An Improved Method for the Purification and Characterization of a 54 kDa Protein in Rat Liver Which Has Recently Been Identified as a Selenium-Binding Protein

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A 54 kDa protein, which is induced by treatment with 3,3′,4,4′,5-pentachlorobiphenyl, 3-methylcholanthrene and butylated hydroxytoluene, was purified from rat liver by conventional methods involving DEAE-Sephacel column chromatography and processing using a Rotofor Cell™. This method does not require a denaturation or solubilization step and yields a single protein on SDS-PAGE. The molecular mass of the purified protein was 54.7 kDa. We also characterized the 54 kDa protein by immunoblot analysis and measured the binding ratio of selenium. The purified 54 kDa protein was stained with antiserum which had been previously obtained, and the molecular ratio of selenium to the 54 kDa protein was 0.014. Glutathione peroxidase (GPx) is a typical selenoprotein which has a selenocysteine amino acid sequence. We measured the binding ratio of selenium with commercial GPx as a positive control and obtained a value of 1.214. The binding ratio of selenium to 54 kDa protein was much lower than that of GPx, and this led us to believe that there is no selenocysteine in the 54 kDa protein. In this report, we have developed a convenient purification procedure and measured the purification factors and binding ratio of selenium associated with a 54 kDa protein in rat liver which is homologous with a selenium-binding protein in the mouse.

Key words —— selenium-binding protein, acetaminophen-binding protein, purification, Rotofor Cell™

INTRODUCTION

Selenium-binding protein (SeBP) is one of the cytosolic proteins first reported by Medina *et al.*[1] SeBP is known to be present in a variety of tissues in mice and rats. In particular, there are high levels in the testes, kidney, ovary, mammary gland and liver of mice.[2,3] SeBP is also believed to bind selenium in a manner different from selenoproteins containing selenocysteine.[4] Although the physiological role of SeBP in the mouse is not yet completely understood, one possible function has been discussed involving the inhibition of cell proliferation or an anti-carcinogenic action.[2,5]

In addition, acetaminophen-binding protein (APBP), which is also a cytosolic protein in the mouse, is highly homologous with SeBP.[3] In fact, the deduced APBP amino acid sequence differs from SeBP only at 14 residues.[3] APBP is known to be present in the liver,[3] and although its physiological role is not yet completely clear, it is regarded as an important protein associated with hepatic necrosis in patients who have taken an acetaminophen overdose. Acetaminophen is widely used as an analgesic and antipyretic and is considered safe at a therapeutic dose; however, many reports have shown that an acetaminophen overdose causes hepatic centrilobular necrosis.[6] Although the exact mechanism of this toxic effect is still unknown, it is widely accepted that an active metabolite of acetaminophen, *N*-acetyl-*p*-quinone imine (NAPQI), which is formed by cytochrome P-450, depletes glutathione and binds to hepatic macromolecules.[7–9] Some of the hepatic macromolecules suggested as binding to NAPQI have been identified, and one of those is APBP.[10]

The cDNA cloning of mouse SeBP and

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APBP has been reported by Bansal *et al.*[4] and Lanfear *et al.*[3], respectively. Recently, a homologous human cDNA clone of SeBP was isolated by Chang *et al.*[11] In that report, they described that the human DNA sequences had 87.2 and 86.6% similarity when aligned with those of mouse SeBP and APBP, respectively.[11]

We previously reported that 3,3′,4,4′,5-pentachlorobiphenyl (PenCB), 3-methylcholanthrene (MC) and butylated hydroxytoluene (BHT) induce a 54 kDa protein in rat liver.[12–14] We have also shown that this 54 kDa protein is homologous with SeBP from its partial amino acid sequence.[12]

In spite of this information about SeBP and/or APBP, its physiological role remains to be unclear. One reason this has not yet been achieved is that there are no methods for processing native SeBP or APBP to afford high purity preparations. Although mouse SeBP and APBP have been purified by Bansal *et al.*[1] and Bartolone *et al.*,[15] respectively, they were electro-eluted using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We have previously purified the 54 kDa protein from rat liver treated with MC,[16] but our procedure included a denaturation step using urea and then a solubilization step involving detergent. In addition, there have been no reports of the purification factors and binding ratio of selenium to SeBP. This limited information is one reason for our limited understanding of the physiological role of SeBP. At purification, understanding the induction of a target protein is very useful. To study the function and properties of SeBP, a convenient and mild purification method is required. This paper is the first description of such a convenient purification procedure, and also reports the purification factors and binding ratio of selenium to the electrophoretically pure 54 kDa protein which is highly homologous with SeBP in the mouse.

