PURIFICATION OF RECOMBINANT HSP70 BY IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC) AND ITS ROLE IN IMMUNE ENHANCEMENT AND ANTIOXIDANT ENZYME PROTECTION

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ABSTRACT

By utilizing Ni-NTA affinity chromatography, the expressed recombinant HSP70 protein was purified, and the SDS-PAGE analysis was used to assess its purity. Purified recombinant HSP70 was used to immunize BALB/c mice, and this had a considerable impact on the anti-HSP70 antibody titers. The results of this study show that the adjuvanted hsp70 injected into the mice had a good immunogenic property. In this study, we measured the effects of HSP70 on catalase stability after exposure it to different range of temperature (40, 45, 50, 55, 60) ˚C, and found that the catalase kept its remaining activity% (99% and 17%) when incubating it in 40 and 60ºC respectively in the present of HSP70, compared to its remaining activity % without HSP70 which were 95%, 10% respectively. Also noticing that the enzyme kept 90% of its remaining activity % when incubating it with buffer at pH 8.0 and 95% of it at pH 6.0 comparing with its remaining activities % (81%, 90%) respectively without HSP70. while the activity was completely eliminated in pH 2.0. Also studied the inhibition effect of some heavy-metals with different concentrations (10, 20,30mg/L) on catalase activity with and without HSP70.

Keywords: Heat shock protein 70 (HSP70); Immunogenicity; Catalase; Enzyme activity.

تنقية بروتين الصدمة الحرارية المؤتلف 70 بواسطة كروماتوغرافيا تقارب المعادن الثابتة ودوره في تحسين المناعة وحماية انزيمات الاكسدة

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المستخلص

نقي بروتين الصدمة الحرارية (HSP70) بواسطة كروموتوغرافيا الألفة (Ni-NTA affinity chromatography), وحددت نقاوتة بأستخدام الترحيل الكهربائي بهلام الاكريل امايد SDS-PAGE. ودرست قابلية بروتينات الصدمة الحرارية HSP70 في تمنيع الحيوانات المختبرية بصورة جيدة ولوحظ زيادة في مستويات المستضدات عند تمنيع فئران الـBALB/c بالبروتين المعبر النقي خاصة عند أضافتة كمعقد من (HSP70+Adjuvant). لوحظ أيضاً تأثير بروتين الصدمة الحرارية قيد الدراسة على ثباتية انزيم الكاتاليز catalase عند حضنة بدرجات حرارة مختلفة (40-60) مº. حيث وجد ان الانزيم قد احتفظ بفعاليتة المتبقية عند حضنه بدرجتي 40 و 60 مº لمدة 30 دقيقة حيث كانت 99 % و17 % بالترتيب بوجود بروتين الصدمة الحرارية HSP70 مقارنة مع فعاليتة المتبقية 95% و10% عند حضن الأنزيم بمفرده بالدرجات الحرارية قيد الدراسة. كذلك لوحظ أحتفاظ الأنزيم بحوالي 90% من فعاليتة المتبقية % عند حضنة بوجود بروتين الصدمة الحرارية HSP70 مع داري بالرقم الهيدروجيني 8.0 كما احتفظ الانزيم ب95% من فعاليتة المتبقية % عند حضنة مع داري بالرقم الهيدروجيني 6.0 وبوجود بروتين الصدمة الحرارية مقارنة مع الانزيم بمفردة والتي كانت فعاليتة المتبقة % 81% و 90% فقط على التوالي. بينما اختفت الفعالية كلياً عند الرقم الهيدروجيني (2.0). لوحظ أيضاً التأثير المثبط لبعض المعادن الثقيلة وبتراكيزمختلفة (10, 20, 30) ملغم/لتر على فعالية أنزيم الكاتاليز الحرومع وجود بروتينات الصدمة الحرارية HSP70.

كلمات مفتاحية: بروتين الصدمة الحرارية. المناعة. الكاتاليز. الفعالية الانزيمية.

