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PURIFICATION OF HUMAN ERYTHROCYTE CATALASE BY ION EXCHANGE CHROMATOGRAPHY: A FEW MODIFICATIONS

MADHUR M. GOYAL*, ANJAN BASAK
Department of Biochemistry, J. N. Medical College, Datta Meghe Institute of Medical Sciences (Deemed University), Wardha-442004, India
*Corresponding Author: Email- monusvm@yahoo.com

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Abstract- Catalase is well known antioxidant enzyme to protect cells from the toxic effects of hydrogen peroxide. Though the enzyme has been studied a lot, research continues to reveal different characters of mammalian catalase. We purified the human erythrocyte catalase by ion-exchange chromatography as per available methods. Present article discuss the problems faced during the purification with the way out. The troubleshooting and modifications collectively made the purification work easy and quick to be standardized.
Key words - Human erythrocyte catalase; purification; ion-exchange chromatography
Abbreviations BLC, Bovine liver catalase; CV, Column volume; EDTA, Ethylenediaminetetraacetic acid; HEC, Human erythrocyte catalase; PAGE, Polyacrylamide gel electrophoresis; PB, Phosphate buffer; pI, Isoelectric point; PMSF, Phenylmethanesulfonylfluoride; RBC, Red Blood Cell; SDS, Sodium dodecyl sulfate.

Introduction
Catalase is well known enzyme that plays critical role in protecting cells against the toxic effects of hydrogen peroxide. Catalase removes over half of the hydrogen peroxide generated in normal human erythrocytes [1]. The altered catalase activity has been observed in wide spectrum of pathological conditions from cataractogenesis to atherosclerosis [2-5].
Though the role of catalase in mammals has been studied a lot but still there are some gray areas associated with its functions and mechanism of action [5,6]. We purified the human erythrocytes catalase following the method described by Goth [7]. Few modifications were adopted from the method described by Ko et al. [8]. Both methods are modified form of procedure described by Morikofer-Zwez et al. [9]. In this article, we discussed the problems faced during the standardization of entire procedure with the way out. These troubleshooting and modifications collectively made the purification work easy and quick to be standardized.

Material & Methods
DEAE- and CM-Cellulose (GeNei, India), BLC (Sigma, USA), dialysis and ultra-filtration membranes (HiMedia Pvt. Ltd., India) were purchased. The ion-exchange and size-exclusion chromatography were performed on software operated Biologic LP system with fraction collector (BioRad, USA). Spectrophotometer BL-198 (ELICO, India) was used for the measurement of enzyme activity and protein concentration. Analytical grade water and chemicals were used during the entire procedure.

Sample preparation
Outdated blood was received from the blood bank. It was centrifuged at 2500g for 15 minutes to separate pack cell volume. Supernatant plasma and buffy coat were discarded. RBCs were washed with 3 volumes of normal saline and haemolysed by adding 5 volumes of water. After 20 minutes, it was centrifuged at 3500g for 20 minutes. The transparent supernatant was collected and filtered through qualitative filter paper (Whatmen-1) to remove left out cell debris. It was followed by precipitation of proteins with 50% ammonium sulphate. For precipitation equal volume of saturated solution of $(NH_4)_2SO_4$ was mixed with filtrate and after 15 minutes, it was centrifuged in 20ml test tubes at 3500g for 10 minutes. All the pellets were pooled and washed with 45 % $(NH_4)_2SO_4$ solution. Finally, the pellet was dissolved in minimum volume of 1.5 mM Na-K PB at pH 7.3.

Dialysis
The dissolved pellet was dialysed for 72 hrs against 1500 ml dialysis buffer (1.5 mM PB, 10mM EDTA, 1 mM PMSF and 10 mM β-mercaptoethanol; pH 7.3) in cold room. Buffer was changed three times during 72 hrs.

Chromatography on DEAE-cellulose
Preswollen gel were packed in glass column (28 X 3.5 cm) and equilibrated with 3 CV of 1.5 mM PB (pH 7.3). The dialysed protein sample was filled in the column and kept for 4 hrs so that catalase molecules bind to the gel. After that, column was washed with 4-5 CV of starting buffer for complete removal of trace amount of

haemoglobin and other unwanted proteins. Catalase was eluted with increasing concentration (10-100 mM) of PB at pH 6.8 “Fig. (**1b**)”. Flow rate was maintained at 1.5-1.8 ml/min throughout the procedure.
Elutes were collected by fraction collector. Fraction size of elutes were 4 ml per test tube. The fractions having maximum catalase activity “Fig. (**4c**)” were pooled and subjected to 50 % $(NH_4)_2SO_4$ precipitation. Pellets were collected and kept for dialysis as described earlier. 1500 ml 1.5 mM PB (pH 6.5) alongwith 0.2 mM EDTA, 5 mM β-mercaptoethanol were used for dialysis.

