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Purification and Properties of Z Protein from Rabbit and Rat Liver[1)]

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Z protein was purified from the rabbit and rat liver by fractionation with ammonium sulfate. The physico-chemical properties of Z protein was studied by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis, polyacrylamide gel electrofocusing, amino acid analysis, and sedimentation analysis. The molecular weight was determined to be 1.2×10^4 by gel filtration on Sephadex G-75 and 1.1×10^4 by SDS-polyacrylamide gel electrophoresis, and also 8872 and 8639 from amino acid analysis with the rabbit and rat liver, respectively. Z protein lacked tyrosine and tryptophan residues, and had an absorption maximum at 260—270 nm. The isoelectric point was at pH 7.0. The sedimentation coefficient of Z protein was *ca.* 0.7S at a concentration of 0.04—0.06%. Physical and chemical properties of rabbit and rat liver Z protein were closely alike.

Keywords——Z protein; Y protein; rabbit liver; rat liver; organic anion binding protein; liver cytoplasmic protein; purification of protein; hepato-biliary excretion; characterization of protein

In recent years, the investigation of the organic anion binding protein has been a field of considerable activity, particularly in regard to the mechanism of the hepatic uptake of organic anions from plasma.[3-8)] Two cytoplasmic protein fractions, Y and Z, that bind bilirubin, BSP (bromosulfophthalein), and ICG (indocyanine green) *in vivo* and *in vitro* were recognized from the rat liver by the use of gel filtration.[3)] Of the two kinds of proteins, Y protein was first purified and its properties were investigated by Arias, *et al.*[9-11)]

The purpose of this report is to isolate Z protein from the rabbit and rat liver, and to disclose the characteristics of the protein by gel filtration, SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis, polyacrylamide electrofocusing, sedimentation analysis, and amino acid analysis.

Experimental

Materials——Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Co. Ovalbumin (Grade III) and Coomassie brilliant blue R were obtained from Sigma Chemical Co. Ampholine (pH range 3.5—10.0) was purchased from LKB Instruments Co. Cytochrome c (horse) and pepsin were obtained from Miles Laboratories Ltd. Sephadex G-75 was purchased from Pharmacia Fine Chemicals. All other chemicals were of reagent grade from various commercial sources.

Preparation of Liver Cytoplasmic Protein——Supernatant fractions were prepared from the homogenate of the liver of adult male Donryu rats (200—250 g) and male rabbits (3.0—3.5 kg) in the following manner.

1) Presented in part before the 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April, 1976.
2) Location: *Shirokane, Minato-ku, Tokyo.*
3) A.J. Levi, Z. Gatmaitan, and I.M. Arias, *J. Clin. Invest.*, **48**, 2156 (1969).
4) H. Reyes, A.J. Levi, Z. Gatmaitan, and I.M. Arias, *J. Clin. Invest.*, **50**, 2242 (1971).
5) R.I. Levine, H. Reyes, A.J. Levi, Z. Gatmaitan, and I.M. Arias, *Nat. New Biol.*, **231**, 277 (1971).
6) K. Takada, M. Ueda, M. Ohno, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1477 (1974).
7) G. Fleischner, J. Robbins, and I.M. Arias, *J. Clin. Invest.*, **51**, 677 (1972).
8) Y. Matsushita, S. Nakagawa, H. Umeyama, and I. Moriguchi, *Chem. Pharm. Bull.* (Tokyo), **24**, 1650 (1976).
9) G. Litwack, B. Ketterer, and I.M. Arias, *Nature*, **234**, 466 (1971).
10) K. Kamisaka, I. Listowski, and I.M. Arias, *Ann. N.Y. Acad. Sci.*, **226**, 148 (1973).
11) R. Kirsch, G. Fleischner, K. Kamisaka, and I.M. Arias, *J. Clin. Invest.*, **55**, 1009 (1975).

