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Comparison of different transition metal ions for immobilized metal affinity chromatography of selenoprotein P from human plasma

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Abstract

Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} were evaluated in metal ion affinity chromatography for enrichment of selenoprotein P, and immobilized Co^{2+} affinity chromatography was found to be the most selective chromatographic method. The chromatography was performed by fast protein liquid chromatography and the fractionation was followed by analysis of the collected fractions for selenium by inductively coupled plasma mass spectrometry. By the combination of immobilized Co^{2+} affinity chromatography and heparin affinity chromatography a simple method was developed yielding a 14 800-fold enrichment of selenoprotein P. The purity of the protein was determined by SDS–PAGE and by sequencing from polyvinylidene diflouride blots of SDS–PAGE gels.

Keywords: Selenoprotein P; Transition metal ions

1. Introduction

The essential trace element, selenium, is incorporated into proteins as the amino acids selenomethionine and selenocysteine. In animals, selenomethionine seems to be incorporated non-specifically into proteins in place of methionine and to have no selenium-related function. Selenocysteine containing proteins, however, apparently have a selenium related function [1]. In selenocysteine containing proteins the incorporation of selenocysteine is encoded by an in-frame UGA codon [2,3], whereas in non-selenocysteine containing proteins the UGA codon is a signal for polypeptide chain termination. Two selenoproteins have been localized in human plasma: extracellular glutathione peroxidase and selenoprotein P. Glutathione peroxidase is a well characterized protein with a known function and structure [4]. Selenoprotein P has not been characterized that intensively, and the three-dimensional structure is unknown. The function of selenoprotein P is not known precisely but several recent reports all suggest an antioxidant function for the protein [5–8].

Selenoprotein P cDNA from several species including human has been sequenced [9–12]. The deduced amino acid sequences for the human, rat and mouse selenoprotein P contain 10 selenocysteines and a histidine rich motif. The mature protein has a molecular mass (M_r) of 41 000, but migrates close to an apparent M_r of 60 000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). In rat and also in human at

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least two isoforms are present [13–15]. Two isoforms from rat plasma have been purified and characterized: one full length isoform and one isoform with identical N-terminal but C-terminal truncated [16]. This truncation is believed to be the result of premature termination of protein translation just before the second UGA codon located in the C-terminal part of the protein [13,16]. Thus, the full length isoform contains 10 selenocysteines, whereas the truncated isoform contains only one. The truncated isoform has a mobility in SDS–PAGE corresponding to an apparent M_r of 55 000. Both isoforms contain the histidine rich motif.

So far, it has not been possible to produce recombinant selenoprotein P by in vitro expression. Therefore, the only possibility for studying structure/function relationship of selenoprotein P is to purify the protein from the natural source. Previously, methods involving either immunoaffinity chromatography [14,17] or laborious multi step column chromatography [18,19] have been used. This kind of chromatography is not easily adoptable for purification of human selenoprotein P, since only antibodies against murine selenoprotein P are commercially available and these antibodies do not cross react with the human orthologue. As human plasma contains only about 80 μg selenium/l, a large volume of starting material is needed [20,21]. Therefore a simple and fast purification protocol is required, a requirement that is further enhanced by the lability of the protein [14]. Recently, several methods involving immobilized metal ion affinity chromatography (IMAC) have been published [7,8,15]. The purpose of the present investigations was to evaluate different transition metals in IMAC of selenoprotein P in order to develop a simple and fast method for the purification of selenoprotein P from human plasma.

2. Materials and methods

2.1. Materials

Frozen citrate stabilized human plasma was obtained from the blood-bank at the Central Hospital in Nykøbing Sjl., Denmark. The columns: 5 ml HiTrap Heparin, 5 ml HiTrap Chelating, Fast Desalt HR 10/10 (G-25). Column material: Chelating Sepharose Fast Flow and Coomassie R-350 were from Pharmacia Biotech, Uppsala, Sweden. Pre-cast Tris–glycine PAGE gels were from Novex, Frankfurt/Main, Germany and pre-stained electrophoresis standards were from Bio-Rad, Hercules, CA, USA. All other chemicals were of analytical grade from either Sigma, St. Louis, MO, USA, or Merck, Darmstadt, Germany. Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used throughout.

