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TEMPERATURE EFFECTS ON THE STABILITY OF MYCOBACTERIUM TUBERCULOSIS SHIKIMATE KINASE (MtSK): STRATEGIES FOR SECURE TRANSPORT

Pengaruh Suhu Pada Stabilitas *Mycobacterium tuberculosis* Shikimate Kinase (*Mt*SK): Strategi untuk Transportasi yang Aman

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ABSTRACT

Mycobacterium tuberculosis Shikimate Kinase (*Mt*SK) has a crucial role in the shikimic pathway, which is essential for this bacteria but is absent in humans, making it a potential target for novel anti-tuberculosis drugs. This study used enzyme-coupled fluorescence to examine the stability of *Mt*SK stored in 50% glycerol at -30 ℃, 4 ℃, and ±28 ℃ for six days. Results showed stable enzyme activity values (α=0.05) at all temperatures. This research underscores that *Mt*SK's stability depends on its molecular properties, including GC content, hydrophobic residues, Mg^{2+} binding, and intra-helical salt bridge. Despite some activity decline over time due to glycerol-induced aggregation, *Mt*SK can be safely transported at ±28 ℃ for up to six days without special cooling compartment. Understanding *Mt*SK stability ensures its active conformation remains consistent, reducing off-target effects on drug design and enhancing drug efficacy. This insight ultimately leads to high-quality and commercially viable tuberculosis treatment development. Future research should explore *Mt*SK stability at higher temperatures and assess the optimal glycerol content for cryopreservation.

***Keywords**: Glycerol, MtSK, Stability, Temperatures, Transportation*

ABSTRAK

Mycobacterium tuberculosis Shikimate Kinase (MtSK) memiliki peran penting dalam jalur shikimat, yang esensial bagi bakteri ini namun tidak ditemukan pada manusia, menjadikan enzim ini sebagai target yang potensial dalam pengembangan obat anti-tuberkulosis baru. Penelitian ini menggunakan metode fluoresensi enzyme-coupled untuk menguji stabilitas MtSK yang disimpan dalam larutan 50% gliserol pada suhu -30 ℃, 4 ℃, ±28 ℃ selama enam hari. Hasil menunjukkan nilai aktivitas enzim yang stabil (α=0.05) pada semua suhu. Penelitian ini menekankan bahwa stabilitas MtSK dipengaruhi oleh beberapa sifat molekulernya, termasuk kandungan GC, residu hidrofobik, pengikatan Mg^{2+}, dan jembatan garam intra-heliks. Meskipun aktivitas enzimatik menurun seiring waktu akibat agregasi yang diinduksi oleh gliserol, MtSK tetap dapat dimobilisasi dengan aman pada ±28 ℃ untuk durasi perjalanan hingga enam hari tanpa memerlukan penggunaan kompartemen pendingin. Pemahaman stabilitas MtSK dapat memastikan konsistensi konformasi aktifnya, sehingga dapat mengurangi pengaruh off-target dalam desain obat tuberkulosis dan meningkatkan efikasi obat tersebut. Pengetahuan ini dapat mendukung pengembangan obat tuberkulosis yang berkualitas tinggi dan layak jual di pasaran. Studi lanjutan diperlukan untuk mengetahui stabilitas MtSK pada suhu yang lebih tinggi dan menentukan konsentrasi gliserol yang optimal untuk kriopreservasi.

Kata kunci: *Gliserol, MtSK, Stabilitas, Suhu, Transportasi*

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INTRODUCTION

Mycobacterium tuberculosis shikimate kinase (*Mt*SK) is particularly significant for the survival of tubercle bacillus (Simithy et al. 2014), a pathogenic agent causing human tuberculosis (TB), which remains the most common infectious disease in Indonesia with 354 cases per 100,000 population (WHO, 2023). The shikimate kinase (SK) is the fifth enzyme in the shikimate pathway, and it plays a role in synthesizing numerous essential substances, including ubiquinone, folic acid, and aromatic amino acids. This pathway is exclusive to bacteria, including *M. tuberculosis*, fungi, and plants; it is not present in humans (Bentley, 1990). As a result, it has emerged as a promising target for antitubercular drug development.

In recent years, much research has focused on characterizing *Mt*SK and identifying its inhibitors. Studies have employed various approaches, including structural analysis, kinetic studies, and high-throughput screening, to understand the enzyme's function and identify potent inhibitors (Simithy et al. 2014; Gordon et al. 2015; Rajput et al. 2016; Simithy et al. 2018b; Simithy et al. 2018a; Alhaji Isa 2020; Rahul Reddy et al. 2020; Freitas de Freitas et al. 2023; Kawamoto et al. 2023). The accumulated knowledge from studies on *Mt*SK is invaluable for advancing tuberculosis drug research, highlighting the need for continued development and exploration.