Rats and rabbits were anesthetized with ether and intraperitoneal injection of pentobarbital sodium, respectively. The liver was perfused with ice-cold 0.25 M sucrose solution through the portal vein to remove the blood. The 33% homogenate was prepared in 0.25 M sucrose–10 mM phosphate (pH 7.4) using teflon pestle homogenizer. The homogenate was centrifuged at 10000 *g* for 20 min, and the supernatant was centrifuged again at 105000 *g* for 90 min in a Hitachi 65P ultracentrifuge at 4°. After the surface lipid was removed, the supernatant fraction was carefully collected. The final supernatant was used as the starting material for the purification of Z protein.

Method for the Purification of Z Protein——The clear supernatant was fractionated with ammonium sulfate at concentrations of 50, 70, and 100% saturation. The precipitates (50%, 70%, and 100% ppt.) were dissolved in 10 mM phosphate (pH 7.4) separately. The supernatant with 100% saturated ammonium sulfate (100% sup.) was dialyzed against the same buffer solution and concentrated by ultrafiltration using G-05T membrane filter (BEC diafilter) under positive pressure of nitrogen. Each fraction (50, 70, 100% ppt. and 100% sup) was dialyzed against the same buffer. BSP was added to each dialyzate, and the mixture was placed on a Sephadex G-75 column (3.2 × 98 cm) equilibrated with the same buffer at 4°. Elution was performed using the same buffer solution with a flow rate of 23—25 ml/hr. Each tube in the collector contained 4.0 ml. The elution patterns were monitored using a Hitachi 200—20 type spectrophotometer: protein at 280 nm and BSP at 580 nm (after alkalization with 2N NaOH). All the fractionation and purification procedures were performed at 2—4° unless otherwise indicated. Z protein was pooled as fraction Z indicated in Fig. 2. This fraction was used for the electrophoresis, electrofocusing, and amino acid analysis.

Molecular Weight Determination by Gel Filtration——The molecular weight of Z protein was estimated with a 3.2 × 98 cm column of Sephadex G-75 using bovine serum albumin, ovalbumin, cytochrome c as marker proteins.

SDS-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis of Z protein was carried out according to the procedure of Weber and Osborn[12] under a constant current (6 mA/gel) for 6 hr. Protein were stained with 0.25% Coomassie brilliant blue R. The determination of the molecular weight of Z protein was based on a linear plot of the relative mobility of bovine serum albumin, ovalbumin, pepsin, and cytochrome c in gel electrophoresis.

Isoelectric Focusing in 5% Polyacrylamide Gel——This was performed as described by Gronow and Griffiths.[13] A gel mixture (3.0 ml) was made up in 5 M urea solution containing 0.3 mg of protein and 2% Ampholine (pH 3.5—10.0). The mixture was poured into tubes (14 × 0.6 cm). After focusing at a constant voltage of 150V for 24 hr at 4° the gels were fixed in 10% trichloroacetic acid and stained with Coomassie brilliant blue R. Destaining was achieved by soaking in several changes of ethanol: acetic acid: water (25: 10: 65 v/v) mixture until the background became clear. In order to determine the pH gradient established in the gels a sample was taken immediately after focusing and sliced manually into 5 mm section. These were placed in 1.0 ml of degassed freshly distilled water for 2 hr and then the pH was determined.

Amino Acid Analysis——A sample of Z protein was dialyzed for 48 hr against distilled water and then lyophilized. The protein sample was then dissolved in 2.0 ml of 6N HCl, and 1.0 ml aliquots were distributed to hydrolysis tubes. The tubes were sealed under vaccum, hydrolyzed at 105—110° for 24 or 48 hr, and analyzed for amino acid composition on a automatic amino acid analyzer (JEOL JLC-5AH type).

Measurement of the Sedimentation Coefficient——Sedimentation analysis was carried out at 60000 rpm and 25° with a Hitachi Model 282 preparative ultracentrifuge, equipped with absorption optics at 280 nm, with 0.04—0.06% protein in 10 mM phosphate buffer (pH 7.4).

