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Biochemical Evaluation Of Selected Heavy Metal-Induced Inhibition On Chicken Liver Sorbitol Dehydrogenase

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Highlights:
- SDH
- Inhibition
- Heavy metal

Keywords:
- Polyol pathway,
- Sorbitoldehydrogenase,
- Chicken liver,
- Heavy metal,
- Enzyme inhibition

ABSTRACT:

Sorbitol dehydrogenase (SDH) serves as a critical enzyme in the polyol pathway, an alternative metabolic route diverging from glycolysis. In this study, SDH was extracted and purified from chicken liver using a two-step chromatographic procedure involving DEAE-Sephadex anion exchange chromatography followed by Sephadex G-100 gel filtration. Molecular characterization determined that the enzyme's subunit weight is approximately 41.4 kDa, while the native enzyme exists as a tetramer with a total molecular weight of 169 kDa, as confirmed by SDS-PAGE and gel filtration analyses. The enzyme exhibited peak catalytic activity under alkaline conditions, with an optimal pH of 9.0 and a temperature optimum of 50°C. The inhibitory impact of several heavy metal ions—including Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+}, Zn^{2+}, and Ni^{2+}—on SDH activity was evaluated. Among these, cadmium ions demonstrated the most potent inhibition, with an IC_{50} value of 0.006 mM. Further studies using Lineweaver-Burk plots showed that the inhibition values were 0.07033 ± 0.00287 mM for Cd^{2+}, 0.18033 ± 0.04879 mM for Pb^{2+}, and 2.112 ± 0.03716 mM for Zn^{2+}. The data unequivocally identify Cd^{2+} as the most effective inhibitor of sorbitol dehydrogenase (SDH) among the heavy metals analyzed, characterized by its remarkably low IC_{50} value.

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This study is derived from Musa AKKUŞ's Master's thesis.

INTRODUCTION

Enzymes facilitate biochemical reactions in living organisms, making the inhibition of enzymatic activity a critical regulatory mechanism within biological systems. Therefore, research on enzyme inhibition holds significant importance. Numerous pharmacologically active substances and toxic agents exert their effects by interfering with enzymatic functions, a process that also serves as a valuable approach for elucidating enzyme catalytic mechanisms and functional dynamics (Keha & Küfrevioğlu, 2005).

Heavy metals, defined as metals with densities exceeding 5 g/cm³, include more than 60 elements in this category (Kahvecioglu et al., 2009). Unlike many toxic substances of anthropogenic origin, heavy metals naturally occur in the Earth's crust and are persistent environmental pollutants, as they cannot be synthesized or broken down by human activity. When heavy metal enter the human body through contaminated food, water, or air, they tend to bioaccumulate, resulting in a condition known as "metal burden." This accumulation, amplified by bioconcentration in living organisms, is linked to various chronic and degenerative diseases. Moreover, heavy metals can disrupt essential biochemical processes—such as core metabolism and ATP production—by binding to functional protein groups and interfering with enzymatic reactions (Ozbolat et al., 2016).

Heavy metals are categorized as essential or non-essential based on their physiological roles and biological impact. Essential heavy metals, such as Fe, Cu, Zn, Ni, and Se, serve as enzymatic cofactors and are necessary at specific levels, but they can become toxic if their concentrations exceed certain limits (1–10 ppm). In contrast, non-essential metals, like mercury (Hg), cadmium (Cd), and lead (Pb), are associated with severe physiological and neurological toxicity, even at low concentrations (Jarup, 2003; Bliefert, 2004).

The polyol pathway is an alternative metabolic route that bypasses glycolysis and is initiated by aldose reductase (AR), which reduces glucose to sorbitol. Subsequently, sorbitol dehydrogenase (SDH) catalyzes the oxidation of sorbitol to fructose. This pathway becomes significantly activated under chronic hyperglycemia and plays a pivotal role in the progression of diabetes-related complications affecting ocular, neural, renal, and vascular tissues (Kinoshita et al., 1988; Demir et al., 2021; Sujayev et al., 2024; Akarova et al., 2024). The polyol pathway is considered a primary mechanism underlying cellular toxicity in diabetic hyperglycemia. Elevated blood glucose levels stimulate the pathway, resulting in increased intracellular sorbitol and fructose concentrations. SDH (EC 1.1.1.14), a zinc-dependent tetrameric enzyme belonging to the alcohol dehydrogenase family, is essential for regulating metabolic flux through this pathway (Alım & Beydemir, 2012).

