Original Article

Biochemistry

Buffalo Kidney L-Gulonate Dehydrogenase: Isolation and Kinetic Characterization

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ABSTRACT [ENGLISH/ANGLAIS]

L-Gulonate dehydrogenase (L-GuDH) from buffalo kidney has been investigated with regard to the feasibility of purification and some kinetic properties. Salt fractionation with ammonium sulfate, SP-Sephadex chromatography, Hydroxyapatite chromatography, among the resins tested, have shown promise for enrichment of this enzyme. Pseudoaffinity chromatography on immobilized Cibacron Blue F3GA was not useful for enrichment of L-GuDH from buffalo kidney. L-Gulono-γ-lactone and L-Ascorbic acid were found to inhibit the enzyme activity. The physiological significance of these observations to diabetic nephropathy has been discussed.

Keywords: Buffalo kidney, Gulonate dehydrogenase, Gulono lactone

RÉSUMÉ [FRANÇAIS/FRENCH]

L-gulonate déshydrogénase (L-GuDH) à partir de rein de buffle a été étudiée en ce qui concerne la faisabilité de purification et de certaines propriétés cinétiques. Fractionnement de sel avec du sulfate d'ammonium, SP-Sephadex, Chromatographie d'hydroxyapatite, parmi les résines testées ont montré des résultats prometteurs pour l'enrichissement de cette enzyme. Pseudoaffinity chromatographie sur immobilisée F3GA Cibacron Blue n'était pas utile pour l'enrichissement de la L-GuDH de rein de buffle. L-gulono-γ-lactone et d'acide L-ascorbique ont été trouvés pour inhiber l'activité de l'enzyme. La signification physiologique de ces observations pour la néphropathie diabétique a été discutée.

Mots-clés: Rénale Buffalo, gulonate déshydrogénase, gulono lactone

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Accepted/Accepté: February, 2013

Full Citation: Sharma V, Hussain SS, Pooja, Sonia, Nirmala K, Muralihar K.. Buffalo Kidney L-Gulonate Dehydrogenase: isolation and kinetic characterization. World Journal Life Science and Medical Research. 2013;3(1):8-14.

INTRODUCTION

L-Gulonate dehydrogenase (EC 1.1.1.45) is a key enzyme of the pentose phosphate cycle. The substrate of this enzyme i.e. L-Gulonic acid occupies a unique position in a metabolic traffic junction. L-Gulonic acid hypothetically has equal choice to enter into either the pentose phosphate metabolic pathway to be metabolized into L-xylitol or get transformed to L-ascorbic acid. Ascorbic acid is considered one of the water-soluble vitamins required for the successful completion of the reproductive processes in majority of mammals. It is found stored in endocrine tissues like adrenals and in reproductive tissues [1] attesting to its possible metabolic role in gonadal physiology [2]. Biosynthesis of ascorbic acid seems to be directly related to the expression of the L- Gulonate dydrogenase. During starvation, the metabolite flux is found shifted towards L-xylulose formation, rather than ascorbic acid formation, owing to increase in gulonate dehydrogenase activity [3]. Hence to understand the physiological significance of this metabolite flux regulation, purification and characterization of GuDH from buffalo kidney has been attempted by investigating its interaction with different chromatographic matrices.

MATERIALS AND METHODS

L-Ascorbic acid, β-hydroxy butyrate and L-Gulonic acid-Y-lactone were purchased from Sigma Aldrich, USA. NAD^+ and NADH were procured from Spectrochem.

Preparation of L-Gulonic Acid

200 mM L-gulonic acid-Y-lactone was made in 50 mM phosphate buffer, pH 7.5. The pH of the solution was brought to 12.0 by adding 1M NaOH drop wise and left for one hour with constant stirring. Finally, the pH of the

solution was brought back to 7.5 by adding 1N HCl drop wise. This solution is used as the enzyme substrate in the enzyme assays.

