A SIMPLE PROCEDURE FOR THE PURIFICATION OF PORCINE LIGANDINE (Y-PROTEIN)

J. A. J. TRIP, J. VAN DAM, T. TEPPER and G. S. QUE
Department of Internal Medicine, Division of Hepatology, University Hospital, Groningen, The Netherlands

Received 19 April 1974

1. Introduction

Ligandine or Y-protein is a non-specific anion-binding protein in liver cytosol, probably concerned with the intracellular transport of different anionic substances [1]. In rats in vivo binding has been described of bromosulphthalein, bilirubin, indocyanine green [2], hydrocortisone metabolites [3], azodye carcinogens and some of their conjugates [4, 5], and in vitro binding of radiographic contrast materials [6].

Isolation methods of rat ligandine have been reported in detail by Ketterer [4] and Morey [7], and in brief by Arias et al. [8]. Based on these techniques a simplified method is described for the purification of porcine ligandine. The isolation was established by gel filtration, electrophoresis on polyacrylamide, immunoelectrophoresis, and ultracentrifugation. The molecular weight and the amino acid composition were determined and found to be different from rat ligandine.

2. Materials and methods

A fresh porcine liver was immediately washed and perfused with an ice-cold isotonic NaCl-solution. Pieces of about 20 g were stored at −20°C.

Homogenate was prepared according to Levi [2]. The liver pieces were homogenized in 3 vol of 0.25 M phosphate buffer (pH 7.4) by means of a Waring blender for 1 min at 15 000 rpm. Subsequently the homogenate was centrifuged for 120 min at 4°C and at 110 000 *g* in a Christ Omega II ultracentrifuge.

Eight ml of the supernatant, corresponding to 3 g of liver tissue, were incubated with 2 mg BSP (bromosulphthalein) for 15 min and then placed on a Sephadex G-75 superfine column, equilibrated with 0.01 M phosphate buffer pH 7.4. Elution was performed with the same buffer in an upward pump-driven flow system. Protein concentration in the eluate was measured by a LKB Uvicord apparatus. BSP concentrations were determined at 580 nm in a Beckman PMQ II spectrophotometer after alkalinization with NaOH.

Chromatography on DEAE-Sephadex A-50 was performed on a column equilibrated with 0.01 M phosphate buffer pH 7.4. Linear gradient elution from 0 to 0.5 M KCl was applied by mixing equal volumes (500 ml) of 0.01 M phosphate buffer pH 7.4 and 0.01 M phosphate buffer pH 7.4 containing 1 M KCl.

The next step was chromatography on a column of cellulose phosphate (No. C-2383, Sigma Chem. Co., USA), equilibrated with 0.01 M phosphate buffer pH 7.4. A linear gradient system was performed as described in the former paragraph.

After each chromatography the fractions of each protein peak were pooled and concentrated by ultrafiltration through Amicon filters No. UM 2. Because the binding of BSP to ligandine is lost during passage through an anion-exchanging column, a pre-incubation with 2 mg BSP was necessary. Afterwards the peaks were placed on a Sephadex G-75 column as described above for localization of the ligandine.

Immunoelectrophoresis was carried out in a Gelman electrophoresis chamber (No. 51211). The antiserum was prepared by immunization of a rabbit with pooled and concentrated (10 mg/ml) elution fractions of the

 North-Holland Publishing Company – Amsterdam

ligandine-containing protein peak after gel filtration on Sephadex G-75.

Electrophoresis on polyacrylamide with sodium dodecyl sulphate (SDS) was performed according to Weber [9].

Ultracentrifugal experiments were done in a Spinco model E ultracentrifuge at a speed of 59 780 rpm and a mean temperature of 20°C. The protein concentration was 2 mg/ml, as measured by the method of Lowry [10].

Amino acid analysis was performed with a Technicon Auto Analyzer (Technicon Chromatography Corp., USA), using a modification of the ninhydrin method of Stein and Moore [11,12].