Chromatography on CM-cellulose

Preswollen gel (CM-cellulose) were packed in glass column (35 x 2 cm) and equilibrated with 3 CV of 1.5 mM PB (pH 6.5). Flow rate was maintained at 0.5 ml/min. At pH 6.5, catalase was bound to CM-cellulose and rests of the proteins were washed out “Fig. (**2a**)”.
These washed out proteins were collected and concentrated by ultrafilteration. Catalase was eluted with increasing gradient of PB (pH 6.5) from 50 – 100 mM. Peaks were collected and purity of protein was analyzed on SDS-PAGE. Gel was stained by silver staining method [10].

Other procedures

If required, the eluted proteins can further be purified by size exclusion chromatography using Sepharose 6B [7] and be concentrated by MICROSEP centrifugal devices (from PALL Life Sciences, USA). Protein concentration was measured by Bradford’s method [11].
Catalase activity was estimated by Luck’s method [12]. 10µl of the sample was mixed with 990µl of 30mM H_2O_2 and the rate of decomposition of H_2O_2 was measured at 240nm. The corresponding running buffer was taken as blank and readings (if any) were subtracted. The assay was done in triplicate.
The enzyme units/ml was calculated using the definition “decomposition of 1µM H_2O_2 in 1 minute corresponds to one unit of catalase”. Specific activity (units/µg protein) was also calculated. For spot assay of catalase activity, 10 µL of sample was mixed with 50 µL of 6% H_2O_2 on glass slide. Immediate production of bubbles was indicative of catalase activity. The samples produced maximum bubbles were pooled.

Results

Catalase was purified from 80 ml blood. The short description of entire procedure is given in table-1. Table-2 shows the activity of catalase, concentration of protein and specific activity at different level of purification. Specific activity continuously increased after each purification step along with purity of catalase. Fig. (**1-2**) are the graphs recorded in Biologic LP. In stained SDS-PAG, number of protein bands was measure of purity of catalse shown in figure-3.

Discussion

Catalase has been purified from variety of sources and by various methods [13-16]. Many of the described methods are the procedure at a glance and without discussion on the troubleshooting. Overcoming the onsite problems is important to get success in any procedure. We described here our experiences in details with the modifications made during the purification of HEC.

Preparation of haemolysate

It is important to remove membrane lipids after RBC haemolysis to avoid interference in successive purification procedures. Goth [7] used 5 volumes water for haemolysis followed by centrifugation, whereas Ko et. al. [8] homogenised packed RBCs in cold toluene. Toluene dissolves membrane lipid, but it is required to be mixed in the ratio of 1:5 with RBC volume.
After centrifugation, toluene is separated out as supernatant. Sometime this procedure needs to be repeated to remove left out lipids. Removal of upper fat layer (in toluene) is cumbersome due to its thick greasy nature. If 3 volumes of water (or less) were used for haemolysate preparation, it requires centrifugation at 23,000g for 1 hr to precipitate RBC ghosts.In haemolysate, Catalase and high molecular weight proteins were co-precipitated by 50% $(NH_4)_2SO_4$. A large amount of haemoglobin [8] and other unwanted stroma proteins remained in solution.

Dialysis

Lipids and unwanted protein-free haemolysate is to be dialysed for 72 hrs. It is very important to maintain the temperature at 4-8°C. At room temperature catalase activity was found to be lost. Sometimes during or after dialysis brown coloured precipitate was encountered. The precipitate was soluble in 250 mM NaOH and it showed low catalase activity by spot test. Though the reason of precipitate formation was not known to us but it was not due to super saturation of proteins in dialysis buffer because it was not soluble in excess PB. It may be due to denaturation of some proteins [7]. The precipitate was filtered out.