2.2. Electrophoresis

SDS–PAGE was run in 10% pre-cast Tris–glycine gels according to the recommendation of the manufacturer and stained with Coomassie R-350. Semi-dry electroblotting from SDS–PAGE gels onto polyvinylidene diflouride (PVDF) membranes was performed using the Hoefer TE70 semi-dry transfer unit (Hoefer, San Francisco, CA, USA) according to Matsudaira [22].

2.3. Concentration of samples

Protein solutions with volumes larger than 10 ml were concentrated at 4°C by ultrafiltration in a stirred cell equipped with a low non-specific protein binding YM30 filter with a M_r cut-off at 30 000 (Amicon, Beverly, MA, USA). Smaller volumes of protein solutions were concentrated in microcentrifuge filters with nominal molecular mass limit of 30 000 (Millipore).

2.4. Glutathione peroxidase assay

The activity of glutathione peroxidase was determined by a coupled enzymatic assay [23].

2.5. Determination of selenium concentrations

The qualitative determinations of selenium from stained bands from PVDF membranes were performed by electrothermal atomic absorption spectrometry (ETAAS) using a Perkin-Elmer Zeeman 5000 instrument equipped with a HGA-500 graphite furnace, pyrolytic graphite tubes without platforms and an AS-40 autosampler (Perkin-Elmer, Norwalk, CT, USA). Relevant bands were cut out from the

membrane, 5 μl of chemical modifier (7.5% HNO_3, 4 g Pd/l) was pipetted onto the membrane and the membrane was allowed to dry for 10 min. The membrane was then manually placed in the graphite tube.

Quantitative determinations of selenium in plasma fractions were performed on a Perkin-Elmer Sciex Elan 6000 instrument equipped with an AS 90 autosampler. A 500-μl volume of sample was mixed with 4450 μl water and 50 μl methanol prior to analysis.

2.6. Chromatography

All chromatographic steps were performed using a fast protein liquid chromatography (FPLC) instrument equipped with the FPLC director software (Pharmacia Biotech). In order to collect the eluate in an inert atmosphere, a tube with an argon flow was mounted next to the outlet sample tube in the fraction collector.

Analytical IMAC for comparison of immobilized ions was performed on 5 ml HiTrap chelating columns packed with Sepharose coupled iminodiacetic acid (IDA). The procedure was as follows: after washing the gel material with two column volumes of water, one column volume of a 0.1 *M* solution of the relevant metal ion was loaded. The column was washed with three column volumes of water, two column volumes of B-buffer [20 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), 20 m*M* (3-(*N*-morpholino)propanesulfonic acid) MOPS, 20 m*M* sodium acetate, 1 *M* NH_4Cl, pH 4.0] and three column volumes of A-buffer (20 m*M* MES, 20 m*M* MOPS, 20 m*M* sodium acetate, 1 *M* NH_4Cl, pH 7.0). All buffer solutions were sterile filtered and saturated with argon before use. After elution, the column was regenerated by washing with two column volumes of a solution containing 50 m*M* ethylenediaminetetraacetic acid (EDTA) and 0.5 *M* NaCl to remove any metal ions from the column. A 5-ml volume of plasma was loaded, and the column was washed with 20 ml of A-buffer before a linear gradient from 100% A-buffer to 100% B-buffer was applied between 25 to 65 ml. Fractions of 1.5 ml were collected and selenium concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS).

