PURIFICATION AND CHARACTERIZATION OF HIGHLY STABLE ALDO-KETO REDUCTASE FROM CAMEL (*CAMELUS DROMEDARIUS)* LIVER

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Abstract - Aldo-keto reductase (AKR) was purified to homogeneity from camel liver by ion exchange on Q Sepharose, affinity chromatography on Blue-Sepharose and 2,5-ADP-Sepharose 4B. The purification procedure resulted in 32.43-fold purification with 0.65% final yield. Subunit and native molecular weights of the purified enzyme determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography, were 33kD and 133kD, respectively. The purified AKR exhibited maximal activity at a temperature of 50°C and pH of 7.0. The K_m values for NADPH and NADH calculated from the Lineweaver-Burk plot were 0.01 mM and 0.083 mM, respectively, whereas the K_m values for m-Nitrobenzaldehyde, 4-Anisaldehyde and P-Benzoquinone were 0.9 mM, 1.11 mM and 0.57 mM, respectively.

Key words: Camel, purification, aldo-keto reductase, NAD(P)H, characterization, m-Nitrobenzaldehyde, 4-Anisaldehyde, p-Benzoquinone.

INTRODUCTION

Carbonyl reductase (CR) (EC 1.1.1.184) comprises a large family of cytosolic NAD(P)H-dependent oxidoreductase which catalyzes the reduction of a variety of carbonyl compounds present in foods and environmental pollutants. CRs are considered as phase I drug metabolizing enzymes for a variety of carbonyl-containing drugs, thereby play a central role in xenobiotic metabolism leading to bioactivation or detoxication of reactive compounds derived from lipid peroxidation and glycation (Yi and Trevor, 2007). Other CR endogenous carbonyls include monosaccharides, steroids, neurotransmitter and prostaglandins (Vincent et al., 2000).

The characterized carbonyl-reducing enzymes are grouped into two distinct protein superfamilies, the short-chain dehydrogenases/reductases (SDR) aldo-keto reductases (AKR), or medium chain dehydrogenases/reductases (MDR) (Oppermann, 2007). Mammalian tissues produce enzymatic systems belonging to the SDR family or members of the AKR family such as aldose reductase, aldehyde reductase, dihydrodiol dehydrogenases, and several other reductases. AKR enzymes act on aldehydes and ketones, where aldehydes are converted to primary alcohols and ketones are reduced to corresponding secondary alcohols (Annerita et al., 1995; Wermuth, 1985; Matsumoto et al., 2006) that are more hydrophilic and can be conjugated and excreted easily. Reactive oxygen species (ROS) production is enhanced during the normal aerobic metabolic process. Heat stress stimulates excessive production of free radicals that are capable of initiating lipid peroxidation, leading to a variety of carbonyl compounds, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Bernabucchi et al., 2002).

The Arabian camel (*Camelus dromedarius*) is able to survive in extremely harsh desert conditions characterized by a hot and dry summer and extremely cold winter seasons. The camel is well adapted to dehydration for relatively long periods (Souilem O., and Barhoumi K., 2009). Metabolic processes in camels may lead to the accumulation of harmful endogenous carbonyl compounds and these compounds can in turn damage healthy cells by forming adduct with proteins and nucleotides. To our knowledge, studies describing AKR from Arabian camel liver are not available. Therefore, in the present work, the purification and biochemical properties of a novel, thermostable AKR from Arabian camel are reported for the first time.

MATERIALS AND METHODS

Chromatographic media and molecular weight markers were from GE Healthcare. Coenzymes, (NADPH and NADH) and EDTA was purchased from Sigma Chemical Co. (USA). All other chemicals were of analytical grade obtained from BDH Chemicals or Merck AG (Germany).