Assay of Protein——Protein was assayed by the method of Lowry, *et al.*[14] using bovine serum albumin as a reference.

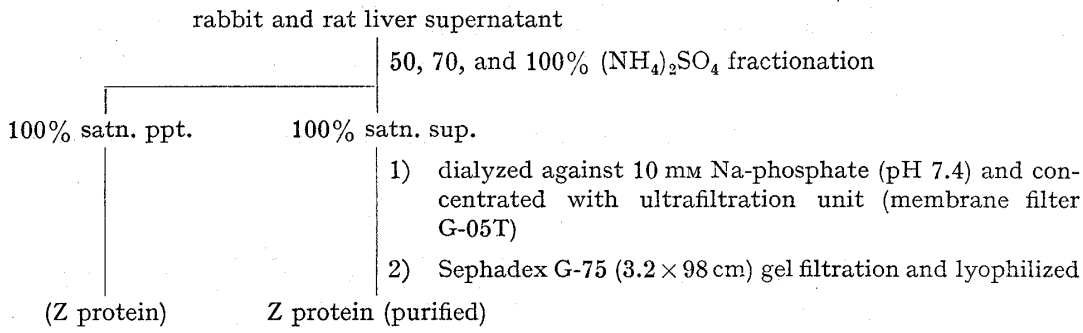

Chart 1. Flow Diagram for the Purification of Z Protein

12) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
13) M. Gronow and G. Griffiths, *FEBS. Letters*, **15**, 340 (1971).
14) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Results

Purification of Z Protein

The process of purification of Z protein was outlined in Chart 1. The Sephadex G-75 gel filtration of 50% and 70% ppt. with BSP added *in vitro* yielded elution patterns, in which the binding of BSP to void volume fraction and Y protein fraction (MW 4.4×10^4) was noticed, but in which the binding of BSP to Z protein was not observed. The elution patterns of 100% ppt. and 100% sup. are illustrated in Fig. 1 and 2 clearly indicating that Z protein is present

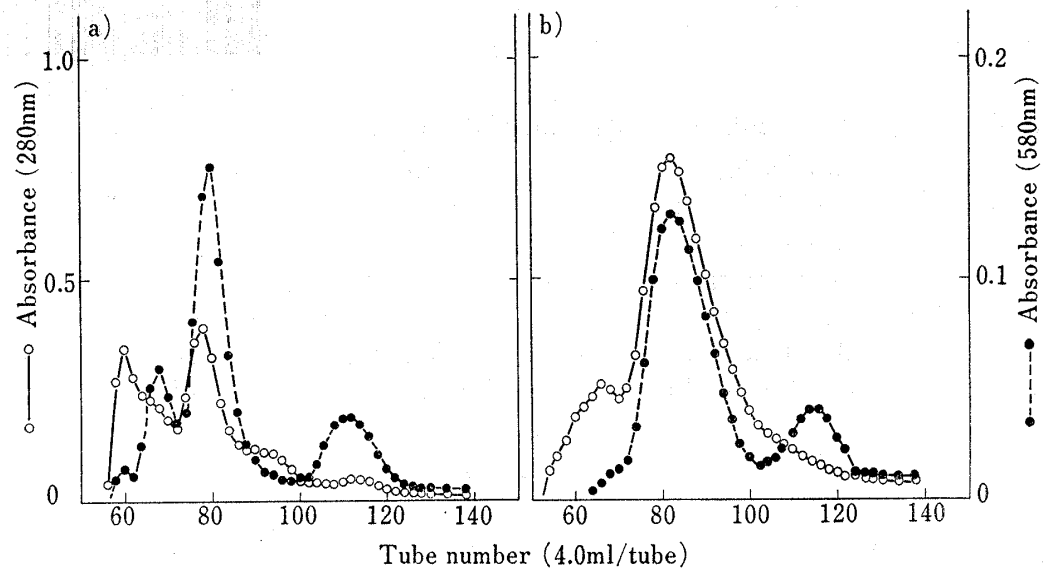

Fig. 1. Gel Filtration and BSP Binding Patterns of $(NH_4)_2SO_4$ 100% saturation Precipitate Fraction from the Rabbit and Rat Liver Cytosol

