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Full Length Research Paper

Purification of human serum paraoxonase: A simple and rapid method

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Paraoxonase/arylesterases (EC.3.1.8.2) is an enzyme found tightly associated with high density lipoprotein particle in serum. Because of its unique enzyme activity, antioxidant property and its role as an anti atherosclerotic molecule, various methods are used for its purification from human serum. Methods involved in its purification are elaborate and complicated. Also the yield and final activity are highly variable. Here, we report a 2 step method of purification involving affinity chromatography on cibacron blue sepharose followed by gel filtration on sephadex G50.The final preparation was 27.7 fold purified compared with the serum and gave a single band in SDS-PAGE by silver staining.

Key words: Cibacron blue sepharose, paraoxonase, phenyl acetate, SDS-PAGE, silver staining.

INTRODUCTION

Paraoxonase (E.C. 3.1.8.2), (PON) is an enzyme ubiquitously distributed in nature (La Du et al., 1992). In animals it is mainly found in blood in association with high density lipoprotein (HDL) particle, by a strong hydrophobic association through the N-terminal hydrophobic peptide (Kuo and La Du, 1995). Consequently methods of purification have employed isolation of HDL, dissociation of HDL-PON and subsequent purification of PON.

PON was first purified by Main (1968). This method involved a series of ethanol precipitation and ammonium sulphate fractionation steps. The purified enzyme had a molecular weight ranging from 35 - 50 KDa.

The other methods of purification involved precipitation of HDL using heavy metal salts (Burstein et al., 1970) or by density gradient centrifugation to separate HDL fraction. More recent methods employ gel filtration of HDL on sepharose or affinity purification of HDL by affinity material like cibacron blue sepharose or heparin-agarose (Chapman et al., 1971) .These methods also use other additional steps of purification.

We report a 2 step method using affinity purification on cibacron blue sepahrose followed by gel filtration on sephadex G-50.

MATERIALS AND METHODS

Materials

Glycine, tris buffer, ammonium per sulphate, acrylamide, bis acrylamide, coomassi brilliant blue, TEMED, SDS and ethylene diaminetetraaceticacid (EDTA) were purchased from SRL chemicals (Mumbai, India). Cibacron blue 3GA and deoxycholate were purchased from Sigma Aldrich company USA. Acetic acid was purchased from Ranbaxy chemicals (New Delhi). All other chemicals, acids, bases, solvents and salts used for the purification were of analytical grade. Dialysis bags used during dialysis was purchased from Thermo scientific life science research products, Rockford, USA.

Human blood was collected from normal healthy volunteers according to guidelines mentioned by the institutional ethical committee for biomedical research.

Methods

Purification of human serum PON on affinity chromatography

Human serum paraoxonase was purified by nonspecific affinity chromatography on cibacron blue 3GA. Cibacron blue sepharose (30 ml) was washed and suspended in column buffer which contained 10 mM tris-HCl, 1 mM $CaCl_2$ and the pH was adjusted to 8.2 (Kuo and La Du, 1995). The ratio of gel to serum was maintained 4:5 (v/v). Serum (45 ml) was mixed with an equal volume of column buffer and the mixture was loaded on cibacron blue gel column, (40 $\times$ 2 cm). The eluate was recycled through the column twice. The eluate was then collected in one fraction. The column was then washed with 30 ml of 4 M NaCl solution. The 4 M NaCl salt solution greatly reduces albumin or other soluble proteins from binding to cibacron blue gel and thus most of the soluble proteins and other lipoproteins are washed out of column. The salt fraction was collected in a separate tube to check whether it contained the esterase activity (Rodrigo et al., 1997).

The column was then eluted with 30 ml of 0.1% deoxycholate and fractions were collected (1 ml). Optical density of the eluted fractions was measured at 280 nm. PON activity in the eluted fractions was monitored by assaying the aryl esterase activity. The fractions containing PON were pooled separately. The pooled frac-

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tions were concentrated and further purified by gel filtration on sephadex G-50 column.