Purification of camel liver carbonyl reductase

Camel liver from a local slaughterhouse was transported to the laboratory on ice. The liver was homogenized by blender in 20 mM Tris-HCl buffer pH 8.0 (buffer A) containing 1 mM EDTA and 10 mM PMSF and then centrifuged at 9000g for 20 min. The cold supernatant obtained was loaded onto a Q Sepharose column (10 x 2.6 cm) previously equilibrated with buffer A containing 1 mM EDTA. The column was eluted first with two bed volumes of the equilibration buffer, followed by linear gradient of 0-500 mM NaCl in buffer A at a flow rate of 2 ml min^{-1}. The fractions were collected using an automated liquid chromatography system (ÄKTA GE Healthcare). Fractions showing carbonyl reductase activity were pooled and concentrated by an Amicon ultra-filtration cell fitted with a PM-10 membrane (Millipore). CR was further purified using an affinity column (15 x 1 cm) packed with Blue Sepharose. The column was pre-equilibrated with 50 mM phosphate buffer pH 7.2 (buffer B), and CR was eluted by 1M NaCl in the buffer at a flow rate of 2 ml min^{-1}. Active fractions eluted as a single peak were pooled, concentrated to 3 ml and dialyzed against buffer B. The dialyzed fraction was applied to 2,5-ADP Sepharose affinity column (5 x 1 cm) equilibrated with buffer B. The column was washed with buffer and CR was eluted by linear gradient of 0-1 mM NADPH in buffer B. The active fractions were pooled and used as the purified enzyme. Unless otherwise noted, all steps of enzyme purification were carried out at 4°C.

Protein Estimation

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Bradford, 1976). Absorbance at 280 nm was used to monitor the protein content in column fractions.

Determination of molecular weight

Subunit molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed with a Bio-Rad mini-protean II electrophoresis unit according to Laemmli (1970) in a vertical slap gel apparatus using 12% separating and 4% stacking gel with 0.3% cross-linking. The gel was stained with Coomassie blue R-250. Protein samples were treated with sample buffer containing 1% SDS and 5% 2-mercaptoethanol at 95°C for 5 min prior to electrophoresis. Phosphorylase b (94 kDa), bovine serum albumin (BSA) (67 kDa), ovalbumin (43kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as standard molecular weight markers.

The molecular mass of the native enzyme was estimated by gel filtration through a column that had previously been calibrated with the following standard protein markers: aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa) carbonic anhydrase (29 kDa) and ribonuclease (13.7 kDa). CR was eluted with potassium phosphate buffer (20 mM, pH 7.2), containing 0.1M NaCl at a flow rate of 1 ml min^{-1}.
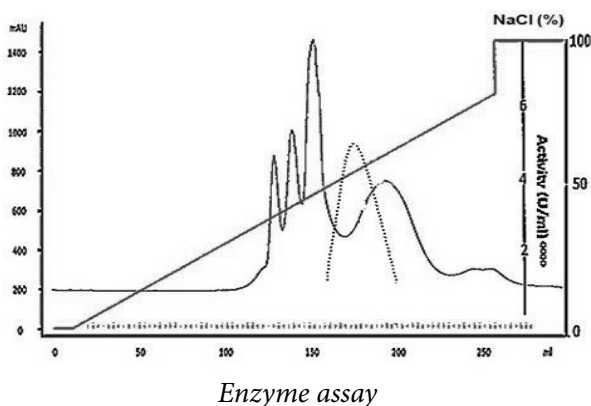
Enzyme assay

Fig. 1. Elution profile of Q Sepharose fast flow ion exchange chromatography observed during purification of camel liver carbonyl reductase.
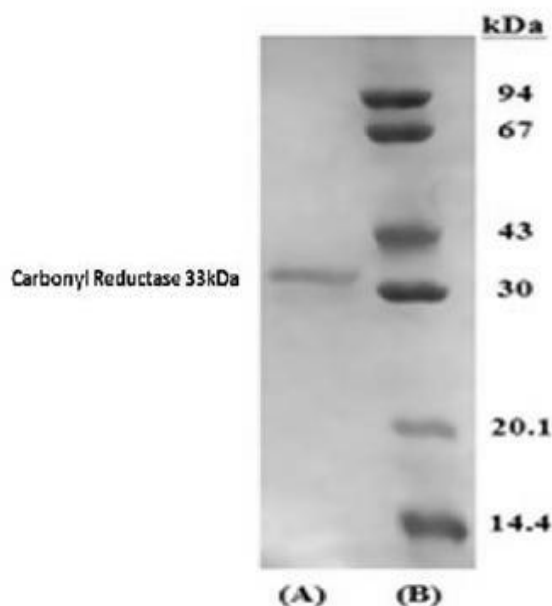