DEAE cellulose

The presence of small particles in DEAE-cellulose “Fig. (**4a**)” reduces the flow rate near to zero. These small particles can be decanted during repeated washing of cellulose beads before packing into the column. During purification of HEC the color of DEAE column may turn to greenish after washing and eluting proteins “Fig. (**4b**)”. It may be due to impurities or adsorption of free iron on DEAE-cellulose [17].
It requires separate procedure to wash the column (described in column washing). A purified BLC was run through DEAE column following the same procedure. The recorded chromatogram “Fig. (**1a**)” provides information regarding efficiency of the method and pattern of catalase elution from DEAE cellulose. We observed that the BLC lost its most of the activity after run whereas activity of HEC remains as such. HEC and BLC have very close similarity in their structure [15] therefore the difference may be due to different functional characteristics of both catalase.

CM Cellulose

In DEAE-cellulose step, fraction size was 4 ml as the peaks were relatively broad. In this step peaks were very sharp therefore 2 ml elute per test tube was collected. More than 2 ml fraction size will cause unwanted dilution and abolition of nearby small peaks.Before elution of adsorbed proteins, column washing should be continued until constant UV-baseline was obtained “Fig. (**2a**)”. To elute catalase, a linear gradient of PB was used and it was prepared by gradient mixture, a part of Biologic LP. This can be observed and followed by upward movement of red line representing conductivity. The rate of change in gradient of PB needs to be controlled. If the gradient get changed at slow rate, protein will come out slowly thus generate broader peak. This will cause unnecessary dilution. A sudden change in gradient will produce sharp peak but other unwanted proteins can also be eluted simultaneously. The protein concentration (blue line) was detected using wavelength 280 nm “Fig. (**2b**)”. All chromatography exercises were performed at 20°C. During catalase elution from CM-cellulose, we obtained two peaks at different PB gradient “Fig. (**2b**)”. Both peaks generate single band on SDS-PAGE. It shows that catalase may have two domains on the surface with different affinity to bind with CM-cellulose. In both reference methods [7,8] catalase was eluted in two parts either with change in pH or buffer strength. In sample-V specific activity of catalase was 198.77 Units/µg of protein which is very close to 206.44 Units/µg of protein reported by Goth [7]. Concentrated washed out proteins “Fig. (**2a**)” produced multiple bands in SDS-PAGE “Fig. (**3**)” but the band corresponds to catalase was lacking. The washed out protein solution gave negative spot test for catalase.

Catalase activity

We did spot test to detect the presence of catalase activity in different elutes. It was assumed that haemoglobin did not interfere as the turn over number of catalase is comparatively very high. This assumption is supported by our previous finding where peroxidase (haem as part of enzyme) activity was reduced to zero in denatured form (haem only) [18].

The turn over number of catalase is much more than peroxidase so haem should not affect catalase activity. Fig. (**4c**) shows that the method can be used for quick assessment of catalase activity in elutes. During the entire procedure the pH of solutions remained in between 6.5-7.3 with PB. Catalase has broad range of pH and stable configuration [5,6]. Both of these properties made spot test successful.

The Ratio of A_{280} and A_{405} was used for this purpose by several workers [7,8]. Estimation of protein in sample-I (table-1) was done after adequate dilution as haemoglobin interfered in Bradford’s assay. That might be reason of more specific activity of sample-I compared to sample-II (table-2). Increase in specific activity is always better indicative of increased purity of enzyme as activity alone may alter at different dilutions. Each step of purification cause different dilution and spot test is a measure of only catalase activity, semiquantitatively. So, spot test should not be used for comparison of elutes from two different steps.

Column washing

After each run, DEAE cellulose column was regenerated as described [17]. In short, 1st and 2nd washing were with 2-3 CV of 2M NaCl followed by 1 CV of 1M NaOH. In 3rd washing 30mM EDTA with 0.2% Triton-x-100 was used. The column was then washed with a mixture of 1.5 mM PB and 20% ethanol to remove any residual Triton X-100. It was used to remove impurities and free iron bound to the beads. After this the column can be stored at 4°C until further use. As most of the proteins were removed in DEAE-cellulose step, CM-cellulose can be washed with 1M NaCl and was kept in refrigerator with 20% ethanol. Before use gel should be equilibrated with 3-4 CV running buffer until a constant UV baseline was obtained.

