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UDP-glucose dehydrogenase from *Capra hircus* liver: Purification, partial characterization and evaluation as a coupling enzyme in UDP-galactose 4-epimerase assay

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ABSTRACT

UDP-glucose dehydrogenase from *Capra hircus* has been purified to homogeneity by salt fractionations, heat treatment and chromatographic steps. It is a homohexamer of about 300 kDa. Though the basic physical and enzymatic properties of the caprine enzyme are comparable to those of the beef liver enzyme, it has lower energy of activation and different entropy and enthalpy for the transition state during catalysis. The caprine enzyme can act suitably as an auxiliary enzyme in the coupled assay system for UDP-galactose 4-epimerase.

Enzymes: UDP-Glc DH, UDP-glucose dehydrogenase (EC 1.1.1.22); Epimerase, UDP-galactose 4-epimerase (EC 5.1.3.2).

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1. Introduction

UDP-glucose dehydrogenase (UDP-Glc DH) oxidizes UDP-glucose (UDP-Glc) to UDP-glucuronic acid (UDP-GA) with the concomitant reduction of two molecules of NAD^+ [1]. The reaction involves two steps; first, reversible formation of an intermediate UDP-α-D-gluco-hexodialdose that remains tightly bound to the enzyme while in the second step, it is irreversibly converted to UDP-GA (Scheme 1) [2]. This enzyme was initially isolated from guinea pig liver [3] and pea seedlings [4]. Since then, it has been isolated ranging from virus [5] to human [6] including bacteria [7] and yeast [8], and they are all well characterized. Even, the X-ray crystal structures of the bacterial [9] and human [10] enzymes have been solved. This enzyme produces UDP-GA, which is an essential precursor for the synthesis of connective tissue glycosaminoglycans in animals [11]. Further, in animals, UDP-GA helps in the solubilization and consequent excretion and detoxification of harmful xenobiotics [12,13]. Thus UDP-Glc DH plays a vital role in the phase II reactions of xenobiotics metabolism occurring primarily in liver [13]. In plants, UDP-GA is the donor of D-glucuronosyl units for the synthesis of several types of structural polysaccharides, *e.g.*, primary cell-wall matrix of higher plants and is the precursor of other nucleotide sugars necessary for the synthesis of pectins and hemicelluloses [14,15]. The specific activity of the enzyme from different sources varies depending on its multimericity [16]. UDP-Glc DH has been hypothesized to be a rate-limiting enzyme, controlling part of the polysaccharide biosynthesis in plants and animals [17–20]. The normal functions of UDP-Glc DH have been studied so far in various organisms. In *Drosophila* the enzyme controls wing formation [21], while in *Caenorhabditis elegans* it helps in embryonic development [22]. Human and mouse proteins are similar and produce hyaluronan at specific developmental stages [23]. Elevated production of hyaluronan has been extensively implicated in the development of epithelial cancers [24,25]. UDP-GA is the precursor of hyaluronan and by restriction of precursor availability by inhibition of UDP-Glc DH may regulate development of tumors [26].

UDP-Glc DH is a conserved homohexameric protein of ~300 kDa in most eukaryotes [27]. However, rat and *Cryptococcus* enzymes are tetramers and dimers, respectively [28,29]. The functional enzyme unit is a dimer irrespective of multimericity, and at saturation, one substrate molecule binds to one dimer [27]. Therefore, the eukaryotic enzyme is inactive in the monomeric state [16]. Though conserved, the total number of amino acids and the sequence of the enzyme vary from one species to another [30]. Usually little differences in the primary sequence, secondary and tertiary structure, or multimericity do not contribute significant variation of stability and other physico-chemical properties of the enzymes

Abbreviations: GG, glycylglycine; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; UDP-Xyl, UDP-xylose; UDP-Man, UDP-mannose; UDP-GA, UDP-glucuronic acid; D(+)-Gal, D(+)-galactose; D(+)-glc, D(+)-glucose.

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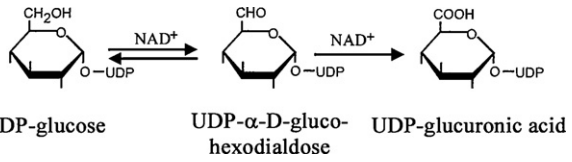


Scheme 1. Mechanism of action of UDP-glucose dehydrogenase.

that are conserved. Preference of substrate or inhibitor of the enzymes also remains unaltered. But properties like kinetic and thermodynamic parameters can differ considerably even in very closely related species. Therefore, comparative studies on the same enzyme from different sources are ongoing practice. UDP-galactose 4-epimerase (epimerase), which reversibly converts UDP-Gal to UDP-Glc, is closely related to UDP-Glc DH in the metabolic pathway [31]. These two enzymes together maintain the pool of these sugar derivatives *in vivo*. Since both UDP-Gal and UDP-Glc are devoid of chromophoric groups, it is difficult to follow their inter-conversion by epimerase. UDP-Glc DH from beef liver is conventionally used as an auxiliary (coupling) enzyme to quantify oxidation of UDP-Glc formed from UDP-Gal by epimerase (Scheme 2) [32–34]. The oxidation reaction being NAD^+ dependent, it can be followed spectrophotometrically at 340 nm [35].