Interaction of L-Gulonate Dehydrogenase from Buffalo Kidney with Various Matrices

Buffalo kidney was brought from the Gazipur abattoir (Delhi government unit near Noida) to our laboratory in an ice box. The adhering membranous covering was removed. The cortical portion was cut into small pieces and later homogenized in 50 mM PB, pH 7.5 containing 1 mM PMSF (Buffer A) using a kitchen blender. Finally the homogenate was centrifuged at 20,000 g for 30 min at 4°C. The clear supernatant was termed as "crude extract" which was ultimately subjected to ammonium sulfate precipitation (0-30% and 30-60% saturation). The pellets recovered from various steps were assessed for enzyme activity and for protein concentration. Semi-pure preparation was finally used for the investigation of the interaction of buffalo kidney L-Gulonate dehydrogenase with various types of chromatographic matrices including Hydroxyapatite, Blue-sepharose, Yellow-Sepharose, SP-Sepharose, CM-sepharose, DEAE-Sephadex, Phenyl-Sepharose, and IMAC matrices.

In dye affinity chromatography (Blue-Sepharose and Yellow-Sepharose), the protein solution was loaded on the Blue-Sepharose/Yellow-Sepharose at pH 5.0 and bound proteins were eluted with 50 mM citrate buffer, pH 5.0, 50 mM phosphate buffer,

pH 6.0, 50 mM phosphate buffer, pH 7.5 and 50 mM phosphate buffer, pH 7.5 containing 1 M NaCl. The same preparation was also loaded at pH 7.5 and bound proteins were eluted with 50 mM phosphate buffer, pH 7.5 and 50 mM phosphate buffer, pH 7.5 containing 1M NaCl.

Semi-pure preparation of the Gulonate dehydrogenase was also loaded on various ion exchangers. It was loaded onto various cation exchangers (SP-Sephadex/CM-cellulose) which were previously equilibrated with 50 mM citrate buffer (pH 5) having 50 mM NaCl. Bound proteins were eluted with 50 mM phosphate buffer, pH 6, 50 mM phosphate buffer, pH 7.5 and 50 mM phosphate buffer, pH 7.5 having 500 mM NaCl. Gulonate dehydrogenase was also loaded on anion exchanger like DEAE-Sephadex at pH 7.5, equilibrated with 50 mM phosphate buffer (pH 7.5) having 50 mM NaCl. The bound proteins were eluted with discontinuous gradient of salt (50 mM-1M NaCl). Hydroxyapatite chromatography was also used to purify Gulonate dehydrogenase from buffalo kidney. The enzyme extract was loaded at 20 mM phosphate buffer, pH 7.5 and bound proteins were eluted with increasing discontinuous concentration of phosphate buffer (20-200 mM).

Gulonate dehydrogenase was also loaded on Phenyl-sepharose at high salt concentration (50 mM phosphate buffer, pH 7.5, 1M NaCl) and later bound proteins were eluted with decreasing concentration of salt and ethylene glycol.

The semi-pure preparation was also loaded on immobilized metal affinity chromatography (IMAC) by using different metal ions namely nickel and cobalt. IDA-Sepharose matrices were recharged with 1M metal salt solutions ($NiCl_2.6H_20$; $CoCl_2.6H_20$). Columns were equilibrated with 20 mM phosphate buffer, pH 7.5; 0.5 M NaCl and enzyme preparation were loaded at the same pH on the column. After removing unbound fractions, bound proteins were eluted out by 300 mM imidazole.

Biochemical and Zymogram Assays of L-GuDH

Enzyme activity of Gulonate dehydrogenase was assayed by measuring the rate of change in NADH absorbance at 340 nm. Km and Vmax was calculated by keeping increasing Gulonate concentration between 1-40 mM [4].. Enzyme inhibition studies were also performed by incubating enzyme with increasing inhibitor concentration and Ki was calculated from the graph between K_M ^{apparent} Vs inhibitor concentration and from the graph between $K_M^{apparent}$ Vs inhibitor concentration. The in-gel enzyme activity staining method was also used to detect enzyme activity qualitatively [5]. Protein concentration was determined by Lowry's method using BSA as a standard [6].

RESULTS AND DISCUSSION

L-Gulonate dehydrogenase (L-GuDH) is a physiologically important enzyme catalyzing the NAD^+-linked dehydrogenation of L-Gulonate into dehydro-L-Gulonate in the uronate cycle [7]. Regarding this enzyme, particular areas of interest include details of substrate catalysis, active site modification, substrate-binding-site specificity, kinetics, and stability. To get more understanding of its structural features, interaction behavior of buffalo kidney L-Gulonate dehydrogenase with different chromatographic matrices have been attempted. This can lead to the formulation of a purification procedure for the Gulonate dehydrogenase.