Gel staining and Image analysis

To check the purity of catalase, samples and elutes were collected during the procedure (table-1). Each sample (50 µg of protein) was mixed with equal amount of sample buffer (for SDS-PAGE) and after boiling was loaded on SDS-polyacrylamide gel. Protein bands were visualized by silver staining instead of CBB staining. Molecular weight of BLC is almost similar to HEC [15] so it was run as standard. Change in background and hue of the image help to visualize very faint bands which is useful for analysis and future experiment designing. Fig. (**3**) shows that the procedure was successful as sample-V,VI & VII produced single band after SDS-PAGE. Further purification increases specific activity by 1.6- 4.7% [7] so it was avoided. In ion exchange chromatography the binding of protein with DEAE- or CM-cellulose depend on charge present on protein molecule as well as the ionic strength of running buffer. Both variables are decided by pI of the protein which is 6.5 for native catalase enzyme [19]. Difference between pH of buffer from the pI increases the protein binding with oppositely charged gel beads due to increased surface charges. Increase in ionic strength of buffer will reduce the binding between protein and cellulose beads (DEAE- or CM-). Goth [7] in his method used the variation in pH for purification. He initially adjusted pH of haemolysate at 6.8 with 1.5 mM buffer. Catalase was adsorbed on DEAE sephadex and eluted by 60 mM buffer. The pH of this elute was then adjusted to 4.8 with 60 mM buffer and poured into CM-sephadex for binding.

It was further eluted with 60 mM buffer at different pH, 5.8 and 6.2. Ko et. al. [8] did not change pH that much. For DEAE cellulose, pH of sample was 7.2 with 10mM buffer. It was eluted with gradient NaCl (0-250mM). For cation exchange chromatography pH was adjusted to 6.5 with 10 mM buffer. Around 40 % of catalase was eluted as unbound protein and rest of catalase was eluted with buffer gradient (0-250mM). In our method we maintained the strength of running buffer at 1.5 mM and gradient (10-100mM) were used for elution of catalase. pH was adjusted to 7.3 and 6.5 in anion and cation exchange chromatography, respectively.

Conclusion

Modifications are mandatory part of work when problems are faced during the standardization of a method. We adopted some modification and developed different strategies from that of others. The purity and yield of HEC was good and it can be used for different experiments.

Acknowledgment

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Conflict of Interest

None

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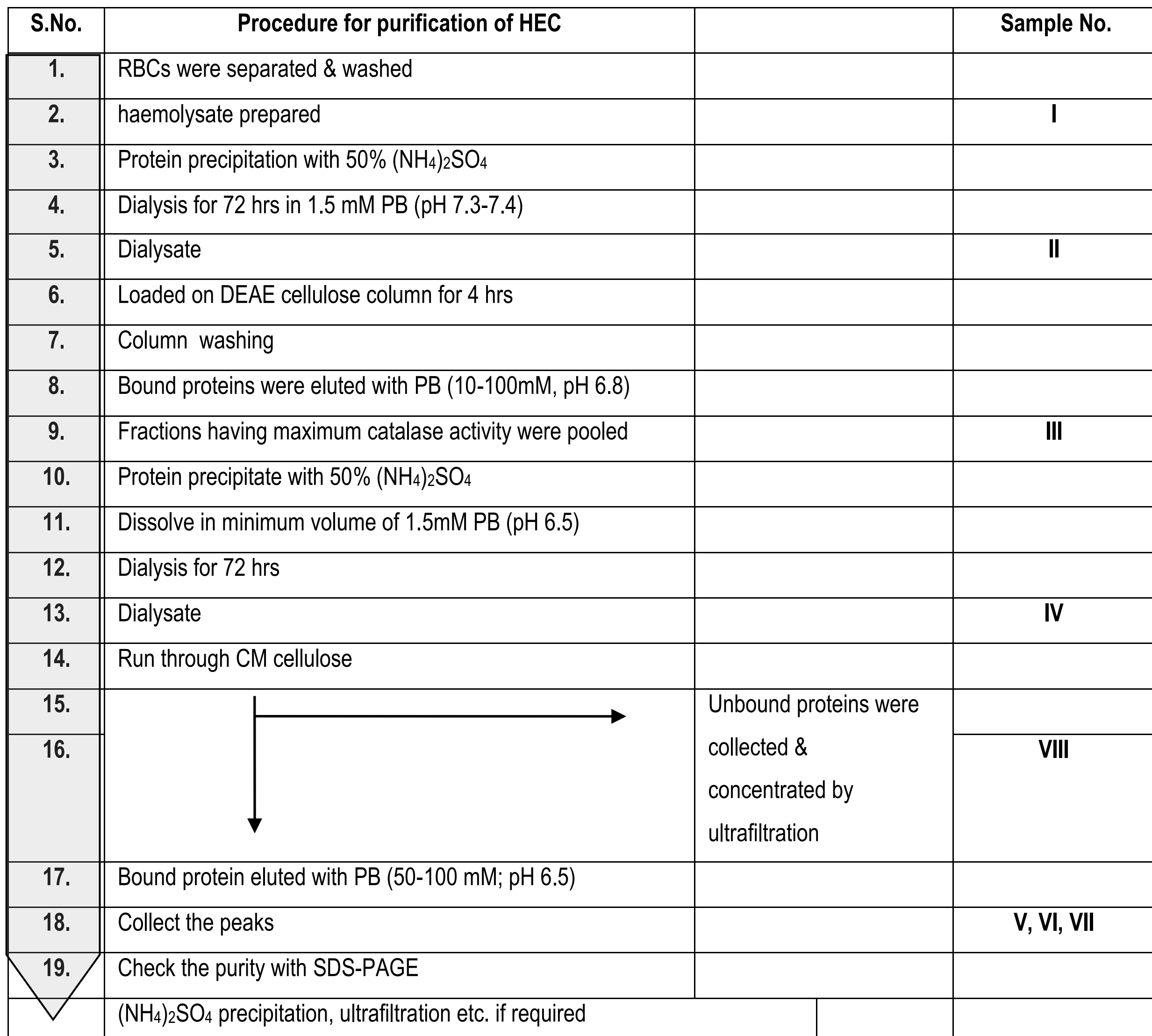
Table 1-Stepwise purification of HEC. The sample numbers (in roman numerals) represent aliquots from the corresponding steps that were preserved to evaluate specific activity of catalase.