In Indian subcontinent beef is not raised in farms and only the condemned animals are being slaughtered. Further the slaughterhouses being limited in numbers are distantly located leading to a difficulty in collection of beef liver immediately after slaughtering of the animals. Thus, the quality of the bovine liver as a source for UDP-Glc DH always remains questionable. Further, often the yield of the enzyme is very low. Combination of these makes beef liver as an undependable source for enzyme purification. Thus an alternative source like caprine liver is required for isolation of UDP-Glc DH to be used in epimerase coupled assay in the laboratories like ours. Since young goats are easily available in local markets, it is easy to collect the liver fresh immediately after the animals are slaughtered. The enzyme purified from caprine liver is better in respect of both quality and quantity than the bovine enzyme in our country. Thus, caprine liver serves as a reliable source of UDP-Glc DH. Here we report purification of caprine liver UDP-Glc DH and compare its properties with the bovine enzyme as reference. We also verify suitability of the caprine UDP-Glc DH as an auxiliary enzyme for the conventional epimerase coupled assay system.

2. Experimental

2.1. Reagents

GG, β-NAD^+, $NADP^+$, NADH, UDP-Gal, UDP-Glc, UDP-Xyl, UDP-Man, UDP-GA, 5′-UMP, UDP, UTP, D(+)-Gal, D(+)-Glc, Sephadex G-200, CNBr-activated Sepharose, CM-sephadex C-25, UDP-Glc DH (bovine liver) and M_w-markers were from Sigma, USA. Blue Dextran-Sepharose for affinity chromatography was prepared in the laboratory by coupling CNBr-activated Sepharose beads with Blue Dextran [36]. Yeast strain *Kluyveromyces fragilis* (ATCC No. 10022) was purchased from Microbial Type Collection Center and Gene Bank, IMTECH, Chandigarh, India.

2.2. Purification of UDP-Glc DH from caprine liver

The initial steps were similar to that of the beef liver enzyme with some modifications [37]. All steps were carried out at 4 °C. Caprine liver (150 g) stored at −20 °C was freeze-thawed and minced; adhering fat and connective tissues were discarded and homogenized with 150 ml of 10 mM Na-acetate, pH 5.4, containing 2 mM EDTA and 10 mM β-mercaptoethanol (extraction buffer) for 2 min. The homogenate was stirred gently for 5 min and the pH was adjusted to 4.9 with ice-cold 0.1N acetic acid. After centrifugation at 7000 rpm for 30 min, the supernatant was filtered through absorbent cotton (previously washed with the extraction buffer) to remove fat. It was subjected to 30% ammonium sulfate saturation. The centrifugation steps thereafter were carried on at 12,000 rpm for 30 min. The pellet was discarded and the supernatant was further fractionated by 55% ammonium sulfate saturation. The precipitate was dissolved in minimum volume of extraction buffer to yield a solution of 25–30 mg/ml of protein. The solution was adjusted to pH 4.9 and quickly immersed into a water bath maintained at 80 °C with constant stirring using a stainless steel beaker. After reaching 52 °C, heating was continued for 1 min whereby it reached 55 °C, and then rapidly cooled to 0 °C in freezing mixture with stirring. The precipitate was removed by centrifugation and the supernatant was subjected to 50% ammonium sulfate fractionation. The pellet was re-dissolved in minimum volume of extraction buffer and dialyzed overnight against the same.

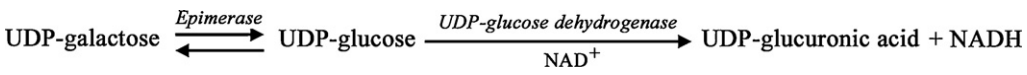
The dialyzed sample was applied to a CM-Sephadex C-25 column (6 cm × 0.5 cm) equilibrated with extraction buffer at a flow rate of 5 ml/min. After washing, UDP-Glc DH activity was eluted with a linear gradient of the buffer containing 0–0.5 M NaCl. Active fractions were pooled and dialyzed against extraction buffer before application to Blue Dextran-Sepharose column (2 cm × 0.5 cm) equilibrated with the same buffer. After washing, bound fractions were eluted with the buffer containing 0.5 M NaCl. Active fractions were pooled, dialyzed and concentrated by lyophilization. The sample (1-ml) was applied to a precalibrated Sephadex G-200 column (0.8 cm × 150 cm) equilibrated with extraction buffer at a flow rate of 12 ml/h. Active fractions were pooled and the homogeneity of the preparation was verified by 10% SDS-PAGE after silver-staining. The enzyme was finally lyophilized and stored in 10 mM Na-acetate, pH 5.4 at −20 °C.

2.3. Purification of epimerase

Growth of yeast cells, extraction and purification of epimerase have been described in details earlier [38,39]. Purification of epimerase involved crude cell extraction, 55% ammonium sulfate fractionation, hydroxyapatite treatment and DEAE-cellulose chromatography. Homogeneity of the preparation was verified by PAGE and SDS-PAGE. Specific activity of the enzyme was 65–75 units/mg. This enzyme preparation showed kinetic lag during the conversion of UDP-Gal because of its association with one molecule of 5′-UMP, an inhibitor of epimerase [40].

