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**EXPRESSION, PURIFICATION AND  
CHARACTERISATION OF RECOMBINANT  
PEPTIDE:N-GLYCOSIDASE F.**

A thesis presented in partial fulfilment of the requirements for  
the degree of Master of Philosophy in Biochemistry  
at Massey University, New Zealand.

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**2000**

“I have not failed. I've just found 10,000 ways that won't work.”

**Thomas Edison.**

**ABSTRACT**

PNGase F (Peptide- $N^4$ -(*N*-acetyl-D-glucosaminyI) asparagine amidase F) is an amidohydrolase isolated from the extracellular medium of the Gram-negative bacterium *Flavobacterium meningosepticum*. The 34.8-kDa enzyme catalyses the complete and intact cleavage of asparagine-linked oligosaccharide chains from their associated proteins. A T7 promoter-based *E. coli* expression system was developed in which PNGase F was expressed as a fusion protein with a leader sequence from the *ompA* gene. The hexa-histidine-tagged PNGase F was correctly processed and exported to the *E. coli* periplasm and had a calculated molecular weight of 36.2 kDa. A single step purification using immobilised metal affinity chromatography yielded 8 mg of pure protein per litre of culture.

The sequence of the PNGase F coding region from the CDC strain 3352 of *F. meningosepticum* was found to differ from a published sequence from another strain of the bacterium (ATCC 33958) in 57 positions. These differences between the two strains result in eight amino acid substitutions, which are mostly conservative in nature and are on the surface of the protein. Moreover, three potential *N*-glycosylation sites not present in the ATCC strain 33958 were detected in CDC strain 3352.

The recombinant enzyme has similar characteristics of the native enzyme with a pH optimum of 8.5 and is strongly inhibited by  $\text{Ag}^+$ ,  $\text{Cu}^{2+}$ , and  $\text{Fe}^{3+}$  ions but not by sulfhydryl-targeting agents such as DTT and NEM. This indicates inhibition by these ions is probably through interactions with a histidine residue at position 193 that may be involved in substrate recognition or catalysis. The specific activity of the native PNGase F is about four times that of the recombinant protein which may be contributed to inhibition by components of the Complete™ protease inhibitor tablets used in the enzyme preparation or due to modifications for cloning and purification. Using a discontinuous assay and a non-labelled 11-mer ovalbumin-derived glycopeptide as substrate, a rough estimate of the Michaelis constant ( $K_m$ ) for the recombinant PNGase F was determined to be 2.1  $\mu\text{M}$ . An intriguing observation with the activity assays was the apparent product inhibition of enzyme activity and the inhibitor may be either peptide and/or glycan components, which require further investigations into the cause of the inhibition.

## ACKNOWLEDGEMENTS

The many staff and students at the Institute of Molecular Biosciences to be thanked for their help and support. The following people deserve special mention:

My supervisor Dr. Gillian Norris for her invaluable advice, infinite patience and enthusiasm throughout the course of my study. Sincere thanks also to my co-supervisors Dr. Mark Patchett and Dr. Shaun Lott for their advice, encouragement and support.

I would also wish to thank my friends in X-lab: Deborah Frumau for her assistance, Julian Adams for his advice and modelling 3D structures, and F. Y. Chai for his humour and assistance. Dr. Cristina Weinberg for her encouragement as a caring friend who was forever patient with my questions, who kept me company late at night, and made me eat healthy.

Joanne Mudford for her friendship and giving me a “crash course” about her job before moving to Dunedin. Carmen Norris and David Elgar from NZDRI for giving me permission to use their HPLC system, Dr. Robert Norris for proof-reading my thesis, Associate Professor Geoff Jamerson for his generosity in equipment purchase and consumables, Associate Professor David Harding and Dick Poll for their valuable advice on chemistry and equipment loans, Dr. Catherine Day and Mrs. Carole Flyger for advice and assistance when I was working on my expression constructs, and Professor Pat Sullivan for his interest and encouragement for this work.

I should like to thank my long-suffering flatemate Desmond for enduring the chaos and his infallible efforts to keep the house habitable during house renovations and my long absences in writing of this manuscript.

Finally, thanks to my family who has always encouraged me to pursuit my interests regardless how bizarre they seemed.

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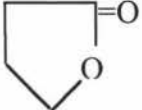
## ABBREVIATIONS

amu	Atomic mass units
Amp	Ampicillin
ATCC 33958	American Type Culture Collection No. 33958; sequence from this strain was published by Tarentino <i>et al.</i> , 1990.
AUFS	Absorbance units at full scale
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
Caps	3-Cyclohexylamino-1-propanesulfonic acid
Capso	3-Cyclohexylamino-2-hydroxy-1-propanesulfonic acid
CDC strain 3352	United States Communicable Disease Centre culture collection strain 3352; the strain used in this study.
CDI	1,1'-carbonyldiimidazole
cfu	Colony forming units
CIAP	Calf intestinal alkaline phosphatase
CNBr	Cyanogen bromide
CTB	<i>N,N'</i> -diacetylchitobiose
Dabsyl	4-(dimethylamino)-azobenzene-4'-sulfonyl
DIG	Digoxigenin
DNA	Deoxyribose nucleic acid
DNTPs	Deoxyribose nucleotide triphosphates
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid (di-sodium salt)
EGCase	Endoglycoceramidase
ENGase	Endo- <i>N</i> -acetyl- $\beta$ -D-glucosaminidase or endoglycosidase
EPPS	N-[2-Hydroxyethyl]piperazine- <i>N'</i> [3-propanesulfonic acid]
ER	Endoplasmic reticulum
ES-MS	ElectroSpray Mass Spectrometry
EtBr	Ethidium bromide
FPLC	Fast protein liquid chromatography
L-GLDH	L-Glutamic dehydrogenase
GdmHCl	Guanidine hydrochloride