Although SDH has been purified and characterized from various tissues, its instability has posed challenges in isolating the enzyme from mammalian sources. This instability has been consistently reported in purification studies (O'Brien et al., 1983; Karacaoğlan & Ozer, 2005; Kim, Lee, & Lee, 2016; Fouché, Tornquist, & Horney, 2021).

In diabetes, elevated SDH activity disrupts the cytosolic NAD^+/NADH balance, leading to oxidative stress and early microvascular and neural dysfunction (Yan et al., 2018). By converting sorbitol to fructose and generating NADH, SDH further exacerbates metabolic stress under hyperglycemic conditions. Inhibition of SDH has been shown to restore redox balance and mitigate hyperglycemia-induced impairments, including increased vascular permeability, reduced tissue perfusion, and delayed nerve conduction (Yang et al., 2025).

Moreover, SDH inhibition may enhance glucose oxidation and ATP production in ischemic cardiac tissue, indicating a potential cardioprotective effect (Caturano et al., 2023). These results

suggest that SDH is crucial in diabetes-related metabolic imbalance and may serve as a target to prevent early complications.

The primary aim of this study is to isolate sorbitol dehydrogenase (SDH) from chicken liver and to characterize its enzymatic properties. Furthermore, the study seeks to examine the inhibitory effects of selected heavy metals on SDH activity, providing valuable insights into how environmental or dietary exposure to these metals may impact enzyme function and potentially affect metabolic processes.

MATERIALS AND METHODS

Materials

Fresh chicken liver samples were obtained from a regional supplier under appropriate handling conditions. Chromatographic materials, including DEAE-Sephadex and Sephadex G-100, as well as D-sorbitol, β-NAD^+, and all reagents required for electrophoresis and protein quantification, were supplied by Sigma-Aldrich Chemical Co. Additionally, all other analytical-grade chemicals used throughout the experimental procedures were procured from the same manufacturer.

Procurement and preparation of the tissue

Initially, fresh chicken liver specimens were rinsed in an isotonic 0.09% sodium chloride solution to ensure complete removal of blood contaminants. The tissue was then minced into small pieces and homogenized under chilled conditions in a 50 mM Tris-HCl buffer (pH 7.6) supplemented with 2 mM β-mercaptoethanol, using a buffer-to-tissue ratio of 2 ml per gram to optimize extraction efficiency. The homogenate was filtered through filter paper to remove larger debris and centrifuged at 13000 rpm for 20 minutes. The resulting supernatant was collected and prepared for the subsequent purification stage

Protein precipitation using ammonium sulfate and dialysis

The chicken liver SDH enzyme was isolated by ammonium sulfate precipitation in the 35–60% saturation range, according to the method described by Karacaoğlan and Özer (2005). The precipitate was re-dissolved in a small volume of 50 mM Tris-HCl buffer (pH 7.6) containing 2 mM β-mercaptoethanol and dialyzed for 2 hours to remove low-molecular-weight contaminants. Upon completion of dialysis, the sample was subjected to ultracentrifugation at 100000 × g for 1 hour. The resulting supernatant was carefully collected and retained for subsequent analytical applications.

Enzyme activity assay

Absorbance changes resulting from NAD^+ reduction in the sorbitol-to-fructose reaction were monitored at 340 nm over a three-minute period, with measurements recorded every minute. The reaction mixture, prepared according to Linstad et al. (1992), consisted of 50 mM glycine/NaOH buffer (pH 9.9), 10 mM sorbitol, and 470 µM NAD^+ in a 1 mL quartz cuvette.

Elution through DEAE-Sephadex column and collection

Following ultracentrifugation, the enzyme-containing solution was applied to the column. As sorbitol dehydrogenase did not bind to the DEAE-Sephadex column, elution was carried out using 50 mM Tris-HCl buffer (pH 7.9), the same buffer employed for column equilibration. Fractions of 3 mL were collected systematically, and the enzymatic activity of each fraction was determined by measuring absorbance at 340 nm. Fractions exhibiting detectable activity were pooled together. The combined sample was then placed in a dialysis bag and dialyzed for two hours against 50 mM Tris-

HCl buffer (pH 7.9) containing 2 mM β-mercaptoethanol to remove low-molecular-weight contaminants

Enzyme purification and native molecular weight determination

Following dialysis, the enzyme sample was supplemented with 0.125 mL of glycerol to facilitate its passage through the column. The sample was then applied to a Sephadex G-100 gel filtration chromatography column pre-equilibrated and maintained with 50 mM Tris-HCl buffer at pH 7.5. Elution was performed using the same buffer, and the absorbance of each fraction was monitored at 340 nm. Fractions exhibiting significant enzymatic activity were identified and selectively pooled for further analysis.