2.4. Enzyme assays

UDP-Glc DH activity was measured in 1 ml of reaction mixture that contains 100 mM GG, pH 8.8, 0.5 mM NAD^+ and 0.1 mM UDP-Glc at 25 °C and continuously monitoring increase of A_{340}. The assay was initiated by the addition of 0.001–0.01 units of the enzyme.



Scheme 2. Epimerase coupled assay with UDP-Glc DH.

Under these conditions, initial reaction rates were linear for at least 10 min. Activity was calculated on the basis that 2 moles of NADH were produced per mole of UDP-Glc oxidation. Dependence of reaction velocity on substrate concentration was determined by increasing UDP-Glc concentration from 0 to 0.25 mM in presence of 0.5 mM NAD^+. Similarly, dependence of reaction kinetics on co-substrate was determined by increasing the concentration of NAD^+ from 0 to 0.3 mM and holding the UDP-Glc concentration constant at 0.1 mM.

Epimerase activity was measured under conditions as stated above with 0.1 mM UDP-Gal as substrate and 0.01 unit of UDP-Glc DH in presence of 0.4 mM NAD^+ (Scheme 2). The assay mixture was preincubated for 5 min to oxidize any contaminating UDP-Glc in UDP-Gal by UDP-Glc DH and the epimerization reaction was initiated by the addition of 0.001–0.01 units of epimerase. Under these conditions, initial reaction rates were linear for at least 10 min. In absence of the preincubation, an initial burst phase may occur [40].

2.5. Thermodynamic properties

Reaction rates of UDP-Glc DH under assay conditions were measured within 20–38 °C at an interval of 2 °C. The energy of activation (E_a) was calculated from the Arrhenius equation,

$$\ln(k) = -\frac{E_a}{R}\left(\frac{1}{T}\right) + \ln(A) \quad (1)$$

where, 'k' is the rate constant, 'R' is gas constant, 'T' is absolute temperature in K and 'A' is pre-exponential or frequency factor. Other related thermodynamic parameters were calculated from Eyring equation, the linear form of which is,

$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H^{\ddagger}}{R}\left(\frac{1}{T}\right) + \ln\left(\frac{k_B}{h}\right)\frac{\Delta S^{\ddagger}}{R} \quad (2)$$

where, 'k' is the rate constant, '$\Delta H^{\ddagger}$' is enthalpy change for activation, 'k_B' is Boltzmann constant, 'h' is Planck's constant and '$\Delta S^{\ddagger}$' is entropy change for activation. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of activation can be derived from the slope and intercept of the linear plot of $\ln(k/T)$ versus $1/T$. The Gibbs free energy change ($\Delta G^{\ddagger}$) for catalysis can be calculated at any constant temperature as follows:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (3)$$

2.6. Lag period for epimerase coupled assay

In a coupled enzyme assay of the type: $A \xrightarrow{E_1} B \xrightarrow{E_2} C$, conversion of $A \rightarrow B$ by the primary enzyme E_1 is followed from conversion of $B \rightarrow C$ by the auxiliary (coupling) enzyme E_2. Thus, initiation of the reaction occurs under a condition where the substrate concentration of the coupling enzyme E_2 is zero. This leads to an obligatory kinetic lag. A number of approaches describing ways to ensure valid coupled assays and to calculate the amount of coupling enzymes required for a theoretically correct assay are known [33]. Duration of the lag can be conveniently calculated using McClure's relation [41]:

$$t = \frac{-2.303[\log(1 - F_p)]K_M}{V_2} \quad (4)$$

where, 't' is the lag period by which the rate of substrate conversion by E_2 becomes identical to the rate of any desired fraction of E_1, 'F_p' is the desired fraction of the steady-state reaction of E_1 to be measured where a value of 0.99 is reasonably acceptable, K_M and V_2 are the Michaelis–Menten constant and maximum velocity of E_2, respectively.

Alternately the lag (t) can be calculated from relation proposed by Storer and Cornish-Bowden [32]:

$$t = \frac{\Phi K_M}{v_1} \quad (5)$$

where 'Φ' is defined as:

$$\Phi = \frac{V_2 v_1}{(V_2 - v_1)2}\ln\left[\frac{v_1(V_2 - v_2)}{V_2(v_1 - v_2)}\right] - \frac{v_1 v_2}{(V_2 - v_2)(V_2 - v_1)} \quad (6)$$

'Φ' is a dimensionless value and a function of 'v_2/v_1' and 'v_1/V_2', where 'v_1', 'v_2' and 'V_2' stand for velocity of first and second reactions and maximum velocity of second reaction, respectively. The values can be calculated from a Table provided by Storer and Cornish-Bowden [32]. The desirable value of v_2/v_1 should be at least 0.99 in such assays and amount of E_2 required should be estimated for this.