Fig. 2. (A) SDS-polyacrylamide gel electrophoresis (12%) of the purified CR from camel liver. Lane 1 shows, purified CR (0.5 μg protein) in final step and lane 2, contained a set of marker proteins along with their corresponding molecular masses.

Fig. 3. Effect of pH on the activity of camel liver carbonyl reductase.

Fig. 4. Effect of temperature on the activity of camel liver carbonyl reductase; the enzyme activity was assayed by incubating the reaction mixtures at 25-80°C.

CR activity was determined spectrophotometrically by monitoring the NADPH oxidation at 340 nm. The enzyme was assayed routinely at room temperature using a reaction mixture containing 50 mM potassi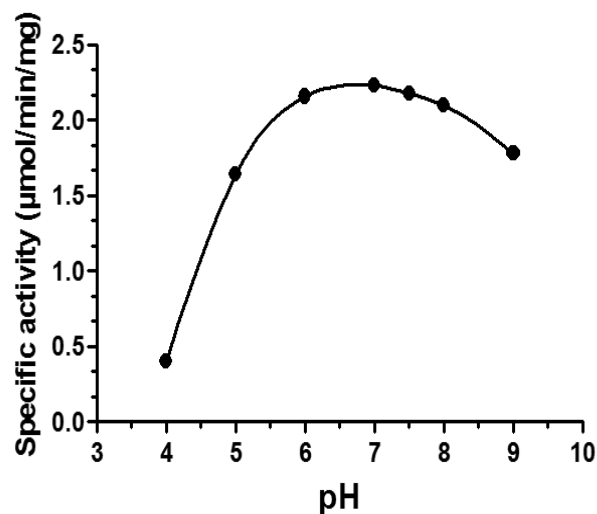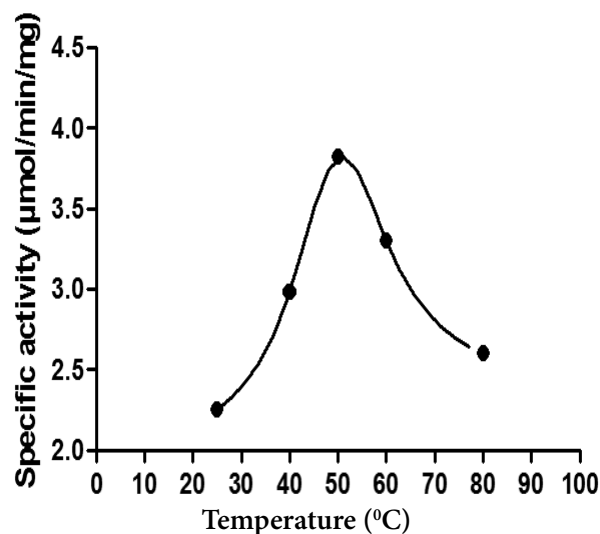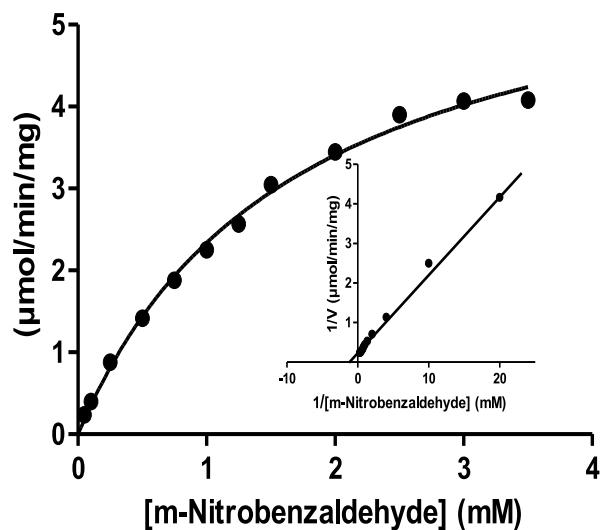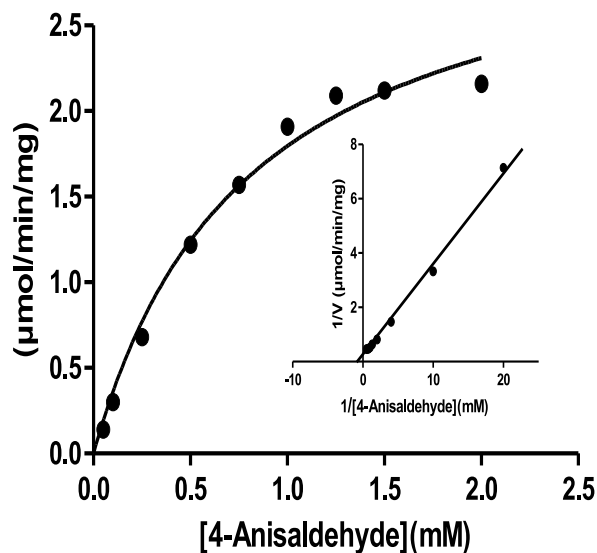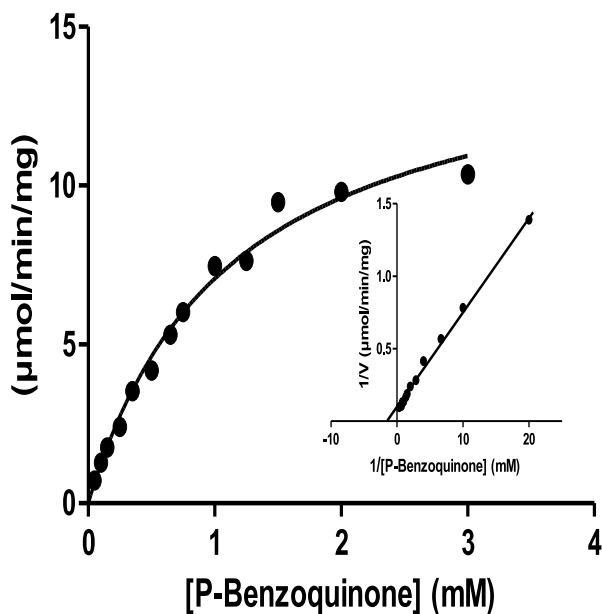um phosphate buffer (pH 7.2), 0.1 mM NADPH, and 1 mM m-Nitrobenzaldehyde in a final volume of 1.0 ml. The reaction was initiated by the addition of an appropriate volume of enzyme solution. One unit (U) of activity was defined as the amount of enzyme required to oxidize one mole of NADPH per minute under the assay conditions. Specific activity was calculated as activity units per mg of protein.

(A)

(B)

(C)

Fig. 5. Substrate saturation curve and Lineweaver-Burk plot of CR activity in presence of different concentrations of substrate (A) for p-benzoquinone, (B) for m-nitrobenzaldehyde and (C) for 4-anisaldehyde, using NADPH coenzyme at constant concentration. The Lineweaver-Burk plot (inset Fig. 5A, B and C) was generated by plotting 1/velocity vs. 1/substrate.