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INTRODUCTION

When normal cells are exposed to heat, they begin to synthesize stress proteins, such as heat shock proteins (HSPs), which function as chaperones. As a result, they are referred to as chaperones (1, 3). Hsps are primarily categorized according to the sizes of their molecules. (22, 27). They often belong to seven large families. DnaK can be purified using well-known techniques, however preparation quality is in dispute. A purification method known as "affinity chromatography" hinges primarily on a very particular interaction between the purge-target molecule and the solid phase that will separate the impurities. DnaK preparations are typically regarded as pure if just one band can be seen on an SDS-PAGE. These formulations aren't particularly pure though. However, because Hsp are molecular chaperones that can interact with a variety of proteins, isolating a "pure" Hsp may be problematic because they usually appear paired with other heat shock and non-heat shock proteins. In the past, conflicting information has been presented on the immunological characteristics of HSP, particularly DnaK. It facilitates the processing of antigens by the class I or class II Major Histocompatibility Complex (MHC) on antigen-presenting cells. (29). Affinity chromatography (also called affinity purification) makes use of specific binding interactions between molecules (30). The majority of Hsps are stress-induced, making them crucial disease indicators (15). The majority of Hsp70s are released into the extracellular environment from inside the cell (6). Mustafa 2019 investigate the effects of Eucalyptus Camaldulensis leaves powder (EUP) and oil (EUO) to alleviate cold stress and their effects on oxidative index expression levels of heat shock protein (hsp: 40, 70, and 90). Hsp70 also makes it easier for antigen to reach T cells (25). Furthermore, the circulatory system of the host has been shown to contain parasite Hsps and antibodies that detect them (20) Thus, Hsps of both host and parasite origin that end up in the host circulatory system eventually interact with immune cells to influence their function. Because of their great conservation, Hsps are expected to play a role not only in host immune regulation but also in the development of autoimmune diseases. As a result of their secondary responsibilities outside their primary functions as molecular chaperones, Hsps are thought to be moonlighting molecules. Hsps are implicated in a variety of diseases, owing to their increased interest as immunological modulators (7). Hsps are engaged as a defense mechanism against damaging stimuli as part of the stress response (16). Increased generation of reactive oxygen species (ROS) causes oxidative stress, which raises the risk of chronic diseases such as diabetes, cancer, neurological disease, and liver cirrhosis (8). Kun-peng Fan et al., 2021 analyzed the expression levels of TrHSP70 under different temperatures, three experimental groups were established: H1 (20 °C), H2 (24 °C), and H3 (28 °C). Expression analysis revealed that temperature stress significantly affected the mRNA levels of the HSP genes. Specifically, TrHSP70 expression peaked at 12 h in the H1 group and at 48 h in the H3 group (17). To safeguard cells from oxidative stress, living things have developed an antioxidant defense system that includes catalase, superoxide dismutase (SOD), glutathione synthase (GST), and glutathione peroxidase (GPX) (31)(28). An indication of oxidative stress and a strong ability to counteract it by removing ROS from cells is the substantial rise in the activities of antioxidant enzymes (34). According to Miao et al., the LbHsp70s and LbHsp110 genes are involved in Liposcelis bostrychophila's tolerance to thermal stress, and antioxidant enzymes and heat shock proteins may work in concert to increase psocids' tolerance to thermal stress (19). According to Humam et al. (14), adding various postbiotics to the feed of broilers increases the expression of acute phase proteins, HSP70, and overall antioxidant capacity while also reducing the negative effects of heat stress. The ultimate goal was to test recombinant HSP70 in BALB/c mice as a potential vaccination candidate and as a defense mechanism for the antioxidant enzyme catalase against many stressors.

MATERIALS AND METHODS

Unless, specified chemical reagents used in this study including the complete Freund's Adjuvant (CFA), incomplete Freund's Adjuvant (ICFA) were purchased from Hi-

media/India, catalase enzyme from Merck limited / India, Nickel NTA resin and column was purchased from Merck limited/India,

Animal: Male BALB/c mice were obtained from the Center for Cellular and Molecular Biology (CCMB) Hyderabad, India.

Purification of recombinant protein: Inoculate Bl21culture containing the recombinant plasmid (pBAD TOPO vector) in 20ml of LB broth containing ampicillin overnight at 37°C, agitated at 200 rpm. Next day reinoculated the overnight grown culture in 1000ml LB broth containing ampicillin until O.D reaches 0.4-0.5 at 37 °C, 200 rpm. The recombinant culture was established, and the optimal concentration of IPTG was used to trigger expression for 5hr. The cells were harvested by Centrifugation at 6000rpm for 20min, and cooled on ice. Carefully discard the supernatant and cell pellet was resuspended in Lysis buffer pH 8.0 (50mM tris HCL, 500mM Nacl, 0.05% Triton X). The cells suspended in lysis buffer were allowed to thaw on ice for 1 hr. Sonicate the dissolved pellet very carefully for 20min. The cell lysate was centrifuged at 6000rpm for 30minutes at 4°C to pellet the cellular debris. Add nickel beads to the sonicated solution and keep if for rocking at 4°C for 3hr. Take the column purifier and wash with distilled water and add the nickel beads along with solution to the column slowly by inverting it and place at 4°CFollowing the manufacturer's instructions, protein was purified using a HisPur nickel-charged nitrilotriacetic acid (Ni-NTA) immobilized metal affinity chromatography column (IMAC). Purity of the recombinant protein was analyzed on 10% SDS-PAGE and the protein concentration was measured by Nanodrop2000 reading (35).