3. Results

The elution pattern after gel filtration on Sephadex G-75 is shown in fig. 1. In full accordance with the studies of Arias et al. [2], four peaks were found by estimation of the elution fractions at 580 nm. First a yellow-brownish coloured X peak in the void volume, containing only a very small amount of BSP. Secondly we find the Y peak, containing the ligandine. Next the Z peak can be seen, constituted by another anion-binding protein [2], and finally a large bulk of free BSP leaves the column. Repeated experiments with the whole supernatant as well as with the Y peak alone on the same Sephadex G-75 column and in unaltered conditions, gave fully identical results. The Y peak was always eluted in the same fractions. Once localized in two or three runs, it was possible to elute ligandine on guidance of the E 280 nm without the necessity of the addition of BSP.

Chromatography of the Y peak fraction (No. 41–44) on DEAE-Sephadex A-50 resulted in the chromato-

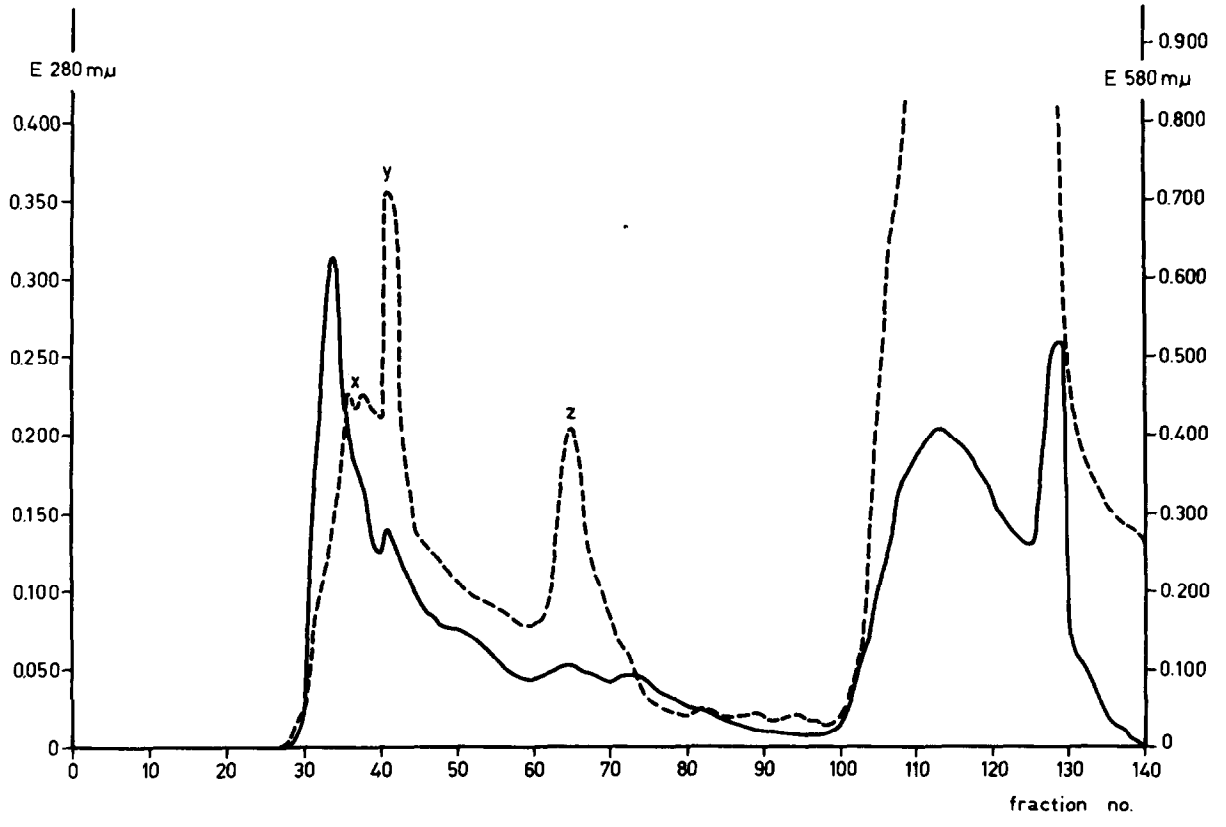

Fig. 1. Gel filtration on Sephadex G-75 of homogenate supernatant after incubation with BSP. A column of 70 × 2.5 cm and a 0.01 M phosphate buffer pH 7.4 were used. Flow rate 8 ml/hr, fraction volume 4 ml. ——, elution pattern of protein (E 280 nm); – – – – – elution pattern of BSP (E 580 nm).