Preparative IMAC for plasma purification was performed on a XK26/20 column packed with 50 ml Chelating Sepharose Fast Flow and thermostated at 4°C. The procedure was the same as for analytical IMAC until the plasma application. Plasma was filtered through a paper filter before it was diluted twice with a mixture of 60 m*M* MOPS, 60 MES, 60 m*M* sodium acetate and 3 *M* NH_4Cl, pH 7.0. The mixture was loaded with a flow-rate of 2 ml/min. Before elution the column was washed with 30% B-buffer until the A_{280} signal was less than 0.02. Fractions of 10 ml were collected and selected fractions were analyzed by ICP-MS. Fractions containing selenium were pooled and concentrated by ultrafiltration with a molecular mass cut-off at 30 000. The buffer of the concentrate was changed into 60 m*M* MOPS, pH 7.0 by gel filtration on a Fast Desalt column. A 5-ml volume of buffer exchanged concentrate was loaded on a 5 ml HiTrap Heparin column equilibrated with a 60 m*M* MOPS, pH 7.0 buffer. After loading, the column was washed with 60 m*M* MOPS, pH 7.0 before a gradient of 60 m*M* MOPS, pH 7.0, from 0 to 1 *M* NH_4Cl was applied. Fractions of 2 ml were collected and selected fractions were analyzed by ICP-MS. Fractions containing high amounts of Se were pooled and concentrated in microcentrifuge filters with a nominal molecular mass limit of 30 000. The purified selenoprotein P was analyzed by SDS–PAGE, ETAAS and amino acid sequencing from blots of SDS–PAGE gels.

3. Results and discussion

3.1. Analytical IMAC

The presence of a histidine-rich motif in the amino-acid sequence of selenoprotein P makes IMAC a well suited candidate for chromatographic purification of selenoprotein P. It has previously been shown that IMAC with immobilized Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} are selective for exposed histidine residues [24], although these metal ions also are capable of coordinating to the side chains of tryptophan and cysteine [25]. We investigated the following transition metals: Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} in the analytical IMAC experiments. Five

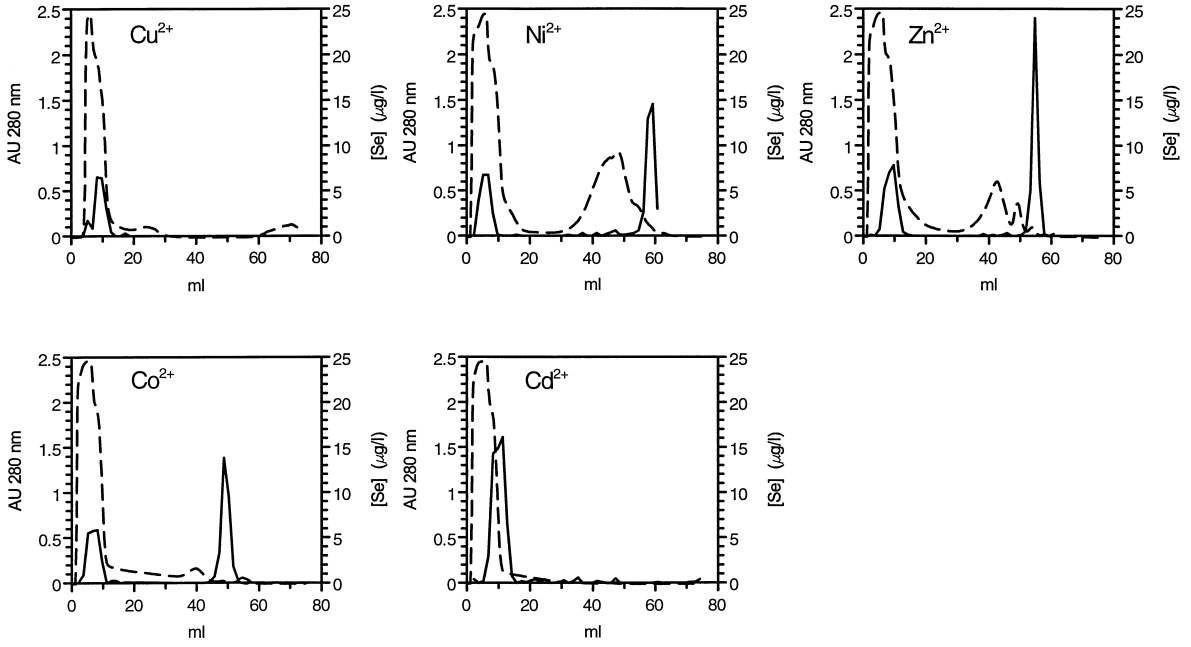

Fig. 1. Chromatograms from the analytical IMAC with five different metal ions as indicated in the upper left corner of each chromatogram. The absorbance readings at 280 nm from the FPLC (- - -) and the concentration of selenium (—) are shown. The elution gradient is linear (100% A-buffer to 100% B-buffer) and applied from 25 ml to 65 ml. The first eluting selenium peak corresponds to glutathione peroxidase and the second eluting peak to selenoprotein P.