In drug discovery and development, it is necessary to involve scientific collaborations between laboratories or even cross inter-institutional boundaries among industrial and academic institutions (Cheng et al. 2020). Thus, the utilization of *Mt*SK in therapeutic research development requires stability during storage and transportation among collaborators. The stability of the enzyme is crucial to maintaining its functionality and effectiveness in the application. Temperature remains essential in managing protein stability throughout production and distribution processes. Proteins are often exposed to relatively warm conditions (20 °C to 30 °C) for several hours or days during unit operations and under refrigerated storage (4 °C to 8 °C) for weeks or months, or even years below-freezing temperatures. In general, protein stability will decrease as temperature increases (Correia et al. 2020). Sharma et al. 2019 stated that most enzymes lose their activity at temperatures between 25°C to 37°C. However, there are thermostable enzymes that can resist high temperatures, typically between 45°C to 120°C (Rigoldi et al. 2018). Moreover, the enzyme stability may also be improved by using additive mixtures such as glycerol (Braham et al. 2021). This polyol has been described to stabilize some enzymes since it can shift the native protein structure to a more compact (Romero and Albis 2010; Kumar et al. 2011; Pazhang et al. 2016; Khoshnevisan et al. 2018). Nevertheless, the effects of glycerol on enzyme stability depend on its concentration and the nature of the enzyme itself (Chen et al. 2019).

Despite extensive studies on protein stability, there is a lack of detailed information on the stability of *Mt*SK under various storage temperatures in a specific time frame. Therefore, this study aims to investigate how different storage temperatures impact the stability of *Mt*SK. The primary objective of this research is to evaluate the stability of *Mt*SK when stored at -30 °C, 4 °C, and room temperature (±28 °C) over a specified period. Understanding the stability profile of *Mt*SK at different temperatures will help establish best practices for its storage and transportation, ensuring its effectiveness in research and therapeutic applications. This study utilizes a new one-step, enzyme-coupled assay for ADP detection that reads the fluorescence intensity of resorufin produced by the coupling reaction (Imamura et al. 2019; Nurkanto et al. 2022).

In summary, this study seeks to provide an evaluation of *Mt*SK stability under different storage temperatures, contributing valuable insights that will support the reliable use of this enzyme in scientific and medical fields.

MATERIALS AND METHODS

Location and time

This study was conducted in June – August 2023 at the Biotechnology Laboratory, the National Research and Innovation

Agency (BRIN), BJ Habibie Science and Technology Park, South Tangerang, Banten, Indonesia.

Production of the recombinant enzyme *MtSK*

The *E.coli* BL21 Star (DE3) pCold *MtSK* strain was developed by BRIN and the University of Tokyo. The *E.coli* glycerol stock was cultured for 24 hours at 37°C in Luria-Bertani (LB) medium containing 100 µg/ml carbenicillin. After being inoculated, single colonies were cultivated overnight at 37^0C with 200 rpm shaking in 25 mL of LB broth medium containing 100 µg/ml carbenicillin. Optical density (OD600) was measured, then the culture was transferred to 500 mL of fresh LB broth medium containing 100 µg/ml carbenicillin and incubated for 2 hours at 37°C with 200 rpm of shaking. Enzyme expression was induced by adding 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was then shaken at 200 rpm for 18 hours and incubated at 15^{o}C. Centrifugation at 7500 × g for 10 min at 4°C was used to harvest the cells. The pellet was then resuspended in a lysis buffer that contained 50 mM Tris-HCl pH 8, 150 mM NaCl, and 0.1% Triton X-100. Sonication was used to lyse the cell pellet for a total of 7 minutes (5 seconds of pulses and 20 seconds of rest) (Rajput et al. 2016).

Isolation of the recombinant enzyme *MtSK*

The lysed cells were centrifuged at 14,000xg, 4˚C for 60 minutes to separate the remaining cells. The protein-containing supernatants were equilibrated with Ni-NTA (Nitrilotriacetic acid matrix (NTA)) resin by gently shaking the process for an entire night at 4 ˚C. The protein-binding resin was then introduced into the exposed column. The liquid that comes out of the column was collected as flow-through (FT). After that, the resin was rinsed twice with a lysis buffer that contained, respectively, 20 mM and 50 mM imidazole. Proteins were eluted by a lysis buffer containing 300 mM imidazole. Imidazole elimination and protein concentration were carried out by ultrafiltration using a membrane with a Molecular Weight cut-off (MWCO) of 10,000 Da. The purified MtSK protein was added with glycerol in a ratio of 1:1. After that, the enzymes were divided into three groups and kept for six days at three different temperatures: room temperature (± 28 ˚C), 4 ˚C, and 30 ˚C (Rajput et al. 2016).

Quantification of Protein Concentration

The Bradford assay determined protein concentration (Bradford 1976) with Bovine Serum Albumin (BSA) as the standard. A total of 200 µL of Bradford reagent was added to 2 µL of a sample solution containing 1 to 10 µg of protein. Measurements were carried out using spectrophotometry at a wavelength of 595 nm.