Gel filtration on G- 50

The pooled deoxycholate fraction (containing the aryl esterase enzyme activity) was loaded on sephadex G-50 column (50 × 2 cm) pre-equilibrated with 0.5 M NaCl solution. The column was also eluted with 0.5 M NaCl. 20 fractions (1 ml) were collected and the optical density was measured at 280 nm (Cordle et al., 1985).

Dialysis

The diluted protein fractions from G-50 with esterase activity were pooled (about 10 ml) and kept for dialysis in a dialysis bag with 8 KDa cut-off. Dialysis was carried out overnight against double distilled water with 2 changes. This process facilitates removal of deoxycholate which was used during the elution of HDL fraction from affinity column and the salt from gel filtration column. Long time association of deoxycholate with paraoxonase affects its activity as well as its half life.

Protein estimation by Lowry's method

Protein estimation was carried out according to Lowry's method (Lowry, 1951) to estimate serum protein as well as protein in purified fractions.

Assay of enzyme activity

Synthesis of phenyl acetate

10 ml of distilled phenol was taken in a round bottom flask and 20 ml acetyl chloride was added and refluxed at 70°C for 1 h. The contents of the flask were cooled and 200 ml of ice cold water was added. Free acid was neutralized by adding calculated amount of NaOH. The oily layer was separated by dissolving it in petroleum ether. Petroleum ether layer was evaporated to yield Phenyl acetate (Gan et al., 1991).

Aryl estrase activity

PON 1 activity towards phenyl acetate was measured spectrophotometrically at 270 nm in an automated shimadzu UV-1601, UV visible spectrophotometer. Buffer substrate was prepared by adding 50 mM Tris-HCl buffer pH 8.0 containing 2 mM Calcium. Phenyl acetate in Isopropyl alcohol was added such that the Phenyl acetate concentration was 2 mM and the isopropyl alcohol concentration was less than one percent. To 2.99 ml of buffered substrate 10 µl of the sample from different fractions was added and the optical density was measured at 270 nm continuously at intervals of 30 sec for 3 min. PON 1 activity was calculated from the linear part of the curve by calculating the change in OD per min. One unit of activity was defined as that amount of enzyme which produced 1 micromole of phenol per min. The molar extinction coefficient of phenol was 1310 M^{-1} cm^{-1} (Gan et al., 1991).

Inactivation of PON

Heat inactivation

PON activity in serum samples was also confirmed by the heat inactivation of PON. Selected serum, HDL or purified protein samples were heated at 80°C for 15 min prior to determining their PON activity as described above. There was no precipitation of protein by this treatment and the activity was essentially zero

Electrophoresis

SDS –page

Polyacrylamide gel electrophoresis (10% cross linking) was carried out for purified human serum paraoxonase as described by Davis (1964) using an alkaline buffer. (Tris-glycine buffer pH 8.8) The electrophoresis was performed at constant voltage for 2 - 4 h. The gels were stained using coomassi brilliant blue dye.

Silver staining

Silver staining was carried out essentially by the method of Switzer et al. (1980). Protein detection by silver staining depends on the binding of silver ions to the amino acid side chains, the sulphydryl and carboxyl groups of proteins followed by reduction to free metallic silver. As a result, the image of protein distribution within the gel is based on the difference in oxidation-reduction potential between the gel's area occupied by proteins and the free adjacent sites. It is sensitive enough to detect nanogram level of contaminating proteins if any.

Regeneration of cibacron blue gel

This is the most important and necessary step employed in the purification of PON from human serum sample. Initially the cibacron blue gel was washed with 3 M NaCl to remove any protein which was bound to cibacron blue gel. The gel was then washed with excess of water to remove chloride ions. This facilitated rapid purification and higher yield. The gels could be regenerated to maximum of 4 times.

RESULTS

The total protein concentration in the partially purified paraoxonase (Deoxycholate fraction) was 0.7 g/l with a specific activity of 1.4 mU/mg and total protein concentration of purified paraoxonase was 0.023 g/l with a specific activity of 4.6 mU/mg.

The elution profile of PON activity from cibacron blue affinity column is shown in Figure 1. Protein was eluted in 5 protein peaks while the enzyme activity was eluted in only 2 peaks namely peak 2 and 3. The protein elution and enzyme elution did not overlap. The elution of PON activity from sapahadex G-50 column is shown in Figure 2. The protein and enzyme activity elution overlapped. However a single symmetrical peak was not obtained.