chromatograms were obtained as shown in Fig. 1, one for each metal ion used. As can be seen from the first four chromatograms, approximately 50% of the total amount of selenium elutes between 2 and 12 ml, i.e., it flows through the column without binding. The other half is retained on the column, and only elutes upon applying an eluting gradient from pH 7 to pH 4. The non-retained selenium correlates with the glutathione peroxidase activity (data not shown), whereas no glutathione peroxidase activity could be detected in the retained selenium eluate. These results show that under the applied conditions none of the tested metal charged gel materials bind glutathione peroxidase.

In the Cd^{2+}-IMAC experiment, an amount of selenium corresponding to the total loaded amount is eluted at ~10 ml, indicating this column does not retain any of the two selenoproteins. In the Cu^{2+}-IMAC, however, the retained selenoprotein P is bound very tightly and is not eluted during the gradient but only after adding 50 m*M* EDTA (data not shown). In the chromatograms from the Ni^{2+}-IMAC, Zn^{2+}-IMAC and Co^{2+}-IMAC (Fig. 1) two selenium peaks are observed, corresponding to the non-retained glutathione peroxidase and the retained selenoprotein P. Thus, under the applied conditions the Cu^{2+}-, Ni^{2+}-, Zn^{2+}- and Co^{2+}-IDA gel materials are all capable of separating the two selenoproteins in human plasma, whereas the Cd^{2+}-IDA gel material is not. Fig. 2 shows a SDS–PAGE gel of the most selenium rich fractions from the Ni^{2+}-IMAC, Zn^{2+}-IMAC and Co^{2+}-IMAC together with a low-molecular-mass standard. Selenoprotein P expected at M_r 60 000 and 55 000 cannot be seen from the gel because the concentration is too low to be visualized by Coomassie staining. However, the gel gives an impression of the amount of contaminating proteins in the selenoprotein containing fractions. When comparing the Ni^{2+}-IMAC, Zn^{2+}-IMAC and Co^{2+}-IMAC it is seen that the eluted selenoprotein co-elutes with decreasing amounts of contaminating protein and thus becomes increasingly purified when

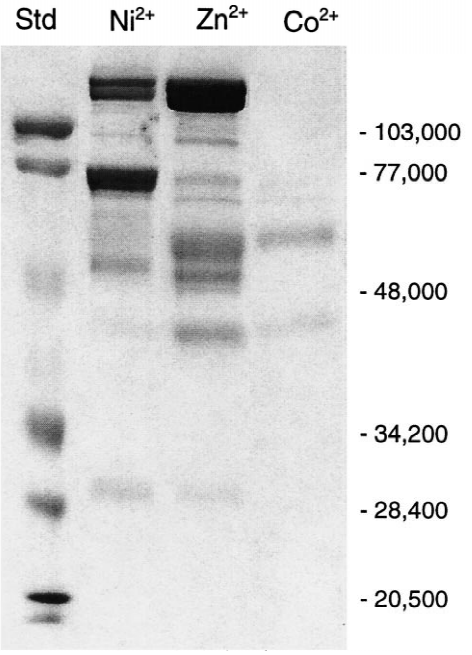

Fig. 2. SDS–PAGE gel of the most selenium rich fractions from the Ni^{2+}-, Zn^{2+}- and Co^{2+}-IMAC together with a low-molecular-mass standard in lane 1 with the indicated molecular masses.