2.7. Other methods

Optical measurements were done by a Specord 200 (Analytic Jena, Germany) recording spectrophotometer connected to a water-bath (Polyscience, USA). Proteins were quantified with Bio-Rad Protein Assay Reagent (Bradford) using BSA as Ref. [42]. Centrifugation steps were carried out using a refrigerated table-top centrifuge (Eppendorf, Model No. 5810 R). The following buffers of 0.5 M were used: Glycine–HCl, pH 2.2; Na-acetate, pH 3.6–6.0; Na-phosphate, pH 6.5–8.0; Tris–HCl, pH 8.4–9.0 and Na-carbonate, pH 9.2–10.7. The Sephadex G-200 column was calibrated using Blue Dextran (void volume); β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (67 kDa) and ovalbumin (45 kDa). Linear dependencies of $\log M_w$ *versus* V_e/V_0 was observed where V_e and V_0 indicate the elution volume and the void volume, respectively. The following extinction coefficient values were used: NADH, $\varepsilon_{340\,nm} = 6.3 \times 10^3\ M^{-1}\ cm^{-1}$; NAD, $\varepsilon_{260\,nm} = 17.8 \times 10^3\ M^{-1}\ cm^{-1}$, uridine nucleotides and its derivatives, $\varepsilon_{260\,nm} = 10 \times 10^3\ M^{-1}\ cm^{-1}$. All data were plotted and analyzed using the Origin (version 6.0) software.

3. Results and discussion

3.1. Purification of UDP-Glc DH

The enzyme from caprine liver was purified to homogeneity by ammonium sulfate fractionation, heat treatment and chromatographic steps using CM-Sephadex C-25, Blue Dextran-Sepharose and Sephadex G-200 columns (Fig. 1A–C). Corresponding SDS-PAGE profiles have been shown in Fig. 1D. Homogeneity was achieved by 17.2-fold purification from the cytosolic fraction to a specific activity of 342.9 units/mg-protein having an overall recovery of 10.8% (Table 1).

The final preparation was free from colored proteins, proteases and of high specific activity. In all chromatographic steps, single peak of activity was eluted suggesting the absence of isoenzyme or proteolytically cleaved functional components. The initial abundance of UDP-Glc DH was as high as 14.9% but even higher abundance of hemoglobin made the purification difficult. The first four steps (up to the 2nd ammonium sulfate fractionation) were somewhat modified from the conventional protocol of beef liver enzyme to get a better yield. The sequence of steps applied for purification was optimized from trial and error method. It is evident that the yield was sacrificed for purity, mainly at the heat treatment step. The specifications of this step was somewhat modified from that used in case of bovine enzyme purification. Such modifications increased the yield up to 6-fold and the interfering hemoglobin was removed mostly by the second ammonium sulfate step. However, as a reagent for epimerase assay, purification of DH was continued