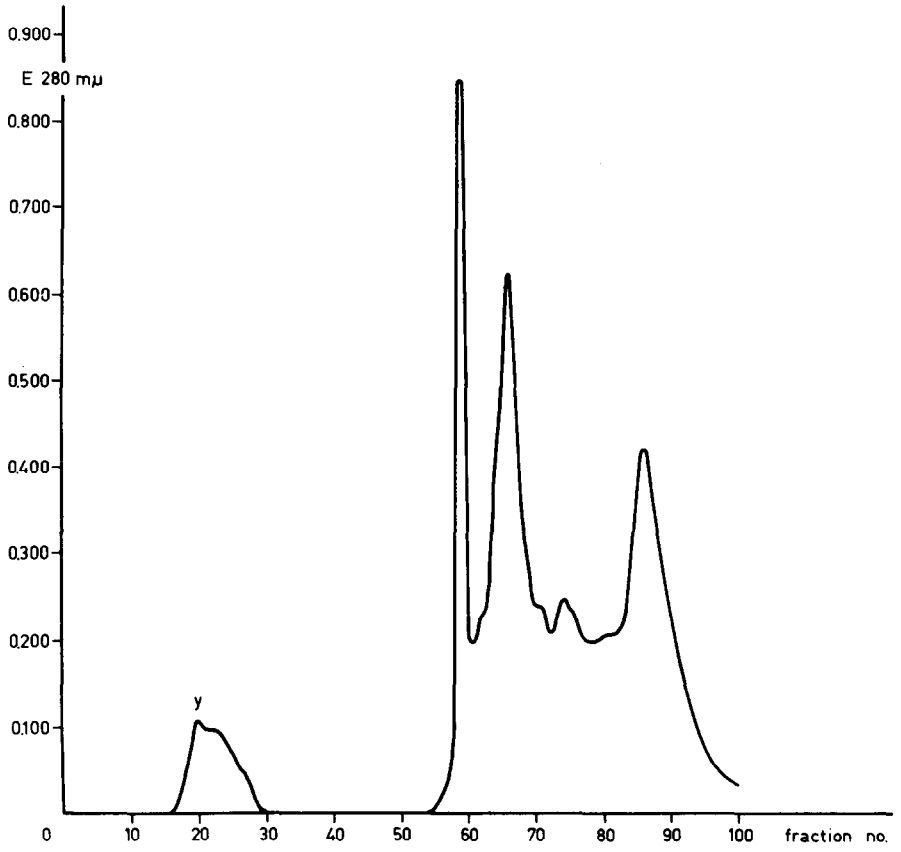

Fig. 2. The elution of the ligandine containing protein peak of fig. 1 (fractions 41–44) on a DEAE-cellulose column (70 × 2.5 cm) with 0.01 M phosphate buffer pH 7.4 and a linear gradient from 0 to 0.5 M KCl. Flow rate 20 ml/hr, fraction volume 6 ml. Ligandine was found to be present in the first protein peak.

gram shown in fig. 2. Among the 5 peaks at 280 nm only the first one was able to bind BSP, indicating the presence of ligandine.

This peak (fractions 17–28) was submitted to chromatography on cellulose phosphate (fig. 3). Only the second of the 2 protein peaks obtained in this way was capable of BSP-binding. The elution fractions of this peak (No. 16–26) were again pooled and concentrated to 10 mg/ml for further investigation.

With immunoelectrophoresis only one precipitation line was visible (fig. 4).