S.No.	Procedure for purification of HEC		Sample No.
1.	RBCs were separated & washed		
2.	haemolysate prepared		I
3.	Protein precipitation with 50% $(NH_4)_2SO_4$		
4.	Dialysis for 72 hrs in 1.5 mM PB (pH 7.3-7.4)		
5.	Dialysate		II
6.	Loaded on DEAE cellulose column for 4 hrs		
7.	Column washing		
8.	Bound proteins were eluted with PB (10-100mM, pH 6.8)		
9.	Fractions having maximum catalase activity were pooled		III
10.	Protein precipitate with 50% $(NH_4)_2SO_4$		
11.	Dissolve in minimum volume of 1.5mM PB (pH 6.5)		
12.	Dialysis for 72 hrs		
13.	Dialysate		IV
14.	Run through CM cellulose		
15.		Unbound proteins were collected & concentrated by ultrafiltration	
16.			VIII
17.	Bound protein eluted with PB (50-100 mM; pH 6.5)		
18.	Collect the peaks		V, VI, VII
19.	Check the purity with SDS-PAGE		
	$(NH_4)_2SO_4$ precipitation, ultrafiltration etc. if required		

Table 2-Specific activity of HEC during various steps of purification. T_{10-12} are test tube number shown in figure-2b from which sample was taken to measure specific activity of catalase.

S.No.*	***Purification Steps***	***Protein (µg/ml)***	***Catalase (Units/ml)***	***Specific activity (Units/ µg protein)***
I.	*RBC Haemolysate*	*18432.0*	*93577.0*	*5.076*
II.	*Dialysate*	*1872.0*	*6422.0*	*3.43*
III.	*Pooled DEAE cellulose elutes*	*1960.0*	*33027.4*	*16.85*
IV.	*Dialysate*	*53.0*	*2752.0*	*51.929*
V.	*CM cellulose elute (T_{10})*	*60.0*	*11926.0*	*198.77*
VI.	*(T_{11})*	*111.0*	*1834.8*	*16.68*
VII.	*(T_{12})*	*110.0*	*1376.0*	*6.008*