Investigation of immunological enhancement of recombinant HSP70: Ten male BALB/c Mice weighted 25–28g, 7–8 weeks, used in this study, five of them used as control and 5 of which were vaccinated. Purified recombinant HSP70 was filtered through 0.22µm filters and mixed with complete Freund's adjuvant at the rate of (40µg/dose) for mouse. Blending equal amounts of HSP70 and CFA by using Silverson L5M-A mixer set at 12000 rpm in a small vial dipped into crushed ice, the adjuvanted hsp70 volume that contain (40µg) of the hsp70 was adjusted to be in 100µl of the injected material per each mouse, the injection was subcutaneously with recombinant protein70 with complete Freund's adjuvant on day 1. A booster dose was given with incomplete Freund's adjuvant (ICFA) after (14^{th} day) and the second booster dose was given with incomplete Freund's adjuvant (ICFA) after (28^{th} day). Sera were collected after (42^{th} day). ELISA method used to measure of HSP70 antibody, 96 well plates covered with 100µl antigen mixture (specified concentration of antigen in 50mM carbonate–bicarbonate buffer, pH 9.6) for overnight at 4°C. washed the wells three times with PBST (PBS containing 0.05% (v/v) Tween20), and then blocked with 1% fetal bovine serum buffer (FBS) at 37°C for 1hr. and repeat the washing cycle again. 0.2ml of 1:100 dilution of the serum of mice were added in triplicate and again incubate the plate for another 2 hr. at 37ºC, after that the plate was washed three times with PBS tween20 solution before adding an alkaline phosphate-tagged anti mouse IgG antibody (1:1000) dilution with PTB buffer, then incubated the plate at 37ºC for 2hr. and repeat the washing with PBS tween20. Add 0.2ml of p-nitrophenol (1mg/ml) to each well and incubate the plate for 15min. at room temperature, then stopped the reaction with 50µl of 1M NaOH solution. And then read the plate at 450nm in a Thermomax ELISA (24).

protection properties of HSP70 of enzymes stability (Catalase): The catalase (CAT) activity was determined by Beers & Sizer method (4) using pure enzyme supplied as a crystalline suspension by (Merck limited / India). Protein concentrations were determined according to the Bradford method (5), with bovine serum albumin as the standard. By detecting the drop in absorbance at 240 nm brought on by H_2O_2 breakdown, CAT activity was ascertained. The amount of H_2O_2 that is decomposed at a rate of 1 mol per minute at pH 7.0 and 25°C, while the H_2O_2 concentration decreases from 10.3 mM to 9.2 mM, is considered to be one unit of CAT activity. The rate of decline in absorbance at 240 nm is used to track the pace of disappearance of H_2O_2.

Thermal stress: Because the human body also performs best at 37°C, it is hypothesized that 37°C will be the ideal temperature where catalase activity will occur at the fastest pace. therefore, different ranges of temperatures including (40, 45, 50, 55, 60) ˚C keeping the 37ºC as control were used to measure the effect of increasing temperature on catalase stability, following the same procedure recommended by Beers & Sizer (4), incubate the enzyme in multiple thermal range for 30minute, cool it down to reach 25ºC and read the catalase activity each. Then using the HSP70 which incubated with enzyme, repeat the procedure to measure the differences between the remaining activity (100%) with and without HSP70.

pH effect: Each enzyme stabilizes bests at a specific pH value. To investigate the direct effect of pH on enzyme stability, the enzyme was incubated in different range of pH values (2.0, 4.0, 6.0, 8.0, 10.0) for potassium phosphate buffer, keeping pH 7.0 as control for 30minute to estimate the changes of enzyme stability for catalase. And again calculated the differences between the remaining activity% with and without HSP70 presence.

Heavy metal exposure: To study the effects of heavy metals exposure on catalase stability, we exposed six metals (manganese, cadmium, copper, zinc, Iron, and nickel) to catalase solution. All the chemicals were purchased from Sigma (Sigma-Aldrich, Inc., commercial supplier- India), the effect of heavy metals on catalase stability was determined spectrophotometry by using Phosphate buffer (50mM, pH 7.0excluding copper, cadmium, and zinc tests since those elements accumulated in phosphate buffer. Imidazole-HCI buffer (0.2M, pH 7.0) was used for the test with copper and zinc, while Tris-HCI buffer was used for the assay with cadmium and lead (0.1M, pH 7.0). The heavy metal ions supplied to the buffer for in vitro experiments were salts of cadmium chloride, copper chloride, ferric chloride, manganese chloride, nickel chloride, and zinc sulphate at varying concentrations (10, 20, and 30 mg/L) (26). The control enzyme activity was the untreated enzyme, the relation between the concentrations of heavy metals and remaining enzyme activity % was drawn to determine the effect of heavy metals on catalase stability and the remain activity % was calculated. 1ml of HSP70 was added to the 1ml catalase solution, then 1ml of that solution added to the second 3 set of tubes with a ratio of 1:1 (V/V), incubated for 30min. at room temperature and the remaining activity% was calculated.

RESULTS AND DISCUSSION

For the production of recombinant *DnaK, E. coli*, was selected and the *DnaK* gene that was cloned in the pBAD expression vector, which produced C-terminal 6xHis fusion protein with high yield was purified by using Ni-NTA super flow column chromatography (affinity chromatography). The resulted purified protein was analyzed on 10% SDS-PAGE and showed high purity of the recombinant protein for the last 4 elution fractions by using the elution buffer pH 6.0 (50mM tris HCL, 500mM Nacl, 300mM imidazole) figure (1).

