

CHARACTERISATION OF STRUCTURAL AND FUNCTIONAL CHANGES OF  
HUMAN HEAT SHOCK PROTEIN 47 WILD-TYPE AND HIS-TO-ALA  
MUTANTS

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## DEDICATION

*“My dearest family”*

I dedicate this thesis to all of you

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## ABSTRACT

Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that localises in the endoplasmic reticulum (ER). The HSP47 is essential for a proper formation of the collagen triple helix. The HSP47 binds to the completely-folded collagen molecule and accompanies it from the ER to the Golgi apparatus (GA), where dissociation occurs and HSP47 is recycled back to the ER. The binding and release behaviour are triggered by the lower pH in the GA or the ER-Golgi intermediate compartment (ERGIC). Histidine has been suggested to be the trigger residue due to the side chain  $pK_a$  value of around 6.1, similar to the HSP47-collagen dissociation pH. In this study, Histidine-to-alanine (HA) mutants of human HSP47 were constructed to elucidate the specific mechanism that governs HSP47 release from collagen at the molecular level, known as the “pH-switch mechanism”. The binding of three mutants were found to be affected, namely H220A, H335A and H368A. This study investigated the effects of these mutations to the human HSP47 binding strength, using collagen type I extracted from rat tail tendon and modified enzyme-linked immunosorbent assay (ELISA)-based binding assay. The secondary structure of mutant proteins was investigated using circular dichroism (CD) spectroscopy. The result showed that mutants were found to retain their binding ability to collagen, except for H220A. The H335A and H368A were found to have slightly lower binding affinity to collagen relative to the wild-type (WT) ( $K_D = 50.76$  nM), with a dissociation constant ( $K_D$ ) of 152.9 nM and 131.3 nM respectively. In addition to the lost in collagen binding, H220A was also significantly more thermostable compared to the other mutants, that have similar thermostability to WT. The secondary structure of the HA mutants at alkaline pH was found to differ slightly from WT, with H368A showed a perturbed pH-induced secondary structural changes. In conclusion, the binding strength of the human HSP47 HA mutants was successfully elucidated and the structural changes caused by the mutations were described. The H220 was suggested to be important for binding with collagen while H368 was important for the release mechanism.

## ABSTRAK

Protein Kejutan Haba 47 (HSP47) adalah protein pendamping kolagen dan dijumpai di dalam retikulum endoplasma (ER). HSP47 sangat penting untuk pembentukan heliks tiga serangkai yang berfungsi sebagai peneman dari ER ke badan Golgi sebelum pemisahan berlaku dan kembali semula ke ER. Tingkah laku pengikatan dan pelepasan ini dicetuskan oleh pH rendah di dalam GA atau di ruang perantaraan ER-Golgi (ERGIC). Histidina telah dicadangkan sebagai residu pencetus kerana nilai  $pK_a$  rantai sisinya iaitu sekitar 6.1 yang sama seperti nilai pH di mana pemisahan kolagen daripada HSP47 berlaku. Dalam kajian ini, mutan histidina-ke-alanina telah dibangunkan untuk HSP47 manusia bagi menjelaskan mekanisma khusus yang mengatur pelepasan HSP47 dari kolagen pada tahap molekul, yang dikenali sebagai “mekanisma pertukaran pH”. Tiga mutan sebelum ini didapati mempunyai pengikatan lebih rendah dengan kolagen, iaitu H220A, H335A dan H368A. Kajian ini bertujuan menyelidik kesan mutasi ini kepada kekuatan pengikatan HSP47 manusia. Kekuatan pengikatan dikaji menggunakan kolagen jenis I yang diekstrak daripada tendon ekor tikus dan kaedah berasaskan Imunoserap Berkait Enzim (ELISA). Struktur sekunder protin mutan seterusnya dikaji menggunakan spektroskopi dwikroisme membulat (CD). Keputusan menunjukkan semua mutan didapati masih mampu mengikat kepada kolagen, kecuali H220A. H368A dan H335A didapati mempunyai kemampuan mengikat kolagen yang lebih rendah, dengan pemalar pengasingan ( $K_D$ ) bernilai 131.3 nM dan 152.9 nM, berbanding protein jenis asal (WT) ( $K_D = 50.76$  nM). H220A bukan sahaja hilang kemampuan mengikat kolagen malah mempunyai tahap kestabilan haba yang lebih tinggi berbanding WT dan mutan yang lain. Struktur sekunder pada pH alkali untuk semua mutan didapati sedikit berbeza daripada WT, dengan H368A berbeza dari segi perubahan pertukaran struktur yang dicetuskan oleh pH. Kesimpulannya, kekuatan pengikatan di antara mutan HSP47 manusia dan kolagen telah berjaya dikaji dan perubahan struktur disebabkan oleh mutasi telah dicadangkan di dalam kajian ini. Kajian ini mencadangkan yang H220 adalah penting bagi pengikatan HSP47 dengan kolagen, manakala H368 penting bagi mekanisma pemisahan.