Buffalo kidney L-Gulonate dehydrogenase was subjected to ammonium sulfate precipitation and was found to concentrate mostly in the 30%-60% ammonium sulphate

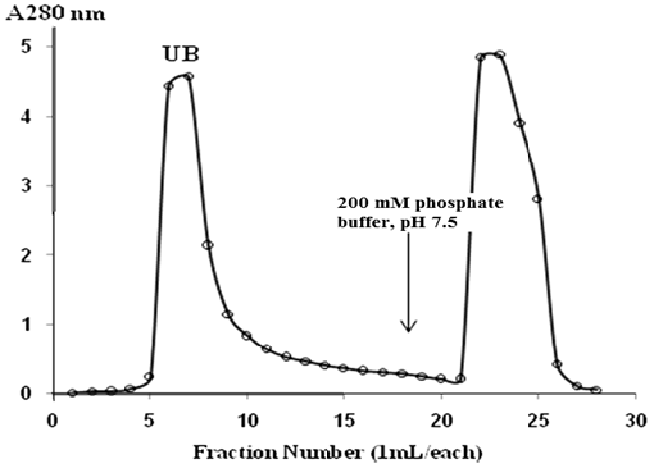
pellet (>82% of the starting enzyme units) fraction. Different fractions were assayed for the enzyme activity by using classical spectroscopic dehydrogenase assay. Specific activity of the final preparation was found to be 0.002051 U/mg with 82.71% yield. Gulonate dehydrogenase has been found to be purified by 3.53 fold in comparison to crude extract, while very little enzyme activity has been observed in other side fractions of salt fractionation step (Table 1). One unit of the enzyme was defined as the amount of the enzyme required to reduce 1µmol of NAD.

Hydroxyapatite chromatography was found to concentrate enzyme activity in unbound fractions and bound fractions were found to be devoid of any enzyme activity (Fig 1). Buffalo kidney extract has been loaded on to SP-Sephadex and Gulonate dehydrogenase activity has been found to be solely restricted to unbound fractions (Fig 2 and Table 2). Interestingly Gulonate dehydrogenase has been found to bind CM- Sephadex and elute at pH 7.5 (Fig 3). Presence of the enzyme activity in unbound fractions can be ascribed as overloading of the protein on the column (Table 3).

Table 1: This table shows summary of the partial purification of the Gulonate dehydrogenase from buffalo kidney

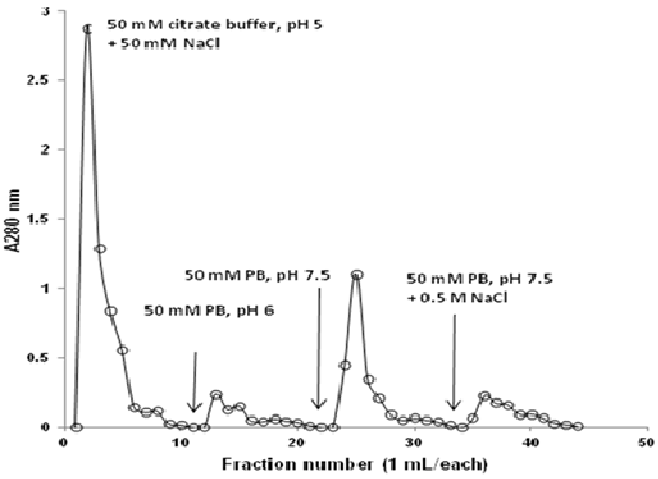
S no.	Steps	Total Volume (mL)	Total Protein (mg)	Total Activity (µmoles/min)	Specific activity (µmoles/min/mg)	Purification Fold	Yield %
1	Crude	100	9000	7.0425	0.000783	1	100
2	30 P	3.4	214.2	0.1245	0.000581	0.742789	1.77
3	60 P	40	2840	5.825	0.002051	3.528805	82.71
4	60 S	6.8	17	0.000	0	0	0

Figure 1: This figure shows hydroxyapatite chromatography elution profile of buffalo kidney extract for the interaction studies of buffalo kidney L-Gulonate dehydrogenase with hydroxyapatite



Hydroxyapatite chromatography has been reported as a purification step for the hog kidney L-Gulonate dehydrogenase (K. Muralidhar- personal communication). When kidney extract was subjected to anion exchanger (DEAE-Sephadex), bound Gulonate dehydrogenase was found to be eluted in two fractions, mostly with buffer 2 (50 mM PB, pH 7.5, 100 mM NaCl), while another isoform eluted later with buffer 3 (50 mM PB, pH 7.5, 200 mM NaCl). No enzyme activity appeared in the fractions eluted with high salt concentration suggesting presence of the less positive charge on Gulonate dehydrogenase at pH 7.5. (Fig 4; Table 4).