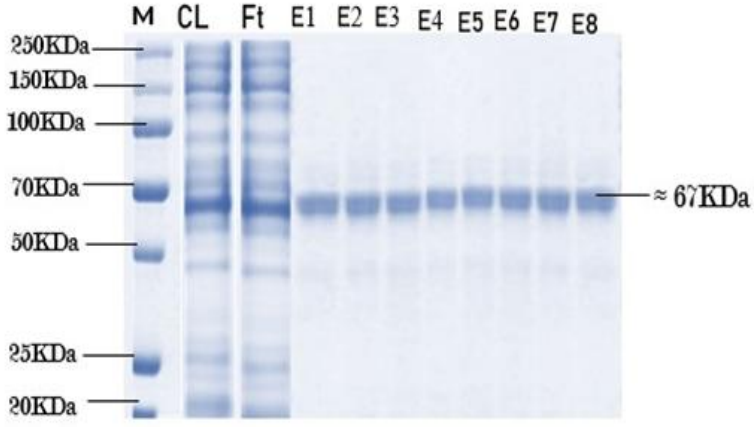


Figure1. SDS-PAGE (10% w/v) analysis of the optimum IPTG concentration (0.05mM) used to induced the expression of the recombinant *DnaK* purification by using Ni-NTA (affinity chromatography) at 100 volts, for 1hr

The total protein concentration of HSP70 was determined by spectrophotometry with NP-2000 the concentration of protein was 9.7mg/ml figure (2)

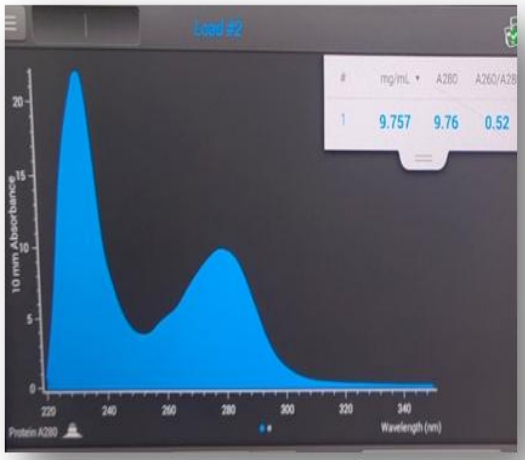


Figure 2. protein peak in Nanodrop2000 spectrophotometer showing the HSP70 concentration of the purified recombinant *DnaK* protein by using Ni-NTA super flow column affinity chromatography. In terms of absorbance, there are two peaks, one at 230 nm and the other at 280 nm. The peak at 230 nm is caused by peptide bond absorption, while the peak at 280 nm is caused by the absorbance of aromatic amino acid rings (tryptophan, tyrosine, and phenylalanine).

A quick and flexible method for affinity purifying recombinant proteins and antigenic peptides is the 6xHis NI-NTA system. It is based on the extremely selective contact that results from the high affinity binding of six consecutive histidine residues (the 6xHis tag) to immobilized nickel ions. This interaction enables the purification of tagged proteins. (18). Under rigorous circumstances, impurities can be rapidly removed thanks to the tag's strong bond with the resin, but the attached proteins may be softly released by competition with imidazole or a minor drop in pH. In order to improve binding to the Ni-NTA matrix and increase the effectiveness of the purification process by minimizing the possibility of nonspecific binding, the 6xHis-tag on a protein will be fully exposed during denaturing conditions. (11). Recently, further investigators used the 6xHis-tag at the C or N-terminal of the protein of interest in order to purify the recombinant protein to homogeneity by one step using the Ni-NTA affinity chromatography because this 6xHis-tag dose not interfere with structure or function of the purified protein and its poorly immunogenic. This purification technique used to purify HSP70from S. Typhi also. And the gene was cloned and expressed in E. coli BL21 and purified by affinity chromatography (21).

HSP70-specifi antibodies measurement in vaccinated mice sera by ELISA

The results of ELISA tests during 6 weeks of vaccination shown in (**figure 3**). the points represent the mean triplicate values from the 5 vaccinated mice, which where (0.387) for the first dose and (0.478) for the second and (0.790) for the last one. The trend suggests a progressive rise in the titer of the anti-hsp70 antibodies to a significant level by the end of the vaccination periods (6 weeks) this shows that the adjuvanted hsp70 injected into the mice had a good immunogenic property. Also there was no results of any significant level of the anti-hsp70 antibodies in the sera taken from the control mice.

