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Isolation and properties of lactate dehydrogenase isoenzyme from buffalo liver: Application in AST and ALT assay diagnostic kits

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ABSTRACT

Lactate dehydrogenase (LDH) enzyme is a major component of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) diagnosis kits. In this work, the LDH enzyme was purified and characterized from buffalo liver for direct application in the preparation of AST and ALT diagnosis kits. One major LDH (BLLDH) isoform and two other secondary LDH peaks were analyzed for buffalo liver by diethylaminoethyl (DEAE) cellulose chromatography. BLLDH was obtained by ammonium sulfate sedimentation and chromatographically separated on ion exchange and size-exclusion matrices. The isolated BLLDH has a specific activity of 17.6 units/mg proteins represented 16 folds and 32% recovery. BLLDH was manifested homogeneous on native and SDS gels with 35 kDa native mass. Optimum pH of BLLDH was displayed at pH 7.6. BLLDH activity was diminished by $FeCl_2$ and SDS. The produced BLLDH is utilized in constructing of AST and ALT diagnosis kits that were sensible and analogous to trade ready kits.

INTRODUCTION

Lactate dehydrogenases (L-lactate: NAD oxidoreductase, EC 1.1.1.27; LDH) are glycolytic enzymes that initiate the pyruvates reduction to lactates under anaerobic cases (Li *et al*., 2013). LDHs are present in almost all living organisms since the enzyme was separated and described from animals, plants, and bacteria by diverse chromatographic techniques (Qian *et al*., 2014). LDH is detected in nearly whole creatures since it is playing a significant part in the metabolism of carbohydrates. Through situations when pyruvate manufacture in glycolysis surpasses the cell capability in pyruvate oxidizing, LDH transforms pyruvate to lactate and hence repeats the oxidized Nicotinamide adenine dinucleotide (NAD) necessary for more glycolysis. Furthermore, LDH permits the lactates diversion to pyruvates; and the obtained pyruvates and Nicotinamide adenine dinucleotide reduced (NADH) can then be employed for different operations (Kadiyala, 2015). In animal tissues, LDH is generated by two genes, called LDH A and LDH B. LDH A gene is strongly expressed in muscles and livers (M isozyme), while LDH B gene is strongly expressed in hearts (H isozyme). Nearly in whole kinds, the gene yields compose tetramer compounds with different properties slightly relying on the proportional quantities of various isoenzymes that exist in the tetramer (Schurr, 2017). The two common LDH subunits (LDH-M) and (LDH-H) found in circulation can compose five tetramers (isoforms): 4H, 4M, 3H1M, 2H2M, and 1H3M (Nadeem *et al*., 2014). High level of LDH in serum was found in myocardial infarction, liver disease, renal disease, malignant diseases, progressive muscle dystrophy, and certain forms of anemia (Holmes and Goldberg, 2009; Ketchum *et al*., 1984). LDH can be used as a marker for tumor, heart attack, hypothyroidism, anemia, preeclampsia, meninges and brain inflammations, severe pancreatitis, HIV, lungs and liver diseases, and tissues collapse (Butt *et al*., 2002; Kim *et al*., 2014; Xu *et al*, 2014). LDH and malate dehydrogenase (MDH) enzymes are major constituents in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) diagnosis kits that are very precise in the determination of AST and ALT enzymes levels in the blood, serum, and plasma. AST and ALT kits are at most employed as livers job marker tools in diagnostics, controlling, and treating hepatic illnesses (Darwish *et al*., 2018; Huijgen *et al*., 1997). Thus, the leading goal of this search is purifying, describing, and producing LDH enzyme from

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buffalo livers as the home available origin for use in constructing AST and ALT diagnosis kits.

MATERIALS AND METHODS

Liver tissues

Water buffalo (*Bubalus bubalis*) liver tissue samples of six different animals were gained from a local slaughter-house and stocked at −40˚C.

Chemicals

Bovine serum albumin, DEAE cellulose, NADH, Nitroblue tetrazolium salt (NBT), Sodium pyruvate, Lithium lactate, Phenazine methosulfate (PMS), Sephacryl S-300, Standard proteins for molecular weights, and electrophoresis chemicals were bought from Sigma-Aldrich Chemical Co. All other chemical compounds were of analytical degree.