The native molecular weight of the enzyme was determined by gel filtration chromatography using standard proteins with known molecular masses. During this procedure, Blue dextran (2,000 kDa) was first applied to the column to determine its void volume. Subsequently, the standard proteins β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa) were applied to the column in sequence. These standards were followed by the enzyme sample from chicken liver, each eluted in 2 mL volumes. Absorbances were measured at 280 nm, and the corresponding values were recorded and plotted against tube number. K_{av} values and log Mw values were calculated for each protein. The molecular weight of the native SDH enzyme was determined by constructing a plot of log Mw versus K_{av}.

Verification of enzyme purification by sodium-polyacrylamide gel electrophoresis (SDS-PAGE)

The enzyme purified from chicken liver was evaluated for purity using 3–8% SDS-PAGE, following the method described by Laemmli (1970). Molecular weight determination was performed with a panel of standard proteins, including myosin (200 kDa), β-galactosidase from *E. coli* (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). Following electrophoresis, R_f values for the standards were calculated, and a logarithmic calibration curve correlating molecular weight with R_f was was plotted. The R_f of the purified SDH enzyme was then measured, and its logarithmic molecular weight was extrapolated using the standard curve. The molecular mass of the SDH subunits was subsequently calculated by taking the antilogarithm of the inferred value.

Quantitative protein determination by the bradford method

Protein quantification of enzyme preparations obtained through sequential purification steps—including chicken liver homogenization, ammonium sulfate precipitation, DEAE-Sephadex ion-exchange chromatography, and gel filtration—was carried out using the Bradford assay, with bovine serum albumin employed as the calibration standard to ensure accuracy, in accordance with the method described by Bradford (1976).

Kinetic studies for SDH enzyme purified from chicken liver

Optimum temperature

Each enzyme exhibits a specific temperature at which its activity is maximized, known as its optimum temperature. In this study, enzyme activity was measured spectrophotometrically at 10°C intervals using a digital water bath to maintain precise temperature control. The optimum temperature of the enzyme was then determined based on these measurements.

Optimum pH

The optimal pH of SDH purified from chicken liver was determined by assessing enzyme activity across a broad pH range using three buffer systems: potassium phosphate (pH 5.0–8.0), Tris-

HCl (pH 7.5–9.0), and glycine–NaOH (pH 9.0–10.5). Buffers were prepared at 0.5-unit intervals and used in place of the assay buffer to accurately identify the pH at which SDH activity is maximal.

RESULTS AND DISCUSSION

The purification of chicken liver SDH enzyme in this study was accomplished in three stages: ammonium sulfate precipitation, DEAE-Sephadex anion exchange chromatography, and Sephadex G-100 gel filtration chromatography. By employing these techniques, the SDH enzyme was purified 266.95-fold from chicken liver, achieving a specific activity of 3.152 EU/mg and a yield of 3.65%. All purification steps were performed in a cold environment to prevent the loss of enzyme activity due to temperature. (Table 1).

Table 1. Purification Steps of Chicken Liver Sorbitol Dehydrogenase Enzyme

Purification Steps	Activity (EU/mL)	Total Volume (mL)	Protein (mg/mL)	Total Protein (Mg)	Total Activity (EU)	Spesific Activity (EU/mg)	Yield %	Purification Fold
Homogenate	0.468	29	39.5	1145.5	13.572	0.0118	100	1
$(NH_4)_2SO4$ Precipitation and Dialysis	0.571	8.5	29.6	251,6	4.85	0.0193	35.7	1.64
Deae-Sephadex A-50	0.300	14	0.372	5.208	4.2	0.806	30.98	68.3
Gel filtration (Sephadex G-100)	0.198	2.5	0.063	0.157	0.495	3.152	3.65	266.95

SDS-PAGE analysis showed that the purified enzyme produced a single clear protein band, indicating that it was isolated in a pure form. The monomeric molecular weight of SDH obtained from chicken liver was determined to be approximately 41.4 kDa. In addition, using the Sephadex G-100 gel filtration chromatography method, the native molecular weight of the enzyme was calculated to be around 169 kDa (Figure 1,2).

