HETEROLOGOUS PRODUCTION AND CHARACTERISATION OF A YEAST PEPTIDE:*N*-GLYCANASE

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Abstract

Peptide:*N*-glycanases (PNGases) removes *N*-linked glycans from glycoproteins. Three distinct families of PNGases have been characterised, although all of them not completely. Some of these PNGases are cytosolic, others are secreted. Cytoplasmic PNGases (Png1p) are implicated in the proteasomal degradation of newly synthesized misfolded or unfolded glycoproteins that are exported from the endoplasmic reticulum (ER). Cytoplasmic PNGases are encoded by the *PNG1* gene and have been classified as members of transglutaminase-like superfamily based on the sequence analyses. There has, however, been no report of transglutaminase activity in any PNGase. The three-dimensional structures of recombinant PNGases from yeast (*S. cerevisiae*) and mouse have been determined in complex with the XPCB domain of Rad23 and mHR23B respectively. These PNGases were both produced as insoluble proteins, and could only be refolded and crystallised in the presence of their physiological binding partners.

In this study, the gene encoding for *S. pombe* PNGase has been cloned and heterologously expressed as a soluble thioredoxin-fused protein. The proteolytic cleaved recombinant protein (rPNGase Sp) remained soluble as a monomer and retained its deglycosylating activity. It did not have, however any transglutaminase activity despite its homology to the transglutaminase family of proteins. The activity of rPNGase Sp *in vitro* is both reductant and Zn^{2+} dependent. rPNGase Sp showed apparent heterogeneity on SDS-PAGE, which was characterised by the appearance of two bands differing in their molecular weights by an ~ 2.3 kDa. This heterogeneity was eventually shown to be the result of two different local conformations that were dependent on disulfide bond and/or Zn^{2+}. The enzyme was shown to only deglycosylate the denatured glycoproteins, not their native counterparts. Moreover, it preferred to deglycosylate glycoproteins with high mannose-type glycan chains, both of which are consistent with the biological function of cytoplasmic PNGases.

Compared to bacterial PNGase F, rPNGase Sp is not very active, at least on the substrate used in this study. A higher K_m (186 μM) determined for rPNGase Sp using a FITC-labelled glycopeptide which carries a complex-type glycan as the substrate also suggests that complex glycans are not favoured substrates for these PNGases. rPNGaseSp has similar characteristics to the yeast (*S. cerevisiae*) and mouse PNGases; it has a neutral pH optimum and is strongly inhibited by Cu^{2+}, Cd^{2+} and Ni^{2+}. EDTA treatment deactivates it, and the addition of Zn^{2+} could not restore its activity. Interestingly, addition of exogenous Zn^{2+} was found to strongly inhibit rPNGase Sp.