Figure 2: This figure shows SP-Sephadex chromatography elution profile of buffalo extracts for the interaction studies of buffalo kidney L-Gulonate dehydrogenase with SP-Sephadex.



In dye affinity chromatography, protein binding was found to be dependent on the initial loading pH. At pH 5.0 Gulonate dehydrogenase seems to bind the Blue-Sepharose, while at pH 7.5 almost nothing bound (data not shown). Bound Gulonate dehydrogenase eluted with

phosphate buffer pH 7.5 but there was not much increase in specific activity.. Interestingly contrast observations were reported with regards to binding behavior of L-Gulonate dehydrogenase towards Blue-Sepharose [7]. Ionic interaction seems to dominate these binding interactions. Similar interaction behavior has been observed when Gulonate dehydrogenase was subjected to reactive Yellow-sepharose (data not shown).

Table 2: This table shows summary of the purification profile of the Gulonate dehydrogenase from buffalo kidney by using SP-Sephadex Buffer 1: 50 mM citrate buffer pH 5 + 50 mM NaCl ; Buffer 2: 50 mM phosphate buffer pH 6; Buffer 3: 50 mM phosphate buffer pH 7.5 and Buffer 4: 50 mM phosphate buffer pH 7.5 + 500 mM NaCl

S no.	Steps	Volume (ml)	Total Protein (mg)	Total Activity (umoles/min)	Specific activity (umoles/min/mg)	Purification fold
1	Crude	2	180	0.05634	0.000313	1
2	Buffer 1 (F1)	2	4.32	0.007918	0.001833	5.855821
3	Buffer 1 (F2)	2	3	0.009084	0.003028	9.673697
4	Buffer 2 (F1)	2	0.3	0	0	0
5	Buffer 3 (F2)	2	3	0.00004	0.000013	0.041534
6	Buffer 4 (F2)	2	0.3	0	0	0

Table 3: This table shows summary of the purification profile of the Gulonate dehydrogenase from buffalo kidney by using CM-Sephadex. (Buffers 1-4 mean the same as in Table 2).

S/ No	Sample	Volume (ml)	Total Protein (mg)	Total Activity (umoles/min)	Specific activity (umoles/min/mg)	Purification fold
1	Crude	2	180	0.05634	0.000313	1
2	Buffer 1 (F1)	2	1.66	0.002532	0.001525	4.873464
3	Buffer 1 (F2)	2	0.66	0.001206	0.001827	5.836905
4	Buffer 2 (F1)	2	0.3	0.00004	0.000133	0.425985
5	Buffer 3 (F1)	2	0.5	0.000924	0.001849	5.906948
6	Buffer 3 (F2)	2	4.32	0.001286	0.000298	0.951199
7	Buffer 4 (F2)	2	0.44	0.00008	0.000182	0.580889

Table 4: This table shows summary of the purification profile of the Gulonate dehydrogenase from buffalo kidney by using DEAE-Sephadex. (Buffers mean the same as in Table 2).

S/ No	Sample	Volume (ml)	Total Protein (mg)	Total Activity (umoles/min)	Specific activity (umoles/min/mg)	Purification fold
1	Crude	2	180	0.05634	0.000313	1
2	Buffer 1 (F1)	2	11.2	0.000563	0.00005	0.159744
3	Buffer 1 (F2)	2	9	0.002814	0.000312612	0.998759
4	Buffer 1 (F3)	2	5	0.003296	0.000659164	2.105955
5	Buffer 2 (F3)	2	0.54	0.003095	0.005731214	18.31059
6	Buffer 2 (F4)	2	0.76	0.002371	0.00312024	9.968819
7	Buffer 3 (F3)	2	0.9	0.000884	0.000982494	3.138958
8	Buffer 3 (F4)	2	0.76	0.000603	0.000793281	2.534445
9	Buffer 4 (F2)	2	1	0	0	0
10	Buffer 5 (F2)	2	0.44	0	0	0

Semi-pure preparation was loaded on immobilized metal affinity chromatography (IMAC) by using different metal ions namely nickel and cobalt. Both metal ions were found to bind protein significantly, while significant enzyme activity eluted in unbound fractions. Nickel-Sepharose was shown to bind certain proteins (Fig 4).