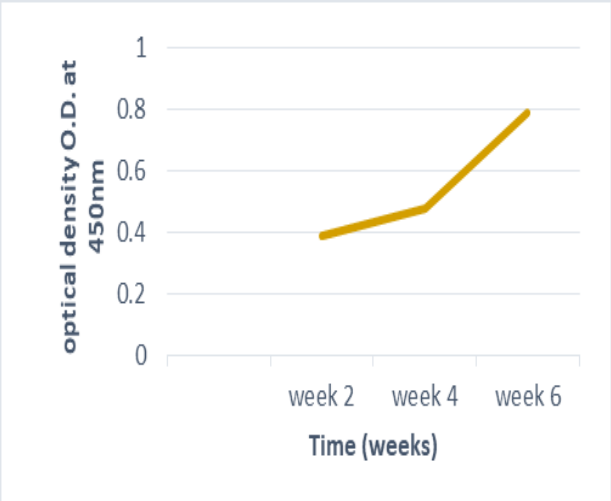


Figure 3. The immune response time curse study of BALB/c mice immunized with 40µg HSP70 at 2 weeks' intervals, each point represents the mean of 5 mice IgG sera measured by ELISA

Several similar studies had been done in recent past years, in the beginning of 20th Ovalbumin (OVA) hsp70 fusion protein was observed to activate anti-OVA CD8+ CTLs in C57BL/6 mice with CD4+ knockout and wild-type cells almost equally effectively, moreover when the hsp70 was derived from murine (self) cells. Hsp70 fusion proteins have the ability to elicit CD4-independent CTL responses, which makes them useful for the prevention and

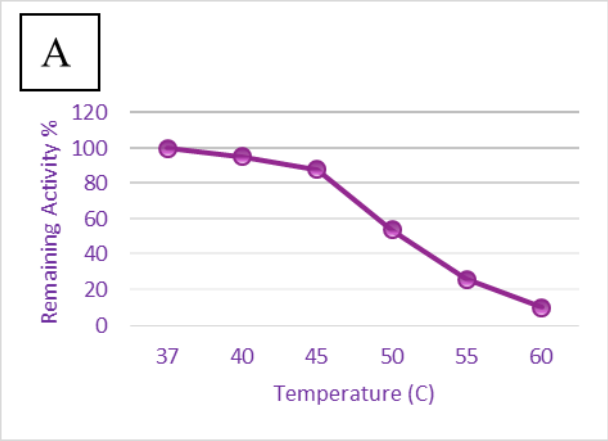
therapy of immunological diseases in patients with low levels of CD4+ T cells. (13). More recent similar study was the study of searching for potential vaccine candidates, so in their search for genes that code for proteins with potential use in the creation of an effective vaccine to control piscirickettsiosis, in 2005, Wilhelm and associates identified and sequenced the Piscirickettsia salmonis genes encoding the heat shock proteins 60 (Hsp60) and 70 (Hsp70) (salmonid rickettsial septicemia SRS). The coding areas had been translated into thioredoxin fusion proteins and produced in *E. coli.* When administered intraperitoneally to Atlantic salmon, both recombinant proteins were shown to induce a humoral response. They also provided protection to fish that had been exposed to *P. salmonis*. (32)(9).

protection properties of HSP70 for enzyme stability (Catalase

The activity was (5.5U/ml) at 37ºC and pH 7.0 and the protein concertation of the catalase was (1.26mg/ml) assayed in Bradford method and so, the specific activity was (4.36U/mg protein) to this study. The highest catalase activity was defined as 100% of the activity.

Effect of temperature on Catalase stability

Catalase stability was assayed at various temperatures (40, 45, 50, 55, 60) ˚C and was compared with control one, the temperature dependence of catalase stability was not great, the results in (**Fig.4a**) shown decreasing in remaining activity % by increasing the temperature when it reached to maximum 95% of the control activity in 40˚C, then it begins to decline with increasing temperature until reached to 10% in 60˚C. The second trials with Hsp70 were shown a slight different in the increasing of the activity comparing with the results we obtained without it. The maximum activity 99% was at 40˚C, and its start to decrease until reaching to 17% in 60˚C (**Fig.4b**). These results may give a sight to the possibility of Hsp70 protectively function for the enzymes and help to maintain their stability at high temperatures.



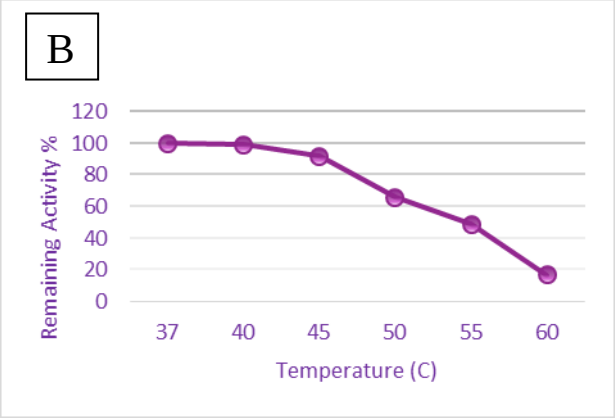


Figure 4. Effect of different temperature (40, 45,50,55,60) ºC on the catalase stability, a- the results without adding the Hsp70 solution, b- the results were after added the Hsp70.