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Table of Contents

Page

Abstract i

Acknowledgements ii

Table of Contents iii

List of Figures vii

List of Tables x

Abbreviations xi

Chapter 1 — Introduction

1.1 *N*-linked Glycosylation of Proteins 1

1.2 De-*N*-glycosylation 1

1.3 Peptide:*N*-Glycanase (PNGase) Overview 2

1.4 Potential Functions of Cytoplasmic PNGase 3

1.5 Cytoplasmic PNGases

1.5.1 Discovery of cytoplasmic PNGases 4

1.5.2 Conservation in eukaryotes 5

1.5.3 Similarity to proteins of the transglutaminase-like superfamily 6

1.6 Crystal Structure of the Png1p 8

1.7 Png1p Proteins are Cytoplasmic PNGases 13

1.8 The Protein Interactions of Cytoplasmic PNGase 14

1.9 Common Enzymatic Properties of Png1p

1.9.1 Effect of pH and Temperature 16

1.9.2 Effect of metal ions and dithiothreitol 17

1.9.3 Substrate specificity 17

1.10 Aims of This Investigation 19

Chapter 2 — Materials and Methods

2.1 Materials and Equipment 21

2.2 General Methods

2.2.1 Agarose gel electrophoresis 23

2.2.2 SDS polyacrylamide gel electrophoresis 23

2.2.3 Native polyacrylamide gel electrophoresis 23

2.3 Cloning

2.3.1 Cloning *PNG1Sp* into different vectors 24

2.3.2 Transformation, colony screening and plasmid DNA preparation 25

2.3.3 Sequence analyses of DNA 26

2.4 Protein Expression in *Escherichia coli*

2.4.1 Small-scale expression trials 27

2.4.2 Large-scale expression in Origami™ B (DE3) 28

2.5 Purification of Thioredoxin-His_6-tagged PNGase Sp

2.5.1 Cell lysis 29

2.5.2 Immobilised metal affinity chromatography (IMAC) 29

2.5.3 Proteolytic removal of fusion tag 30

2.5.4 Removal of tag using IMAC 31

2.5.5 Size exclusion chromatography (SEC) 31

2.5.6 Protein quantitation 31

2.6 PNGase Activity Assay 32

2.7 Transglutaminase Activity Assay 33

2.8 Mass Spectrometry Analyses

2.8.1 Peptide-mass fingerprinting 34

2.8.2 Electrospray Mass Spectrometry 36

2.9 Characterisation of Recombinant PNGase Sp

2.9.1 Substrate specificity 37

2.9.2 Effects of reductant 38

2.9.3 Effects of pH 39

2.9.4 Determination of the Michaelis Constant (K_m) and k_{cat}/K_m 39

2.9.5 Effects of metal ions 41

2.10 Crystallisation Trials

2.10.1 Basic principle 42

2.10.2 Setting up crystallisation trials 44

Chapter 3 — Results and Discussion

3.1 Molecular Cloning 48

3.2 Expression in Origami B (DE3) Strain 50

3.3 Purification of rPNGase Sp 53

3.4 The Use of Different Expression Hosts and Constructs to Solve the Heterogeneity problem

3.4.1 Premature translation termination 57

3.4.2 Proteolytic degradation 57

3.4.3 Stop codon read-through 59

3.4.4 The use of alternative vectors and fusion partners 63

3.5 Anomalous SDS-PAGE Behaviour of rPNGase Sp

3.5.1 Mass Spectrometry analyses of rPNGase Sp. 66

3.5.2 Experiments with different reducing agents and reduction regimes 68

3.5.3 Analyses of expression product 72

3.6 Transglutaminase assay 73

3.7 Substrate Specificity of rPNGase Sp 78

3.8 Effects of reductant concentration on PNGase Activity 82

3.9 Sensitivity to nickel Ions 83

3.10 Determination of optimum pH 85

3.11 Kinetic Studies of the rPNGase Sp-catalysed Deglycosylation

3.11.1 Determination of Michaelis constant (K_m) for FITC-Ova 87

3.11.2 A comparative study on the kinetics of unlabeled and FITC-labeled substrates 90

3.12 Effects of Metal Ions

3.12.1 Effects of metal ions on the activity of rPNGase Sp 97

3.12.2 Effect of EDTA, and subsequent addition of metal ions 100

3.12.3 Effect of Mn^{2+} on activity 103

3.13 Crystallisation Trials

3.13.1 Initial crystallisation trials 105

3.13.2 Optimisation of crystallisation conditions 105

Chapter 4 — Summary 107

Appendices

Appendix I : *PNG1Sp* sequence 110

Appendix II : pET32a_HTBH vector information 111

Appendix III: Preparation of the ovalbumin glycopeptide substrate 112

Appendix IV: In-gel tryptic digest for protein identification by peptide mapping . . . 114

