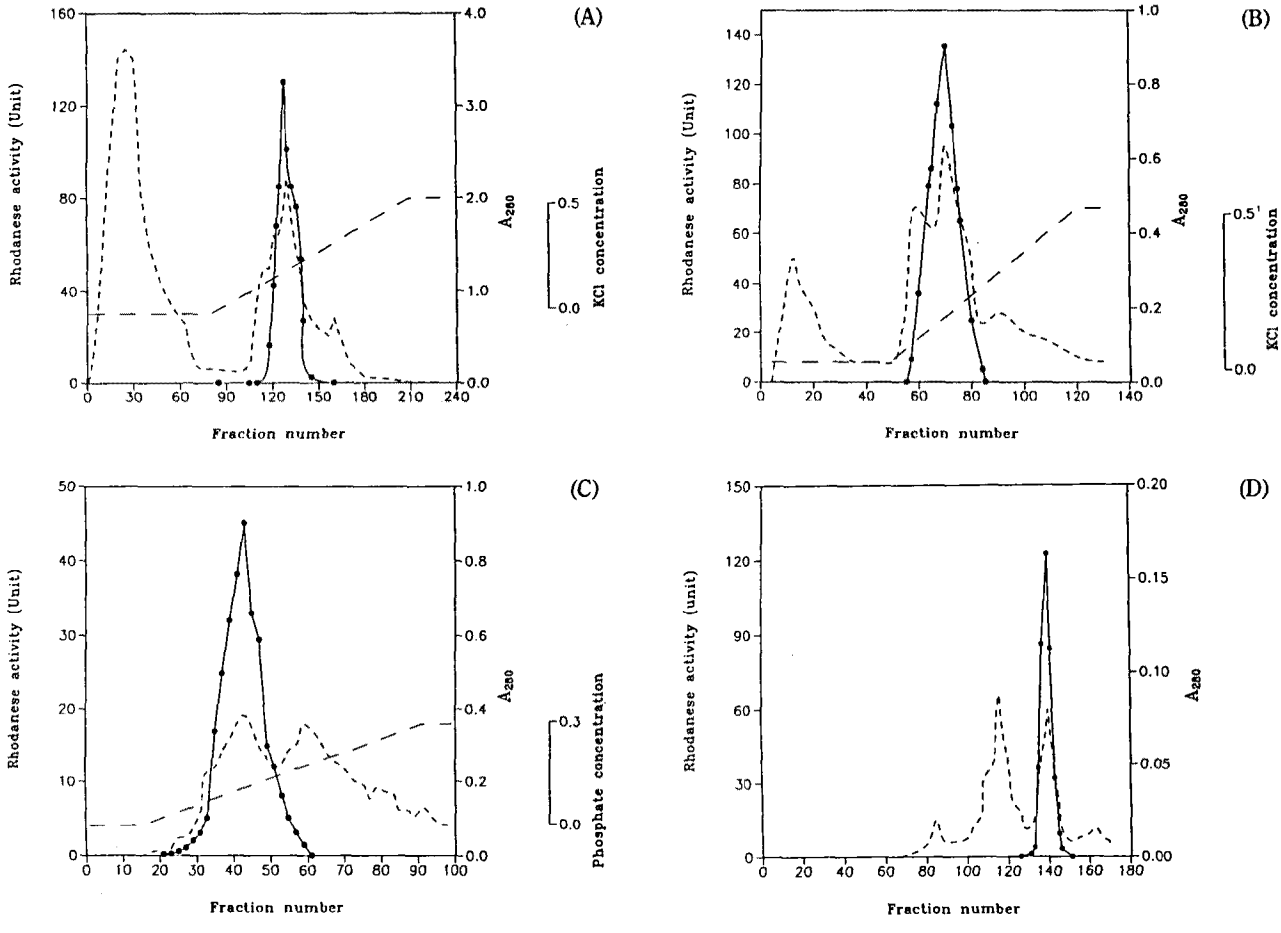


Fig. 1. Chromatographic purification of mouse liver rhodanese. (A) CM-Sephadex ion exchange chromatography, (B) Affi-gel blue chromatography, (C) Hydroxyapatite chromatography, (D) Sephacryl S-200-HR gel filtration. ●—● : Rhodanese activity, ----- : protein concentration, – – – : KCl concentration.