The mixture was incubated for 1 hr at 4° and eluted from a column (3.2×98 cm).
a) rabbit *b*) rat

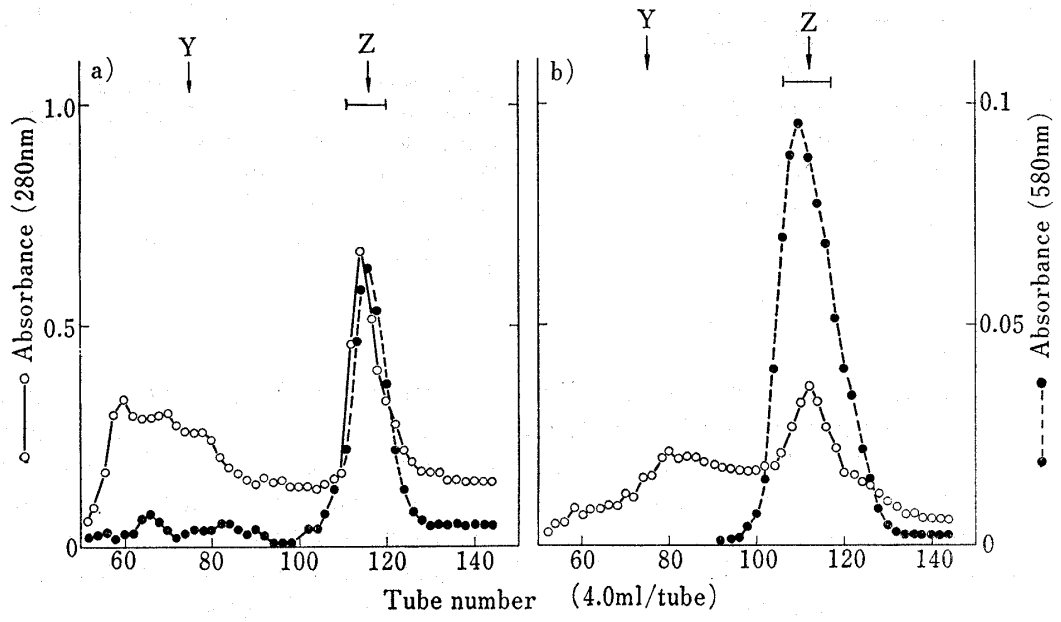

Fig. 2. Gel Filtration and BSP Binding Patterns of $(NH_4)_2SO_4$ 100% saturation Supernatant Fraction from the Rabbit and Rat Liver Cytosol

The mixture was incubated for 1 hr at 4° and eluted from a column (3.2×98 cm).
a) rabbit *b*) rat

in 100% ppt. and 100% sup. Z protein was almost recovered from 100% sup., while the content of Z protein seemed much lower in 100% ppt. In these figures, a lag in the elution peak of BSP appeares. This retardation may be caused by a partial dissociation of the dye bound to Z protein during the elution. The binding of BSP to Z protein was not covalent but reversible (probably electrostatic and/or hydrophobic). Z protein was obtained in a yield of approximately 0.08—0.1 per cent of the total cytoplasmic protein from rabbit and rat. The purified Z protein migrated as a single band in electrophoresis in SDS-polyacrylamide gel (Fig. 3).