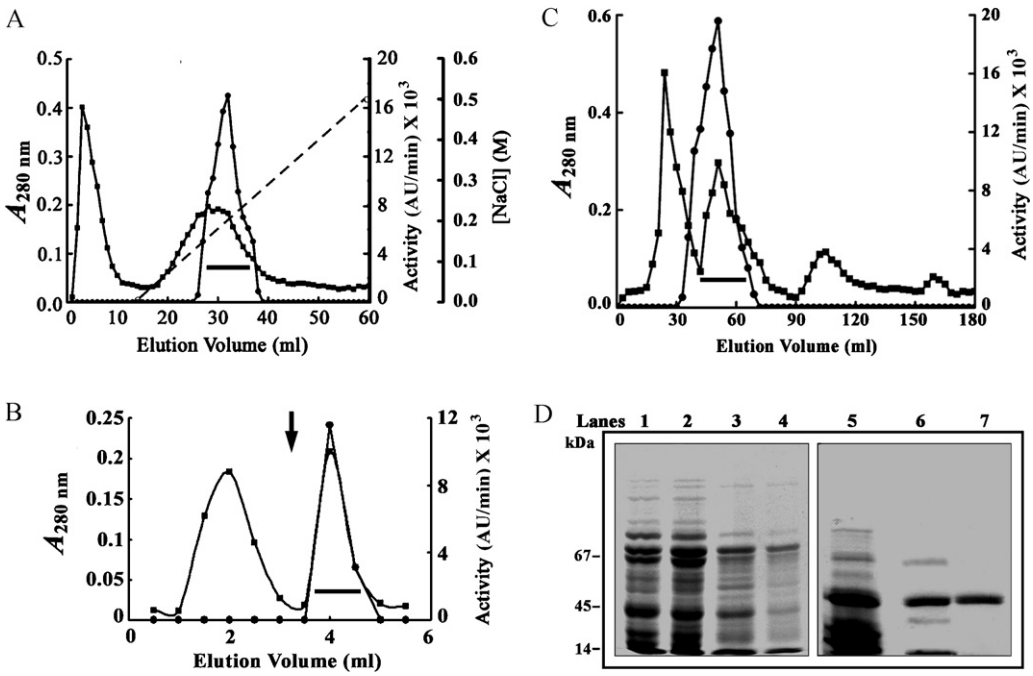


Fig. 1. Purification of caprine UDP-Glc DH. (**A**) Chromatogram of CM-Sephadex C-25. (**B**) Chromatogram of Blue Dextran-Sepharose. Downward arrow indicates initiation of elution of the bound fractions with buffer containing 0.5 M NaCl. (**C**) Chromatogram of Sephadex G-200. In each step of chromatography, elution of proteins was followed at 280 nm (■) and UDP-Glc DH activity assay was followed at 340 nm (●) in absorption Unit/min (AU/min). The dashed line (○–○) indicates NaCl concentration of the eluting buffer. The transverse bars indicate the fractions collected for further studies. (**D**) 10% SDS-PAGE patterns of liver extract after each step of purification. Lane 1, crude extract; lane 2, dissolved pellet after 1st ammonium sulfate fractionation; lane 3, protein after heat treatment; lane 4, dissolved pellet after 2nd ammonium sulfate fractionation; lane 5, active fractions of CM-Sephadex chromatography, lane 6, active fractions of Blue Dextran-Sepharose chromatography; lane 7, purified protein. 20 μg of proteins had been applied in each lane. Position of marker proteins, *viz.*, BSA (67 kDa), ovalbumin (45 kDa) and lysozyme (14 kDa) has been indicated.

up to the heat denaturation step where contaminating epimerase was removed.

3.2. Physical properties

The native molecular mass of UDP-Glc DH as estimated from gel filtration experiment was 300 kDa whereas the subunit molecular mass was revealed as 50 kDa from SDS-PAGE (Fig. 1D). This indicated that the caprine enzyme is a homohexamer as in the cases of UDP-Glc DH from other sources [27,43].

The optimum temperature required for the caprine enzyme was found as 25–45 °C (Fig. 2A). Residual activities were calculated considering the maximum activity as 100%. UDP-Glc DH was incubated at different temperatures for 30 min to determine thermal stability (Fig. 2A, inset). Stability in term of residual activity (%) was compared to that of enzyme stored at −20 °C. The optimum pH required for the reaction of UDP-Glc DH was measured as 8.4 by replacing 100 mM GG, pH 8.8 by buffers of pH 2.2–10.7 in the assay mixture, as compared to 9.25 for the bovine enzyme [44]. Activity decreased almost symmetrically on either side of this point, being approximately 80–83% of maximum at pH 8.8 (Fig. 2B). Since the enzyme loses activity rapidly at pH 9.25, the assay was routinely run at pH 8.8 as for the epimerase assay. Therefore, it can be suitably used for epimerase coupled assay. Catalytic stability of the enzyme was assessed after exposing to 0.05 M buffers of pH 2.2–10.0 for 42 h at 4 °C. The enzyme retained 93–97% residual activity between pH 5.4–6.5 (Fig. 2B, inset). Therefore, for long term storage of UDP-Glc DH, buffer of pH 5.4 was used.

Caprine UDP-Glc DH was found to be stable in 1 M urea for 2 h. However, there was complete inactivation due to unfolding of UDP-Glc DH within 10 min in presence of 3–4 M urea (results not shown).

3.3. Catalytic properties

The K_M and V_{max} for UDP-Glc and NAD^+ were determined under assay conditions. The enzyme displayed typical hyperbolic Michaelis-Menten dependencies for both the substrates (Fig. 3A and B). Corresponding Lineweaver-Burk plots were linear (Fig. 3A and B, insets). The apparent K_M values (mean ± SE of duplicate experiments) obtained for UDP-Glc and NAD^+ were 21 ± 1 μM and 132 ± 10 μM, respectively. Corresponding V_{max} was 24.4 ± 0.4 μmoles/min/mg of enzyme (mean ± SE of duplicate experiments), when both UDP-Glc and NAD^+ were in saturating concentration, *i.e.*, 0.1 mM and 0.5 mM, respectively.

The reported K_M of UDP-Glc DH for UDP-Glc greatly varies from 10 μM to 8.4 mM, likewise the K_M for NAD^+ varies between 20 μM

Table 1
Purification of UDP-Glc DH from caprine liver (enzyme was extracted as described in the text).

Purification step	Total protein (mg)	Total activity (kU)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	2226	44.3	19.9	1	100
1st $(NH_4)_2SO_4$ fraction	1106	35.2	31.8	1.6	79.5
Heat treatment	332	28.3	85.2	4.3	64.0
2nd $(NH_4)_2SO_4$ fraction	279	28.2	101.1	5.1	63.7
CM-Sephadex C-25	72	13.0	180.6	9.1	29.3
Blue dextran-sepharose	37	8.8	237.8	12.0	19.9
Sephadex G-200	14	4.8	342.9	17.2	10.8