The Heat Shock protein 70 genes (***Hsp70*** genes) expression as virulence factor was detected by (2) in variable temperature activation include 28 ºC, 37 ºC and 45 ºC by real time PCR. The results of ***HSP70*** gene expression showed the level increased at 37 ˚C but decreased when the temperature increases to 45 ˚C.

Effect of different pH range on Catalase stability: The best pH for enzyme stability for purified Catalase was found to be 7.0. This is a characteristic of mono-functional catalases that is prevalent, yet catalase has a limited catalytic pH range (33). The ideal pH range, as illustrated in Figure (**5a**), was between pH 6.0 and 8.0. and the remaining enzyme activity was reduced at pH 4.0 and 10.0, whilst pH 2.0 saw a total elimination of the activity. When the Hsp70 solution was added after the enzyme had been incubated in a buffer solution (pH range, 2.0 to 10.0) at 37°C for 30 minutes (**Fig.5b**), the enzyme was stable in the pH range from 6.0 to 8.0 and more than 95%

of the activity remained at pH 6.0. Enzymatic activity was completely eliminated at pH 2.0 & only 3% of activity remain at pH 10.0. Even with the presence of heat shock protein as a protective agent, the differences in Catalase stability with the different pH values return primarily to changes in the ionic state of the enzyme molecule, so it is important to be aware that the different pH values of stability may change depending on some compounds (12).

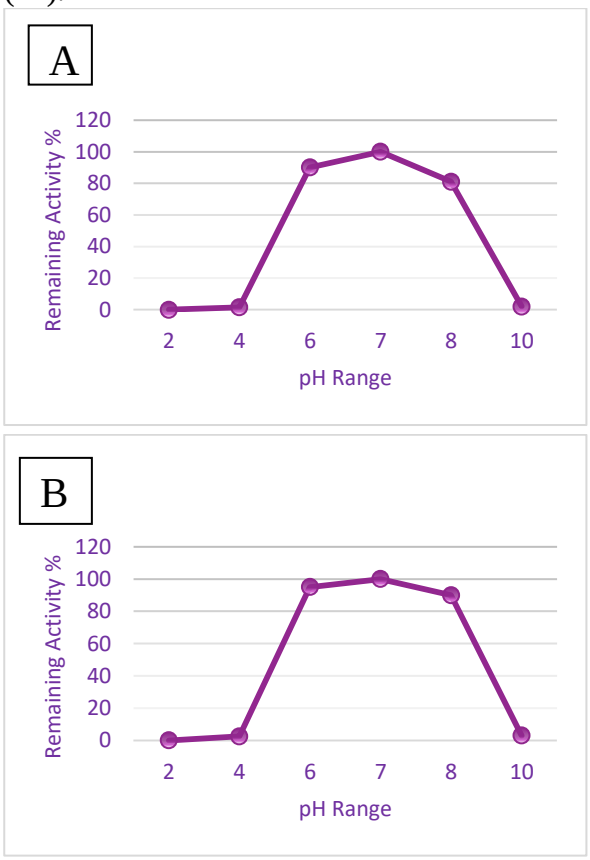


Figure 5. Effect of different range of pH (2.0, 4.0, 6.0, 8.0, 10.0) on the catalase stability a- the results without adding the Hsp70 solution, b- the results were after added the Hsp70

Effect of Heavy metals on Catalase stability

Table (**1**) shows the effect results of some metallic chlorides on catalase stability after incubated it with these solutions for 30min. at 25˚C, the differences of the materials effect depend on the different of type and concentration, when it found that the adding of $MnCl_2$, $NiCl_2$, $CdCl_2$, $FeCl_3$, $CuCl_2$, and $ZnSO_4$ with 10, 20, 30mg/L leads to inhibit part or complete activity of the catalase depending on the amount of concentration added of these metals. The $CuCl_2$, showed inhibition effect of enzyme when the enzyme lost complete activity when incubated with all three different concentrations (10, 20, 30mg/L), while the catalase kept a slight amount of its activity when treated with Hsp70 in 10, and 20mg/L concentration of $CuCl_2$, (**table 2**), while only 20% from activity of enzyme remained when it treated with 30mg/L from $ZnSO_4$, even after adding Hsp70 solution to it, the remaining activity raised only 3%. That proved that copper, which has a 100% inhibition of catalase activity at concentrations of 10, 20, and 30 mg/L, is the strongest inhibitor. Copper is followed by zinc, iron, and cadmium, respectively. Nickel and manganese as its shown by their results considered as a very weak inhibitor at least at these concentrations.