MATERIALS AND METHODS

Materials —— Bovine erythrocyte glutathione peroxidase was purchased from Toyobo (Osaka, Japan). Deoxycholic acid sodium salt (DOCNa) was purchased from Sigma Chemical Co. Ampholine was purchased from Pharmacia LKB (Uppsala, Sweden). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, U.S.A.). All other materials were of the highest quality commercially available.

Animals and Treatment —— Male Wistar rats (3 weeks old) purchased from Charles River Japan (Tokyo, Japan) were housed in stainless-steel cages for one week prior to treatment. These rats were given MC at a dose of 20 mg/kg in 3 ml corn oil, by i.p. injection. The day after this treatment, the livers were removed and cytosolic fractions prepared.[16]

Purification of 54 kDa Protein —— (1) DEAE-Sephacel Fractionation: The liver cytosol from rats treated with MC was fractionated with ammonium sulfate (50—70%) and dialyzed against 10 mM Tris–HCl buffer, pH 7.5. Six ml of dialyzed sample (about 250 mg protein) was applied to a DEAE-Sephacel (2 × 21 cm) column equilibrated with 10 mM Tris–HCl buffer, pH 7.5. The column was then washed with 100 ml primary buffer (10 mM Tris–HCl, pH 7.5) followed by a 150 ml linear gradient of NaCl in primary buffer (0—0.3 M). The flow rate was 12 ml/h, and 4 ml fractions were collected.

(2) Rotofor fractionation: Partial purification was performed using a Rotofor Cell™ (Bio Rad). The 54 kDa protein obtained from the DEAE-Sephacel column was concentrated by ultrafiltration (cut-off 50 kDa). Ten ml of 2% Ampholine (pH range 6—8) in 10 mM Tris–HCl buffer was added to 25 ml of sample and the protein solution was loaded into the Rotofor Cell™. Focusing was carried out at 12 W constant power at 4℃ for 3 h (until the voltage reached a plateau). Fractions were then collected, and aliquots were subjected to SDS-PAGE analysis. Major fractions containing 54 kDa protein were collected and to separate Ampholine from protein, 1 M NaCl was added and dialyzed for 6 h against 100 volumes of water.

Protein Assay —— Protein was assayed by the method of Lowry *et al.*[17] However, after the Rotofor Cell™ procedure, protein was assayed by the method of Bensadoun and Weinstein[18] because of interference from Ampholine.

Electrophoresis and Immunoblotting —— SDS-PAGE was carried out as described by Laemmli.[19] The protein was stained with 0.1% Coomassie brilliant blue R-250. Immunoblot analysis was performed according to a method of Blake *et al.*[20] Electrophoresed cytosolic proteins in gel were transferred to PVDF membrane according to the method of Towbin *et al.*[21] The membrane was then precoated with a phosphate-buffered saline–Triton (PBS–Triton) buffer containing 5% skimmed milk for 1 h at 37℃. Next, it was incubated with 500-fold diluted rabbit

anti-54 kDa protein antiserum in PBS–Triton for 1 h at 37℃. After washing it with six changes of PBS–Triton for 30 min, the membrane was incubated with 1000-fold diluted alkaline phosphatase conjugated mouse IgG against rabbit IgG in PBS–Triton for 1 h at 37℃. Finally, the membrane was washed as described above and incubated with a reaction buffer. The composition of the reaction buffer was as follows: 9μl NBT stock buffer (173 mM nitrobluetetrazorium–70% dimethylformamide) and 7μl BCIP stock buffer (115.3 mM 5-bromo-4-chloro-3-indolyl phosphate–100% dimethylformamide) in 10 ml buffer (100 mM Tris – 100 mM NaCl–50 mM $MgCl_2$, pH 9.5 with HCl). Until required, the reaction buffer was kept in ice in the dark, and during the reaction, it was protected from light.

Estimation of the 54 kDa Protein Using NIH-Imaging —— Estimation of the band intensity of the 54 kDa protein was performed using a GT-8000 scanner (Epson, Tokyo, Japan) and NIH image software (version 1.52, Wayrve Rasband, Bethesda, MD). Electrophoresed cytosolic proteins in the gel were transferred to a PVDF membrane, then immunoblot analysis was performed. After staining, the membrane was dried and the digital images computed with the scanner. The scan data were saved and imported into the NIH image software for subsequent analysis.