Assay of Lactate dehydrogenase activity

The activity LDH assay is measured by the method described by Nadeem *et al*. (2011). The test mixture consists of 3 ml 0.1 M phosphate buffer pH 7.0, 0.22 mM NADH, 0.2 mM sodium pyruvate, and enzyme solution. The variation in absorbance at 340 nm was followed for 5 minutes and LDH activity units were calculated by Beer–Lambert law, utilizing 6,220 M^{-1} cm^{-1}extinction coefficient value of NADH. For LDH isoenzyme patterns on polyacrylamide gels, the gels were flooded in 50 ml 0.1M Tris-HCl, pH 8, 288 mg lithium lactate, 14 mg NAD, 3.5 mg NBT, and traces of PMS. In dark, gels were incubated in this staining mixture at 37˚C until appearing of dark blue bands (Whitt, 1970).

Purification of buffalo liver lactate dehydrogenase (BLLDH)

Preparation of crude extract

Buffalo liver tissues were homogenized in 0.05 M Na phosphate buffer pH 7.4 including 10 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM β-mercaptoethanol by Omni mixer homogenizer. Cells debris and not soluble materials were segregated with centrifuging at 5,000 × *g* for 20 minutes with saving the supernatant as a crude extract.

Ammonium sulfate precipitation

Solid $(NH_4)_2SO_4$ was progressively added to the crude extract till be 40% saturated and stirred for 30 minutes at 4 °C followed by 20 minutes centrifugation at 8,000 × *g*. The pellets from this stage were thrown out and the supernatant was 80% Solid $(NH_4)_2SO_4$ saturated followed by 30 minutes centrifugation at 12,000 × *g* and the precipitate was gained and dissolved in 0.02 M Na phosphate buffer pH 7.4 including 10 mM EDTA and 1 mM β-mercaptoethanol and dialyzed extensively versus the same buffer.

DEAE-cellulose and Sephacryl S-300 column chromatography

The 40%–80% $(NH_4)_2SO_4$ portion was loaded on DEAE cellulose column (12 cm × 2.4 cm i.d.) already equilibrated with 0.02 M sodium phosphate buffer pH 7.4 containing 10 mM EDTA and 1 mM β-mercaptoethanol. For further purification, the DEAE cellulose portions retaining LDH activity were pooled and concentrated by lyophilization. The concentrated DEAE-cellulose material was loaded over Sephacryl S-300 column (142 cm × 1.75 cm i.d.) equilibrated by 0.02 M sodium phosphate buffer pH 7.4 including 10 mM EDTA and 1 mM β-mercaptoethanol.

Electrophoretic analysis

Electrophoretic analysis of the purified LDH enzyme was performed on 7% PAGE (Smith, 1969) and 12% SDS-PAGE (Laemmli, 1970), while its molecular mass was detected according to Weber and Osborn (1969).

Staining of proteins was carried out utilizing 0.25% Coomassie Brilliant Blue R-250.

Protein determination

Proteins concentration was detected by Bradford method (1976) utilizing albumin from bovine serum as a standard.

Preparation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) diagnostic kits

The purified buffalo liver LDH and the previously purified sheep liver MDH (Darwish *et al*., 2018) are employed in constructing AST and ALT diagnosis kits. The AST kit consists of two solutions; 80 mM Tris-HCl buffer pH 7.8, 240 mM L-aspartate, 900 U/L LDH and 600 U/L MDH and 12 mM 2-oxoglutarate and 0.18 mM NADH. ALT kit consists of two solutions; 80 mM Tris-HCl buffer pH 7.5, 500 mM L-Alanine, 1200 U/L LDH and 15 mM 2-oxoglutarate and 0.18 mM NADH (Thomas, 1998).

RESULTS

Purification of buffalo liver lactate dehydrogenase (BLLDH)

The procedure of the major buffalo liver LDH purification is summarized in Table 1. The LDH specific activity of buffalo liver crude extracts was detected as 1.1 units/mg

Table 1. A typical purification scheme of BLLDH.

Purification steps	Total protein (mg)	Total activity (unit)	Specific activity	Yield (%)	Fold purification
Buffalo liver crude extract	400	440	1.1	100.0	1.0
40-80 % $(NH_4)_2SO_4$ Fraction	144.6	350	2.4	79.5	2.2
DEAE-cellulose fractions					
0.0 M NaCl	62.9	90	1.4	20.0	1.3
0.05 M NaCl	40.9	180	4.4	40.9	4.0
0.1 M NaCl	30.3	40	1.3	9.1	1.2
0.05 M NaCl Sephacryl S-300 fraction	8.0	141	17.6	32.0	16.0

protein. The chromatographic pattern of the buffalo liver LDH on DEAE cellulose matrix showed one major LDH activity peak collected by 0.05 M NaCl and designated BLLDH and other two minor LDH activity peaks (Fig. 1a). The major BLLDH fractions were gathered, lyophilized, and furthermore loaded on Sephacryl S-300 column (Fig. 1b) which showed one peak of LDH. After chromatography, BLLDH specific activity was elevated to 17.6 units/mg showing 16-folds and 32% outputs (Table 1).