* *Sample number shown in table-1*

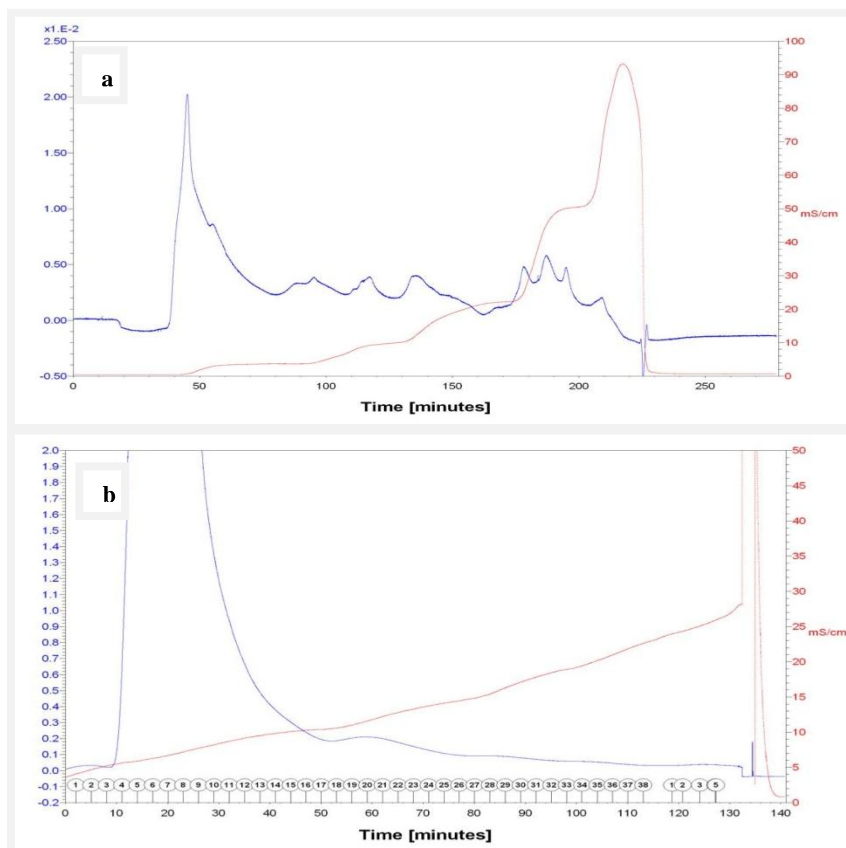


Fig. 1- Chromatogram of a) standard BLC and b) HEC eluted from DEAE-cellulose. Purified BLC (from sigma) or haemolysate (after step-5) was introduced into the column. Blue line represents the absorbance at λ_{280} and red line is for conductivity which increases with increasing molarities of PB. Fractions were collected in test tubes marked with numbers in circles (b). Fractions in set of 3 test tubes were pooled and catalase activity was tested by spot test.

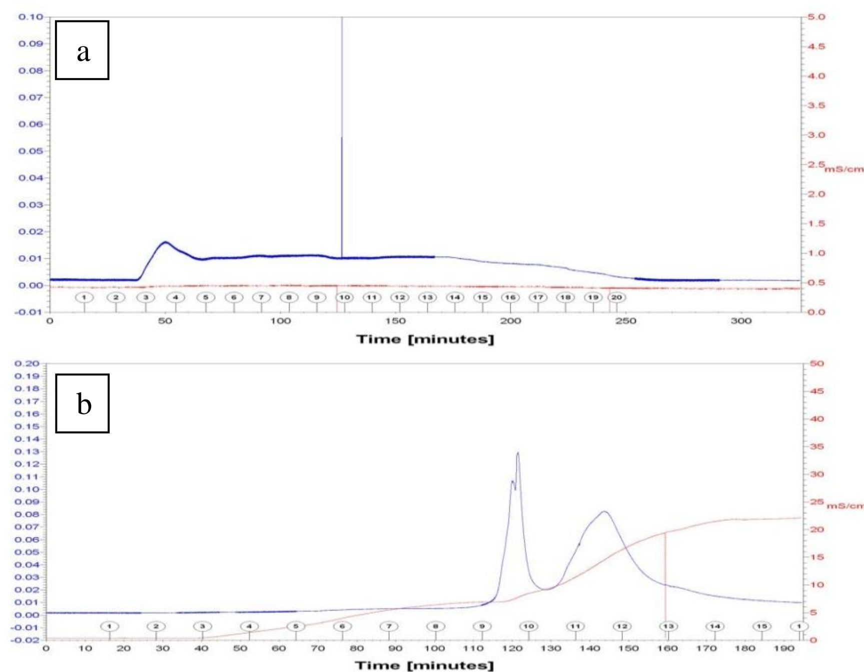
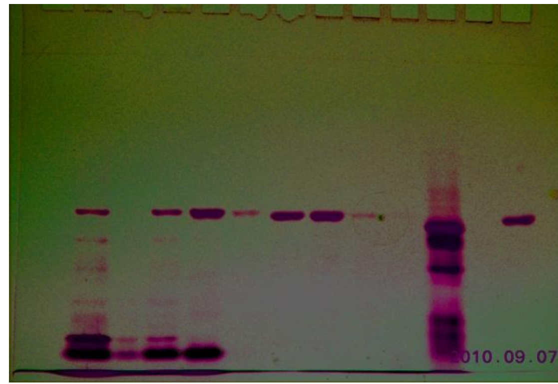


