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Unique Peptide:*N*-glycanase of *Caenorhabditis elegans* has Activity of Protein Disulphide Reductase as well as of Deglycosylation

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Peptide:*N*-glycanase (PNGase) is the enzyme responsible for de-*N*-glycosylation of misfolded glycoproteins in the cytosol. Here, we report the molecular identification and characterization of PNGase (*png-1*, F56G4.5) from *Caenorhabditis elegans*. This enzyme released both high mannose- and complex-type *N*-glycans from glycopeptides and denatured glycoproteins. Deglycosylation activity was inhibited by Zn^{2+} and z-VAD-fmk, but not by EDTA. PNG-1 has a thioredoxin-like domain in addition to a transglutaminase domain, the core domain of PNGases, and exhibited protein disulphide reductase activity *in vitro*. Our biochemical studies revealed that PNG-1 is a unique bifunctional protein possessing two enzyme activities.

Key words: *Caenorhabditis elegans*, deglycosylation, peptide:*N*-glycanase, protein disulphide reductase, thioredoxin.

Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; MBP, maltose-binding protein; PNGase, peptide:N-glycanase; SGP, sialylglycopeptide; z-VAD-fmk, carbobenzyloxy-Val-Ala-Asp-α-fluoromethylketone.

Peptide:*N*-glycanase (PNGase, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase) (EC 3.5.1.52) is a deglycosylation enzyme that hydrolyzes the β-asparatyl-glucosaminyl bond of asparagine-linked (*N*-linked) sugar chains on glycopeptides or glycoproteins (*1*). The gene encoding the cytoplasmic PNGase was first identified in yeast *Saccharomyces cerevisiae*, and this enzyme was found to be widely conserved among eukaryotes (*2*). Various lines of evidence have demonstrated that this enzyme is involved in the deglycosylation of misfolded glycoproteins in the cytosol during the process of ER-associated degradation (ERAD) (*3–6*). The enzyme removes bulky *N*-glycans from ERAD substrates before proteasomal degradation, and released *N*-glycans are then sequentially hydrolyzed by cytosolic endo-β-*N*-acetylglucosaminidase and α-mannosidase. We previously identified endo-β-*N*-acetylglucosaminidase from the nematode *Caenorhabditis elegans* (Endo-CE) (*7*), and have studied the processing of free *N*-glycan in the cytosol of the worm. In this study, we report the identification and characterization of PNGase from *C. elegans*. Worm PNGase has a unique feature; namely, it contains an apparent thioredoxin-like domain near the N-terminus. This domain is found in many oxidoreductases as well as in thioredoxin itself, which is an evolutionarily conserved small protein with important functions in many cellular processes including gene expression, signal transduction and proliferation (*8*, *9*). Since there is no report describing the presence of a thioredoxin-like domain in glycan-processing enzymes, we wondered whether this domain in worm PNGase has any role or not. Here, we report that worm PNGase shows protein disulphide reductase activity *in vitro*, and thus, is a unique bifunctional enzyme.

MATERIALS AND METHODS

Cloning of png-1 From C. elegans—A full-length cDNA of F56G4.5 gene from *C. elegans* was obtained by RT-PCR using Sepasol RNA I Super (Nacalai Tesque, Japan)-purified RNA from wild-type N2 worms, a ReverTra-plus kit (Toyobo, Japan), and the primer pair SL2 (ggttttaacccagttactcaag) and F56G4.5rev (ttaatttttcatccaaattttgaccg). An ~1.9 kb amplified fragment was cloned into the SmaI site of the pUC119 vector (pUC119-*png-1*) and sequenced.