Molecular weight, electrophoretic analyses, and Optimum pH of BLLDH

In this study, the BLLDH pureness was inspected by analyzing on 7% native-PAGE. BLLDH isoenzyme demonstrated a unique protein band identical with the LDH isozyme band corroborating the pureness of the preparation (Fig. 2a and 2b). The molecular weight of BLLDH was deduced from both gel filtration column as well as SDS-PAGE to be 35 kDa (Fig. 1b and 3a). The maximum activity of BLLDH enzyme was investigated with

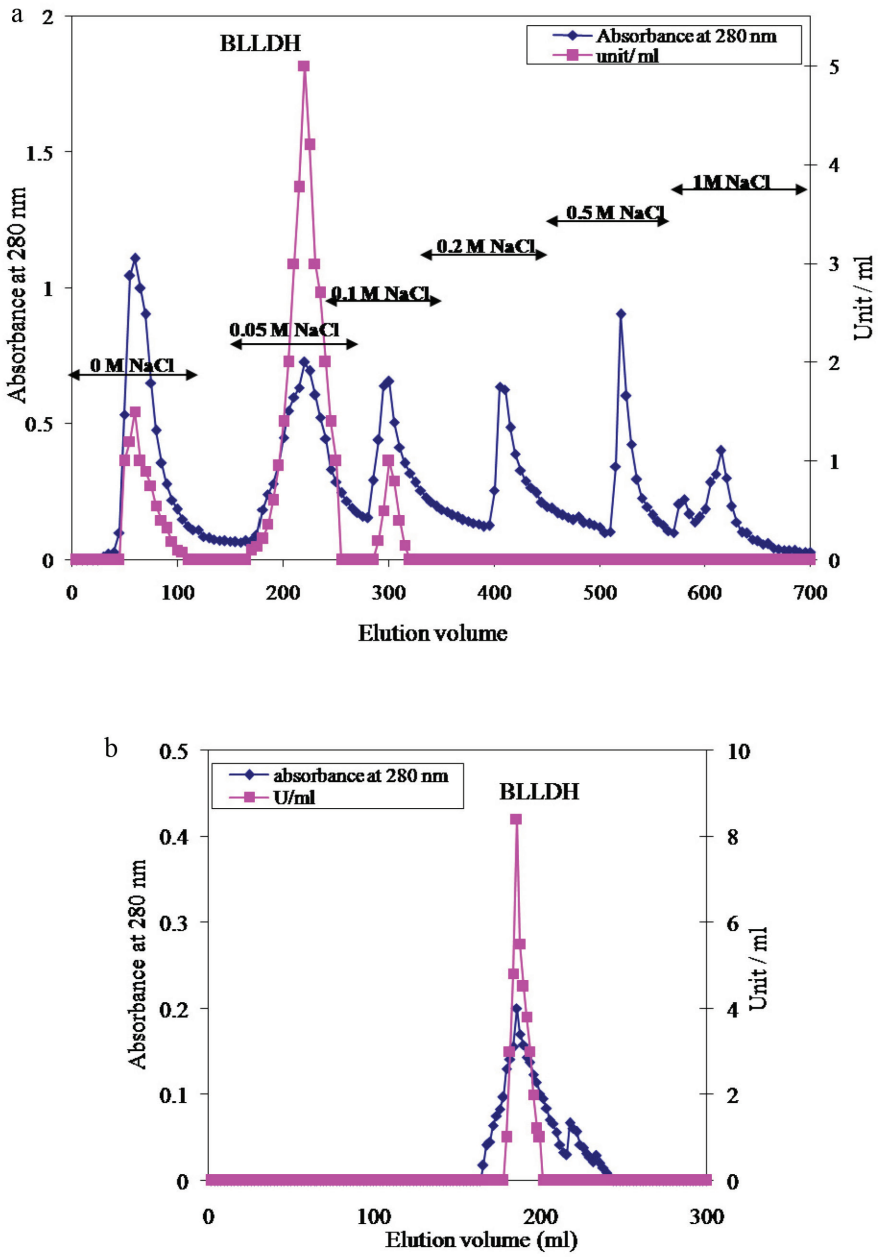

Figure 1. (a) A typical elution profile for the chromatography of the buffalo liver ammonium sulfate fraction on DEAE-cellulose column (6 cm × 2.4 cm i.d.) previously equilibrated with 0.02 M sodium phosphate buffer pH 7.4 containing 10 mM EDTA and 1 mM β-mercaptoethanol. (b) A typical elution profile for the chromatography