Figure 3: This figure shows CM-Sephadex chromatography elution profile of buffalo kidney extract for the interaction studies of buffalo kidney L-Gulonate dehydrogenase with CM-Sephadex.

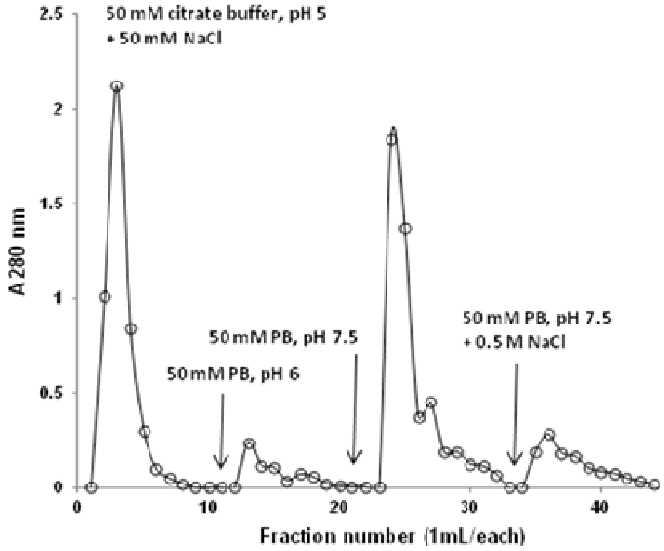
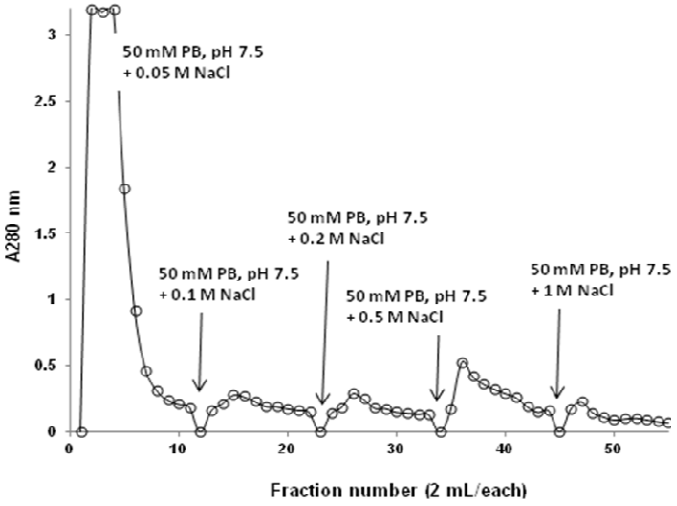


Figure 4: This figure shows DEAE-Sephadex chromatography elution profile of buffalo extracts for the interaction studies of buffalo kidney L-Gulonate dehydrogenase with DEAE-Sephadex.



As per zymogram studies most of the enzyme activity was retained in the unbound fractions while some enzyme activity was also retained in bound fractions. 36.62% of the total proteins were found to interact with Ni-IDA sepharose (9.4 X 10^{18} molecules Ni2+/mL matrix). Cobalt-Sepharose was shown to interact differently in comparison to nickel. As per protein absorbance whole protein came out as unbound fractions (data not shown).

Figure 5: This figure shows Nickel-IDA Sepharose chromatography elution profile of buffalo kidney extract for the interaction studies of buffalo kidney L-Gulonate dehydrogenase with Ni-IDA sepharose.