Measurement of *Mt*SK Dose Response

Measurement of *Mt*SK dose response was measured in a total reaction volume of 40 µL in 384 well plates (Corning, USA). The *Mt*SK enzyme was added to a solution containing 100 mM Tris-HCl, 10 mM MgCl2, 200 µM shikimate, and ATP. The enzyme tested was in the range of 0.01 – 6.40 µM with a two-fold dilution. The test was carried out by following changes in the concentration of the ADP product formed by adding Kumagai reagent (20 mM Tris HCl, 10 mM MgCl2, 0.02% Triton-X 100, 0.01% BSA, 200 µM glucose, 20 µM NADP, 200 u/ml ADP hexokinase, 200 u/ml G6P dehydrogenase, 200 u/ml diaphorase I, 10 µM Resazurin 2 mM N-ethylmaleimide) (Imamura et al. 2019). The reaction is measured by enzyme-coupled fluorescence assay through reaction kinetics for 40 minutes (40 cycles) at 28 °C using a multi-plate reader (Thermo Scientific Varioskan LUX multimode microplate reader), with excitation and emission at 540 nm and 590 nm. Enzyme-coupled fluorescence assays utilize fluorescence intensity to provide a sensitive and specific method for monitoring enzyme activity through a linked reaction. The signal amplification from the secondary enzymatic reaction enhances detection sensitivity, allowing for real-time monitoring of enzyme kinetics and continuous data collection. This approach ensures high specificity, as the fluorescent signal is only generated when both enzyme reactions occur, minimizing non-specific interference (Imamura et al. 2019; Moisă et al. 2020).

Measurement of *Mt*SK stability

*Mt*SK enzyme stability was determined by measuring the *Mt*SK activity against ATP and Sikimate using a fluorescence spectrophotometer with excitation and emission at 540 nm and 590 nm. The activity assays were carried out in 4 replications for each sample. Samples were stored in 50% glycerol. Then, samples were aliquoted in the vial tubes and stored at three distinct temperatures: -30 ˚C, 4 ˚C, and ±28 °C for six days. The choice of 50% glycerol as a cryoprotectant was made to effectively prevent and minimize freezing, thus eliminating the need for repeated thawing and refreezing of the enzyme during usage. It is known that the freezing point of a 50% glycerol solution is approximately -23 °C (Lane 1925), which is close to our storage temperature of -30°C and provides a suitable balance without excessive viscosity. From our observations, no freezing occurred at -30°C, likely due to the presence of proteins and buffer components, which may lower the freezing point of the solution. While higher concentrations, such as 60% or 70% glycerol, could theoretically provide additional cryoprotection, they may also increase the solution's viscosity (Uribe and Sampedro 2003a). This heightened viscosity can restrict molecular movement and potentially result in enzyme aggregation or decreased activity over time. Moreover, a previous study from our research group stored its enzyme using 50% glycerol (Hartuti et al. 2018).

This test measured how the concentration of the ATP substrate changed when it reacted with ADP after the addition of the Kumagai reagent (20 mM Tris HCl, 10 mM $MgCl_2$, 0.02% Triton-X 100, 0.01% BSA, 200 µM glucose, 20 µM NADP, 200 u/ml ADP hexokinase, 200 u/ml G6P dehydrogenase, 200 u/ml diaphorase I, 10 µM Resazurin, 2 mM N-ethylmaleimide) (Imamura et al. 2019) and read the reaction kinetics for 40 minutes (40 cycles) at 28 °C using a multiplate reader (Thermo Scientific Varioskan LUX multimode microplate reader). The Z factor (Bradford 1976) was computed using the equation below to determine the screening quality. The standard deviations (σ) and averages (µ) of the positive (p) and negative (n) controls are represented by the variables σp, σn, µp, and µn:

$$Z\ factor = 1 - \frac{3(\sigma p + \sigma n)}{|\mu p + \mu n|}$$

Statistical data analysis

All statistical analyses were performed with one-way ANOVA and 'Tukey' *post-hoc* tests to determine statistically significant differences among groups, as this is the most prevalent post-hoc analysis. It uses the "Honest Significant Difference," a metric that quantifies the disparity between groups, to evaluate each means against all other means. The level of significance was set at α = 0.05 (IBM SPSS Statistics for Windows, v.25 (IBM Corp., Armonk, NY, USA). The overall process of this study is shown as a flowchart in Figure 1.

RESULT AND DISCUSSION

Quantification of Protein Concentration

The Bradford test was performed on samples of enzymes that had been mixed with 50% glycerol; the absorbance was measured at a wavelength of 595 nm. The BSA protein standard curve was then plotted against the sample absorbance value (shown in Figure 2). The test resulted in the concentration of purified enzyme was 2.3 g/L. In this study, the molecular mass of *Mt*SK is 71.8 kDa. Thus, the concentration of *Mt*SK is equal to 32 µM.