RESULTS AND DISCUSSION

Enzyme purification

CR was purified from camel liver by three steps, anion exchange on Q Sepharose, Blue-Sepharose affinity and ADP-Sepharose-4B affinity column chromatography. A typical elution profile of CR on Q Sepharose column chromatography is shown in Fig. 1. The scheme utilized resulted in almost 32.43-fold purification of CR with a recovery of 0.65%, and the final specific activity of the purified enzyme was 1.2 units per milligram of protein (Table 1). The binding of the enzyme to Blue Sepharose was found to be very tight and high ionic strength was required to liberate the enzyme, therefore the enzyme was eluted by 1 M NaCl. The purity of the enzyme was shown by SDS-PAGE and a single band with a molecular weight of 33 kDa was observed (Fig. 2). This molecular weight is in the range of CR subunits from other mammalian sources, including dog liver (Endo et al.,

Effect of cations on CR activity

The effect of divalent metal ions on CR activity was examined by preincubating CR for 15 min at room temperature with the cations to be tested (Ba^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}) in 50 mM Tris-HCl buffer (pH 7.2) at 0.1 mM final concentration. The residual activity of CR was determined by the assay procedure presented above.
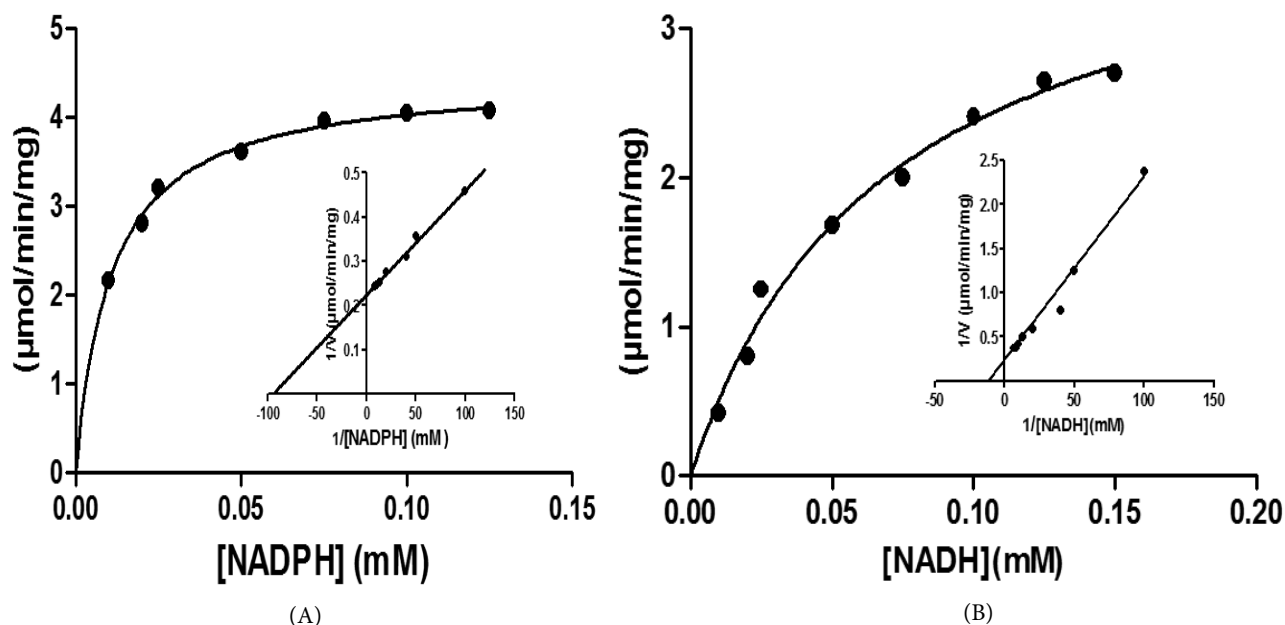
(A)

(B)

Fig. 6. Coenzyme saturation curve and Lineweaver–Burk plot of CR activity in presence of different concentrations of coenzyme (A) for NADPH and (B) for NADH, using m-nitrobenzaldehyde substrate at constant concentration. The Lineweaver-Burk plot (inset Fig. 6A and B) was generated by plotting 1/velocity vs 1/substrate.