Determination of Selenium Content —— The selenium content was determined by a fluorometric method as described by Kawamoto *et al.*[22] After wet ashing of the purified 54 kDa protein with nitric acid and perchloric acid at 160℃ for about 3 h, reduction with 6 M hydrochloric acid was carried out. Selenium was determined from the fluorescence produced by reaction with 2,3-diaminonaphthalene (λ_{EX}=377 nm, λ_{EM}=520 nm)

RESULTS

Purification of the 54 kDa Protein

Purification of the 54 kDa protein from the liver cytosol of rats treated with MC was performed using a DEAE-Sephacel column and Rotofor Cell™. Table 1 summarizes the protein content of the different fractions that were obtained during purification of the 54 kDa protein. Fig. 1 shows the elution profile of the DEAE-Sephacel column chromatography. The 54 kDa protein from the DEAE-Sephacel column was enriched to the greatest extent at 0 M NaCl (fraction numbers 13–25). Although the 54 kDa protein was also

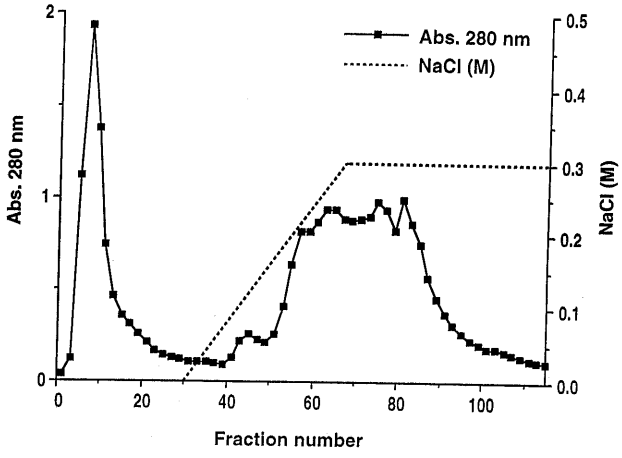

Fig. 1. Elution Profile of DEAE-Sephacel Chromatography Using an NaCl Gradient (0–0.3 M)

Table 1. Protein Content During the Different Stages of 54 kDa Protein Isolation

Fraction	Sample volume (ml)	Protein concentration (μg/ml)	54 kDa protein content (× 100 pixel/μg)[c]	Purification factor[d] (% of Cytosol)
Cytosol (MC 20 mg/kg)	220	16590	5.135	100
Ammonium sulfate (50–70%)	20	41450	8.716	169.7
DEAE-Sephacel[a] Chromatography	50	507	14.560	283.5
Rotofore cell™[a,b] separation	39	12.87	91.500	1781.9

a) Protein content was measured by the method of Lowry *et al.*[17] after concentration.
b) Protein content was measured by an improved method, details of which are given in Materials and Methods.
c) Measurement of band intensity was performed using NIH image software. The unit of band intensity is represented by pixel. Details are described in Materials and Methods.
d) Figures mean the purity of 54 kDa protein compared to the cytosol sample.

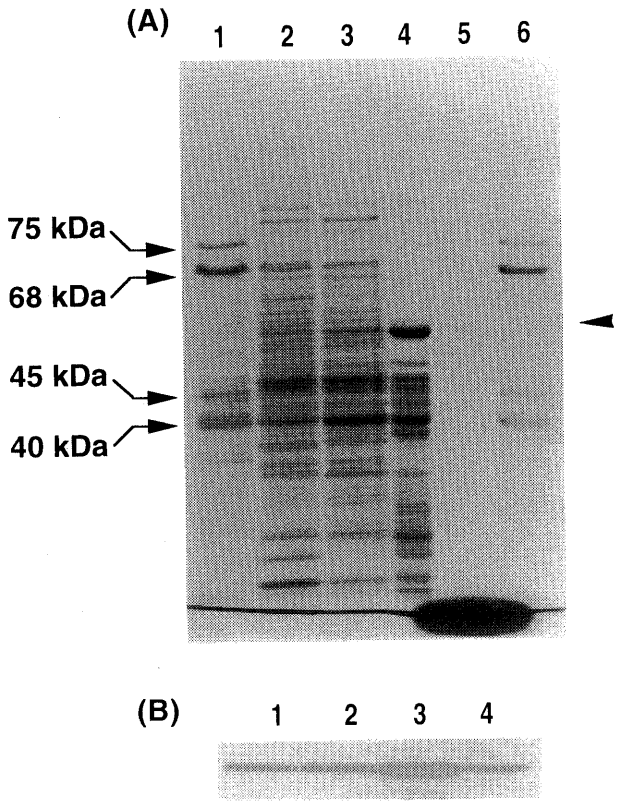

Fig. 2. SDS-Polyacrylamide Gel (10%) Electrophoresis (A) and Immunoblot Analysis (B) of 3-Methylcholanthrene-Inducible 54 kDa Protein Purified from Rat Liver Cytosol