of the buffalo liver DEAE-cellulose fraction (BLLDH) on Sephacryl S-300 column (142 cm × 2.4 cm i.d.) previously equilibrated with 0.02 M sodium phosphate buffer pH 7.4 containing 10 mM EDTA and 1 mM β-mercaptoethanol.

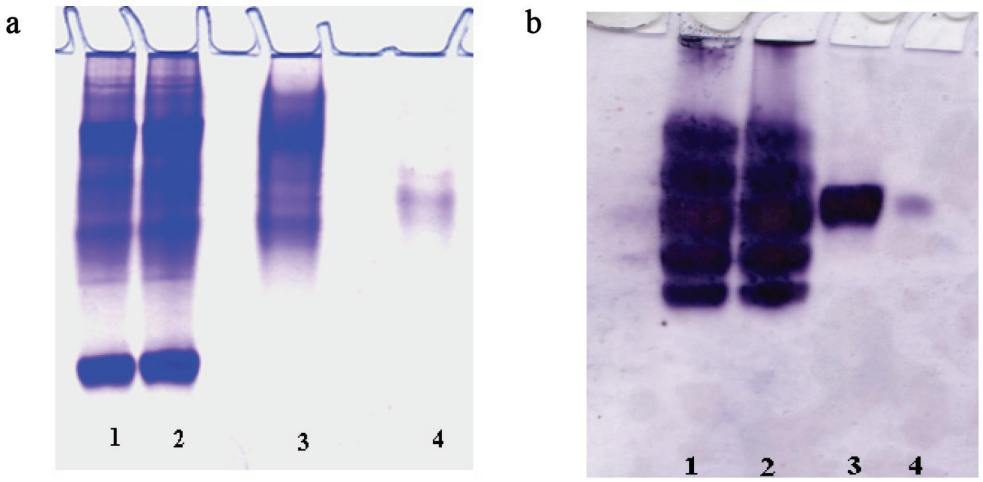

Figure 2. Electrophoretic analysis of BLLDH on 7% native PAGE: (1) crude extract, (2) ammonium sulfate fraction, (3) 0.05M NaCl DEAE-cellulose fraction, and (4) Sephacryl S-300 purified fraction (a) protein pattern and (b) LDH isoenzyme activity pattern.

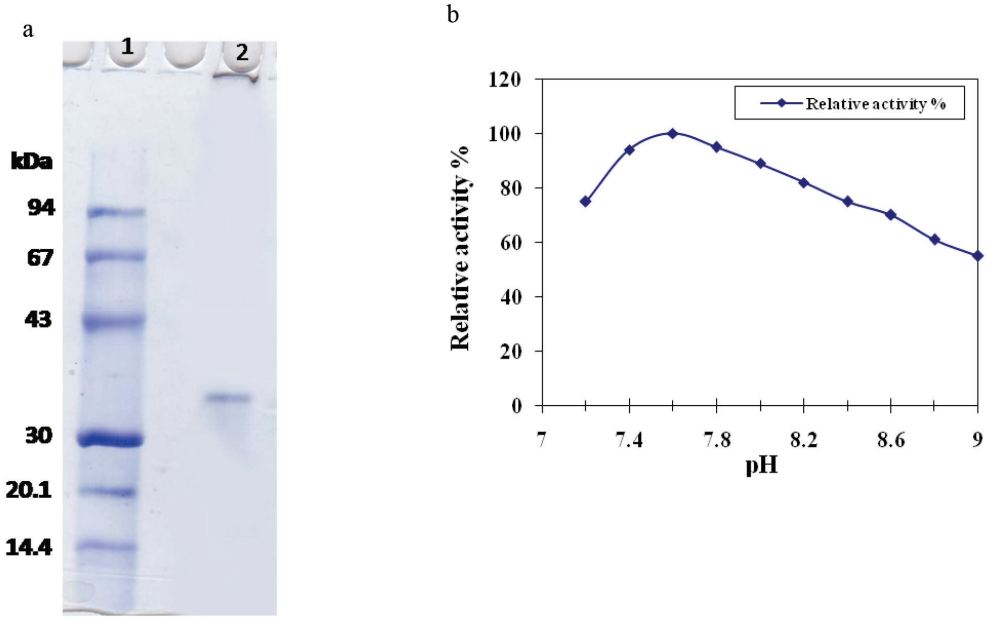

Figure 3. (a) Subunit molecular weight determination by Electrophoretic analysis of BLLDH on 12% SDS-PAGE (1) Low molecular weight marker proteins and (2) purified BLLDH. (b) Effect of pH on the purified buffalo liver lactate dehydrogenase (BLLDH) using phosphate buffer, pH 7.2–9.0.