Repeated ultracentrifugation experiments showed one protein peak. An S-value of 3.6 ± 0.2 was calculated.

Chromatography on a calibrated Sephadex G-100 column resulted in the elution of one protein peak; the elution volume corresponded to a mol. wt. of 55 000.

From electrophoresis on polyacrylamide-SDS a mol. wt. was found of about 25 000.

From amino acid analysis a mol. wt. of 23 696 can be calculated (table 1). However, two corrections are necessary. Because proline and tryptophan were not determined, the acceptance of 10 residues of each results in a contribution of about 3000. Secondly a correction of about 3600 has to be made for approximately 200 peptide bonds. The two corrections are roughly equal to each other and act in opposite direction. We propose therefore an approximated mol. wt. of 23 000.

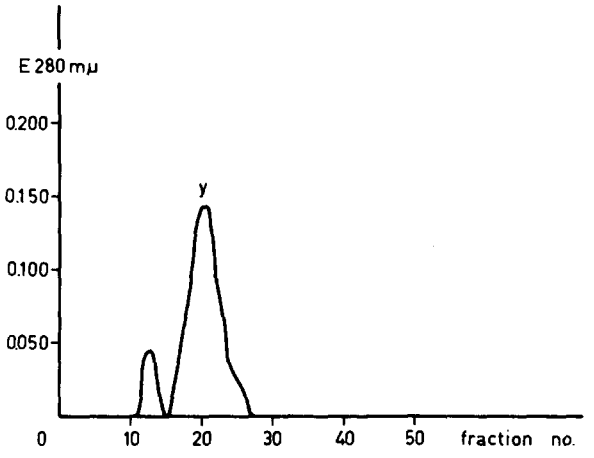

Fig. 3. Chromatography of the fractions 17–28 of fig. 2 on a cellulose phosphate column (35 × 2.5 cm) with 0.01 M phosphate buffer pH 7.4 and a linear gradient from 0 to 0.5 M KCl. Flow rate 20 ml/hr, fraction volume 6 ml. Ligandine was present in the second protein peak.

4. Discussion

As the first step in the isolation procedure we preferred the preparation of the homogenate and the filtration on Sephadex G-75 according to Levi [2]. Because of its excellent reproducibility other chromatographic techniques could be essayed for BSP-binding capacity of protein peaks.

Chromatography on CM-Sephadex G-50 [4] or TEAE-cellulose [13] was not satisfactory in our hands. For further purification we tried the DEAE-cellulose and cellulose phosphate columns as described by Morey [7]. In these techniques our results were better with phosphate buffers instead of Tris–HCl.

The whole purification procedure as described here is the simplest method published up till now. It includes only one centrifugation and 3 runs on columns. The result is a single protein peak with

Table 1
Amino acid analysis of porcine ligandine

Amino acid	No. of residues	Mol. wt, × no. of residues
Aspartic acid	12	1584
Threonine	18	2142
Serine	12	1260
Glutamic acid	20	2940
Glycine	19	1425
Alanine	19	1691
Valine	11	1287
Cysteine	6	726
Methionine	1	149
Isoleucine	9	1179
Leucine	17	2227
Tyrosine	3	543
Phenylalanine	6	990
Lysine	22	3212
Histidine	5	775
Arginine	9	1566
Tryptophan	Not determined	
Proline	Not determined	
	Total =	23 696

Fig. 4. Immunoelectrophoresis. Top: homogenate supernatant; bottom: the ligandine containing eluents from the cellulose phosphate column (fractions 16–26).

BSP-binding property on both Sephadex G-75 and Sephadex G-100. Immunoelectrophoresis, ultracentrifugation experiments, and electrophoresis on polyacrylamide-SDS argue for one protein too. From these facts it is concluded that the final solution, as the result of this isolation procedure, contains only one protein.