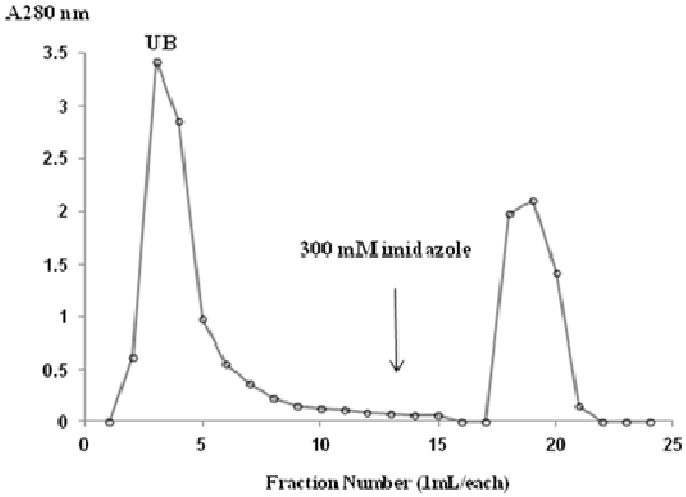
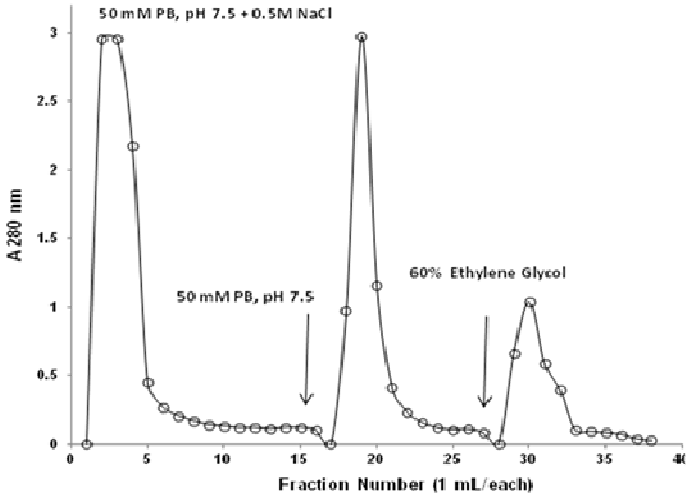


Figure 6: This figure shows Phenyl-Sepharose chromatography elution profile of buffalo kidney extracts for the interaction studies of buffalo kidney L-Gulonate dehydrogenase with Phenyl-Sepharose



The cobalt ions bound to IDA matrix were found to be 5.4 X 10^{19} Co2+ ions/ mL of matrix. Absence of any enzyme activity in bound fraction could be explained in terms of

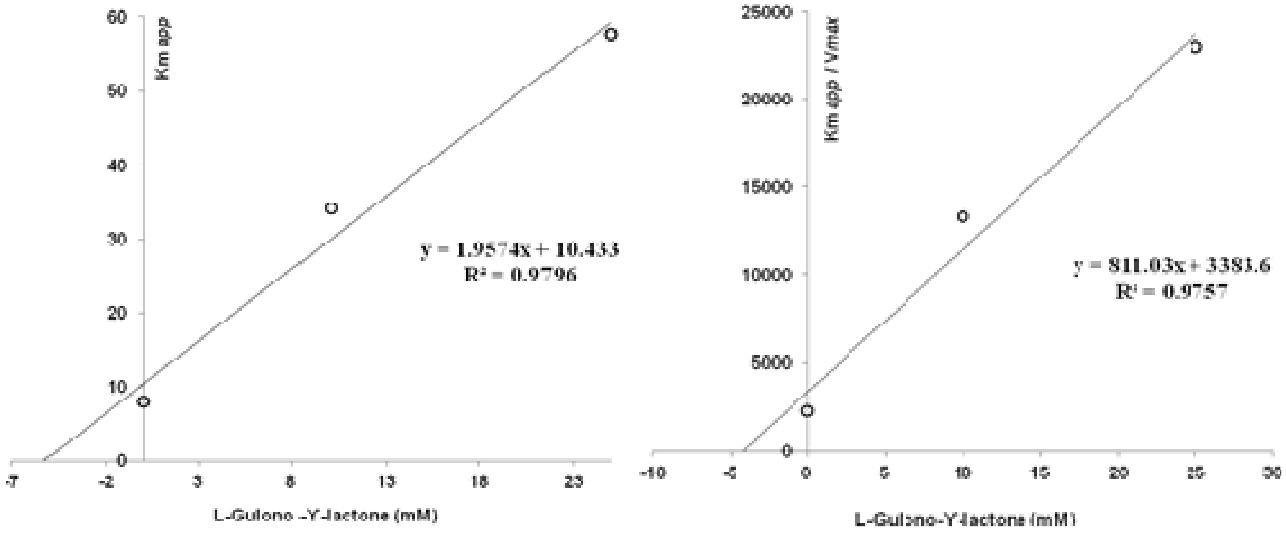
loss of enzyme activity after the interaction of metals with this enzyme as many ions have been shown to affect the enzyme activity of L-GuDH previously [7]. Phenyl Sepharose is a separation medium for hydrophobic interaction chromatography (HIC). Substances are separated on the basis of their different hydrophobicity. Gulonate dehydrogenase has been found to bind Phenyl-Sepharose at high salt concentration and two isoforms were eluted with different eluting buffers. One activity peak eluted with phosphate buffer without any salt while another highly hydrophobic has been eluted with ethylene glycol (Fig 6 and Table 5).