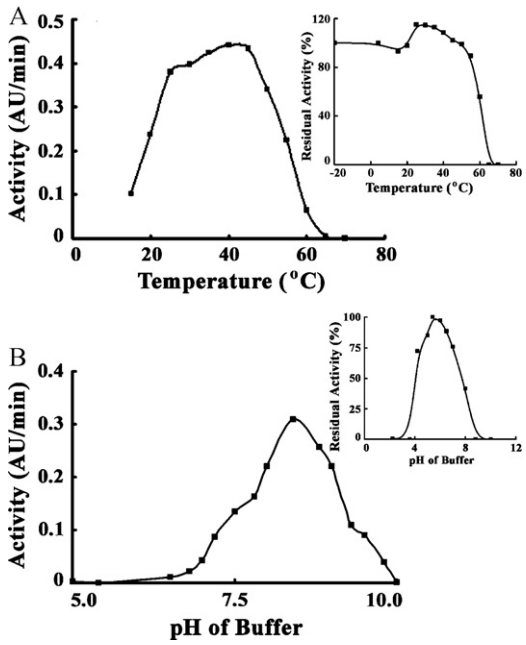


Fig. 2. (**A**) The optimum temperature for caprine UDP-Glc DH assay. Inset: thermal stability of caprine enzyme after 30 min incubation as observed from residual activities. (**B**) The optimum pH required for caprine UDP-Glc DH assay. Inset: pH stability of caprine enzyme after 42 h incubation at 4 °C as observed from residual activities. Enzyme concentration was 0.15 mg/ml in all incubates ($n = 3$).

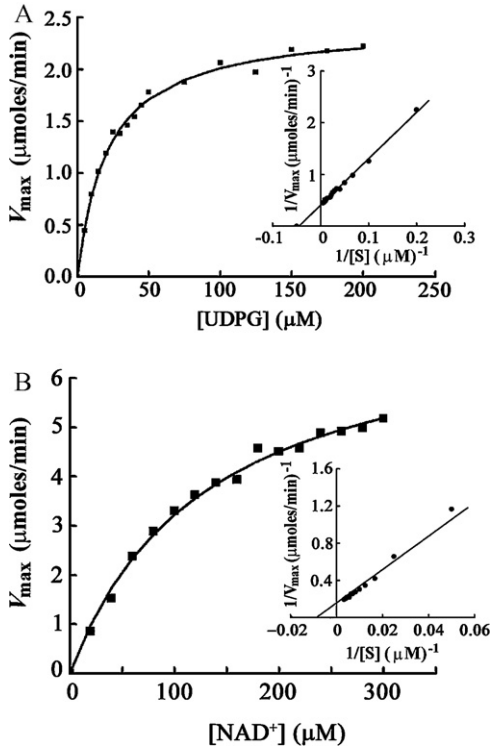


Fig. 3. (**A**) Michaelis–Menten plot of caprine UDP-Glc DH with 0–0.25 mM UDP-Glc as substrate. Enzyme concentration was 0.1 mg/ml and NAD^+ concentration was 0.5 mM. Inset: Lineweaver-Burk plot ($R^2 = 0.9968$). (**B**) Michaelis–Menten plot of caprine UDP-Glc DH with 0–0.3 mM NAD^+ as substrate. Enzyme concentration used was 0.3 mg/ml and UDP-Glc concentration was 0.1 mM. Inset: Lineweaver-Burk plot ($R^2 = 0.9954$). Derived values of K_M and V_{max} have been detailed in the text.

Table 2
Activity of caprine UDP-Glc DH with various substrates.

Substrate (mM), co-substrate (mM)		Activity (%)
UDP-Glc, NAD^+	0.1, 0.5	100
UDP-Gal, NAD^+	0.05, 0.5	0.4
	0.1, 0.5	0
UTP, NAD^+	0.05, 0.5	0.2
	0.4, 0.5	0
5′-UMP, NAD^+	0.05, 0.5	0.2
	0.1, 0.5	0
D(+)-Gal, NAD^+	0.05, 0.5	0
	0.1, 0.5	0
UDP, NAD^+	0.05, 0.5	4.2
	0.4, 0.5	1.8
D(+)-Glc, NAD^+	0.05, 0.5	4.2
	0.2, 0.5	1.8
UDP-Glc, $NADP^+$	0.1, 0.1	5.4
	0.1, 0.2	7.2
	0.1, 0.5	10.8

Results shown are within ±5% error.

and 400 μM [45]. In that scale, these properties of caprine UDP-Glc DH are comparable to the bovine enzyme.

The activity of UDP-Glc DH was measured using nucleotide sugar (UDP-Gal), nucleotides (UDP, UTP, 5′-UMP, $NADP^+$) and reducing sugars (D(+)-Gal, D(+)-Glc) at 0.5 mM under assay conditions. Compared to UDP-Glc (100%), UDP-Gal, UTP, 5′-UMP and D(+)-Gal failed to show any significant activity; whereas the enzyme exhibited activities in the range of 0–5% with UDP and D(+)-Glc. In the assay using $NADP^+$ instead of NAD^+, UDP-Glc DH exhibited 5–12% activity compared to that with NAD^+ (Table 2). The enzyme, however, showed gradual decrease in activity with increasing concentrations of $NADP^+$, D(+)-Glc and UDP. The enzyme is thus catalytically specific for NAD^+ as the co-substrate and is most active with UDP-Glc as the sugar-nucleotide substrate.