The electrophoretic pattern of the purified protein is shown in Figure 3A and B, a single band was obtained both in coomassi blue staining as well as silver staining.

The scheme of purification of PON is summarized in Table 1. The final product was 27.7 fold purified compared with pooled serum.

DISCUSSION

Since PON and HDL are tightly associated, during purifi-

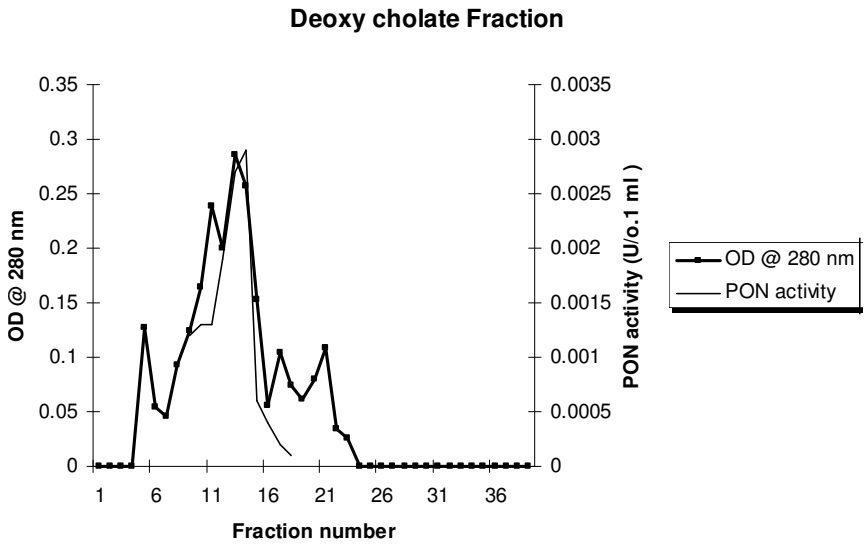

Figure 1. Elution of PON activity from cibacron blue sepharose column by deoxy cholate cibacron blue sepharose (30 ml) was packed in a column (40 × 2 cm). Pooled human serum was diluted with load buffer and passed through the column. Column was washed with 4 M NaCl followed by elution with 0.1% deoxycholate solution. Protein in the eluted fraction was monitored at 280 nm. The activity was monitored by measuring aryl esterase activity.

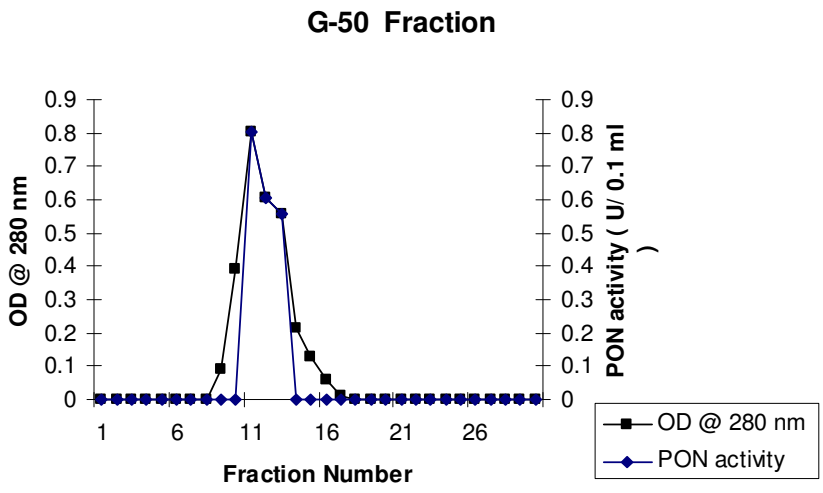

Figure 2. Elution profile of PON activity from sephadex G-50 column.Fraction having PON activity from cibacron blue column was pooled, concentrated and dialyzed against water. It was loaded on a column of sephadex G-50 (50 × 2 cm) preequilibrated and eluted with 0.5 M NaCl. Protein in the fractions (1 ml) was monitored at 280 nm and the activity was monitored by measuring the aryl esterase activity.