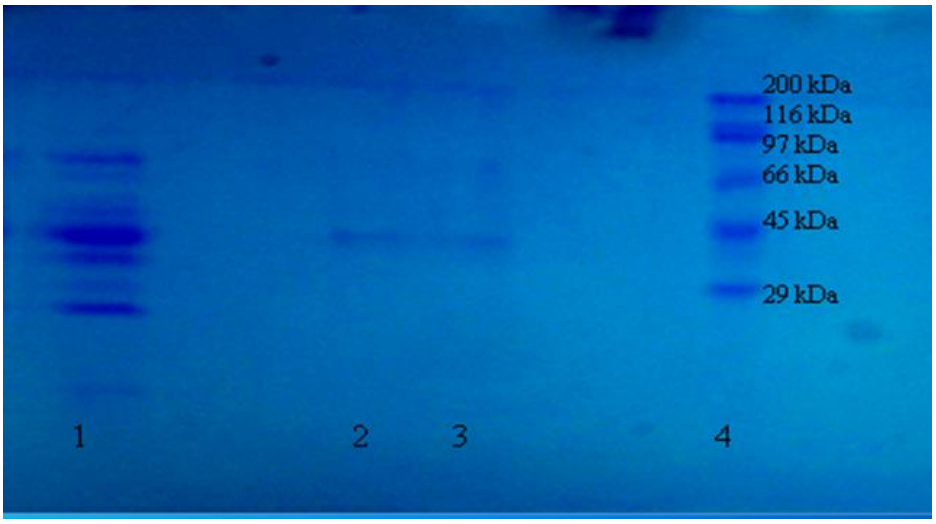

Figure 1. SDS-PAGE profile of chicken liver sorbitol dehydrogenase
(Lane 4 included standard proteins, while lane :1 DEAE, lane: 2,3 Sephadex G-100)