Table 1. Effect of some chlorides on catalase stability

Heavy metals	Concentration (mg/L)	Inhibition %	Remaining activity %
copper chloride	10	100	0
	20	100	0
	30	100	0
cadmium chloride	10	5	95
	20	17	83
	30	20	80
zinc sulphate	10	20	80
	20	25	75
	30	80	20
ferric chloride	10	0	100
	20	23	77
	30	50	50
manganese chloride	10	0	100
	20	0	100
	30	3	97
nickel chloride	10	0	100
	20	0	100
	30	12	88

Table 2. Effect of some chlorides on catalase stability in addition of adding Hsp70 to the reaction solution

Heavy metals	Concentration (mg/L)	Inhibition %	Remaining activity %
copper chloride	10	93	7
	20	98	2
	30	100	0
cadmium chloride	10	0	100
	20	10	90
	30	23	77
zinc sulphate	10	16	84
	20	24	76
	30	77	23
ferric chloride	10	0	100
	20	13	87
	30	42	58
manganese chloride	10	0	100
	20	0	100
	30	0	100
nickel chloride	10	0	100
	20	0	100
	30	0	100

The findings of this investigation were in agreement with those of Sukhdev and

colleagues' 1981 study on the interaction between heavy metals and the catalase activity of Sarotherodon mossambicus (Peters) (23). Om Gandhi recently reported on a further investigation into the impact of metal ions and chelation utilizing antioxidants on catalase activity (10). Additionally, he discovered that zinc was the least inhibitive, followed by copper, aluminum, and then iron. Catalase activity was 34% decreased by copper.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

REFERENCES

1. Alberto, M, J. L. 1995. Heat-shock proteins and molecular chaperones: implications for pathogenesis, diagnostics, and therapeutics. International Journal of Clinical and Laboratory Research, 25, 59-70. https://doi.org/10.1007/BF02592359

2. Al-Barazanchi , T. A., Z. H. Abood., and R.S. Al-rawi, 2022. Molecular investigation of heat shock protein 70 (hsp70) expression levels in aspergillosis patients. Iraqi Journal of Agriculturial Science, 53(3):534_541. https://doi.org/10.36103/ijas.v53i3.1561

3. Bavisotto, C., F. Cappello, J. Alberto, M. de Macario, M. Logozzi, S. Fais, and C. Campanella, 2017. Exosomal HSP60: a potentially useful biomarker for diagnosis, assessing prognosis, and monitoring response to treatment, Expert Review of Molecular Diagnostics, 17:9, 815-822,

4. Beers, R. F., Jr, and I. W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. The Journal of biological chemistry, 195(1), 133–140.

5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.72:248–254.

6. Calderwood, S.K., J. Gong, and A. Murshid. 2016. Extracellular HSPs: The Complicated Roles of Extracellular HSPs in Immunity. Frontiers in Immunology, 7, 9 p.

7. Dreiza, C. M., P. Komalavilas, E. J. Furnish, C. R. Flynn, M. R. Sheller, C. C. Smoke, L. B. Lopes, and C. M. Brophy. 2010. The Small Heat Shock Protein, HSPB6, in Muscle Function and Disease. Cell Stress & Chaperones, 15(1), 1–11.

8. Dubrez, L., S. Causse, N. Borges Bonan, B. Dumétier, and C. Garrido. 2020. Heat-shock proteins: chaperoning DNA repair. Oncogene, 39(3), 516-529.

9. Farhan Y Almalki, A., Arabdin, M., and Khan, A. 2021. The Role of Heat Shock Proteins in Cellular Homeostasis and Cell Survival. Cureus, 13(9), e18316. (Retraction published Cureus. 2021 Nov 18;13(11): r36)

10. Gandhi, Om. 2019. The Effects of Metal Ions and Chelation Using Antioxidants and EDTA on Catalase Activity: Implications in Alzheimer's Treatment.

11. Holzinger, A., K. S. Phillips, and T. E. Waver. 1996. Single-step purification/ solubilization of recombinant proteins: application to surfactant protein B. Biotechniques, 20: 804-808.

12. Hromic-Jahjefendic, A. 2022. Testing temperature and pH stability of the catalase enzyme in the presence of inhibitors. Periodicals of Engineering and Natural Sciences (PEN), 10(2), 18-29.

13. Huang, Q. J.F.L.Richmond, K. Suzue, H.M. Eisen, and R.A. Young. 2000. Elicitation of CTLs by mycobacterial hsp70 fusion proteins maps to discrete domain and is $CD4^+$ T-cell independent, Journal of Experimental Medicine, 191: 403-408.

14. Humam, A. M., T.C. Loh, H. L. Foo, W.I. Izuddin, E. A. Awad, Z. Idrus, and N. M. Mustapha. 2020. Dietary supplementation of postbiotics mitigates adverse impacts of heat stress on antioxidant enzyme activity, total antioxidant, lipid peroxidation, physiological stress indicators, lipid profile and meat quality in broilers. Animals, 10(6), 982.