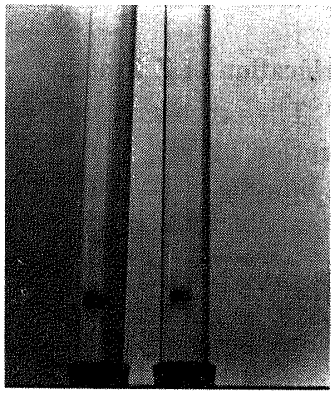

Fig. 3. SDS-Polyacrylamide Gel Electrophoresis of Purified Z Protein from the Rabbit and Rat Liver

left: rabbit right: rat

Molecular Weight and Sedimentation Coefficient of Z Protein

Figure 4 shows a calibration curve for the determination of molecular weight of Z protein on Sephadex G-75 column. It gave a value of 1.2×10^4 for the apparent molecular weight of rabbit and rat Z protein in three repeated runs. From relative mobilities on the electrophoresis, the apparent molecular weight of Z protein from both the species was estimated to be 1.1×10^4 (Fig. 5). The sedimentation coefficient at 25° and 60000 rpm of isolated Z protein from rabbit and rat was *ca.* 0.7 S at a concentration of 0.4—0.6 mg/ml.

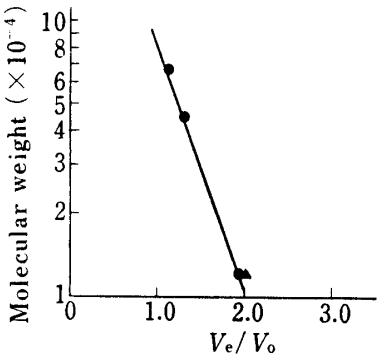

Fig. 4. Calibration Curve for the Molecular Weight of Z Protein on Sephadex G-75 (3.2×98 cm)

Apparent molecular weight of Z protein (indicated by ▲) is 1.2×10^4.

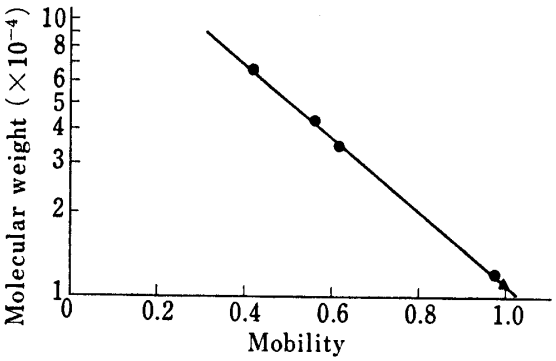

Fig. 5. Semilogarithmic Plot Relating Electrophoretic Mobility to Molecular Weight using Bovine Serum Albumin (6.7×10^4), Ovalbumin (4.3×10^4), Pepsin (3.5×10^4), and Cytochrome c (1.2×10^4)

Apparent molecular weight of Z protein (indicated by ▲) is 1.1×10^4.

Amino Acid Analysis

The amino acid composition of purified Z protein from the rabbit and rat liver is presented in Table I. An attempt to determine the tryptophan content of Z protein using *p*-dimethylaminobenzaldehyde method was unsuccessful presumably owing to a poor content of tryptophan. The lack of tyrosine and tryptophan residues was reflected in the lack of an absorbance maximun of 280 nm (Fig. 6). Calculation of the molecular weight from the estimated amino acid residues gave a value of 8872 (rabbit) and 8639 (rat) which were slightly less than those determined by gel filtration and SDS-polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrofocusing

Polyacrylamide gel electrofocusing of purified Z protein from the rabbit and rat liver was performed as described by Gronow and Griffiths.[13] The isoelectric focusing pattern of Z

Table I. Amino Acid Composition of Z Protein

Amino acid	Number of residues	
	Rabbit	Rat
Lys	10	10
His	1	1
Arg	2	3
Asp	9	9
Thr	7	7
Ser	4	4
Glu	12	11
Pro	2	2
Gly	8	8
Ala	4	4
Val	7	7
Met	1	1
Ile	5	4
Leu	5	5
Tyr	N.D.	N.D.
Phe	4	3
Trp	N.D.	N.D.
Total residues	81	79
Molecular weight	8872	8639