Table 1. Purification of rhodanese from mouse liver tissue.

Step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification fold
Homogenate	8,117.14	11,364	1.38	1
Ammonium sulfate precipitate	514.98	5,400	10.49	7.6
CM-Sephadex	47.28	3,288	69.54	50.4
Affi-gel blue	22.19	2,725	122.80	89.0
Hydroxyapatite	5.35	1,387	259.25	187.9
Sephacryl S-200-HR gel filtration	0.86	925	1,070.60	775.8

[a]One unit of enzyme is defined as the amount of transformation of one micromole of thiocyanate per minute.

Sephadex ion exchange column by eluting with a KCl gradient (0~0.5 M) in 50 mM citrate buffer, pH 5.0 (Fig. 1A). The activity peak of rhodanese was purified sequentially on the following columns; Affi-gel blue column eluted with a KCl gradient (0~0.5 M) in 10 mM phosphate buffer, pH 7.0 (Fig. 1B); Hydroxyapatite column eluted with a phosphate gradient (0~0.3 M) in 10 mM phosphate buffer pH 7.0 (Fig. 1C); finally Sephacryl S-200-HR gel filtration eluted with 50 mM phosphate buffer, pH 7.0 (Fig. 1D). During these four chromatography steps, all buffer solutions used in the chromatography included 10 mM $Na_2S_2O_3$. At the final step, the rhodanese peak coincided with the main protein peak and a nearly homogeneous preparation was obtained.

A summary of the purification is presented in Table 1. The specific activity of the purified enzyme was 1,070 units per mg of protein, a 776 fold purification was achieved and the yield was 8.18%. After the purification of rhodanese in bovine liver was reported (Sörbo, 1953a, b), two main groups of investigators devoted study to the purification and characterization of enzyme from bovine liver and kidney (Westley and Green, 1959; Horowitz and Detoma, 1970; Horowitz, 1978). Rhodanese from bovine liver and kidney were purified by these investigators with 20~176 and 667 folds, respectively. In this study, we purified rhodanese from a mouse liver representing 776 folds purification which is higher than that from bovine liver and kidney.

Determination of molecular weight and isoelectric point

The molecular weight of rhodanese nondenatured by gel filtration on a Sephacryl G-150 column was indicated to be 34.8 KDa (Fig. 2) and the same molecular weight determined by SDS-PAGE indicated approximately 34 KDa, suggesting that the enzyme is a

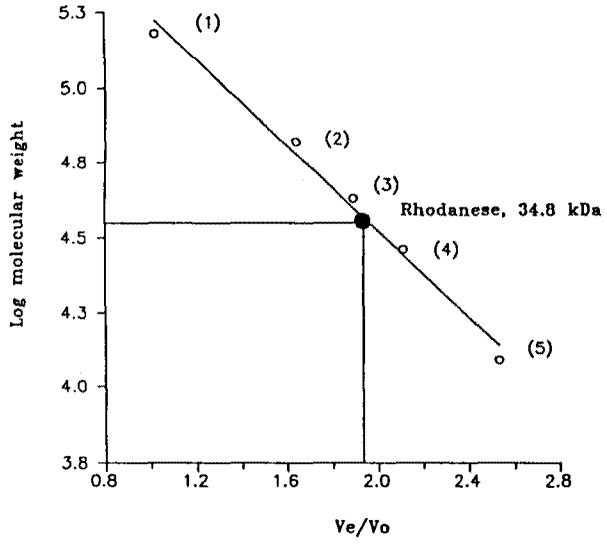


Fig. 2. Determination of molecular weight of rhodanese by Sephadex G-150 gel filtration chromatography. Two mg of marker proteins and rhodanese purified from mouse liver was applied to a Sephadex G-150 column (1.6×90 cm) and eluted by 10 mM phosphate buffer (pH 7.0) at flow rate of 0.13 ml/min. Protein markers were (1) amylase (200 KDa), (2) bovine serum albumin (66 KDa), (3) ovalbumin (43 KDa), (4) carbonic anhydrase (29 KDa), and (5) cytochrome C (12.4 KDa).