The commonly known inhibitors of UDP-Glc DH are substrate analogs like UDP, UDP-Gal, UDP-Xyl, UDP-Man, UDP-GA and NADH. UDP-GA and UDP-Xyl partially inhibited the caprine enzyme activity even at low concentrations of 0.05 mM, which acted as substrate analogs of UDP-Glc having no effect on NAD^+ concentration. UDP, UDP-Gal and UDP-Man were also substrate analogs of UDP-Glc but inhibited the enzyme partially at comparatively high concentrations (0.5 mM). NADH being the analog of NAD^+, inhibited the enzyme at concentration of 0.05 mM. Results of inhibition studies have been represented in Table 3. UDP-Xyl, a potent inhibitor of UDP-Glc DH, is competitive in prokaryotes [46] and is allosteric in eukaryotes having a regulatory role [8,17,47]. Both the caprine and the bovine enzyme are significantly inhibited by UDP-Xyl. The products, UDP-GA and NADH, inhibit UDP-Glc DH from caprine liver to a considerable extent. The basic physico-chemical properties of caprine UDP-Glc DH are somewhat similar to the bovine enzyme, as discussed above, with some insignificant variations.

3.4. Thermodynamic parameters

The Arrhenius relation for the bovine and caprine UDP-Glc DH were constructed at pH 8.8 between 20 and 38 °C. The linear dependencies (R^2 being 0.9781 and 0.9926, respectively) indicated absence of thermal denaturation within the temperature range. The activation energies (E_a) were calculated using Eq. (1). Other thermodynamic parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (at 25 °C) were determined from Eyring plots (Fig. 4A and B) and corresponding $\Delta G^{\ddagger}$ were determined from Eq. (3). Calculation of entropy changes from the Eyring plots requires a large extrapolation, which might cause inaccuracy of the values. But the resulting linear plots were well fitted (R^2 being 0.976 and 0.9913, respectively), which had minimized the uncertainty of the values.

Table 3
Inhibition of caprine liver UDP-Glc DH.

Reagent (mM)	Inhibition (%)
UDP-Glc	
0.1	0
UDP-GA	
0.05	32.2
0.2	58.6
UDP-Xyl	
0.005	38.8
0.05	88.3
UDP	
0.2	22.4
0.5	28.0
UDP-Gal	
0.2	22.1
0.5	27.1
UDP-Man	
0.2	8.3
0.5	21.5
NAD^+	
0.5	0
NADH	
0.05	52.5
0.2	66.1

Results shown are within ±5% error.

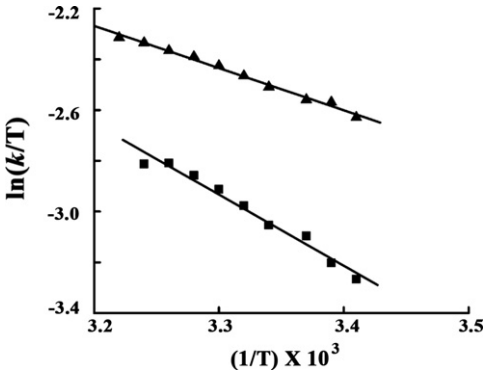


Fig. 4. Eyring plots for UDP-Glc DH from bovine (■) and caprine (▲) liver. Enzyme concentrations were 0.2 mg/ml and 0.15 mg/ml, respectively ($n = 3$). Derived thermodynamic parameters have been summarized in Table 4.

The derived parameters have been compared in Table 4. It shows that the thermodynamic properties of bovine and caprine enzymes differ significantly. The E_a of caprine enzyme is about 40% lower than that of the beef enzyme indicating its faster turnover. The $\Delta H^{\ddagger}$ of the caprine enzyme is also about 40% lower indicating lower number of interaction sites in the transition state complex. Further, corresponding $\Delta S^{\ddagger}$ is again 45% lower than that of the beef enzyme. A lower $\Delta S^{\ddagger}$ term indicates that the transition state configuration is more ordered and favorable for the enzyme-substrate interaction [48]. It is also known that, lower the entropy of catalysis, higher the stability of the transition state [49]. Changes in $\Delta G^{\ddagger}$ for catalysis are found to be comparable for these enzymes. The small difference in $\Delta G^{\ddagger}$ results from large differences in both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, *i.e.*, these values compensate each other to give similar values of $\Delta G^{\ddagger}$ [48].

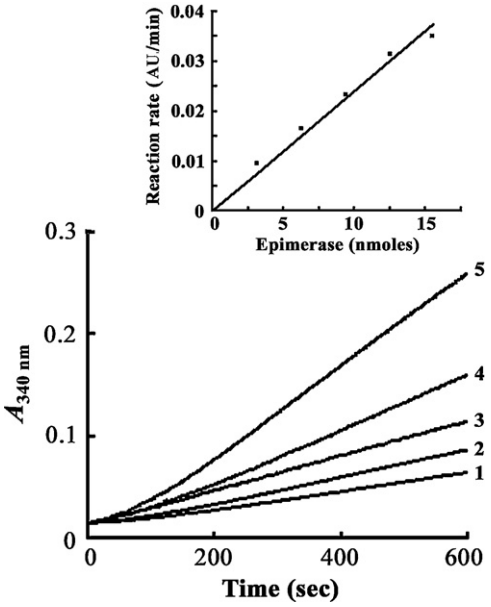


Fig. 5. Time course of catalytic conversion of UDP-Gal to UDP-Glc by epimerase using caprine UDP-Glc DH as coupling enzyme. Epimerase concentration was 2–16 nmoles in sets 1–5. The kinetic lag is an inherent property of epimerase related to association of an inhibitor [40]. The reaction rates were calculated from the steady state kinetics. Inset: linear dependency of the reaction rates with epimerase concentration ($R^2 = 0.983$).