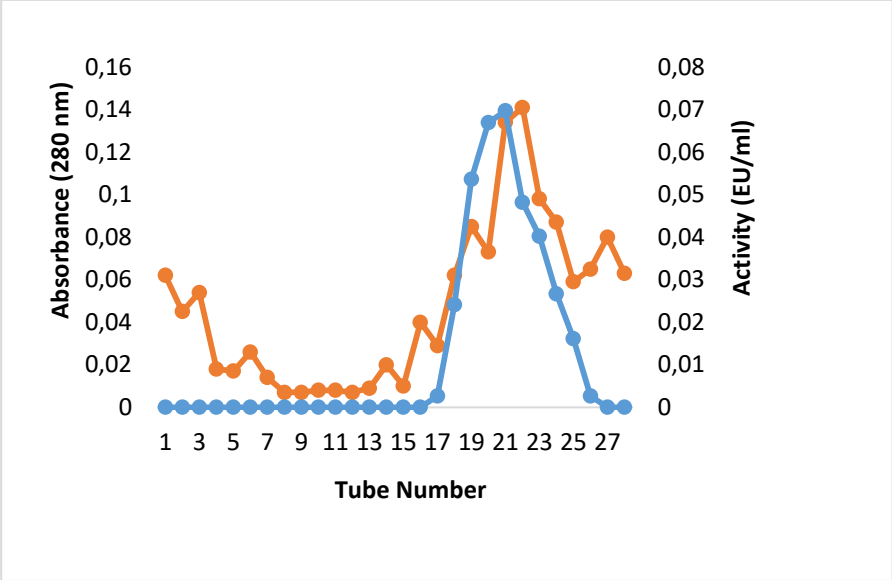

Figure 2. Sephadex G-100 gel filtration results of chicken liver SDH enzyme

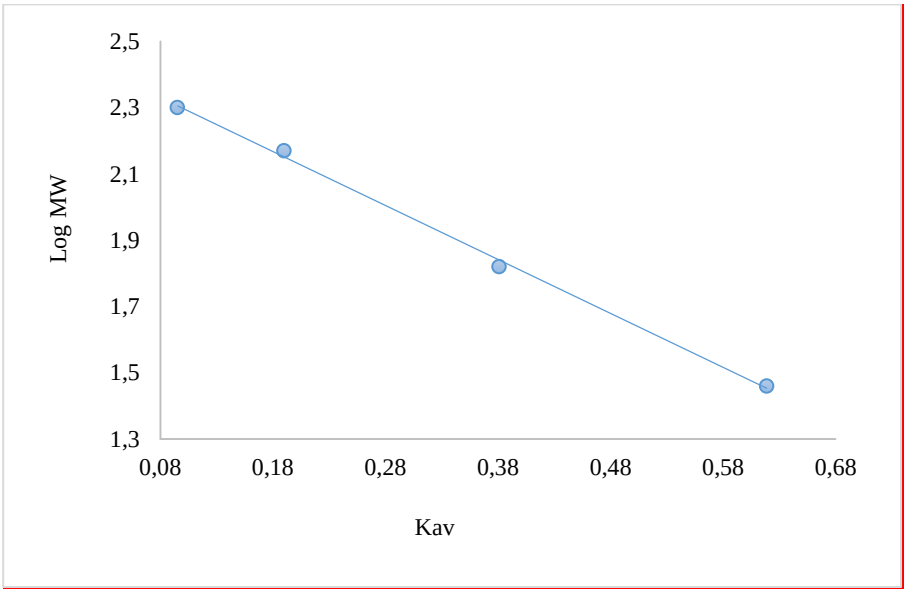

Figure 3. Standard Sephadex G-100 gel filtration graph showing the K_{av} value for SDH (K_{av}: 0.1428)

Assessment of selected heavy metal ions revealed a pronounced inhibitory effect on the activity of SDH isolated from chicken liver, indicating that SDH is highly sensitive to metal-induced inhibition (Table 2).

Table 2. Calculated K_i Values and Inhibition Types of SDH Enzyme Purified From Chicken Liver

Compound	IC_{50} (mM)	Average K_i (mM)	K_i (mM)	Inhibition type
$Pb(NO_3)_2$	0.006	0.18033 ± 0.04879		Competitive
$Hg(NO_3)_2 \cdot H_2O$	0.013	0.07033 ± 0.00287		Competitive
$Zn(NO_3)_2 \cdot 6H_2O$	7.07	2.112 ± 0.037	16.013 ± 1.043	Mixed type

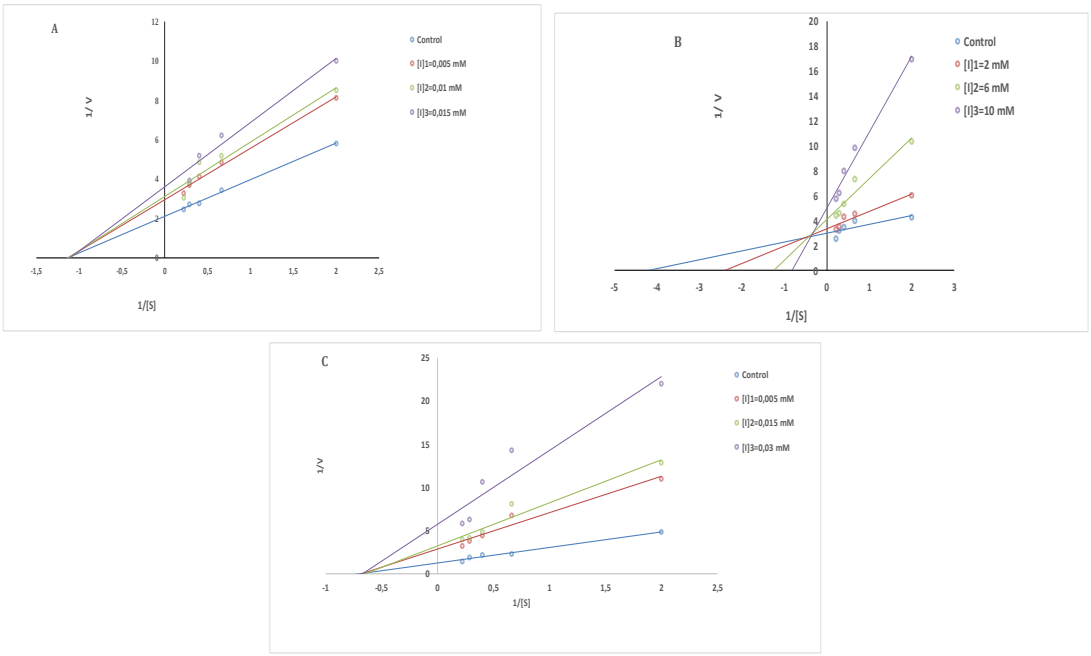

Figure 2. K_i plots of Hg^{2+}(A), Pb^{2+}(B) and Zn^{2+}(C) ions showing the inhibitory effect on the SDH enzyme from chicken liver, respectively.