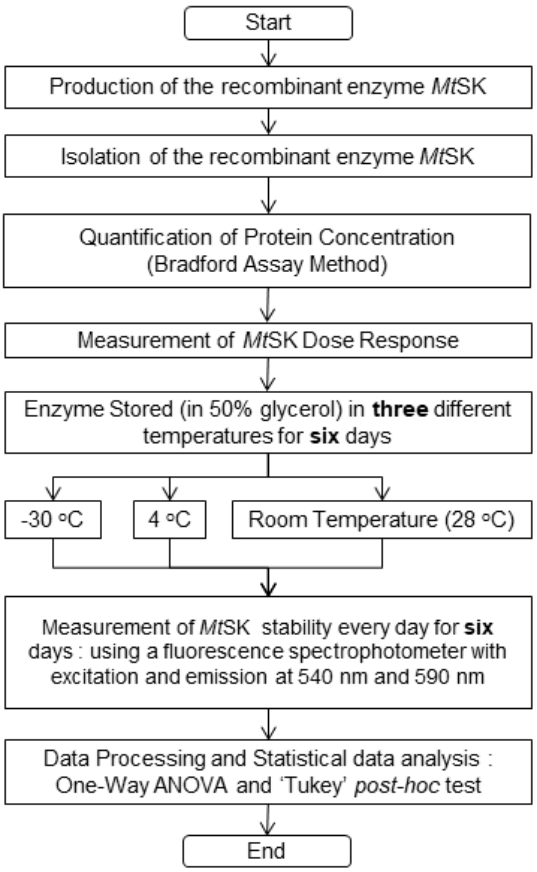

Figure 1. Flowchart of the research process

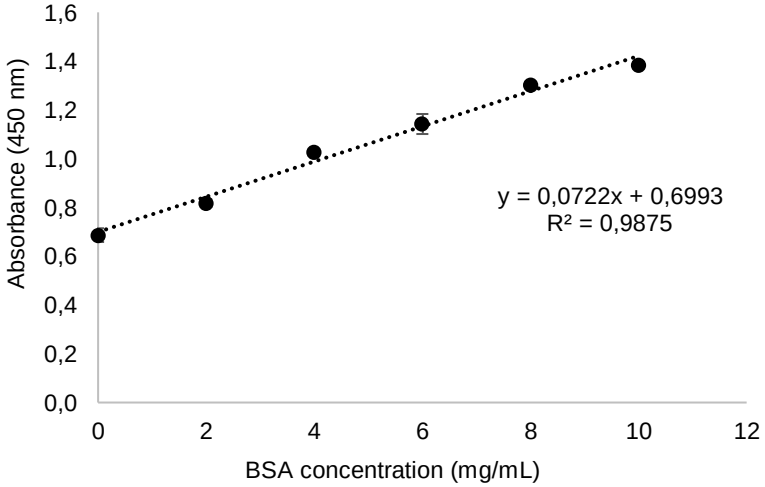

Figure 2. A representative BSA standard curve from the Bradford protein assay. BSA was used as a standard and was diluted to the following concentrations: 0, 2, 4, 6, 8, and 10 mg/mL. Each data point represents an average of duplicate absorbance readings per BSA concentration. The equation of the least square regression line was used to calculate protein concentration from absorbance.

Measurement of *Mt*SK Dose Response

The enzyme dose-response measurements, demonstrate a direct proportionality between enzyme concentration and fluorescence signal increment (shown in Figure 3). Its signal reached a plateau at the two highest concentrations, while an enzyme concentration at the exponential signal was needed to measure its activity. Therefore, 0.8 µM of enzyme was utilized in the subsequent test.

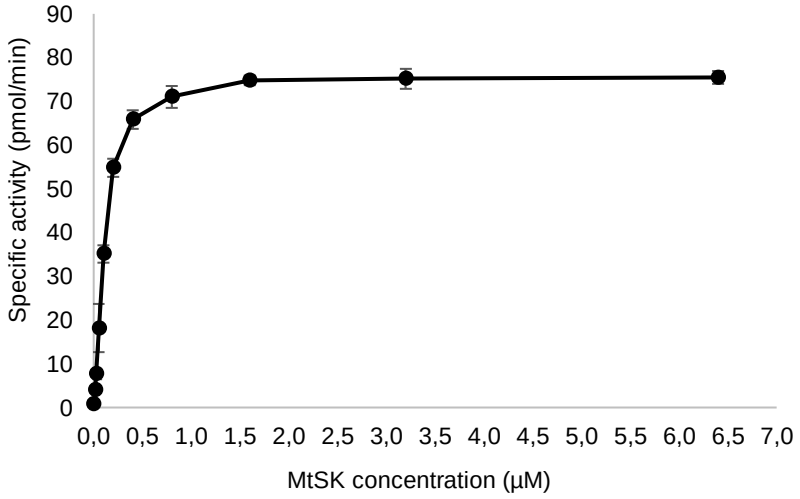

Figure 3. A dose-dependence curve of MtSK was tested by utilizing an enzyme-coupled fluorescence assay for ADP detection with a fixed concentration of substrates (200 µM shikimate and 300 µM ATP). The substrates were reacted with an increasing concentration of enzyme (0.01-6.40 µM). The specific activity of MtSK reached a plateau at its concentration above 1 µM.

Measurement of *Mt*SK stability

The Descriptive Analysis of Variance (ANOVA) Statistics analysis of the experiment with α = 0.05 (shown in Table 1) informs us that distinct temperatures on enzyme storage have no significant difference in *Mt*SK enzyme stability. Meanwhile, the duration of the storage variable demonstrated a significant difference in the stability of the enzyme with F Stat = 54.857; and *p*-Value = 0.000.