Tris-HCl buffer, pH (7.0–9.0) since BLLDH enzyme manifested its optimum activity at pH 7.6 (Fig. 3b).

Influence of divalent cations and different inhibitors on BLLDH

The influence of metal cations on BLLDH activity was tested. $MgCl_2$ and $ZnCl_2$ elevated the activity of BLLDH, while $FeCl_2$, $CuCl_2$, and $NiCl_2$ minimized it (Table 2). The BLLDH inhibition was performed by pre-incubating inhibitors with BLLDH at 37˚C for 5 minutes and inhibition percent was derived as a rate of a non-inhibited specimen. SDS and N-ethylmaleimide inhibited the purified enzyme vigorously (Table 3).

Comparison of the constructed AST and ALT kits with commercially available kits

BLLDH is employed in constructing the ALT diagnosis kit. Also, BLLDH and the purified sheep liver malate dehydrogenase SLMDH previously purified in our lab (Darwish *et al*., 2018) are employed in constructing the AST diagnosis kit. The manufactured kits have been compared with commercially

Table 2. Effect of divalent cations on the purified BLLDH.

Cation	Concentration (mM)	Residual activity (%) of BLLDH
Control	---	100.0
$CoCl_2$	2	101.5
	5	85.0
$CaCl_2$	2	89.6
	5	91.3
$FeCl_2$	2	52.4
	5	1.7
$ZnCl_2$	2	106.5
	5	135.0
$CuCl_2$	2	74.1
	5	35.9
$NiCl_2$	2	82.0
	5	61.6
$MgCl_2$	2	110.4
	5	110.2

Table 3. Effect of inhibitors on the purified BLLDH.

Inhibitor	Concentration (mM)	Inhibition (%) of BLLDH
Control	—	0.0
EDTA	5	0.0
NaN_3	5	18.7
SDS	5	100.0
β-Mercaptoethanol	5	0.0
DTT	5	0.0
PMSF	5	49.2
KCN	5	9.7
Iodoacetamide	5	36.9
N-Ethylmaleimide	5	78.9

available kits utilizing 30 different individual samples (Tables 4 and 5).

DISCUSSION

LDH enzyme can be considered as a bio-indicator for liver, muscular, and cancer troubles (Rao *et al*., 2017; Zheng *et al*., 2017). LDH and MDH enzymes are predominant constituents of AST and ALT diagnosis kits, which are used as liver function biomarkers (Darwish *et al*., 2018; Huijgen *et al*., 1997). In this study, the purification process of buffalo liver LDH (BLLDH) included crude extraction, $(NH_4)_2SO_4$ fractionation, DEAE-cellulose column, and Sephacryl S-300 column. This purification method is considered to be appropriate and proportionally short and consists only of two chromatographic steps. Diverse LDH purification processes were reported, LDH from liver of *Uromastix hardwickii* (Javed *et al*., 1995), LDH from yak *Hypoderma sinense* larva (Li *et al*., 2013), LDH from pig heart (Karamanos, 2014), LDH from heart ventricles of river buffalo (Nadeem *et al*., 2011), and LDH from bovine heart (Labrou and Clonis, 1995). The

Table 4. Comparison of the constructed AST kit with a commercially available kit.

Serum samples	AST (GOT) (U/L)	
	Constructed GOT Kit	Commercial GOT Kit
1	18.7	19.4
2	14.2	16.7
3	11.3	10.8
4	16.9	17.4
5	23.0	24.1
6	53.7	55.2
7	350.9	345.2
8	49.3	46.1
9	86.8	87.3
10	33.9	38.1
11	177.3	174.6
12	48.9	45.6
13	108.2	101.9
14	692.4	699.5
15	51.2	55.9
16	128.5	122.2
17	66.7	69.5
18	403.1	408.2
19	59.3	55.3
20	94.4	91.2
21	31.6	34.5
22	57.5	56.8
23	120.0	117.2
24	75.8	77.4
25	42.2	46.6
26	77.4	80.1
27	222.7	219.5
28	38.4	31.6
29	72.6	75.5
30	64.4	61.2

BLLDH enzyme obtained from the gel filtration column with 17.6 units/mg protein specific activity, 16 purification folds, and 32% output (Table 1). Large diversity of LDH purification folds and yield ratios were notified. LDH of *H. sinense* larva by 280-folds and 195 U/mg protein (Li *et al*., 2013), LDH of pig heart by 54.96 folds and 26.93 U/mg protein (Karamanos, 2014), and LDH of river buffalo heart ventricles with 48-fold and 410 U/mg protein (Nadeem *et al*., 2011).

