CHARACTERIZATION OF STRUCTURE AND FUNCTION OF SMALL HEAT SHOCK LIKE-PROTEINS FROM PSYCHROPHILIC YEAST, *Glaciozyma antarctica* PI12 IN RESPONSE TO THERMAL STRESS

FARHAN NAZAIE BIN NASIB

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BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2023

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FARHAN NAZAIE BIN NASIB

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
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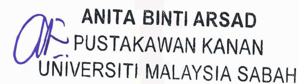

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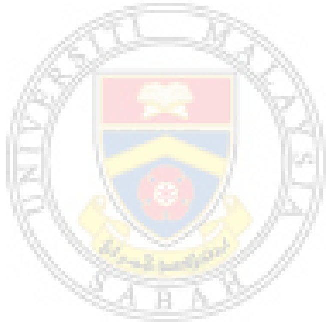

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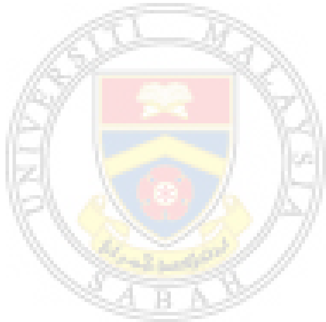

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Farhan Nazaie bin Nasib
11 January 2023

ABSTRACT

Antarctica, with its unique geography and extreme climate, serves as the primary habitat for bacteria. Among these microorganisms, Antarctic subglacial species have developed the ability to endure high pressure and severe cold conditions. Research has shown that molecular chaperones play a crucial role in preventing protein degradation and facilitating protein refolding under heat stress. Specifically, small heat shock like-proteins (sHSPs) have been found to interact with partially unfolded proteins prone to aggregation, thereby reducing cellular damage. The exceptional functionality of psychrophilic sHSPs at low temperatures presents an opportunity to explore the relationship between protein structure, stability, flexibility, and dynamic conformation. This study aims to investigate the role of sHSPs derived from Glaciozyma antartica and examine the connection between their molecular structure and heat adaptation. Out of the four sHSP genes identified in *G. antarctica*, two namely GasHSP07-010 and GasHSP12-338, were amplified and cloned using *E. coli* BL21(DE3). The proteins encoded by these genes were expressed at 37°C overnight and subsequently purified using immobilized metal chelate affinity chromatography (IMAC). The purified proteins underwent both a citrate synthase assay and a thermotolerance assay. Furthermore, comparative modeling of these genes was performed using CHIMERA, aligning them against the *Homo sapiens* (2YRT) and *Schizosaccharomyces pombe* (3W1Z) strains. The quality of the modeled structures was evaluated using the Ramachandran plot, errat, and verify3D. Results from the *in vitro* thermotolerance assay demonstrated that GasHSP07-010 and GasHSP12-338 protected *E. coli* cells from lethal temperatures of 55°C for up to 30 and 60 minutes, respectively. An aggregation assay using citrate synthase (CS) further revealed the chaperone activity of both sHSPs, as they effectively protected CS from complete aggregation. The sHSP:CS at a ratio of 2:1 was found to be more effective than the 1:1 ratio for both *G. antarctica* sHSP proteins. The 2:1 ratio might have functioned better than the 1:1 ratio because sHSP requires a specific ratio of protein concentration and non-native protein to generate stable and effective complexes. Additionally, real-time PCR analysis showed that *gashsp12-338* expression increased by 1.38-fold under high heat stress and 2.33-fold under cold stress compared to the control temperature of 12°C. As a result of exposure to the fatal temperature of 20°C, both *gashsp07-010* and *gashsp12-338* expression levels were downregulated. Interestingly, at 30°C, both *gashsp07-010* and *gashsp12-338* levels were upregulated 2-fold compared to the expression at 20°C. It was possible that at 30°C, the presence of non-native proteins such as aggregates at a certain level triggered the expression of both sHSP. These findings reflect the diverse function of sHSP in *G. antarctica* that may play different roles in thermal adaptation. Comparative modeling of *G. antarctica* sHSP structures uncovered noteworthy alterations in the amino acid composition. In the tertiary structure of GasHSP07-010, an amino acid transition from non-charge to polar resulted in reduced interactions and increased stability. Conversely, GasHSP12-338 exhibited an amino acid change to a non-polar form, leading to diminished amino acid interactions and enhanced structural stability. These modifications loosen the strong ionic interactions and create a flexible connection which allows conformation change in the protein structures similar to the cold-adapted proteins

in hypersaline conditions which play an important role in protein solubility and flexibility to increase the speed of enzymatic bindings and reactions. These structural adaptations likely contribute to the flexibility and stability required for the functional activity of these proteins at low temperatures and their ability to protect other proteins during heat stress. The findings of this study shed light on the thermal protection mechanisms employed by sHSPs and offer valuable insights into their functionality.