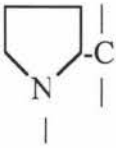
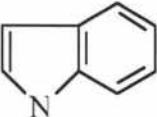
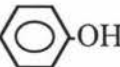
GPI	Glycosylphosphatidylinositol
HCl	Hydrochloric acid
IMAC	Immobilised metal ion affinity chromatography
IPTG	Isopropyl-1-thio- $\beta$ -D-galactopyranoside
kb	kilo base pairs
kDa	kilo daltons
$\alpha$ -KG	$\alpha$ -Ketoglutaric acid
LB	Luria broth
MW	Molecular weight
MWCO	Molecular weight cut-off
Mes	Morpholinoethane sulfonic acid
MOPS	3-[N-Morpholino] propanesulfonic acid
NaAc	Sodium acetate buffer
NaCl	Sodium chloride
NADPH	$\alpha$ -Nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	<i>N</i> -Ethylmaleimide
NGase	$\beta$ -aspartyl- <i>N</i> -acetylglucosamine hydrolase
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ammonium sulphate
NMR	Nuclear magnetic resonance
<i>O</i> -GlcNAcase	Cytoplasmic $\beta$ -GlcNAcase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Phosphatidyl inositol
PMF	Proton motive force
PMSF	Phenylmethylsulfonyl fluoride
PNGase	Peptide- <i>N</i> <sup>4</sup> -( <i>N</i> -acetyl- $\beta$ -D-glucosaminyl) asparagine amidase F
POGase	Peptide- <i>O</i> -glycanase
Psi	Pounds per square inch
PVDF	Polyvinylidene difluoride
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SPR	Surface plasmon resonance

Taps	(N-tris[Hydroxy-methyl]methyl-3-amino propanesulfonic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl)-aminomethane
TFA	Trifluoroacetic acid
Thesit	Polyoxyethylene 9-laurylether
TSK	Chromatography matrix copolymer of oligoethylene glycol, glycidylmethacrylate and pentaeryhtrol-dimethacrylate
UV	Ultra violet
X-Gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside

## Amino Acid Abbreviations

Amino acid	Three Letter Symbol	One Letter Symbol	MW	Side Chain Structure
Alanine	Ala	A	89	-CH <sub>3</sub>
Arginine	Arg	R	174	-(CH <sub>2</sub> ) <sub>3</sub> -NH-C-NH <sub>2</sub>    NH
Asparagine	Asn	N	132	$\begin{array}{c} \text{O} \\ // \\ -\text{CH}_2-\text{C}-\text{NH}_2 \end{array}$
Aspartic Acid	Asp	D	133	-CH <sub>2</sub> -COOH
Asparagine or Aspartic acid	Asx	B		
Cysteine	Cys	C	121	-CH <sub>2</sub> -SH
Glutamic Acid	Glu	E	147	-(CH <sub>2</sub> ) <sub>2</sub> -COOH
Glutamine	Gln	Q	146	$\begin{array}{c} \text{O} \\ // \\ -(\text{CH}_2)_2-\text{C}-\text{NH}_2 \end{array}$
Glutamine or glutamic acid	Glx	Z		
Glycine	Gly	G	75	-H
Histidine	His	H	154	-CH <sub>2</sub> - 
Homoserine	Hs	Hs	119	-CH <sub>2</sub> -CH <sub>2</sub> -OH
Homoserine Lactone	Hsl	Hsl	101	
Isoleucine	Ile	I	131	-CH-CH <sub>2</sub> -CH <sub>3</sub>   CH <sub>3</sub>



Leucine	Leu	L	131	$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH}_2-\text{CH} \\   \\ \text{CH}_3 \end{array}$
Lysine	Lys	K	146	$-(\text{CH}_2)_4-\text{NH}_2$
Methionine	Met	M	149	$-(\text{CH}_2)_2-\text{S}-\text{CH}_3$
Phenylalanine	Phe	F	165	$-\text{CH}_2-$ 
Proline	Pro	P	115	
Serine	Ser	S	105	$-\text{CH}_2\text{OH}$
Threonine	Thr	T	119	$\begin{array}{c} -\text{CH}-\text{CH}_3 \\   \\ \text{OH} \end{array}$
Tryptophan	Trp	W	204	$-\text{CH}_2-$ 
Tyrosine	Tyr	Y	181	$-\text{CH}_2-$ 
Valine	Val	V	117	$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CH} \\   \\ \text{CH}_3 \end{array}$

## Sugar Abbreviations

Sugar	Three Letter Symbol
Fucose	Fuc
Galactose	Gal
Mannose	Man
N-acetylgalactosamine	GalNAc
N-acetylglucosamine	GlcNAc
N-acetylneuraminic (sialic) acid	NeuNAc

Note: Sugar linkages are described using conventional carbon ring numbers connected by a slash and anomericity is denoted by  $\alpha$  or  $\beta$ . For example, galactose  $\beta$ 1-4 linked to N-acetylglucosamine is written as Gal $\beta$ 1-4GlcNAc.