15. Jugueira, L.C., J. Carmeiro, and RO. Kelley. 1995. Basic histology, A Large madical board 1995.

16. Katschinski, D.M. 2004. On heat and cells and proteins. Physiology, 19(1), 11-15.

17. Kun-peng Fan, Ya-fang Liu, Zhi-qiang Zhang, Ying Liu, Peng-fei Liu, 2021. Molecular cloning, characterization and expression patterns of HSP70 and HSP60 in the pufferfish (Takifugu rubripes), Aquaculture Reports, Volume

21,100865,ISSN 2352-5134, https://doi.org/10.1016/j.aqrep.2021.100865.
18. Kuo, W.H. and H.A. Chase. 2011. Exploiting the interactions between poly-histidine fusion tags and immobilized metal ions. Biotechnology, 10: 63-68.
19. Miao, Z. Q., Tu, Y. Q., Guo, P. Y., He, W., Jing, T. X., Wang, J. J., and Wei, D. D. 2020. Antioxidant Enzymes and Heat Shock Protein Genes from Liposcelis bostrychophila Are Involved in Stress Defense upon Heat Shock. Insects, 11(12), 839.
20. Multhoff, G.; and L.E. Hightower. 2011. Distinguishing integral and receptor-bound heat shock protein 70 (Hsp70) on the cell surface by Hsp70-specific antibodies. Cell Stress Chaperones, 16, 251–255.
21. Paliwal, P.K., A. Bansal, S.S.K. Sagi, and M. Sairam. 2011. Intraperitoneal immunization of recombinant HSP70 (DnaK) of Salmonella Typhi induces a predominant Th2 response and protective immunity in mice against lethal Salmonella infection. Vaccine, 29: 6532-6539.
22. Rappa, F.; F. Farina, G. Zummo, S. David, C. Campanella, F. Carini, G. Tomasello, P. Damiani, F. Cappello, E.C. de Macario, et al. 2012. HSP-molecular chaperones in cancer biogenesis and tumor therapy: An overview. Anticancer Res., 32, 5139–5150.
23. Rita, E.-H., K. Carla, T. Thierry, A.A. Adam, and V. Errol. 2017. Indicators for Ecosystem Conservation and Protected Area Designation in the Mediterranean Context. Conservation and Society, 15(2), 217–231.
24. Saied IF, GM. Aziz, AH. Ad'hiah, and A. Mahdi Saeed. 2014. Production of Recombinant Heat Shock Protein 60 (HSP60) From Salmonella enterica serovar Typhimurium ATCC 19585 and Its Evaluation as a Vaccine Candidate in BALB/c Mice. J Microb Biochem Technol 6: 346- 350.
25. Shevtsov, M.; and G. Multhoff. 2016. Heat shock protein–peptide and Hsp-based immunotherapies for the treatment of cancer. Front. Immunol. 7, 171.
26. Singh, S.M. and P.M. Sivalingam. 1982. In vitro study on the interactive effects of heavy metals on catalase activity of Sarotherodon mossambicus (Peters). Journal of Fish Biology, 20: 683-688.
27. Stuart, C, K., Md Abdul Khaleque, Douglas B. Sawyer, and Daniel R. Ciocca. Heat shock proteins in cancer: chaperones of tumorigenesis. Trends in biochemical sciences, 31(3), 164-172. https://doi.org/10.1016/j.tibs.2006.01.006
28. Szyller, J., and I.Bil-Lula, 2021. Heat Shock Proteins in Oxidative Stress and Ischemia/Reperfusion Injury and Benefits from Physical Exercises: A Review to the Current Knowledge. Oxidative medicine and cellular longevity, 2021, 6678457.
29. Tobian, A. A., D.H. Canaday, W.H. Boom, and C.V. Harding. 2004. Bacterial heat shock proteins promote CD91-dependent classI MHC cross-presentation of chaperoned peptide to CD8+ T cells by cytosolic mechanisms in dendritic cells versus vacuolar mechanisms in macrophages. The Journal of Immunology, 172(9), 5277-5286.
30. Walker J.M. (ed.) 2009. The identification of protein S-nitrosocysteine New York: Springer, Humana Press.
31. Wang, Y.; L. Oberley, and D. Murhammer. 2001. Antioxidant defense systems of two lipidopteran insect cell lines. Free Radic. Biol. Med., 30, 1254–1262.
32. Wilhelm, V., C. Soza, R. Martínez, M. Rosemblatt, L.O. Burzio, and P.D. Valenzuela. 2005. Production and immune response of recombinant Hsp60 and Hsp70 from the salmon pathogen Piscirickettsia salmonis. Biological research, 38(1), 69-82.
33. Zeng, HW., Cai, YJ., Liao, XR. et al. 2010. Optimization of catalase production and purification and characterization of a novel cold-adapted Cat-2 from mesophilic bacterium Serratia marcescens SYBC-01. Ann Microbiol **60**, 701–708.
34. Zhang, S.; Fu, W.; Li, N.; Zhang, F.; and Liu, T.X. 2015. Antioxidant responses of Propylaea japonica (*Coleoptera: Coccinellidae*) exposed to high temperature stress. J. Insect Physiol, 73, 47–52.
35. Zincing, T., I. Achilonu, H. Hoppe, E. Prinsloo, H.W. Dirr, and A. Shonhai. 2015. Overexpression, purification and characterisation of the Plasmodium falciparum Hsp70-z (PfHsp70-z) protein. PLoS One, 10(6), e0129445.