Table 1
Retention volumes of selenoprotein P and selenium concentrations in the relevant fractions from the analytical Ni^{2+}-, Zn^{2+}-, Co^{2+}-IMAC experiments (the bold data represent the highest enrichment achieved for the individual chelate columns)

Metal ion	Elution volume (ml)	A_{280} [a]	[Se] [b] (μg/l)	[Se]/A_{280} [c] (μg/l)
Ni^{2+}	54.75	1.038	0.6	0.6
	56.25	0.631	2.6	4.2
	57.75	0.411	13	32
	59.25	**0.22**	**15**	**66**
	60.75	0.18	3	17
Zn^{2+}	51.75	0.391	0.5	1.4
	53.25	0.307	5.1	17
	54.75	**0.33**	**24**	**73**
	56.25	0.136	5.9	43
	57.75	0.106	0.2	2
Co^{2+}	45.75	0.104	0.7	7
	47.25	0.093	3.4	36
	48.75	0.116	14	120
	50.25	**0.077**	**9.6**	**124**
	51.75	0.056	1.8	32

[a] A_{280} is a measure of total protein concentration in the fraction.
[b] [Se] is the selenium concentration in the fraction.
[c] [Se]/A_{280} is an indication of purity.

changing the metal ion from Ni^{2+} to Zn^{2+} to Co^{2+}. Furthermore, the retention times are decreasing in the same order, as shown in Table 1.

This corresponds to a weaker interaction between the proteins and the metal charged gel material when changing the metal ion from Ni^{2+} to $Zn^{2+}$to Co^{2+}. These results are in accordance with the Irving–Williams series for the stability of bivalent metal ion complexes of the first transition series with a given ligand [26] which is also reflected in the strength of protein binding to immobilized metal ions which usually decreases in the order: $Cu^{2+}>Ni^{2+}>Zn^{2+}>Co^{2+}$ [25]. Comparing these four metal ions for IMAC purification of selenoprotein P, it can be seen from the chromatograms and the SDS–PAGE gel in Fig. 2 as well as from the data in Table 1 that as the strength of the protein–metal ion–IDA interactions increases, the selectivity of metal ion–IDA towards selenoprotein P decreases. Thus, among the tested metal ions, Co^{2+} is the one best suited for IMAC purification of selenoprotein P.

3.2. Heparin-Sepharose chromatography

Since it was shown that selenoprotein P binds to heparin [27], this interaction has been used numerous times for chromatography of selenoprotein P on matrix-bound heparin [13,15,20]. In our experiment, the selenium containing eluate from the Co^{2+}-IMAC was concentrated and buffer exchanged prior to loading on a 5 ml HiTrap Heparin-Sepharose column. The retained protein was then eluted with a 0 to 1 *M* NH_4Cl gradient. The chromatogram is shown in Fig. 3. Selenoprotein P elutes before the major protein peak, and thus an excellent purification is achieved in this step. A shoulder is seen on the Se peak at a retention volume about 46 ml, indicating that isoforms of selenoprotein exist.

An SDS–PAGE gel of the eluate before the heparin purification compared to the eluate after the purification illustrates the purification achieved in the heparin-Sepharose chromatography. This is shown in Fig. 3. The major part of the proteins appear between