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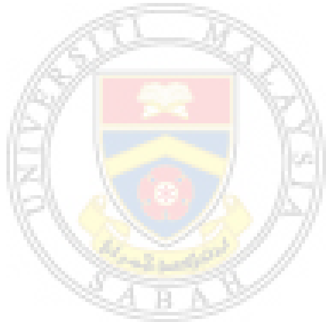
ABSTRAK

PENCIRIAN STRUCKTUR DAN FUNGSI PROTEIN KEJUT HABA KECIL DARIPADA *Glaciozyma antarctica* PI12 SEBAGAI TINDAK BALAS TERHADAP TEKANAN HABA

Antartika,mempunyai geografinya yang unik dan iklim yang melampau, merupakan habitat utama untuk banyak bakteria. Antara mikroorganisma ini, spesies subglasial Antartika telah berupaya untuk mengawal tekanan tinggi dan keadaan sejuk lampau. Hasil penyelidikan telah menunjukkan bahawa protein pengiring memainkan peranan penting dalam mencegah degradasi protein dan memudahkan lipatan semula protein di bawah tekanan haba. Khususnya, protein seperti protein kejut haba kecil (sHSP) telah didapati berinteraksi dengan protein yang sebahagian permukaannya terdedah kepada agritasi, dengan itu mengurangkan kerosakan selular. Kefungsian luar biasa sHSP psikrofilik pada suhu rendah memberikan peluang kepada penyelidik untuk meneroka hubungan antara struktur protein, kestabilan, fleksibiliti dan konformasi dinamik. Kajian ini bertujuan untuk menyiasat peranan sHSP yang diperoleh daripada Glaciozyma antartica dan mengkaji hubungan antara struktur molekul dan penyesuaian haba. Daripada empat gen sHSP yang dikenal pasti di G. antarctica, dua iaitu GasHSP07-010 dan GasHSP12-338, telah diklon menggunakan E. coli BL21(DE3). Protein yang dikodkan oleh gen ini dihasilkan pada 37 °C selama semalaman dan kemudiannya ditulenkan menggunakan kromatografi afiniti kelat logam tidak bergerak (IMAC). Protein yang telah ditulenkan menjalani kedua-dua ujian sintase sitrat dan ujian termotoleransi. Tambahan pula, pemodelan perbandingan gen ini dilakukan menggunakan CHIMERA, menyelaraskannya dengan strain Homo sapiens (2YRT) dan Schizosaccharomyces pombe (3W1Z). Kualiti struktur yang dimodelkan telah dinilai menggunakan plot Ramachandran, errat, dan verify3D. Keputusan daripada ujian termotoleransi in vitro menunjukkan bahawa GasHSP07-010 dan GasHSP12-338 melindungi sel E. coli daripada suhu maut 55°C sehingga 30 dan 60 minit, masing-masing. Ujian pengagregatan menggunakan sintase sitrat (CS) seterusnya mendedahkan aktiviti protein pengiring untuk kedua-dua sHSP, kerana mereka melindungi CS secara berkesan daripada pengagregatan lengkap. sHSP:CS pada nisbah 2:1 didapati lebih berkesan daripada nisbah 1:1 untuk kedua-dua protein G. antarctica sHSP. Nisbah 2:1 mungkin berfungsi lebih baik daripada nisbah 1:1 kerana sHSP memerlukan nisbah khusus kepekatan protein dan protein bukan asli untuk menjana kompleks yang stabil dan berkesan. Di samping itu, analisis PCR masa nyata menunjukkan bahawa ekspresi gashsp12-338 meningkat sebanyak 1.38 kali ganda di bawah tekanan haba tinggi dan 2.33 kali ganda di bawah tekanan sejuk berbanding dengan suhu kawalan 12°C. Hasil daripada pendedahan kepada suhu tinggi 20 °C, kedua-dua tahap ekspresi gashsp07-010 dan gashsp12-338 telah dikurangkan. Menariknya, pada 30 °C, kedua-dua tahap gashsp07-010 dan gashsp12-338 telah dikawal 2 kali ganda berbanding dengan ungkapan pada 20 °C. Ada kemungkinan bahawa pada 30 °C, kehadiran protein bukan asli seperti agregat pada tahap tertentu mencetuskan ekspresi kedua-dua sHSP. Penemuan ini mencerminkan kepelbagaian fungsi sHSP di G. antarctica yang mungkin memainkan peranan berbeza dalam penyesuaian terma. Permodelan perbandingan struktur G.