The S-values found for rat liver ligandine are 3.5 [1], 3.5 [5], and 3.47 [7], and are closely related to our finding of 3.6 ± 0.2 for porcine ligandine. The molecular weights for rat liver ligandine range from 36 000 to 50 000 ± 6000 [1,5,7,14]. Our molecular weight 55 000 corresponds only with the value of 50 000 ± 6000 found by Litwach [14]; in both instances estimation was performed by gel filtration on Sephadex. Litwack also found a lower molecular weight of 37 250 by amino acid analysis. Our result by this method was 23 000, in close agreement with the 25 000 obtained by electrophoresis on polyacrylamide-SDS. But we want to stress again that molecular weights acquired by amino acid analysis have to be considered with caution. In contrast to Morey [7], Ketterer [5] obtained in 8 M urea subunits with mol. wt.of 23 000 from rat ligandine. At this moment we are not sure whether we are dealing with large molecules and subunits, or with small molecules and dimers, or with asymmetrical molecules.

The results of Ketterer [4] and Morey [7] of the amino acid analysis of rat liver ligandine are in close agreement. Proportionally the amino acid composition of porcine ligandine is different: almost equal quantities of serine, glycine, alanine, histidine, and lysine, more threonine, while the other amino acids are present in a lesser amount.

On the amino acid chromatogram we observed a peak in the ornithine position, with a surface of similar order of magnitude as the amino acid peaks. One may speculate if this peak represents an amino sugar for the following reasons: ornithine is not a constituent of proteins, the isolated ligandine was highly purified and the elution of amino sugars in the region of basic amino acids has been published by Steele [15].

The observed differences in amino acid composition and molecular weight between rat and porcine ligandine are not totally unexpected. Fleischner [13] demonstrated that with a specific antiserum against rat ligandine no identifiable precipitation bands could be obtained with liver superantants, Y-fraction, or purified Y-protein of 20 other animal species, including different mammels and man. So ligandine has to be considered to have species specific antogenic activity and it is probable, that this phenomenon is accompanied by a specific different structure.

Acknowledgement

We are much indebted to Dr. G. Ab (Laboratory of Biochemistry, State University, Groningen) for his advice and his help with the polyacrylamide electrophoresis, and to Mr. J. Alserda for sedimentation velocity measurements.

References

[1] Litwack, G., Ketterer, B. and Arias, I. M. (1971) Nature 234, 466–467.
[2] Levi, A. J., Gatmaitan, Z. and Arias, I. M. (1969) J. Clin Invest. 48, 2156–2167.
[3] Fiala, E. S. and Litwack, G. (1966) Biochim. Biophys. Acta 124, 260–266.
[4] Ketterer, B., Ross-Mansell, P. and Whitehead, J. K. (1967) Biochem. J. 103, 316–324.
[5] Ketterer, B., Beale, D., Litwack, G. and Hackney, J. F. (1971) Chem.–Biol. Interactions 3, 285–286.
[6] Sokoloff, J., Berk, R. N., Lang, J. H. and Lasser, E. C. (1973) Radiology 106, 519–523.
[7] Morey, K. S. and Litwack, G. (1969) Biochemistry 8, 4813–4821.
[8] Reyes, H., Levi, A. J., Gatmaitan, Z. and Arias, I. M. (1969) Proc. Natl. Acad. Sci. U.S. 64, 168–170.
[9] Weber, K., Pringle, J. R. and Osborne, M. (1972) in: Methods in Enzymology (Hirs, C. H. W. and Timashers, S. N., eds), Vol. 26 part C, pp. 3–27, Academic Press, New York–London.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Moore, S. and Stein, W. (1954) J. Biol. Chem. 211, 907–913.
[12] Stein, W. and Moore, S. (1954) J. Biol. Chem. 211, 915–926.
[13] Fleischner, G., Robbins, J. and Arias, I. M. (1972) J. Clin. Invest. 51, 677–684.
[14] Fleischner, G., Mishkin, S., Reyes, H., Robbins, J., Levi, A. J., Gatmaitan, Z. and Arias, I. M. (1971) J. Clin. Invest. 50, 31a.
[15] Steele, R. S., Brendel, K., Scheer, E. and Wheat, R. W. (1970) Anal. Biochem. 34, 206–225.