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## LIST OF ABBREVIATIONS

OI	-	osteogenesis imperfecta
HA	-	His-to-Ala
ER	-	endoplasmic reticulum
CD	-	circular dichroism
ELISA	-	enzyme-linked immunosorbent assay
WT	-	wild type
ECM	-	extracellular matrix
FACIT	-	fibril-associated collagens with interrupted triple helices
PTM	-	post-translational modifications
BiP	-	immunoglobulin heavy-chain binding protein
Pro	-	proline
Hyp	-	hydroxyproline
GRP94	-	glucose-regulated protein
CNX	-	calnexin
CRT	-	calreticulin
PDI	-	protein disulfide isomerase
PPIases	-	peptidyl-prolyl cis-trans isomerases
P4H	-	prolyl 4-hydroxylase
SERPIN	-	serine protease inhibitor
RDEL	-	Arg-Asp-Glu-Leu
ERGIC	-	<i>cis</i> -Golgi or the ER-Golgi intermediate compartment
HSCs	-	hepatic stellate cells
ESCC	-	esophageal squamous cell carcinoma
siRNA	-	Small interfering RNA
HNSC	-	head and neck squamous cell carcinoma
LB	-	Luria Bertani
BCIP/NBT	-	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
IPTG	-	isopropyl- $\beta$ -D-thiogalactopyranoside
SDS- PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis

AP	-	alkaline phosphatase
IMAC	-	immobilized metal-ion affinity chromatography
CBB	-	coomassie brilliant blue
SEC	-	size-exclusion chromatography
Asn	-	asparagine
Ala	-	alanine
PAI-1	-	plasminogen activator inhibitor
$pK_a$	-	acid dissociation constant
$k_d$	-	dissociation constant
NMR	-	nuclear magnetic resonance
CpHMD	-	constant pH molecular dynamics
mrm	-	mature recombinant mouse
hPCI	-	human protein C inhibitor
Trp	-	tryptophan
Phe	-	phenylalanine

## LIST OF SYMBOLS

cm	-	centimetre
Da	-	dalton
g	-	gramme
H	-	hour
kDa	-	kilodalton
L	-	litre
M	-	molar
mg	-	milligram
min	-	minute(s)
mL	-	millilitre
mM	-	millimolar
Mw	-	molecular weight
R <sup>2</sup>	-	coefficient of determination
rpm	-	revolutions per minute
U	-	unit of enzyme activity
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume
µg	-	microgram
µL	-	microlitre
µmol	-	micromole
%	-	percentage
xg	-	times gravity
~	-	approximately
°C	-	degree celcius
$\Delta\Delta A_{222}$	-	change in CD signal intensity at 222 nm
$\Delta A$	-	difference in absorbance between CD L and R components
Abs <sub>280</sub> , A <sub>280</sub>	-	absorbance at 280 nm
mdeg	-	milidegrees
T <sub>m</sub>	-	melting temperature