antarctica sHSP menemui perubahan penting dalam komposisi asid amino. Dalam struktur tertier GasHSP07-010, peralihan asid amino daripada tidak bercas kepada kutub mengakibatkan interaksi berkurangan dan peningkatan kestabilan. Sebaliknya, GasHSP12-338 mempamerkan perubahan asid amino kepada bentuk bukan kutub, yang membawa kepada pengurangan interaksi asid amino dan kestabilan struktur yang dipertingkatkan. Pengubahsuaian ini melonggarkan interaksi ionik yang kuat dan mewujudkan sambungan yang fleksibel yang membolehkan perubahan konformasi dalam struktur protein yang serupa dengan protein yang disesuaikan dengan sejuk dalam keadaan hipersalin yang memainkan peranan penting dalam keterlarutan protein dan fleksibiliti untuk meningkatkan kelajuan pengikatan dan tindak balas enzimatik. Penyesuaian struktur ini berkemungkinan menyumbang kepada fleksibiliti dan kestabilan yang diperlukan untuk aktiviti fungsi protein ini pada suhu rendah dan keupayaannya untuk melindungi protein lain semasa tekanan haba. Penemuan kajian ini memberi penerangan tentang mekanisme perlindungan haba yang digunakan oleh sHSP dan menawarkan pandangan berharga ke dalam fungsinya.



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LIST OF CONTENTS

	PAGE
TITLE	**i**
BORANG PENGESAHAN STATUS TESIS	**ii**
DECLARATION	**iii**
CERTIFICATION	**iv**
ACKNOWLEDGEMENT	**v**
ABSTRACT	**vi**
ABSTRAK	**viii**
LIST OF CONTENTS	**x**
LIST OF TABLES	**xv**
LIST OF FIGURES	**xvi**
LIST OF ABBREVIATIONS	**xviii**
LIST OF SYMBOLS	**xix**
CHAPTER 1: INTRODUCTION	**1**
1.1 Background of the study	1
1.2 Research questions	4
1.3 Research aims	4
1.4 Research objectives	4
1.5 Research hypothesis	5
CHAPTER 2: LITERATURE REVIEW	**6**