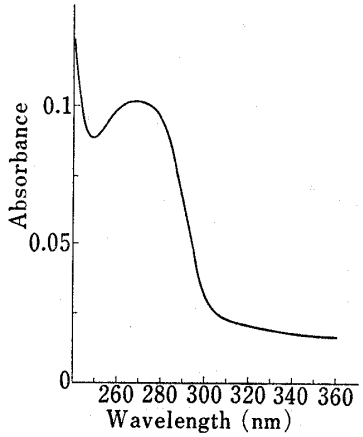

Fig. 6. Ultraviolet Spectrum of Purified Z Protein in 10 mM Phosphate Buffer (pH 7.4)

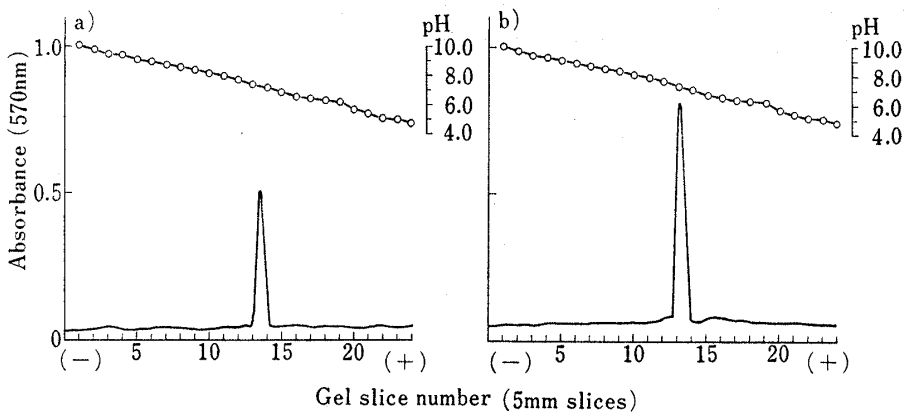

Fig. 7. Densitometric Trace of Coomassie Blue Stained Gel of Z Protein from the Rabbit and Rat Liver

The pH gradient was obtained from the sectioning of blank gel run at the same time (see Experimental).
a) rabbit *b*) rat

Table II. Comparison of Physico-Chemical Characterization of Z Protein isolated from Rabbit with That from Rat

	Rabbit	Rat
Molecular weight		
gel filtration	1.2×10^4	1.2×10^4
electrophoresis	1.1×10^4	1.1×10^4
amino acid analysis	8872	8639
S value	*ca.* 0.7	*ca.* 0.7
Isoelectric point	7.0	7.0

protein is given in Fig. 7. The peak of Z protein was observed at pH 7.0 in the densitometric trace.

Those constants are summarized in Table II.

Discussion

Two kinds of cytoplasmic proteins, Y and Z, are known to bind organic an ions and assumed to play an important role in the hepato-biliary excretion. Arias, *et al.*[3-5,9-11] reported that Y protein is precipitated in 70% saturated ammonium sulfate solution. On the other hand, Z protein is almost soluble in 100% saturated ammonium sulfate solution in this study. This difference in the solubility may indicate that Z protein is less hydrophobic than Y protein. The hydrophobicity is probably originated from aromatic amino acid residues in the protein molecules. The ratio of acidic to basic amino acid residues in Z protein was 0.62, similar to that in Y protein[9,11] (0.63). However, the content of aromatic amino acids in Z protein (5%) are lower than that in Y protein[9,11] (11%). Especially tyrosine and tryptophan were almost absent in Z protein. By comparative gel filtration and acrylamide gel electrophoresis it was shown that Z protein has a molecular weight of approximately 1.2×10^4 and exists as a monomer. On the other hand, Y protein has a molecular weight of 4.4×10^4 with two subunits of 2.2×10^4.[9,11] The difference in those characteristics between Y and Z protein may reflect the difference of their binding characters.[3] It may be assumed that Y protein, which is more hydrophobic, is mainly concerned with the hepatic uptake of unmetabolized drugs from plasma, and that Z protein, which is less hydrophobic, is involved in the hepato-biliary transfer of polar matabolites.

Almost no difference in the physical and chemical characteristics was observed between Z protein isolated from the rabbit liver and that from the rat liver (Table II).