Appendix V : FITC-labeling of ovalbumin glycopeptide 115

Appendix VI: Experimental data sheets 117

References 120

List of Figures

Figure 1.1	The de-*N*-glycosylation reaction catalysed by PNGase	2
Figure 1.2	Schematic representation of the primary structure of various eukaryotic PNGases	5
Figure 1.3	The transamidation reaction catalysed by transglutaminase	6
Figure 1.4	Schematic representation of the yPNGase–yRad23 complex	8
Figure 1.5	The structure of $GlcNAc_2$ bound to Png1p in complex with Z-VAD-fmk (a) and the active site of yPng1p (b)	11
Figure 1.6	Schematic model for Rad23-mediated substrate proteolysis in yeast (a) and mouse (b)	15
Figure 1.7	High-mannose-type glycopeptides/glycoproteins	18
Figure 2.1	Deglycosylation reaction catalysed by PNGases	32
Figure 2.2	Transglutaminase activity assay	34
Figure 2.3	Labeling of ovalbumin glycopeptide with FITC	40
Figure 2.4	The hanging drop (left) and sitting drop (right) methods of protein crystallisation	43
Figure 3.1	Cloning strategy	49
Figure 3.2	(a) PCR amplification of *PNG1Sp* ; (b) Whole cell PCR colony screening	50
Figure 3.3	SDS-PAGE analyses of the whole cell samples of IPTG induced expression of PNGase Sp in Origami B (DE3)	51
Figure 3.4	Solubility analyses of rPNGase Sp produced in Origami B (DE3) cells.	52
Figure 3.5	PNGase activity assay	52
Figure 3.6	SDS-PAGE analyses of the samples taken at various purification stages	54
Figure 3.7	Size exclusion chromatography of rPNGase Sp	56
Figure 3.8	IMAC purification of the fusion rPNGase Sp produced from (a) Origami B (DE3) and Rosetta (DE3), and (b) from Origami (DE3) pLysS and Origami (DE3) pLacI at different temperatures	59
Figure 3.9	Part sequences surrounding the stop codons in the original and newly constructed recombinant plasmids	61

Figure 3.10 Expression trials using Origami B (DE3) with the pET32a_HTBH_*PNG*_STOP vector 62
Figure 3.11 Expression trials using the pSUMO_BXH_*PNG*_STOP and the pMal_CHTBH_*PNG*_STOP vectors respectively 65
Figure 3.12 ESI-mass spectra of the samples containing the double-banded protein and the released fusion tag respectively 67
Figure 3.13 Size exclusion purified recombinant interferon γ receptor. 68
Figure 3.14 Schematic diagram showing possible differences in the structure of a protein in the presence of SDS and reducing agents 69
Figure 3.15 (a) SDS gels of reduced and alkylated rPNGase Sp; (b) Native-PAGE of the fusion rPNGase Sp 70
Figure 3.16 SDS-PAGE analyses of fusion rPNGase Sp samples boiled for varying time (left) or with varying amount of DTT (right) 71
Figure 3.17 MALDI-TOF mass spectra of a tryptic digest of the alkylated rPNGase Sp sample 72
Figure 3.18 The proposed mechanism for the hydrolysis reactions carried out by both the transglutaminases and cytosolic PNGases 74
Figure 3.19 Transglutaminase activity assay of recombinant PNGase Sp 75
Figure 3.20 Gel shift assay showing the substrate specificity of rPNGase Sp 79
Figure 3.21 Effects of TCEP on deglycosylation activity of rPNGase Sp 82
Figure 3.22 The effects of Ni^{2+} ions on rPNGase Sp 84
Figure 3.23 Influence of pH on rPNGase Sp activity 85
Figure 3.24 Primary estimation of optimal pH for rPNGase Sp activity using both FITC-Ova and unlabeled substrates 86
Figure 3.25 (a) The kinetic curve of rPNGase Sp-catalysed deglycosylation of FITC-Ova; (b) Schematic showing the substrate inhibition model 87
Figure 3.26 (a) Visualization of model derived for substrate inhibition of enzyme in Figure 3.22 b; (b) Assessment of the type of inhibition 88
Figure 3.27 (a) Hypothetical model showing the relative arrangements of the core and C-terminal domains of mPNGase; (b) Molecular model of the yPNGase–substrate complex 90
Figure 3.28 K_m estimation of rPNGase Sp for Ova substrate at either pH 7.0 or 6.0 .. 91