UMS UNIVERSITI MALAYSIA SABAH

2.1 Cryospheric Habitats and the Significance of Antarctic Yeasts 6

2.2 Adaptations and Ecological Significance of Cold-Loving Microorganisms 7

2.3 Morphological and Growth Characteristics of Glaciozyma antarctica PI12 9

2.4 Genome data of *Glaciozyma antarctica* 11

2.5 *Studies on Glaciozyma antarctica* 13

2.6 The Role of Molecular Chaperones in Protein Folding and Cellular Adaptation 14

2.7 Small Heat Shock Proteins (sHSPs) in Protein Misassembly and Cellular Protection 15

2.7.1 Characteristics and Roles of Small Heat Shock Proteins (sHSPs) in Protein Folding and Cellular Defense 16

2.7.2 Structural Characteristics and Chaperone Function of Small Heat-Shock Proteins (sHSPs) 17

2.7.3 The Role of Small Heat Shock Proteins (sHSPs) in Cellular Defense and Protein Folding Mechanisms 18

CHAPTER 3: METHODOLOGY 21

3.1 Microorganisms 21

3.2 Target selection for potential sHSP protein- coding genes from G. antarctica genome database for characterization 21

3.3 Oligonucleotide prim 22

3.4 Preparation for stock cultures and competent cells 22

3.4.1 *E. coli and G. antarctica stock cultures preparation* 22

3.4.2 *E. coli competent cells preparation* 23

3.5 Total RNA extraction *G. antarctica* 23

3.6 Preparation of gashsp target genes 24

3.6.1 First-strand cDNA synthesis 24

3.6.2 PCR amplification 25

3.6.3 PCR amplicon clean up using gel purification 25

3.7 Cloning and transformation of the target genes using the E. coli ligation independent cloning system (LIC) 26

UNIVERSITI MALAYSIA SABAH

3.7.1 Vector -specific primer design to amplify shsp genes 27
3.7.2 Annealing of the pET-32 EK/LIC vector and target inserts with compatible overhangs 27
3.7.3 Cloning and transformation 28
3.7.4 Plasmid extraction 28
3.7.5 Validation using PCR 29
3.7.6 Validation of insert by sequencing 30
3.8 Heterologous protein expression in E. coli BL21(DE3) 30
3.9 Buffer preparation for SDS-PAGE analysis 30
3.9.1 10X Running buffer 30
3.9.2 Coomassie blue R350 preparation for gel staining 31
3.9.3 Gel destaining solution preparation 31
3.9.4 Protein sample buffer preparation 31
3.9.5 Lysis buffer 32
3.10 Protein extraction 32
3.11 SDS-PAGE gel preparation 32
3.12 Buffers and solvent preparation for protein purification 35
3.12.1 Preparing Washing Buffer 35
3.12.2 Preparing Elution Buffer 35
3.12.3 Preparing Ethanol 70% 36
3.12.4 Preparing Filtered Water foMolecular Analysis 36
3.13 Metal ion affinity chromatography (IMAC) 36
3.14 Gene Expression Studies Using Quantitative Real-Time PCR (qPCR) 37
3.14.1 Measurement of Target Gene Expression by Quantitative Real Time PCR (qPCR) 37
3.14.2 qPCR procedure 37
3.15 Gene Expression Studies Using Quantitative Real-Time PCR (qPCR) 39
3.15.1 Thermotolerance assay 39
3.15.2 Citrate synthase aggregation assay 40
3.16 Functional Analysis 40

UMS UNIVERSITI MALAYSIA SABAH

3.16.1 Structural Characterization by Comparative Modelling 41
3.16.2 Model building for target proteins 41
3.16.3 Protein models validation 41
3.16.4 Protein structure analysis 42
3.17 Statistical Data 42

CHAPTER 4: RESULT AND DISCUSSION 43
4.1 Sequence characterization analysis of sHSP from G. antarctica 43
4.1.1 Comparative Analysis of sHSP Homologs 43
4.2 Construction of recombinant SHSP from G. antarctica 47
4.2.1 Analyzing RNA for quantity and quality 47
4.2.2 PCR amplification of sHSP G. antarctica 48
4.2.3 Construction of recombinant G.antarctica sHSP 49
4.2.4 Overexpression and purification of recombinant forms of GasHSP07-010 and GasHSP12-338 50
4.3 *G. antarctica sHSP mRNA expression levels* 54
4.3.1 Validation of qPCR primers efficiencies 54
4.3.2 Determining the stability of a reference gene 55
4.3.3 *G. antarctica* sHSP mRNA expression patterns at different temperatures 56
4.4 Functional Analysis 58
4.4.1 sHSP molecular chaperons activity analysis 58
4.4.2 Thermotolerance experiments with transformed E. coli BL21(DE3) 60
4.5 Comparative protein structure analysis 64
4.5.1 Structure model selection 65
4.5.2 Ramachandran plot 69
4.5.3 Structural validation using the ERRAT program 69
4.5.4 Structural validation using Verify3D 70
4.5.5 Structure validation using PROCHECK 71
4.5.6 Amino acid residues substitution analysis 74