mAu - mili absorbance unit

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# CHAPTER 1

## INTRODUCTION

### 1.1 Research Background

Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that assists the maturation and transport of procollagens in collagen biosynthesis. This chaperone can be found in all vertebrates, where collagen is synthesised by the cell. Nowadays, there are a growing body of evidence on the involvement of HSP47 in various types of diseases, such as in osteogenesis imperfecta (OI), fibrosis, and cancer (Syx et al., 2021; Westra et al., 2016; Fan et al., 2020). A close relation between the increased expression of HSP47 and the excessive deposition of collagen has been reported in human, in vivo and in vitro fibrotic disease models (Kim et al., 2019). The increased expression in fibrotic diseases is known to help in the increased assembly of procollagen and thus lead to the excessive accumulation of collagen in fibrotic area (Bellaye et al., 2020). HSP47 is also being reported to be upregulated in various types of cancers such as stomach cancer, lung cancer, head and neck cancer, pancreatic ductal adenocarcinoma and ulcerative colitis-associated carcinomas (Chern et al., 2020; Fan et al., 2020; Zhao et al., 2014). This urges the need to completely elucidate the molecular mechanisms of this chaperone.

Previously, Abdul Wahab and colleagues (2013) have successfully constructed a series of His-to-Ala (HA) mutants targeting all fourteen histidine residues of mouse HSP47, to elucidate the so-called “pH-switch mechanism”. This is the mechanism known to govern the release of HSP47 from collagen. This relates to its cellular behaviour, where HSP47 is recycled back to the endoplasmic reticulum (ER) after assisting the transport of procollagen from the ER to the cis-Golgi. From the study, some HA mutations do not impose significant perturbations in HSP47 function, but several others were found to impair the binding to collagen, which is an unexpected effect.

In 2014, fourteen histidine residues of human HSP47 were successfully mutated to Alanine (unpublished data). This project was therefore proposed to investigate the effects of the selected HA mutations in human HSP47, and to further investigate the pH-switch mechanism. Human HSP47 was used for more physiologically-relevant results as it has 98% of similarities with mouse species. The mutants investigated were H220A, H335A and H368A, based on the perturbed behaviour observed previously (unpublished data). Mutations to alanine abolished the histidine side chain characteristics and the possible bonding with the neighbouring residues, allowing for the assessment of the importance of these target residues.

## **1.2 Problem Statement**

It was found that the HA mutations at specific locations (particularly H220A, H335A, H368A) of HSP47 has significantly affected the binding behaviour based on gelatin agarose pull down assay (unpublished data). These mutations could potentially affect the release mechanism too. The binding study using gelatin pull down assay was only able to predict the conservation of structure and binding interface of the mutants, but unfortunately it does not provide further information on the binding strength. Thus, to investigate further the effects of the mutation on the binding strength, an enzyme-linked immunosorbent assay (ELISA)-based binding assay was proposed. This assay allowed quantification of the binding strength of the mutants, relative to wild type (WT). Consequently, the effects of mutations on HSP47 were investigated based on the secondary structural changes with pH, using circular dichroism (CD) spectroscopy. This technique allowed a more detailed understanding of the pH-induced structural transition of the mutants.

### **1.3 Research Objectives**

The aim of this research project was to study the binding strength and pH-release behaviour of human HSP47 and target mutants (H220A, H335A, H368A) relative to the WT. The specific objectives are as follows:

1. To express and purify the recombinant human HSP47 WT and target mutants.
2. To determine the binding affinity of human HSP47 WT and target mutants using ELISA-based binding assay.
3. To determine the pH-induced structural transition of human HSP47 WT and target mutants using circular dichroism spectroscopy.

### **1.4 Scope of Research**

This project covers a few aspects:

- (a) Preparation of competent cell by using  $\text{CaCl}_2$  method and transformation of competent cells using heat shock protocol.
- (b) Expression and purification of recombinant HSP47 wild type (WT) and target mutants (H220A, H335A, H368A). The purification was done using immobilised nickel affinity column as the first purification step and then using HiTrap<sup>TM</sup> HP desalting column as the second purification step.
- (c) Determination of binding affinity of human HSP47 WT and target mutants by using ELISA-based binding assay.
- (d) Investigation on the changes to pH-induced structural transition of HSP47 WT and target mutants by using circular dichroism spectroscopy.

## **1.5 Significance of Research**

This research is important to discover the key His residue(s) that controls HSP47 pH-switch release, and the involvement in binding or structural integrity. Methods used in this research can directly be applied in collagen-related disease model studies. Therefore, this will increase the understanding on these diseases thus can move towards designing suitable therapeutic strategies.

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