3.5. Coupled assay of epimerase

The steady-state kinetics of epimerase using caprine UDP-Glc DH as coupling enzyme showed increasing rates along with reduction of initial lag as a function of epimerase concentration (Fig. 5). Secondary plot of the rates against epimerase concentration showed linear dependency ($R^2 = 0.983$) and the line passed through the origin indicating that the DH preparation was free from epimerase activity (Fig. 5, inset). The lag is due to the hysteretic character of the primary enzyme arising from binding of an inhibitor [40,50] and does not originate from the coupling enzyme that has been elaborated below.

Since the concentration of the substrate of the auxiliary enzyme is zero at the time of initiation of a coupled assay, kinetic lag is obligatory. Duration of this lag should be minimum and it depends on the K_M and V_2 of the coupling enzyme. The lag for the epimerase assay had been calculated using Eqs. (4) and (5). Usually a lag period of 30 s to 1 min is accepted. But, in case the primary enzyme follows hysteretic kinetics, the lag time must be the shortest possible one [51]. Required amount of auxiliary enzyme can be calculated from Eq. (5) or (6). Therefore, for accurate assays, F_p (Eq. (4)) or v_2/v_1 (Eqs. (5) and (6)) should be 0.99 to validate attainment of 99% of the steady state rate of primary enzyme. To calculate the lag contributed by caprine UDP-Glc DH in epimerase assay, the values of UDP-Glc, $K_M = 0.021$ mM, $V_2 = 2.44$ mmoles/L/min and $F_p = 0.99$ had been substituted in Eq. (4); duration of lag arising from coupled enzyme turned out to be of 2.42 s which was insignificant. Alternately, in Eq. (6), using the K_M and V_2 values as stated above, v_1 (maximum velocity of epimerase to be measured) = 0.56 mmoles/l/min and v_2 should have reached 0.99 v_1

Table 4
Thermodynamic properties of bovine and caprine UDP-Glc DH.

Enzyme	E_a (kJ)	$\Delta H^{\ddagger}$ (kJ)	$\Delta S^{\ddagger}$ (JK^{-1})	$\Delta G^{\ddagger}$ (kJ)
Bovine enzyme	24.42 ± 1.38	23.30 ± 1.38	−33.28 ± 4.60	33.23 ± 2.75
Caprine enzyme	14.95 ± 0.46	13.83 ± 0.46	−60.4 ± 1.51	31.83 ± 0.90

within 1 min, calculations show $v_1/V_2 = 0.23$ which was between the Table values of 0.2 and 0.25, for which the Φ-values (at $v_2/v_1 = 0.99$) were 1.31 and 1.81, respectively [32]. Therefore, the derived values of '*t*' were 2.94 s and 4.08 s, respectively. These values were also insignificant in the time scale of epimerase assay. A safe margin was, therefore, always maintained in coupled assay to eliminate the lag arising from UDP-Glc DH using it in 10-fold excess.

One of the reasons to characterize the caprine enzyme is to evaluate it as a substitute for bovine enzyme as a coupling enzyme for epimerase assay where UDP-Gal is used as substrate. However, the systems may not always work well due to non-specific reactions [52]. The assay times are prepared after the steady-state condition is achieved in a coupled system [41]. K_M values for the measured substrates (here UDP-Gal) and activity of the auxiliary enzyme are compared for proper selection of the system. Here, amount of auxiliary enzyme required is calculated for the caprine enzyme as described in the results section. Reaction mixture is preincubated with the coupling enzyme to confirm whether there is any change and the endogenous product is exhausted prior to the actual assay. It is also checked that inhibitor of the primary enzyme (principally 5′-UMP was used for this study) does not pose any inhibitory effect on the auxiliary enzyme (data not shown). For epimerase assay, therefore, caprine liver UDP-Glc DH should be equally suitable substitute for the beef liver enzyme.

4. Conclusion

We conclude that, caprine liver UDP-Glc DH can be purified to homogeneity using ammonium sulfate fractionation, heat denaturation, affinity chromatography and gel-filtration chromatography up to 17.2-fold with specific activity 342.9 units/mg-protein. Most of the fundamental physical and kinetic properties of caprine liver UDP-Glc DH are comparable with those of the bovine form. Differences in their thermodynamic parameters signify that UDP-Glc is more rapidly converted by caprine UDP-Glc DH than that by the bovine enzyme. It has been as well ascertained that caprine liver UDP-Glc DH can be employed conveniently and with confidence as an auxiliary enzyme for epimerase assay.

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