CHAPTER 5: DISCUSSION 86

UNIVERSITI MALAYSIA SABAH

CHAPTER 6: CONCLUSION **91**

6.1 Conclusion 91

6.2 Future prospects 92

REFERENCES **93**

UMS
UNIVERSITI MALAYSIA SABAH

LIST OF TABLES

			PAGE
Table 2.1	:	List of yeast extracted from Antarctica	12
Table 3.1	:	LIC primers used in the PCR amplification	25
Table 3.2	:	PCR conditions	25
Table 3.3	:	T4 DNA polymerase treatment master mix	27
Table 3.4	:	Ligation of target insert into pET32 Ek/LIC	27
Table 3.5	:	PCR master mix	29
Table 3.6	:	PCR conditions	29
Table 3.7	:	Components used to make 10X running buffer	30
Table 3.8	:	Components used to make a 4X protein sample buffer	31
Table 3.9	:	Lysis buffer components	32
Table 3.10	:	Separation gel (10%)	33
Table 3.11	:	Stacking gel (4%)	34
Table 3.12	:	Washing buffer components	35
Table 3.13	:	running buffer	35
Table 3.14	:	List of qPCR primers	37
Table 3.15	:	Components for qPCR analysis	38
Table 3.16	:	PCR condition	38
Table 3.17	:	Citrate synthase aggregation assay	40
Table 4.1	:	Percent identity of GasHSP07-010 compared to other organisms	44
Table 4.2	:	Percent identity of GasHSP12-338 compared to other organisms	44
Table 4.3	:	Total RNA concentrations and purity values	48
Table 4.4	:	Primer's efficiency for RT-PCR	54
Table 4.5	:	Structures from PDB homolog with GasHSP07-010 amino acids	66
Table 4.6	:	Structures from PDB homolog with GasHSP12-338 amino acids	66
Table 4.7	:	Intraprotein interactions in GasHSP07-010 for substitution of Aspartic acid to Glutamine	75
Table 4.8	:	Intraprotein interactions in GasHSP07-010 for substitution of Serine to Asparagine	76
Table 4.9	:	Intraprotein interactions in GasHSP12-338 for substitution of amino acids (charged/hydrophobic side chains) to Isoleucine	79
Table 4.10	:	Intraprotein interactions in GasHSP12-338 for substitution of Glutamic acid to Valine	82
Table 4.11	:	Intraprotein interactions in GasHSP12-338 for substitution of Glutamic acid to Serine	82
Table 4.12	:	Intraprotein interactions in GasHSP12-338 for substitution of amino acids to Alanine	83

UMS UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

			PAGE
Figure 2.1	:	World map depicting the continents, each represented in a distinct color	7
Figure 2.2	:	The morphology observed under a microscope	10
Figure 2.3	:	Growth curve of Glaciozyma antarctica	11
Figure 2.4		The small heat-shock protein from the archaeon has been determined from Methanococcos jannaschii	17
Figure 4.1	:	In comparison to other organisms, a phylogenetic tree was created for the gashsp07-010	46
Figure 4.2	:	A phylogenetic tree for the gashsp07-338 was developed by comparing it to other organisms	46
Figure 4.3	:	Total RNA was extracted using Trizol and viewed on a 1% agarose gel	48
Figure 4.4	:	Target genes amplified and analysed on 1% agarose gel	49
Figure 4.5	:	Agarose gel of colony-PCR results for gashsp-7-010 and gashsp12-338	50
Figure 4.6	:	Result of SDS-PAGE of protein expression GasHP07-010 and GasHP12-338	52
Figure 4.7	:	Purified GasHSP12-338 protein at the size of 32 kDA and GasHSP07-010 protein at the size of 52kDa	53
Figure 4.8	:	Ni-NTA chromatograms of A) GasHSP07-010 and B) GasHSP12-338	53
Figure 4.9	:	The average Cq values of 18S gene expression collected at 5 different temperatures	56
Figure 4.10	:	Relative expression of target genes under cold stress and high-temperature stress	58
Figure 4.11	:	Citrate synthase was exposed to 53OC for 60 minutes, and the spectrometer measured the aggregates turbidity at 360 nm absorbance wavelength	60
Figure 4.12	:	Percentage of E. coli survival up to 60 minutes following exposure to 55 ^{O}C	63
Figure 4.13	:	E. coli cells colonies without *G. antarctica* sHSP following exposure to 55 ^{O}C for 60 minutes	64
Figure 4.14	:	SWISS model structures for GasHSP12-338with the above picture in monomeric form while the bottom picture shows the oligomers of several sHPS monomers	67
Figure 4.15	:	Superimposed of the modelled structures with their templates	68
Figure 4.16	:	Ramachandran plotted by the software	69
Figure 4.17	:	ERRAT results	70