In (A), Lane 1 and 6, Molecular weight marker proteins; Lane 2, Liver cytosol (10 μg) from the liver of rat treated with 3-methylcholanthrene; Lane 3, Sample obtained by ammonium sulfate (50—70%) separation and dialysis (10 μg); Lane 4, Concentrated 54 kDa protein-enriched fraction from the DEAE-Sephacel column (10 μg); Lane 5, Purified 54 kDa protein (0.3 μg). The arrow head shows the 54 kDa protein. In (B), the loaded samples were as follows: Lane 1, Liver cytosol (3 μg) from rat treated with 3-methylcholanthrene; Lane 2, Sample obtained by ammonium sulfate (50—70%) separation and dialysis (3 μg); Lane 3, Concentrated 54 kDa protein-enriched fraction from the DEAE-Sephacel column (1 μg); Lane 4, Purified 54 kDa protein (0.2 μg).

seen at 0.3 M NaCl (fraction numbers 70—80, approximately), we did not purify this 54 kDa protein since other proteins were present (data not shown). Fig. 2(A) and 2(B) show the SDS-PAGE and immunoblot analysis of fractions representing each purification step. The molecular mass of only one protein was estimated to be 54.7 kDa. This band was recognized by antiserum that we had developed previously.[16] Quantification of the stained band was performed by scanning and subsequent computer analysis using NIH image software. Calculation of the percentage purification yield is shown in Table 1. Thus, we developed a simple method to purify the 54 kDa protein with a high purification factor (about 18 times against cytosol) and without denaturation and solubilization.

Measurement of Selenium Content

To investigate whether the 54 kDa protein was related to selenium, the selenium content of the purified 54 kDa protein was measured. The purified 54 kDa protein (62.4 μg) was concentrated, then wet ashing and reaction with 2,3-diaminonaphtharene was performed. Table 2 shows the selenium content of the purified 54 kDa protein, which was about 19.56 μg/g 54 kDa protein. From this, the binding ratio of selenium to 54 kDa protein was calculated to be 0.014. As a positive control, the selenium content of commercial glutathione peroxidase (GPx) was also measured; in this case it was known that 1 mol selenocysteine is present in 1 mol protein.[23] As we found that the binding ratio of selenium to GPx was 1.214, we concluded that our method was accurate.

DISCUSSION

In this report, we have described a convenient and efficient isolation procedure for rat 54 kDa protein. We previously purified the 54 kDa protein from cytosol using a Rotofor Cell™.[16] In that purification step, because a precipitate was produced, we had to denature and solubilize the cytosol with 4 M urea and 3.16% Nonidet P-40. In contrast to a previously published method, our improved procedure does not require a denaturation or solubilization step and yields a single protein in SDS-PAGE.

We showed that the molecular mass of the purified protein was 54.7 kDa and that it was recognized by the antiserum that we had raised previously.[16] This antiserum recognizes protein with an amino acid sequence very similar to the partial alignment of mouse liver 56 kDa SeBP[4] and the isoform, APBP.[3] In addition, we report for the first time the binding ratio of selenium to 54 kDa protein, 0.014 (Table 2). The present investigation confirms the previous results of Bansal *et al.*,[4] which showed that selenium-binding protein in mouse liver binds selenium but has no selenocysteine in its amino acid sequence. These authors purified mouse SeBP using ^{75}Se, but they did not calculate the binding ratio of selenium to

Table 2. Selenium Content of Purified 54 kDa Protein

	Protein content (ng)	Selenium content (ng)	Se/54 kDa protein ratio[b]
Control[d]	0	N. D.[a]	
Purified 54 kDa protein[c]	62380	1.22	0.014
Glutathione peroxidase	1530	6.99	1.214

a) Not detectable, *b*) This value represents the molecular ratio of selenium to 54 kDa protein. We calculated the mol of 54 kDa protein using a molecular weight of 54700. *c*) Values represents the mean of two samples. *d*) Control was measured with Tris-HCl buffer.

SeBP. Therefore, we calculated the binding ratio of selenium to SeBP from their results and found it to be about 0.004. On the other hand, the ratio of the positive control, selenium to commercial GPx, was 1.214 (Table 2). It is known that GPx has one selenocysteine in its amino acid sequence. If the 54 kDa protein has one or more selenocysteine moieties, then the ratio would be the same or greater than that for GPx. This result suggests that the 54 kDa protein binds to selenium without having a selenocysteine moiety.

Selenium-containing proteins, like GPx, generally have one or more selenocysteine moieties in their amino acid sequence and so the physiological role of those proteins is expected to depend on the nature of the selenium. On the other hand, SeBP has no selenocysteine moiety in its amino acid sequence, and the binding ratio of selenium to protein was very low (Table 2). Although we were unable to explain the reason for the very low binding ratio of selenium to SeBP, one possibility might be that the physiological role of SeBP does not depend on the nature of the selenium.

Various hypotheses about SeBP have been proposed, but its physiological role remains unclear. To achieve a detailed understanding of the role of SeBP, more experiments are required, including gene analysis. The improved method reported here should provide a valuable tool for future experiments.

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