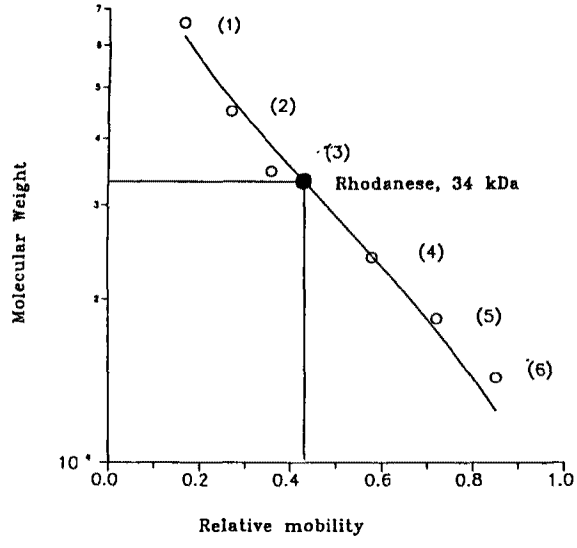


Fig. 3. Determination of molecular weight of rhodanese by SDS-PAGE. Molecular weight standards were (1) bovine serum albumin (66 KDa), (2) ovalbmin (45 KDa), (3) pepsin (43.7 KDa), (4) trypsinogen (24.4 KDa), (5) β-lactoglobulin (18.4 KDa), and (6)lysozyme (14.3 KDa).

monomeric globular protein (Fig. 3). However, rhodanese stored at −70℃ for over 3 weeks showed that the protein split into two identical subunits, Mr. of 17 KDa (Fig. 4). Volini *et al.* (1978) reported the two different molecular weight forms of bovine liver rhodanese, the molecular weights of which are 19 KDa and 33 KDa, respectively. Recently, Horowitz and Bowman

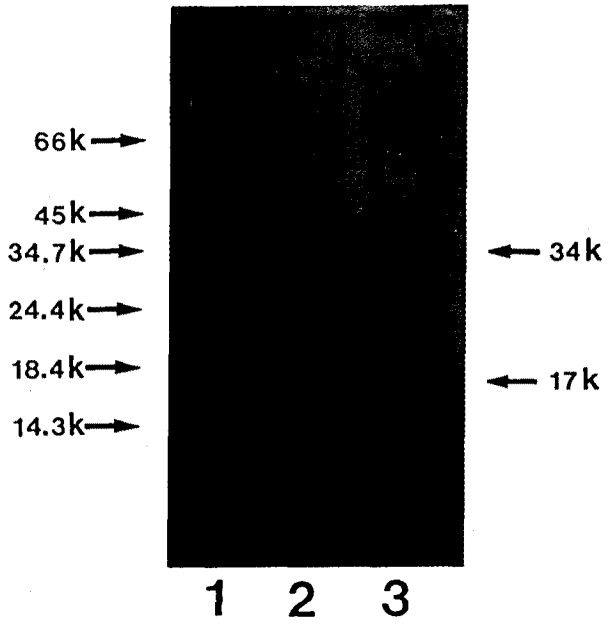


Fig. 4. SDS-PAGE of rhodanese aging for 3 weeks at −70℃. Lane 1 : molecular weight markers (bovine serum albumin : 66 KDa, ovalbumin : 45 KDa, pepsin : 34.7 KDa, trypsinogen : 24.4 KDa, β-lactoglobulin : 18.4 KDa, and lysozyme : 14.3 KDa); Lane 2 : commercial bovine liver rhodanese; Lane 3 : Purified mouse liver rhodanese aged for 3 weeks at −70℃.