UNIVERSITI MALAYSIA SABAH UMS

Figure 4.18 : VERIFY3D results. 71
Figure 4.19 : PROCHECK result for model GasHSP07-010 72
Figure 4.20 : PROCHECK result for GasHSP12-338 73

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LIST OF ABBREVIATIONS

HSP	-	Heat Shock Protein
sHSP	-	Small Heat Shock Protein
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
ATP	-	Adenosine Triphosphate
LIC	-	Ligation Independent Cloning
OD	-	Optical Density
DTT	-	Dithiothreitol
LB	-	Luria-Bertani
TEMED	-	Tetramethylethylenediamine
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
YPD	-	Yeast Extract–Peptone Dextrose
RT-PCR	-	Reverse Transcription Polymerase Chain Reaction
Bp	-	Antibody dependent enhancement
EDTA	-	Base Pair
CS	-	Citrate Synthase
HSR	-	Heat Shock Response
ACD	-	α-crystallin domain

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LIST OF SYMBOLS

Å	-	Angstrom
µM	-	Micro molar
mM	-	Mili molar
°C	-	Degree Celcius
kDa	-	Kilo Dalton
M	-	Molar
Mg	-	Micro Gram
Nm	-	Nanometer
µM	-	Micromolar
µg	-	Microgram

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Antarctica is the world's southernmost continent and the fifth-largest landmass on the planet. Its landmass is nearly covered by a large ice sheet (Buzzini *et al.*, 2017). Additionally, it's home to the world's highest mountain ranges, driest deserts, fiercest winds, and coldest temperatures (Lize, 2021; Satyanarayana & Kunze, 2009). Life on the Antarctic continent depends on the supply of water, which is a freezing, barren desert. More than a thousand distinct species of organisms have been identified in the terrestrial environment, however, microorganisms make up the vast majority (Lize, 2021; Satyanarayana & Kunze, 2009). Several species have been examined for their capacity to cope with the extremes of heat and cold seen in the Antarctic. The development of Antarctic organisms has been influenced by several geological and climatic factors (Boo *et al.*, 2013). Due to the low temperatures, living creatures face several difficulties. Formation of RNA and DNA secondary structure, increase of DNA super-cooling, and decrease in membrane fluid are some of the challenges they may encounter (Jung *et al.*, 2010). Numerous Antarctic organisms have developed a variety of adaptations that enable them to thrive and reproduce in frigid conditions.

Psychrophiles are the group of extremophiles that can survive in extremely cold conditions, such as the oceans and the polar regions. The majority of the species include bacteria, archaea, algae, yeast, plants, and animals, whereas the largest psychrophiles are the polar fish that thrive beneath the icecap. In terms of diversity, biomass, and dispersion, psychrophiles are the most commonly seen microorganisms (Parvizpour *et al.*, 2021). For cold adaptation, a range of structural and functional modifications are required. Low (or even subzero) temperatures

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have a range of effects on psychophilic yeast, including slowed growth rates, changed protein structures, decreased membrane fluidity, increased medium viscosity, and reduced nutrient availability. As a result, they evolved a multitude of adaptation methods, including subcellular, molecular, and metabolic alterations, as well as the synthesis of protective proteins, in response to temperature stress (Buzzini *et al.*, 2012).

Molecular chaperones are categorised into families based on their molecular mass, evolutionary history, and unique features (Haslbeck & Vierling, 2015; Kriehuber *et al.*, 2010; Walter & Buchner, 2002). A member of the stress protein family with one of the most diverse structures and functions is the small heat shock protein (sHSP) (Franck *et al.*, 2004). Due to their ability to selectively bind to unfolded proteins in vitro and inhibit aggregation, the sHSP are classified as molecular chaperones (Walter & Buchner, 2002). The sHSP are defined by the presence of a conserved α-crystallin domain that presents in all three domains of life (Laksanalamai & Robb, 2004; Nakamoto & Vígh, 2007). The sHSP are also linked to a wide range of illnesses, including Alzheimer's and cancer (Haslbeck & Vierling, 2015). In comparison to prokaryotic and unicellular eukaryotic creatures, the sHSP gene is more ubiquitous in multicellular eukaryotic organisms (Kriehuber *et al.*, 2010).