Different biomolecules with similar structure to L-gulonic acid can hypothetically be inhibitors of this enzyme. Metabolic control on the Gulonate dehydrogenase enzyme expression *in vivo* can be achieved by ascorbic acid or L-gulono-Y-lactone. The kinetic parameter like Km was found to be 2 to 7 mM by different reciprocal plots (Table 6). L-Ascorbic acid and L-Gulono–γ-lactone were tested as potential inhibitors. Indeed results indicated that both could inhibit the enzyme activity in a competitive mode.

Table 5: This table shows summary of the purification profile of the Gulonate dehydrogenase from buffalo kidney by using Phenyl-Sepharose.

S/ No	Steps	Volume (ml)	Total Protein (mg)	Total Activity (umoles/min)	Specific Activity (umoles/min/mg)	Purification Fold
1	Crude	2	180	0.05634	0.000313	1
2	Buffer 1 (F1)	2	0.66	0.000121	0.000183	0.58369
3	Buffer 1 (F2)	2	0.16	0	0	0
4	Buffer 2 (F2)	2	0.66	0.017524	0.026552	84.82968
5	Buffer 3 (F2)	2	3.46	0.0041	0.001185	3.785553

Figure 7: This figure shows kinetics of GuDH inhibition by L-gulono-γ-lactone. GuDH was incubated with increasing concentration of gulonic acid at different concentration of inhibitor. Secondary replots of the double reciprocal plots, i.e., KM (B) and slope(C) Vs inhibitor concentrations.



Experimentally determined Ki values obtained from reciprocal plots were found to be 5.332 mM and 4.17 mM for the L- gulono-γ-lactone (Fig 7). The values were 2.83 mM and 6.92 mM for ascorbic acid (data not shown). In summary, purification of Gulonate dehydrogenase can be achieved by coupling salt fractionation, Hydroxyapatite and Phenyl-Sepharose chromatography. Dye affinity chromatography has been not useful for the purification of this enzyme. Metal ions were shown to interact differently with the proteins present in the semi-pure preparation. Many of the matrices have shown the potential of using them as a purification and

characterization step of L-Gulonate dehydrogenase. Inhibition studies of Gulonate dehydrogenase by ascorbic acid and L-gulono-γ-lactone shed light on the possible regulatory mechanism of Gulonate dehydrogenase activity under *in vivo* conditions.

Table 8: This table shows kinetic parameters of Gulonate dehydrogenase deduced from differen t reciprocal plots.

S/No	Kinetic plots	K_M (mM)	V_{max} (µmole NADH/min/mg)
1	*Michaelis–Menten*	3.687	0.002484
2	*Lineweaver-Burk*	7.987	0.003511
3	*Eadie-Hofstee*	7.4392	0.0034
4	*Hanes-Woolf*	2.202	0.002179

CONCLUSION

It is concluded that different chromatography resins were tried to know whether L-GuDH from buffalo kidney could be enriched in specific activity. Hydroxyapatite and Phenyl-Sepharose were useful among these resins. Immobilized textile dye (i.e. Cibacron Blue F3GA) was not found useful for this task.

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ACKNOWLEDGEMENT / SOURCE(S) OF SUPPORT

Nil

CONFLICT OF INTEREST

Nil