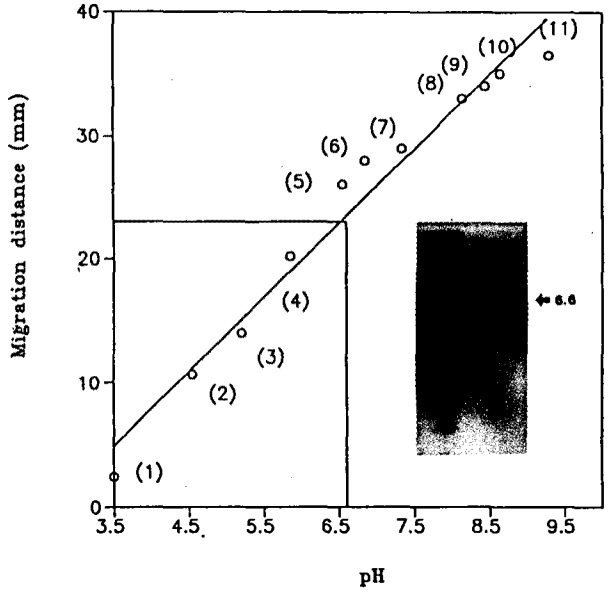


Fig. 5. Determination of the isoelectric point (pI) of mouse liver rhodanese by isoelectric focusing. The standard proteins and their corresponding pI values are: (1) amyloglucosidase (pI 3.5), (2) soybean trypsin inhibitor (pI 4.55), (3) β-lactoglobulin A (pI 5.2), (4) bovine carbonic anhydrase B (pI 5.85), (5) human carbonic anhydrase B (pI 6.55), (6) horse myoglobin (pI 6.85), (7) horse myoglobin (pI 7.35), (8) lentil lectin (pI 8.15), (9) lentil lectin (pI 8.45), (10) lentil lectin (pI 8.65), and (11) trypsinogen (pI 9.3).

(1987) reported that rhodanese can be cleaved by proteolytic endoproteases such as trypsin, chymotrypsin or subtilisin to give a corresponding molecular weight of 16,500 Da. The results presented here are consistent with the idea that the interdomain tether in rhodanese is accessible to proteases or autolysis. The molecular weight and number of polypeptides in rhodanese have been subjects of controversy. Ploegman *et al.* (1978) confirmed that the bovine liver rhodanese is a single polypeptide chain of 293 residues and the molecular weight calculated from this structure is about 32,900 Da. As shown in Fig. 4 lane 2, the main band of commercial bovine rhodanese indicated the Mr. of 33 KDa and a molecular weight smaller than that of mouse liver rhodanese (lane 3) on the SDS-PAGE.

In order to determine the isoelectric point (pI) of purified rhodanese, isoelectric focusing was performed in PhastGel IEF (pH range 3 to 9) together with pI standard markers. The isoelectric point of rhodanese from mouse liver has a pI value of 6.6.

Kinetic constants and physical properties

The K_m and V_{max} values for thiocyanate formation of rhodanese from mouse liver were determined by the modified method described by Sörbo (1953a). The reaction mixtures contained enzyme, saturating concentration of 50 mM KCN, and a varying nonsaturating concentration of sodium thiosulfate, and *vise versa*. The summary of K_m and V_{max} values of rhodanese is shown in Table 2. When the reactions were run for 1 min at 25℃, the experimentally derived values of $K_m^{CN^-}$ and $V_{max}^{CN^-}$ were 12.5 mM and 3.43 μM/min.; $K_m^{S_2O_3^-}$ and $V_{max}^{S_2O_3^-}$ were 8.3 mM and 0.61 μM/min., respectively. This $K_m^{S_2O_3^-}$ value was a little higher when it was compared with those of bovine liver ($K_m^{S_2O_3^-}$: 6.67 mM) and human leukocytes ($K_m^{S_2O_3^-}$: 7.0 mM) (Mintel and Westley, 1966; Pallini *et al.*, 1987).

Table 2. K_m and V_{max} values of KCN and $Na_2S_2O_3$ as substrates for mouse liver rhodanese[a].