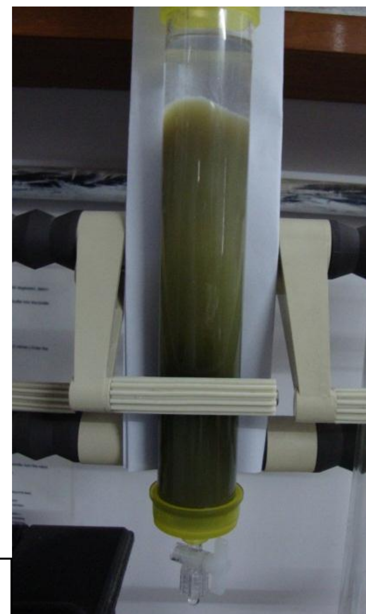
Fig. 2- a) Unwanted proteins came out during washing of CM-cellulose gel. These proteins in 51 ml elute were collected (T_{4-20}). It was concentrated and referred as sample-VIII. Conductivity (red line) remains constant as the molarity of PB was not changed during washing. b) HEC eluted from CM cellulose. 1st peak was collected in single test tube (T_{10}) where as second was in 3 test tubes (T_{11-13}).

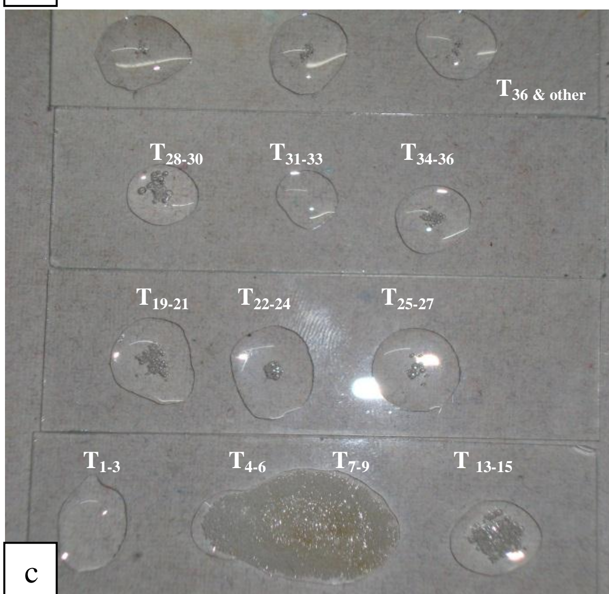


B I II III IV V VI VII T_{13} VIII B BLC

Fig. 3- SDS-PAGE (10% gel) after silver staining. Image is with modification in hue, contrast and background. 12 lanes from left to right represent sample no. collected during the purification of HEC. B-blank, BLC-bovine liver catalase as reference and T_{13} is sample from test tube-13 shown in figure-2b.

a





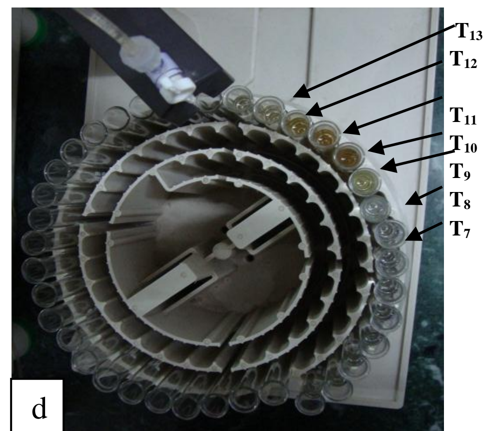


Fig. 4- Cellulose beads (a) should be free from debris. (b) DEAE cellulose gel turned green after RBC haemolysate run. (c) Evaluation of catalase activity by spot test. 'T' is test tube no. shown in figure-1b. Drop second and third were pooled due to very high bubbling. (d) Fraction collector collected elutes in step no.17. Colour of elute (T_{10-12}) was red-brown due to presence of haem containing protein 'catalase'.