Figure 3.29 (a) Active site of the mouse PNGase in complex with a tripeptide inhibitor carbobenzyloxy-Val-Ala-Asp-α-fluoromethylketone (Z-VAD-fmk); (b) Proposed charge-relay system for of the transglutaminase-like catalytic triad 93
Figure 3.30 Comparison of the reaction rates between two substrates at pH 6 and 7 94
Figure 3.31 Multiple ionisation of fluorescein, the parent compound of FITC and the structure of FITC-peptide/protein conjugates 96
Figure 3.32 The effect of metal ions on the activity of rPNGase Sp 97
Figure 3.33 The effects of EDTA and Zn^{2+} on rPNGase Sp activity 100
Figure 3.34 Far UV CD spectra of untreated and EDTA-treated, together with rPNGase Sp that had been treated with EDTA, then exposed to 5 mM Zn^{2+} 102
Figure 3.35 The effects of Mn^{2+} on the activity of EDTA-treated rPNGase Sp 103
Figure 3.36 rPNGase Sp crystals obtained from initial crystallisation screens at room temperature 105

Appendix Figures

Figure 1 pET32a_HTBH vector information 111
Figure 2 SEC purification (a), HPLC purification (b) and ESI mass spectrum (c) of the 11-mer glycopeptide substrate, Ova 113
Figure 3 HPLC-chromatogram for the purification of the labelled product (FITC-Ova) 116
Figure 4 A typical HPLC-chromatogram showing the assay for rPNGase Sp activity with FITC-Ova as substrate 116

List of Tables

Table 2.1 Plasmids used in this study 46

Table 2.2 *E. coli* strains used in this study 47

Table 3.1 Purification table of rPNGase Sp 56

Table 3.2 Observed and calculated masses of the tryptic peptides from the alkylated rPNGase Sp sample 72

Table 3.3 Values of K_m, k_{cat}, and k_{cat}/K_m for rPNGase SP and Ova and FITC-Ova . 92

Abbreviations

~	approximately
Mwt	molecular weight
kb	kilo base
kDa	kilo Dalton
nm	nanometer
ERAD	endoplasmic reticulum associated degradation
DNA	deoxyribose nucleotide triphosphate
PCR	polymerase chain reaction
dNTP	deoxyribose nucleotide triphosphate
Trx	thioredoxin
E. coli	*Escherichia Coli*
Amp	ampicillin
Tet	tetracycline
Kan	kanamycine
Cam	chloramphenicol
LB	Luria Broth
IPTG	iso-propyl-beta-D-thiogalactopyranoside
DNase	deoxyribonucease
RNase	ribonuclease
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
BSA	bovine serum albumin
EDTA	ethylene diamine tetra-acetate
DTT	dithiothreitol
TCEP	Tris (2-carboxy-ethyl) phosphine hydrochloride
IMAC	immobilised metal affinity chromatography
rTEV	recombinant tobacco etch virus
SEC	size exclusion chromatography
FPLC	fast protein liquid chromatography
HPLC	high performance/pressure liquid chromatography
TCA	trichloroacetic acid

TFA	trifluoroacetic acid
MS	mass spectrometry
MALDI	matrix assisted laser desorption ionization
TOF	time-of-flight
PMF	peptide-mass fingerprinting
m/z	mass-to-charge ratio
UV	ultra-violet
HEPES	2-(4-(2-hydroxy-ethyl)-1-piperazinyl) ethane sulfonic acid
MES	2-(N-morpholino) ethane sulfonic acid
Tris	tris (hydroxymethyl) aminomethane
F.C.	final concentration
rPNGase Sp	recombinant Peptide:N-Glycanase from *S. pombe*
TGase	transglutaminase
Ova	hen egg ovalbumin-derived 11-mer glycopeptide
FITC	fluorescein isothiocyanate
FITC-Ova	FITC-dilabeled ovalbumin 11-mer glycopeptide