Substrate	K_m (mM)	V_{max} (μM·min^{-1})
KCN	12.5	3.43
$Na_2S_2O_3$	8.3	0.61

[a]The reaction was carried out at a constant concentration of 50 mM KCN or 50 mM $Na_2S_2O_3$, and other conditions were as described in Material and Methods .

pH dependence of rhodanese showed an optimal activity around pH 9.4 while activity was almost abolished at pH 6.0 as shown in Fig. 6. Data showing an increasing rate of thiocyanate production over the alkaline pH up to pH 9.5 was in good agreement with the other results obtained from bovine rhodanese (We-

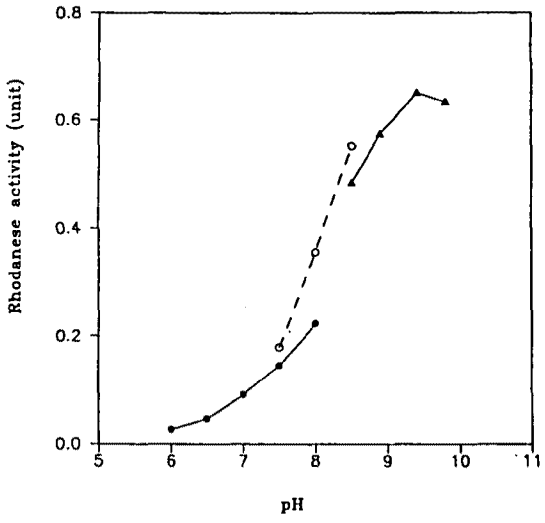


Fig. 6. Effect of pH on the activity of mouse liver rhodanese. One ml of the reaction mixture contained 0.2 ml of 0.05 M KCN and 0.05 M $Na_2S_2O_3$. 0.2 M sodium phosphate buffer (●–●) was used for pH 6.0~8.0, 0.2 M Tris-HCl buffer (○–○) was used for pH 7.5~8.5 and 0.2 M borate buffer (▲–▲) was used for pH 8.5~10.0.

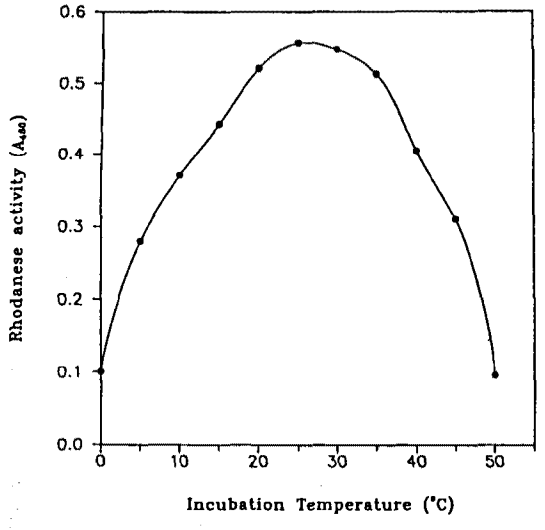


Fig. 7. Optimal temperature and heat stability of rhodanese. The enzyme was incubated at a various temperature as described in the text.

stley, 1959; Wang and Volini, 1968; Weng *et al.*, 1978).

The relation between reaction rate and temperature was studied under temperatures between 0℃ and 50℃ as shown in Fig. 7. Under the assay conditions used, maximal activity showed at 25℃ and a straight line was observed. At 0℃ and 50℃, however, enzyme activity represented only 18% of the total activity.

The formation of thiocyanate from potassium cyanide and sodium thiosulfate by rhodanese over a period of time was measured, and rhodanese activity showed good linearity for 10 min. These results showed that there is no feedback inhibition under the optimal conditions used.

Table 3. Effect of metal ions on Mouse liver rhodanese.

Metal ions	Enzyme activity (%)[a]		
	100 μM	500 μM	1000 μM
None	100	100	100
$MnCl_2$	100	105	110
$MgCl_2$	100	100	101
$CaCl_2$	100	100	95
$FeCl_2$	100	95	90
$NiCl_2$	31	15	10
$ZnCl_2$	30	10	5
$CdCl_2$	22	11	7
$HgCl_2$	20	9	5
$CuCl_2$	10	5	4

[a]Values are the means of triplicate determinations.