Table 1. Descriptive ANOVA Statistics

Tests of Between-Subjects Effects					
Dependent Variable:	**Specific activity**				
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	8855.759[a]	20	442.788	18.055	0.000
Intercept	1202132.626	1	1202132.626	49018.555	0.000
Storage temperature	55.379	2	27.689	1.129	0.339
Day	8071.899	6	1345.316	54.857	0.000*
Storage temperature * Day	131.048	12	10.921	0.445	0.928
Error	637.625	26	24.524		
Total	1366423.530	47			
Corrected Total	9493.384	46			

* Indicated statistical significance

Table 2. Descriptive statistics of 'Tukey HSD' *Post-Hoc* test

Multiple Comparisons

Dependent Variable: Specific activity
Tukey HSD

(I) Day		Mean Difference (I-J)	Std. Error	Sig.
Day1	Day2	10.3917*	2.44046	0.004
	Day3	16.7057*	2.75514	0.000
	Day4	24.0524*	2.75514	0.000
	Day5	32.3517*	2.89970	0.000
	Day6	34.6014*	2.64705	0.000
	Day7	40.7507*	2.75514	0.000
Day2	Day1	-10.3917*	2.44046	0.004
	Day3	6.3140	2.55729	0.212
	Day4	13.6607*	2.55729	0.000
	Day5	21.9600*	2.71242	0.000
	Day6	24.2097*	2.44046	0.000
	Day7	30.3590*	2.55729	0.000
Day3	Day1	-16.7057*	2.75514	0.000
	Day2	-6.3140	2.55729	0.212
	Day4	7.3467	2.85914	0.177
	Day5	15.6460*	2.99869	0.000
	Day6	17.8957*	2.75514	0.000
	Day7	24.0450*	2.85914	0.000
Day4	Day1	-24.0524*	2.75514	0.000
	Day2	-13.6607*	2.55729	0.000
	Day3	-7.3467	2.85914	0.177
	Day5	8.2993	2.99869	0.121
	Day6	10.5490*	2.75514	0.011
	Day7	16.6983*	2.85914	0.000
Day5	Day1	-32.3517*	2.89970	0.000
	Day2	-21.9600*	2.71242	0.000
	Day3	-15.6460*	2.99869	0.000
	Day4	-8.2993	2.99869	0.121
	Day6	2.2497	2.89970	0.985
	Day7	8.3990	2.99869	0.113
Day6	Day1	-34.6014*	2.64705	0.000
	Day2	-24.2097*	2.44046	0.000
	Day3	-17.8957*	2.75514	0.000
	Day4	-10.5490*	2.75514	0.011
	Day5	-2.2497	2.89970	0.985
	Day7	6.1493	2.75514	0.314
Day7	Day1	-40.7507*	2.75514	0.000
	Day2	-30.3590*	2.55729	0.000
	Day3	-24.0450*	2.85914	0.000
	Day4	-16.6983*	2.85914	0.000
	Day5	-8.3990	2.99869	0.113
	Day6	-6.1493	2.75514	0.314

Table 3. Homogenous Subsets – 'Tukey HSD' *Post-Hoc* Test

Treatment (Day)	MtSK Activity (nmol/menit/mg)
1	191.13[a]
2	180.73[b]
3	174.42[b,c]
4	167.07[c]
5	158.77[c,d]
6	156.52[e]
7	150.38[e]

*Means of groups in homogenous subsets

After being stored for seven days on each condition, *Mt*SK activity was not affected by those various storage temperatures. Its mean activity values were 169.05 ± 14.67 nmol/mg/min, 169.53 ± 13.14 nmol/mg/min, and 171.24 ± 14.61 nmol/mg/min for -30 °C, 4 °C, and ±28 °C, respectively. Presumably, this result shows that *Mt*SK is one of the thermostable enzymes. These kinds of enzymes had some characteristics such as a high GC content in their DNA, which causes these enzymes to have distinct hydrogen bonds, electrostatic interactions, hydrophilic contacts, metal binding, and loop deletion that resulting a superior conformational shape (Hickey and Singer 2004). *Mt*SK are characterized by a high guanine-cytosine (GC) content in their DNA with 37% of G and 35% of C, which plays a pivotal role in determining their structural and functional properties. The high GC content promotes stronger and more stable hydrogen bonding between the DNA strands, which in turn enhances the transcriptional efficiency and accuracy of the resulting proteins. This also contributes to distinct electrostatic interactions within the protein, influencing the stability of ionic bonds between charged amino acid residues (Hickey and Singer 2004).

Moreover, thermostable enzymes tend to have large levels of non-polar amino acids (Das and Gerstein 2000). Non-polar amino acids include Alanine (Ala), Valine (Val), Leucine (Leu), Proline (Pro), Methionine (Met), Tryptophan (Trp), Glycine (Gly), Isoleucine (Ile), and Phenylalanine (Phe). Based on peptide analyzing tools by Thermofisher, the amino acid sequence of *Mt*SK contains 61.56% hydrophobic residues, significantly influencing the enzyme's structural properties. The high proportion of hydrophobic residues increases the overall hydrophobicity of MtSK, contributing to its structural rigidity (Goodenough and Jenkins 1991). Hydrophobic interactions between these residues play a critical role in maintaining the protein's core stability as they cluster together to form a tightly packed, water-excluded interior. This rigidity is significant for preserving the enzyme's catalytically active conformation, especially under elevated temperature conditions, where flexible proteins are more prone to thermal denaturation. Increased rigidity helps shield the protein from unfolding by enhancing the compactness of its structure, preventing the exposure of its hydrophobic core to the surrounding aqueous environment(Li et al. 2005). Such stabilization mechanisms allow enzymes like *Mt*SK to maintain their functionality and resist denaturation even in stress-induced conditions, contributing to their resilience and prolonged activity during storage.