Numerous sHSP members are often detected in the same cell compartments, indicating that they have numerous functions (Nakamoto & Vígh, 2007). The sHSP synthesis may have been boosted by the damaged proteins since they had lost their capacity to function and build up in the cell (Walter & Buchner, 2002). Members of this family have core domains known as α-crystallin domains, which are present in all sHSP (Kriehuber *et al.*, 2010; Nakamoto & Vígh, 2007). There are a broad variety of roles in which sHsp may be involved, such as the cellular defences against high temperatures, as well as the ability to bind many distinct cellular substrates (Nakamoto & Vígh, 2007). Many cold-adapted bacteria have been shown to have sHSP downregulation at low temperatures (Martínez-Paz *et al.*, 2014). The cold-induced downregulation of sHSP implies that these folding aids are mostly created at temperatures that are temporarily higher than normal (Feller, 2013).

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The small HSP (sHSP) from the psychrophilic yeast *Glaciozyma antarctica* (GA) was first examined by Yusof *et al*., 2016. *G. antarctica*, a psychrophilic yeast was isolated from sea ice at the Casey Research Station in Antarctica. *G. antarctica* thrives in environments with temperatures no higher than 12°C. Temperature extremes of up to 20°C and more restrict *G. antarctica*'s growth (Alias *et al*., 2014; Boo *et al*., 2013; Koh & Wong, 2017; Koh *et al*., 2019; Turkiewicz *et al*., 2004). In this study, *G. antarctica* was chosen as the subject of study for several reasons. Firstly, the availability of genome data allowed us to perform a thorough investigation of its sHSP. Second, this yeast can be readily grown and maintained in laboratory conditions. Moreover, a study on an sHSP of *G. antarctica* revealed some significant findings on its protein structure and adaptation strategies in extreme temperatures (Yusof *et al*., 2016). Hence, there is a need to study other sHSP in *G. antarctica* to determine the pattern of adaptation strategies acquired by this extreme organism.

To date, there are 9 sHSP genes in *G. antarctica* with one that has been characterised in Yusof *et al*. 2016. Other organisms such as yeast have 2 genes, 12 in *Drosophila melanogaster*, 16 in *Caenorhabditis elegans*, 19 in *Arabidopsis thaliana* and 10 genes in humans (Kappé et al., 2003). The genome of *G. antarctica* (http://www. genomemalaysia.gov.my/glaciozyma_antarctica/) contains 7857 genes, with at least 10% being novel or exhibiting no detectable sequence similarity to known folds. Out of 7 uncharacterised sHSP, two sHSP were able to be fully amplified without any mutations from *G. antarctica* total RNA. Therefore, intrigued by the adaptation strategies acquired by sHSP in *G. antarctica*, this study focuses on two sHSP that we are able to be PCR amplified namely *GasHsp07-010* and *GasHsp12-338*. Based on protein domain analysis, both proteins contained α-crystalline domains that may play important roles in the prevention of protein aggregation during thermal stress. This suggests that these sHSP proteins may acquire function in the cold which reflects protein flexibility and stability. In this study, we characterized both *G. antarctica* sHSP in terms of their protein structures and functions. The outcome of this study is expected to contribute new findings and determine the pattern of thermal adaptation strategies acquired by *G. antarctica*. This important knowledge gathered from this study could be applied in

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various applications such as nanobiotechnology, cryogenic storage for biological materials, proteomics, protein expression system and bioproduction.

1.2 Research Questions

1. Do the selected sHSP genes from *G. antarctica* function similarly to those found in other organisms when exposed to thermal stress?
2. Does the selected sHSP from *G. antarctica* possess specific thermal adaptation strategies at the structural level?
3. What is the relationship between the structures and functions of the selected sHSPs in *G. antarctica* and their role in thermal adaptation strategies?

1.3 Research Aims

The research aims to study the relationship between the function and structure of the selected sHSP proteins from the psychrophilic yeast, *G. antarctica* for further understanding of their protein adaptation strategies in thermal response.

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1.4 Research Objectives

1. To investigate the response of the selected sHSP genes of *G. antarctica* PI12 to various thermal treatments.
2. To analyse the structure of the *G. antarctica* sHSP proteins using comparative homology modelling.
3. To explore the relationship between the structural attributes of the selected sHSP proteins in *G. antarctica* and their role in cellular protection in response to thermal stress.