In this study, the BLLDH pureness was inspected on 7% native-PAGE that manipulated single protein band matched enzyme activity band supporting homogeneity and pureness of BLLDH (Fig. 2). The intact molecular weight of BLLDH obtained from gel filtration was established as 35 kDa. The molecular mass of BLLDH isoenzyme was affirmed by SDS-PAGE revealing that BLLDH is a monomer 35 kDa protein (Fig. 3a). Consistent with BLLDH, LDH from the liver of *U. hardwickii* is a monomer protein of 34 kDa (Javed *et al*., 1995), from *H. sinense* larva is 36 kDa (Li *et al*., 2013), from pig heart is a monomer protein of 36

Table 5. Comparison of the constructed ALT kit with a commercially available kit.

Serum samples	ALT (GPT) (U/L)	
	Constructed GPT kit	Commercial GPT kit
1	11.1	14.6
2	29.7	26.2
3	20.4	23.3
4	18.6	19.2
5	15.1	16.3
6	44.8	48.8
7	36.1	40.7
8	32.0	29.3
9	88.5	91.4
10	34.9	37.8
11	51.8	58.8
12	127.4	131.5
13	42.5	47.1
14	65.2	60.5
15	112.3	105.3
16	30.1	27.4
17	150.2	148.4
18	57.0	61.1
19	70.4	66.3
20	198.3	205.5
21	28.5	22.2
22	41.32	47.25
23	93.7	99.5
24	76.8	72.2
25	305.6	297.4
26	33.2	35.1
27	169.3	174.2
28	430.0	422.5
29	25.0	22.9
30	35.6	39.7

kDa (Karamanos, 2014), and from river buffalo heart ventricles is 36 ± 2 kDa (Nadeem *et al*., 2011). BLLDH showed its maximum activity at pH 7.6 (Fig. 3b) consistent with optimum pH of LDH from the liver of *U. hardwickii* at pH 7.5 (Javed *et al*., 1995), while river buffalo heart ventricles LDH was at pH 7 (Nadeem, et al., 2011). In this study, $MgCl_2$ and $ZnCl_2$ increased the activity of the purified BLLDH. $FeCl_2$ was a potent inhibitor of BLLDH activity while $CuCl_2$ and $NiCl_2$ inhibited BLLDH activity moderately (Table 2). Consistent with this, Cu^{2+}, Co^{2+}, and Mn^{2+} have shown inhibitory effects toward LDH from the liver of *U. hardwickii* (Javed *et al*., 1995). SDS was the potent inhibitor for the purified BLLDH which is also inhibited by PMSF suggesting that BLLDH active site contains a serine residue. BLLDH was inhibited by iodoacetamide indicating the role of methionine, cysteine, and histidine residues on structure and activity of the enzyme. N-Ethylmaleimide inhibited BLLDH indicating the presence of -SH group in enzymatic action (Table 3).

Both AST and ALT diagnosis kits are used to observe the liver job to follow the diverse hepatic diseases (Darwish *et al*., 2018; Zheng *et al*., 2017). Here, BLLDH is employed in the manufacturing of ALT diagnosis kit. Also, BLLDH and the previously purified sheep liver malate dehydrogenase SLMDH (Darwish *et al*., 2018) are utilized in constructing the AST diagnosis kit. The two manufactured kits were found sensitive when compared to ready trade kits. The distinction between structured and trade kits was noticed somewhat within the empirical error.

CONCLUSION

This study presents the first description of BLLDH. It was purified with simple and suitable purification steps from local source contain a large amount of the enzyme. Both BLLDH and SLMDH enzymes were included in the construction of ALT and AST diagnostic kits. The two prepared kits were found sensitive, accurate, and competent with the purchased kits. Production of these two kits on a large scale for commercial use will be our task for the future.

CONFLICTS OF INTEREST

The authors state that there are no conflicts of interest.

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