Furthermore, metal binding is another factor that contributes to enhancing enzyme stability. Magnesium is by far the most frequently found metal ion cofactor in enzymatic systems. This is most likely related to the ability of Mg^{2+} to form a stable metal-enzyme complex with phosphate-containing species such as ATP (Sissi and Palumbo 2009). In the case of *Mt*SK, the binding of Mg^{2+} to specific sites on the enzyme can help maintain its structural integrity and protect it from denaturation or degradation under various environmental conditions, including temperature storage. Hence, supplementation of 10 mM Mg^{2+} ions in the *Mt*SK buffer solution may increase the thermal denaturation temperature of this enzyme. Additionally, Dias et al. (2007) report that Mg^{2+} influences the conformation of the shikimate hydroxyl groups and the position of the side chains of some residues of the active site. The absence of Mg^{2+} changes the positions of shikimate and some active site amino acids, mainly Asp32 and Asp34 (shown in Figure 4). As a non-zymogen enzyme, *Mt*SK typically possesses an active site that should be readily available for substrate binding and catalysis without needing proteolytic cleavage or activation steps. Therefore, the changes in active site conformation can significantly affect its activity, impacting both the catalytic efficiency and structural stability.

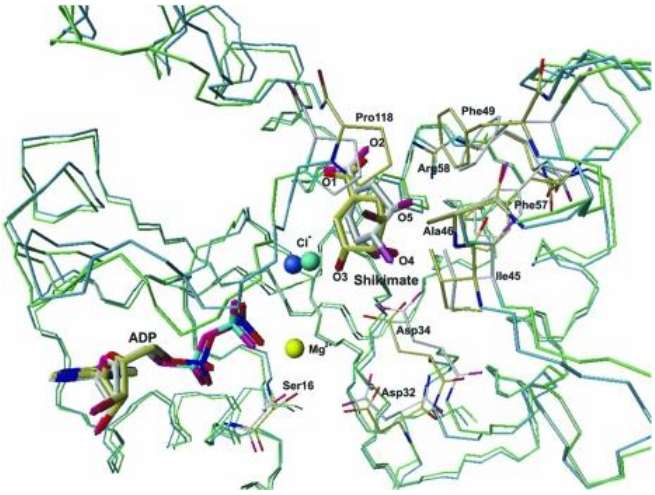

Figure 4. Overlay of the structures of the *Mt*SK–MgADP–shikimate and *Mt*SK–ADP shikimate ternary complexes. The C trace of the *Mt*SK–MgADP–shikimate complex is presented in green and that of the *Mt*SK–ADP–shikimate trace is in blue. The C atoms of *Mt*SK–MgADP–shikimate and *Mt*SK –ADP–shikimate are white and yellow, respectively. The Mg^{2+} shown in yellow (Dias et al. 2007) The figure was generated with the program MolMol (Koradi et al. 1996).

However, the relationship between Mg^{2+} and enzyme stability can be complex. While moderate concentrations of magnesium ions may enhance enzyme stability, excessively high or low concentrations could have detrimental effects. High concentrations of magnesium ions, for example, may lead to protein aggregation or precipitation, ultimately compromising enzyme stability. Conversely, insufficient magnesium levels could result in the destabilization of the enzyme structure. Thus, a stoichiometric amount of Mg^{2+} addition to resulting in the most optimal condition for *Mt*SK stability still needs to be studied in further research.

Furthermore, other divalent metals may not necessarily be able to replace the role of magnesium (Mg^{2+}) in the stability and activity of shikimate kinase. Mg^{2+} plays a crucial role in phosphorylation reactions that involve phosphate transfer. In contrast, other divalent cations, such as calcium (Ca^{2+}), are generally ineffective substitutes for Mg^{2+} in phosphate transfer processes. This inefficacy arises from the larger ionic radius of Ca^{2+} and its differing affinity for nucleotides, which can disrupt the active conformation of the enzyme and decrease the catalyst activity (Knape et al. 2015). Cowan (2002) discusses the differences between Mg^{2+} and other metal ions, including zinc (Zn^{2+}), Ca^{2+}, and manganese (Mn^{2+}). While some of these metal ions can partially replace Mg^{2+}, their efficiency tends to be lower. For instance, Zn^{2+} can inhibit enzymatic activity due to its propensity to form bonds with different electron donors compared to Mg^{2+}, thereby altering the dynamics of substrate interactions with the enzyme. Further studies are necessary to capture the specific effects of alternative metal ions on the structure and activity of *Mycobacterium tuberculosis* shikimate kinase (*Mt*SK) and to quantify the extent of their influence.