purification of PON, it is generally associated with APO-A1 and separating the 2 has been very difficult. APO-A1 is a major protein component of high density lipoprotein (HDL) with molecular weight of 29 KDa. APO-AI promotes cholesterol efflux from tissues to the liver for excretion and also play a major role in preventing accumulation of cholesterol loaded macrophage with in the arterial wall as a foam cells. APO-AI is also a cofactor for LCAT factors that up regulate PON1 gene also up regulate Apo A1 gene (Hirz and Scanu, 1970). Apo A1 up regulation is associated with increase in HDL. (Hesler et al., 1988) PON 1 is also shown to strongly associate with apo A1 (Mackness, 1998) in HDL. This may be responsible for keeping PON completely associated with

Table 1. Scheme for purification of PON activity.

Fraction	Amount of Protein	Specific activity (mU/mg)
Serum	8.750 g/l	0.166
oxycholate fraction (Dialyzed)	0.7 g/l	1.4
G- 50 fraction	0.023 g/l	4.6

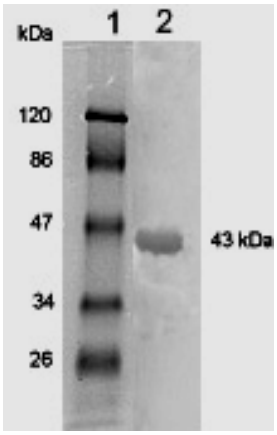

Figure 3a

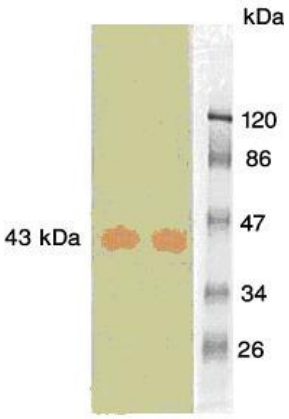

Figure 3b

Figure 3. Electrophoresis –SDS PAGE
(a) Coomassi staining (10%).
(b) Silver staining.

HDL in the serum (Jenne et al., 1991). Separation of APO AI and PON may result in the loss of PON activity. In this study the total recovery of enzyme activity was only 7.3% of the initial activity. It is possible that the entire PON enzyme may not be associated with HDL. It is also possible that there may be pool of modified HDL which does not bind to the affinity matrix. During purification the loss of activity of PON may also be because of the loss of its natural lipid environment. It is also possible that the PON may not be as active in the dissociated form as it in the native form. This is consistent with the report that purified PON loses its activity rapidly (Aviram et al., 1998). However our data show that the specific activity at the cibacron blue step is about 8 fold higher than that of the serum and in the sephadex gel filtration step it is further improved 3 fold.

Since aryl esterase was used to monitor the elution of PON, the enzyme assayed as PON may not be entirely PON, but may have represented other esterases also. For example, serum cholinesterase also shows arylesterase activity but their contribution to the total activity would be less than 5%. However, this esterase would not be active in high salt solution where only PON is active. We have also shown that the non specific esterase hydrolyzes phenyl acetate at high pH and the activity of non specific esterase at pH 8 is negligible. Hence the phenyl acetate hydrolyzing PON activity alone may be seen in the deoxycholate fraction.

It is interesting that a single band was obtained even when the sephadex G-50 fraction did not show a symmetrical peak. It is possible that some of the PON may still attach to lipid non-covalently in the gel filtration step, since PON is known to bind to lipid it may still be associated in the fractionation. However in SDS – page the lipid would be dissociated giving only single band.

It is interesting to note that the PON activity recovered from the purification steps is low. Since Indians have a high prevalence for cardiovascular diseases (Goyal and Yusuf, 2006; Singh et al., 2007). it is tempting to speculate that the active HDL which would be cardio protective is low among Indians while the non functional HDL even though high, may not contain PON or may contain some inactive form of PON.

Conclusion

We report a simple and rapid method of purification of PON from human serum in sufficient quantity in very short time and also we show the regeneration of the affinity gel for reuse.

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