Effect of cations

The effect of various metal ions on the activity of rhodanese was studied as shown in Table 3. At the concentration of 100 μM, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Cu^{2+} inhibited rhodanese by more than 70% of the total activity. Other metal ions, such as Mn^{2+}, Mg^{2+}, Ca^{2+}, and Fe^{2+} were without effect on rhodanese. It has been known that such metal ions inhibiting the rhodanese activity all show a strong affinity for ligands such as phosphates, cysteinly, and histidyl side chains of proteins (Stokinger, 1981). Hg^{2+} and Cd^{2+} are also known to interact with carboxyl groups, tryptophan and tyrosine (Vallee and Ulmer, 1972). Considering the rhodanese contained -SH and -S-S- groups, the biochemical basis of toxicological effects of Hg^{2+}, Cd^{2+} and Cu^{2+} are thought to be due to their interaction with the -SH and -S-S- groups of proteins.

Acknowledgement

This article is offered to commemorate the retirement of professor Chung-No Joo from the Department of Biochemistry, College of Science, Yonsei University.

This study was supported by a grant from the Korea Science and Engineering Foundation, 1992.

References

Aird, B. A. and Horowitz, P. M. (1988) *Biochim. Biophys. Acta.* **956**, 30.

Blumenthal, K. M. and Heinrikson, R. L. (1971) *J. Biol. Chem.* **246**, 2430.

Bradford, M. (1976), *Anal. Biochem.* **72**, 258.
Horowitz, P. M. and Detoma, F. (1970) *J. Biol. Chem.* **245**, 984.
Horowitz, P. M. (1978) *Anal. Biochem.* **86**, 751.
Horowitz, P. M. and Bowman, S. (1987) *J. Biol. Chem.* **262**, 8728.
Lang, K. (1933) *Biochem. Z.* **259**, 243.
Leininger, K. R. and Westley, J. (1968) *J. Biol. Chem.* **243**, 1892.
Mintel, R. and Westley, J. (1966) *J. Biol. Chem.* **241**, 3386.
Ogata, K. and Volini, M. (1986) *J. Protein Chem.* **5**, 239.
Ogata, K., Dai, X. and Volini, M. (1989) *J. Biol. Chem.* **262**, 2718.
Pallini, R., Martelli, P., Bardelli, A. M., Guazzi, G. C. and Ferderico, A. (1987) *Neurology* **37**, 1878.
Ploegman, J. H., Drent, G., Kalk, K. H. Hol, W. G. J., Heinrikson, R. L., Keim, P. S., Weng, L. and Russell, J. (1978) *Nature* **273**, 124.
Russell, J., Weng, L., Keim, P. S. and Heinrikson, R. L. (1978) *J. Biol. Chem.* **253**, 8102.
Sörbo, B. H. (1953a) *Acta Chem. Scand.* **7**, 1129.
Sörbo, B. H. (1953b) *Acta Chem. Scand.* **7**, 1137.
Sörbo, B. H. (1975) in *Metabolic pathways* (Greenberg, D. M., ed.) Vol. 7, pp. 433-456, Academic press, New York.
Stokinger, H. E. (1981) in *Patty's Industrial Hygiene and Toxicology* (Clayton, G. D. and Clayton, F. E., eds.) 3rd Ed., Vol. 2A, pp. 1493-2060, John Wiley and Sons, New York.
Vallee, B. L. and Ulmer, D. D. (1972) *Annu. Rev. Biochem.* **41**, 91.
Volini, M., Detoma, F. and Westley, J. (1967) *J. Biol. Chem.* **242**, 5520.
Volini, M., Craven, D. and Ogata, K. (1978) *J. Biol. Chem.* **253**, 7591.
Wang, S. F. and Violini, M. (1968) *J. Biol. Chem.* **243**, 5465.
Weng. L., Heinrikson, R. L. and Westley, J. (1978) *J. Biol. Chem.* **253**, 8109.
Westley, J. and Green. J. R. (1959) *J. Biol. Chem.* **243**, 2325.
Westley, J. (1959) *J. Biol. Chem.* **243**, 1857.
Westley, J. (1973) *Adv. Enzymol.* **39**, 327.