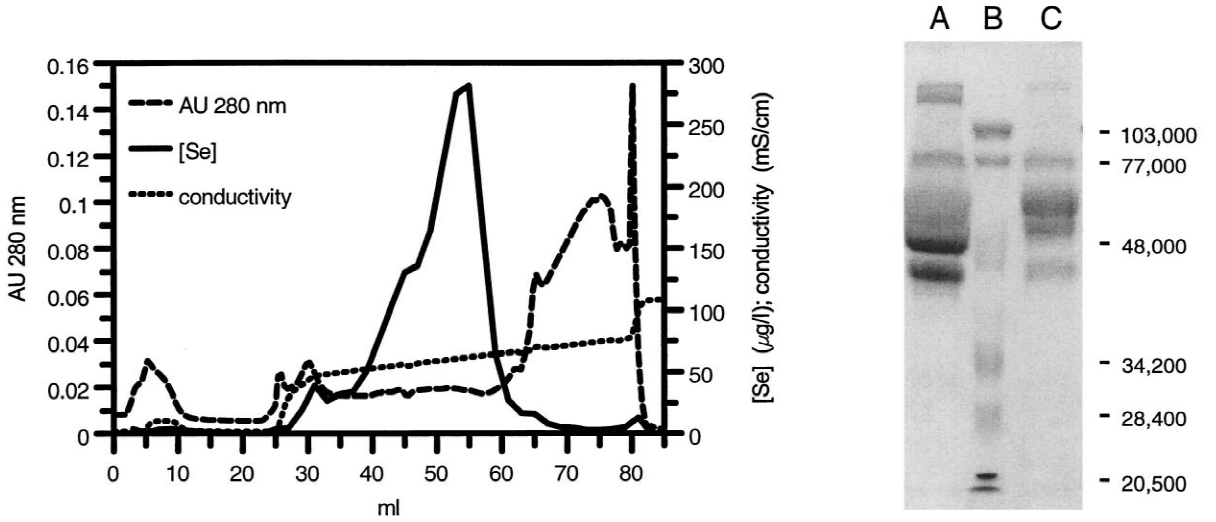

Fig. 3. Chromatogram from the heparin-Sepharose chromatography showing the selenium concentration in assayed fractions (—), the absorbance readings at 280 nm from the FPLC (- - -) and the applied elution gradient (· · ·). To the right is shown a SDS–PAGE gel with three lanes, (A) the concentrate from the Co^{2+}-IMAC experiment loaded on the heparin-Sepharose column, (B) low-molecular-mass standard with the indicated molecular masses, (C) the eluate between 48 and 58 ml concentrated 15 times. The isoforms of selenoprotein P are seen as two bands at M_r 55 000 and 60 000.

48 000 and 77 000. By electroblotting from SDS–PAGE gels onto PVDF membranes and subsequent ETAAS analysis of excised bands, it was shown that only the two bands at M_r of 55 000 and 60 000 (Fig. 3) contained selenium. By 10 cycles of amino acid sequencing of excised bands from the electroblots, the N-terminal sequence of the two bands at M_r 55 000 and 60 000 are determined to be: ESQDQSSL-K. This sequence is identical to the N-terminal part of the amino acid sequence of human selenoprotein P [10]. Amino acid number 9 is a cysteine which is not determined in amino acid sequencing.

The observation of a shoulder on the Se peak from the heparin-Sepharose chromatography together with the appearance of two distinct selenium containing bands in SDS–PAGE gels are in accordance with the existence of isoforms of selenoprotein P. This has previously been shown to be the case for the rat orthologue by heparin chromatography [13,16] and has also been shown for the human orthologue by SDS–PAGE analysis of purified selenoprotein P [14,15].

3.3. Preparative purification

When the Co^{2+}-IMAC method is applied in a preparative scale and combined with an additional heparin-Sepharose chromatographic step, a simple and fast method for enrichment of selenoprotein P is developed. As seen in Table 2 a substantial enrichment is achieved in the first step and less in the

Table 2
Purification factors and yield for the preparative purification of selenoprotein P

Purification step	Volume (ml)	A_{280}	[Se] (μg/l)	[Se]/A_{280} (μg/l)	Purification of selenoprotein P[a] (-fold)	Yield[a] (%)
Native plasma	950	~60	39	0.65	–	–
After Co^{2+}-IMAC	10	2.72	1140	420	1290	62
After heparin-affinity chromatography	20	0.06	290	4800	14 800	31

[a] In calculation of the yield and the purification factor it is assumed that 50% of the plasma selenium is incorporated into selenoprotein P.

second. All though small amounts of impurities are seen at M_r 72 500 and 46 900 when loading the SDS–PAGE gel with a 15-fold concentrate of the eluate from the heparin column, an appreciable purification factor is obtained. As close to 50% of plasma selenium is incorporated into selenoprotein P [28,29], an enrichment factor of 1290 is achieved in the first step and an enrichment factor of 11 in the second step. During the late stages of the experimental work and during the preparation of this manuscript several papers appeared describing purification protocol for selenoprotein P based on IMAC with immobilized Zn^{2+}or Ni^{2+} [7,8,15]. The results presented here gives an overall enrichment factor of 14 800 and a yield of 31%. These results are in the same order of magnitude as the previously published results, which all were obtained using three chromatographic steps. Thus, it seems that a carefully selected metal ion for IMAC may substitute an additional chromatographic purification step.

4. Conclusion

When Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} were compared in IMAC immobilized Co^{2+} affinity chromatography was found to be the most selective chromatographic method for purification of selenoprotein P from human plasma.

With the reported method it is possible to purify selenoprotein P 14 800-fold with a yield in the mg-range within less than one week of work and without the need for production of antibodies.

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