A study by Das and Gerstein (2000) found that intra-helical salt bridges are prevalent in thermostable enzymes and play a crucial role in maintaining their structural integrity under extreme conditions. In MtSK, the Glu61 residue that is strictly conserved in shikimate kinases forms a hydrogen bond and salt bridge with Arg58 and assists in positioning the guanidinium group of Arg58 for shikimate binding (Pereira et al. 2004). Salt bridges, formed between oppositely charged residues within the α-helices of the protein, provide electrostatic stabilization, which helps maintain the enzyme's three-dimensional structure. These ionic interactions are particularly important in environments with elevated temperatures, where increased thermal motion can destabilize weaker non-covalent interactions such as hydrogen bonds or van der Waals forces. The presence of salt bridges in thermostable enzymes contributes to reducing flexibility, enhancing rigidity, and preventing denaturation under stress conditions. Although

several *Mt*SK characteristics follow the thermozyme specifications, more research is needed to obtain a stability profile of *Mt*SK at higher temperature storage conditions. Since a thermostable enzyme usually can withstand elevated temperatures higher than 50 °C (Che Hussian and Leong 2023). Nevertheless, this study proves that even when transported at ±28 °C, *Mt*SK will remain safe and active.

On the other hand, *Mt*SK activity at all temperatures notably decreases over time, as shown in Figure 5. This result probably occurred due to the presence of a high concentration of glycerol in the enzyme solution during the storage period. As previously described, *Mt*SK in this experiment was stored with 50% v/v or 6.8 M of glycerol to avoid freezing. In general, glycerol is a co-solvent that is commonly used in cryopreservation to protect the biological activity of proteins or enzymes during storage from factors such as temperature, contaminants, conformational changes, and others (Braham et al. 2021). However, the stabilizing effect of glycerol is not universal. There are previous studies that report that glycerol has no cryoprotection effect on their enzymes (Anchordoquy et al. 2001; Izutsu et al.1994). It can produce even some destabilization of the immobilized enzyme (Braham et al. 2021). The glycerol effect was strongly concentration-dependent, but not always the most significant results were observed at the highest glycerol concentrations. As Meng et al. (2004) reported glycerol could prevent Creatine Kinase (CK) from thermal inactivation and aggregation in a concentration-dependent manner.

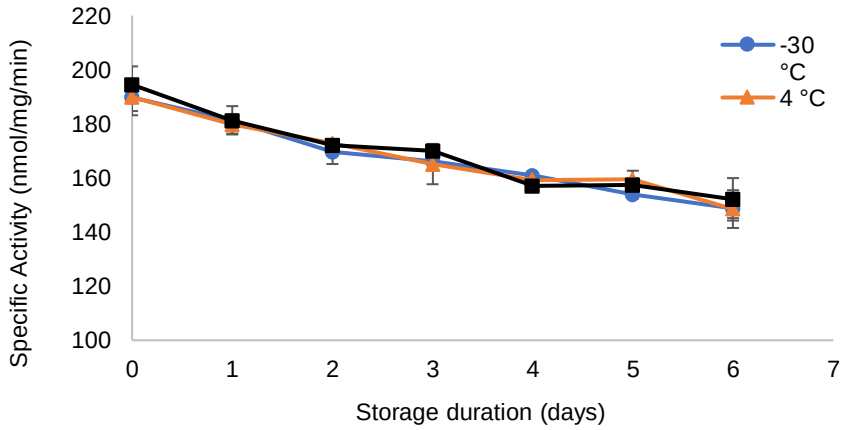

Figure 5. *MtSK*-specific activity decreased over time in all temperatures. The enzyme was stored in each condition with 50% glycerol and aliquoted in a small volume for daily tests to prevent the enzyme from the freeze-thaw process, especially for the enzyme that was stored at – 30 °C. ADP product standard was measured every day, and its equation of the least square regression line was used to calculate *Mt*SK-specific activity. Each data point represents an average value of four replications.

There is an optimum concentration of glycerol to increase the stability of enzymes. A minimum concentration of 0.3M (or ~5%) has been suggested to achieve significant stabilization. As high as 1M sucrose or 10% glycerol have been routinely used to protect the activity of proteins, while sorbitol levels between 20% and 33% have been found to stabilize proteins and suppress aggregation (Chi 2017). We have unpublished data for MtSK preserved in 20% glycerol showing stable activity, with initial activity being 67.10 ± 2.42 pmol/min and 71.07 ± 0.89 pmol/min after 6 months of storage.

In contrast, glycerol has some mechanisms of enzyme destabilization at high concentrations, namely increasing viscosity that causes impeded conformational changes, dehydration effects, protein aggregation, and decreased ligand binding affinity. It has been reported that solution viscosity was increased by adding an increasing concentration of glycerol, resulting in a decrease in the reaction rate of the enzyme (Uribe and Sampedro 2003b). They measured the Vmax of ATPase in the presence of 1 M, 2 M, 3 M, and 4 M of glycerol. The values were 6.24 µmol ATP/mg/min, 4.65 µmol

ATP/mg/min, 4.05 µmol ATP/mg/min, and 1.94 µmol ATP/mg/min, respectively. In this regard, Kramers's theory explains that solvent viscosity causes friction against enzymes in solutions. It can hinder the necessary dynamic movements of the enzyme, leading to decreased enzyme catalytic rate and stability over time (Leiden 1940).