Recent studies indicate that overactivation of the polyol pathway during chronic hyperglycemia contributes significantly to oxidative stress. SDH, the pathway's second enzyme, converts sorbitol into fructose while producing NADH, which reduces NADPH levels and weakens antioxidant defenses. Inhibiting SDH may therefore help restore redox balance and slow the development of diabetic cardiovascular complications (Garg & Gupta, 2022).

Accordingly, considerable efforts have been devoted to the discovery of selective and potent SDH inhibitors. Recent research has identified both natural and synthetic compounds capable of effectively suppressing SDH activity (Ahmad, Khan, & Ali, 2024; Zhu et al., 2023).

The role of SDH in regulating sorbitol levels was investigated in JS1 Schwannoma cells under osmotic stress, with SDH inhibition applied. The results demonstrated that SDH inhibition significantly increased sorbitol concentrations as a consequence of sustained hyperglycemia and osmotic imbalance, indicating that SDH plays a critical role in maintaining sorbitol homeostasis within JS1 cells (Mizisin et al., 1997).

A study by Zhang et al. (2015) reported that treatment with the SDH inhibitor CP-470,711 significantly reduced ischemia/reperfusion-induced liver injury in mice. The inhibitor attenuated histopathological damage, decreased hepatocyte apoptosis and necrosis, and preserved cellular ATP and NAD(H) levels. Moreover, it upregulated SIRT1 protein expression while downregulating caspase-3 activation, indicating a protective effect mediated through enhanced SIRT1 signaling and maintenance of cellular energy homeostasis (Zhang et al., 2015).

Ongoing studies on SDH inhibition may provide valuable insights for structural analyses aimed at understanding the interactions between substrate-like inhibitors and the SDH-NAD^+ complex, including binding at the enzyme's active site. Furthermore, the development of potent and highly specific SDH inhibitors will help clarify the physiological role of this enzyme at the cellular level (Linstad & McKinley-McKee, 1996; Alım et al., 2012; Kim et al., 2016).

Heavy metals occur naturally in ecosystems and enter soil, rivers, and seas through environmental processes. They can accumulate in living organisms via food, water, or respiration and are difficult to eliminate from the body. Studies have demonstrated that heavy metals induce adverse

metabolic effects; therefore, investigating their impact on enzyme activities remains a crucial area of research (Söyüt & Beydemir, 2008; Ekinci et al., 2008, 2014)

The inhibitory effects of Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+}, Zn^{2+}, and Ni^{2+} ions on SDH purified from chicken liver were evaluated. The IC_{50} values were determined as follows: 0.006 mM for Cd^{2+}, 0.012 mM for Pb^{2+}, 0.013 mM for Hg^{2+}, 0.018 mM for Ag^{+}, 7.07 mM for Zn^{2+}, and 35 mM for Ni^{2+}. Among the tested metals, Cd^{2+} exhibited the strongest inhibitory effect on SDH, as indicated by its lowest IC_{50} value.

Additionally, the inhibition mechanisms of Hg^{2+}, Pb^{2+}, and Zn^{2+} were investigated using Lineweaver-Burk plots, yielding inhibition constants (K_i) of 0.07033 ± 0.00287 mM (Hg^{2+}), 0.18033 ± 0.04879 mM (Pb^{2+}), and 2.112 ± 0.03716 mM (Zn^{2+}). Hg^{2+} and Pb^{2+} ions exhibited non-competitive inhibition of SDH, whereas Zn^{2+} showed mixed-type inhibition. Finally, the optimal conditions for SDH activity purified from chicken liver were determined, with maximal enzyme activity observed at pH 9.0 and 50°C.

CONCLUSION

Environmental heavy metals can significantly affect the structure and function of biomolecules, particularly enzymes and polynucleotides, either by displacing essential metal ions or disrupting biomembrane integrity. Our results show that several of the tested heavy metals act as potent SDH inhibitors even at low micromolar concentrations, highlighting their potential to modulate enzymatic activity and impact health. Notably, previous studies suggest that SDH inhibition under hyperglycemic conditions may help delay diabetes-related complications, emphasizing the enzyme's therapeutic relevance. This study is limited by its in vitro design, the small number of metals and concentrations tested, and the lack of assessment of long-term exposure or metabolic factors affecting metal bioavailability. Future research should validate these findings in in vivo models, explore a broader range of metals and concentrations, and investigate the molecular mechanisms of SDH inhibition. Such studies will improve our understanding of metal–enzyme interactions, support the development of targeted SDH modulators, and contribute to protecting both human health and ecosystem integrity.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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