2007), guinea pig liver (Usui et al., 1984), and rabbit heart (Imamura et al., 1999).

Native molecular mass as determined by gel filtration through HiLoad Superdex-200 (16/60) was approximately 133 kDa. Thus, the active purified camel liver CR is homotetramer.

Optimum pH and temperature

The purified camel liver CR, assayed at different pH ranging from pH 4.0 to 9.0, exhibited an optimum pH over a broad range between pH 6.0 and 8.0 with maximum activity around pH 7.0 (Fig. 3). This optimum pH is similar to human liver CR (Doorn et al., 2004). When camel liver CR was assayed at different temperatures, ranging between 25-80°C, it exhibited maximum activity at 50°C (Fig. 4). Studies have reported a wide range of optimum temperature for CRs from different sources. In comparison, CR from human liver and brain showed maximum activity at 37°C and 25°C, respectively (Doorn, 2004 et al; Wermuth, 1981), at 30°C for rabbit liver (Imamura et al.,

2003) and 55°C for *Candida magnoliae* (Wada et al., 1998). The purified CR was stable when stored in 20 mM potassium phosphate buffer (pH 7.2) at 4°C and -20°C for a period of at least two months and was also stable for one week at room temperature.

Kinetic studies

The purified camel liver CR showed activity towards many substrates, including p-Benzoquinone, m-Nitrobenzaldehyde and 4-Anisaldehyde. The apparent K_m and V_{max} values of CR for reduction of p-Benzoquinone as calculated from the double reciprocal plot (Fig. 5A) were 0.57 mM, and 10 U/mg (Table 2), respectively. The K_m and V_{max} values of the enzyme for reduction of m-Nitrobenzaldehyde (Fig. 5B) were 0.90 mM and 4.54 U/mg (Table 2), respectively. The K_m and V_{max} values of the enzyme for reduction of 4-Anisaldehyde were 1.11 mM and 5.26 U/mg (Fig. 5C), respectively. The K_m value for p-Benzoquinone is lower in comparison with m-Nitrobenzaldehyde and 4-Anisaldehyde, so the enzyme has a higher affinity for p-Benzoquinone compared to the others.

In addition, the catalytic efficiency K_{cat}/K_m is higher for p-benzoquinone about 40.4 $M^{-1}S^{-1}$ (Table 2).

The camel liver CR can utilize both NADPH and NADH coenzymes. The apparent K_m and V_{max} values of CR for oxidation of NADPH as calculated from the double reciprocal plot (Fig. 6A) were 0.010 mM, and 4.54 U/mg (Table 2), respectively, which is similar to that reported for CR from human brain (Wermuth, 1981). The K_m and V_{max} values of the enzyme for oxidation of NADH (Fig. 6B) were 0.083 mM and 4 U/mg (Table 2), respectively. The camel liver CR enzyme showed higher affinity for NADPH than NADH, so the camel liver has an efficient mechanism against toxic and carcinogenic agents.

Effect of metal ions on CR activity

The effect of metal ions on CR activity is presented in Table 3. The enzyme activity was enhanced by Ca^{2+} and Ba^{2+} at 0.1 mM. On the other hand, Cu^{2+} ions strongly inhibited the enzyme activity, as reported for CR from other organisms (Kataoka et al., 1992; Yamamoto et al., 2002; Nishinaka et al., 1993). The enzyme activity was inhibited by Co^{2+}, Zn^{2+} and Mg^{2+} ions at 0.1 mM. An inhibitory effect of Zn^{2+} was reported for CR from other organisms (Yamamoto et al., 2002; Kizaki et al, 2005). No change in camel liver CR activity was noticed with Mn^{2+} ions. In conclusion, the properties of camel liver carbonyl reductase appear to be unique because of its good stability, wide pH range, wide substrate specificity and use of both NADPH and NADH coenzymes.

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