Regarding enzyme hydration, glycerol is preferentially excluded from the enzyme surface at below ~40% v/v (Hirai et al. 2018). This exclusion maintains the enzyme's hydration shell, where water molecules remain closely associated with the enzyme surface. The hydration shell is essential for maintaining the enzyme's native structure and stability because water molecules form hydrogen bonds with the enzyme, so it can stabilize the enzyme's conformation. Preservation of the hydration shell can protect enzymes from denaturation and aggregation. On the other hand, Hirai et al. (2018) also reported that glycerol at a concentration above 50% v/v starts to penetrate the hydration shell and disrupt the hydrogen-bonding network between water molecules and the enzyme resulting in the destabilization of the enzyme's structure. The enzyme may lose its native conformation over time and cause partial unfolding or misfolding. In addition, this phenomenon can increase the risk of enzyme aggregation or precipitation during storage.

Moreover, prolonged exposure to high concentrations of glycerol leads to a notable decline in enzyme activity due to the ligand binding affinity interference. As a cosolvent, glycerol can compete with the ligand for hydrogen bonding and other interactions with the protein, reducing the effective concentration of ligand available for binding. A study by Papaneophytou et al. (2014) has shown that increasing solvent viscosity, which is related to high concentrations of glycerol, was detrimental to ligand binding. For instance, the equilibrium dissociation constant (Kd) of ligand-protein TNF-α with SPD304 in a solution containing 5% and 50% glycerol resulted in Kd values of 6.46 ± 0.38 µM and 19.18 ± 1.02 µM, respectively. Where the larger the Kd value, the weaker the binding affinity of the ligand for its enzyme target.

Our findings suggest that glycerol concentration plays a crucial role in *Mt*SK stability during storage. To verify these findings, future research on investigating the precise mechanism of enzyme destabilization at high concentrations of glycerol is needed. Certain analysis techniques, such as Nuclear Magnetic Resonance (NMR) spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy, can be utilized to monitor the changes in an enzyme's structure. Despite decreased activity after 6 days of storage, the S/B value of *Mt*SK was still quite good for screening anti-tuberculosis agents. At the end of the storage period, S/B for each enzyme at these three distinct temperatures was around 7 (shown in Figure 6). Macarrón and Hertzberg, (2009) reported that the minimum S/B value of the screening system to be reliable is 3.

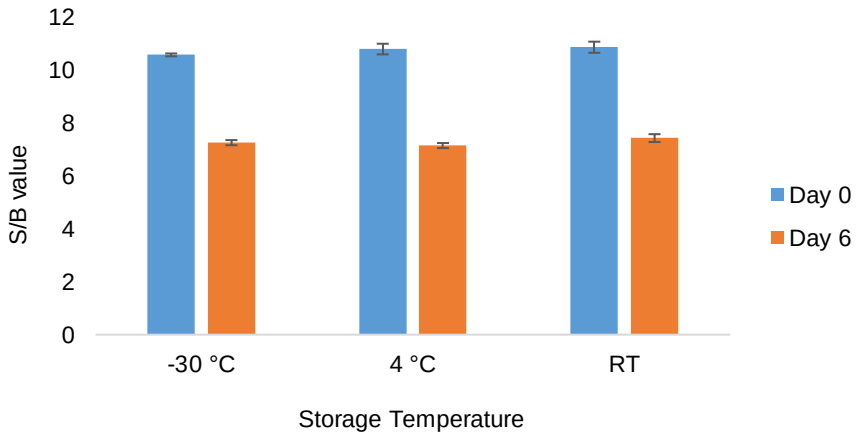

Figure 6. The signal-to-background (S/B) of *Mt*SK was decreased after 6 days of storage in three distinct temperatures. The S/B value was calculated from a ratio of the enzyme reaction signal and the background signal in which the enzyme was absent. The standard deviations were calculated from a quadruplicate data set.

CONCLUSION

This study found that the activity of *Mt*SK is not affected by storage temperatures at -30 °C, 4 °C, and ± 28 °C. The stability of *Mt*SK is probably influenced by the high content of GC in its genome and the hydrophobic residues contained in its amino acids. These characteristics create a rigid structure of *Mt*SK that protects the enzyme from unfolding. Besides that, the presence of Mg^{2+} binding on the *Mt*SK active site can help the enzyme maintain its structural integrity and protect it from denaturation or degradation under various environmental conditions. This result suggests that *Mt*SK is sufficiently safe to be mobilized without using any tools, such as ice gel or dry ice, to keep the temperature low. Even though *Mt*SK potentially remains active when transported at ±28 °C, the activity on this condition decreased over time. Due to the usage of 50% glycerol in *Mt*SK storage, the viscosity solution was increased. It caused impeded conformational changes, dehydration effects, protein aggregation, and decreased ligand binding affinity. A limitation of this study is the focus on a single glycerol concentration used for *Mt*SK storage, which may not exactly represent the effects of other glycerol concentrations on *Mt*SK activity. Additional investigation is required to obtain the optimal glycerol concentration to maintain *Mt*SK activity under various conditions in long-term storage. Overall, our findings highlight the potential of *Mt*SK to become a thermostable enzyme and the need to carefully consider glycerol concentration in *Mt*SK preservation.

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