Expression and Purification of MBP-PNG-1—A cDNA fragment was obtained by PCR using pUC119-*png-1* as template and the primer pair F56G4.5for (atgccggtaacg gaagttggctcactgcc) and F56G4.5rev, and cloned into the XmnI site of the pMAL-c2X vector (New England Biolabs) to be expressed as a maltose-binding protein (MBP)-fusion protein. The *Escherichia coli* DH5α transformed with this construct (pMAL-c2X-*png-1*) were grown in LB medium containing 100 μg/ml ampicillin, at 18°C, until the absorbance at 600 nm reached 0.4, and then further cultured for 48 h at 18°C in the presence of 1 mM IPTG. Cells were disrupted by sonication in 50 mM Tris–HCl buffer, pH 7.5, containing 1 mM DTT, 1 mM EDTA, 1 mM PMSF and 1 μg/ml pepstatin A. The supernatant from cell extract was applied to amylose resin column (New England Biolabs), and the bound fractions were eluted in 20 mM Tris–HCl buffer, pH 7.5, containing 0.2 M NaCl, 1 mM DTT, 1 mM EDTA and 10 mM maltose, and further purified using a

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MonoQ 5/50 GL column on an ÄKTA FPLC system (Amersham Pharmacia). MBP-PNG-1 was eluted with a linear gradient of sodium chloride from 0 to 0.5 M at a flow rate 0.5 ml/min, and from 0.5 to 1.0 M at a flow rate 1.0 ml/min.

Expression of PNG-1 in Mammalian Cells and Microscopic Analysis—To generate the mammalian expression vector pME-*png-1*-FLAG-GST, the *PIG-X* sequence of pME-*PIG-X*-FLAG-GST was replaced with *png-1*. PIG-X is a mammalian ER-resident membrane protein and an essential component of glycosylphosphatidylinositol-mannosyltransferase I (*10*). For the ER marker, the FLAG-GST sequence of pME-*PIG-X*-FLAG-GST was replaced with GFP to generate pME-*PIG-X*-GFP. Chinese hamster ovary (CHO) K1 cells were transiently co-transfected with pME-*png-1*-FLAG-GST and pME-*PIG-X*-GFP using a Gene Pulser (BioRad). Cells cultured on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were stained with a rabbit anti-GST antibody (Bethyl, TX, USA) and Alexa594-conjugated goat anti-rabbit IgG (Invitrogen), and analysed by fluorescent microscopy (Olympus).

Site-directed Mutagenesis—The mutant construct for PNG-$1^{C34S/C37S}$ was generated by PCR using KOD-plus DNA polymerase (Toyobo), pMAL-c2X-*png-1* as template and the following primers: C34S/C37Sfor (attggtctggcccgtcccgtatgatttctc) and C34S/C37Srev (tcggagaaatcatacgggacgggccagacc).

Assays for PNGase Activity—Wild-type N2 worms were disrupted by sonication in 20 mM Tris–HCl buffer, pH 7.5, containing 1 mM PMSF, 1 mM EDTA, 1 mM DTT and 10% glycerol. After centrifugation for 20 min at 16,000*g*, supernatants were obtained as crude worm lysates. Sialylglycopeptide from hen egg yolk [SGP, Lys-Val-Ala-($NeuAc_2Gal_2GlcNAc_2Man_3GlcNAc_2$) Asn-Lys-Thr], a gift from Taiyo Kagaku, Japan, was used as a substrate for PNGase assay. For fluorescent assay, 1 mM dansylated SGP (DNS-SGP), which had been prepared by the method (*11*), was incubated with worm lysates at 25°C for 12 h. The reaction mixtures were analysed by reversed phase HPLC using a Cosmosil 5C18-AR II column (4.6 × 150 mm, Nacalai Tesque) on a Hitachi HPLC system equipped with L-2480 FL detector (*12*). When non-labelled SGP was used for the substrate, SGP was incubated with 1.5-μM MBP-PNG-1 in 20 μl of reaction buffer (20 mM Tris–HCl buffer, pH 7.5, containing 1 mM DTT) at 25°C for appropriate period, and the mixture was analysed by reversed phase HPLC with an L-2420 UV detector. The hydrolysis products were obtained by elution with a linear gradient of acetonitrile (from 0 to 6%) containing 0.1% trifluoroacetic acid for 30 min, at a flow rate of 0.8 ml/min. Elution was monitored by absorbance at 214 nm. Glycan-trimmed SGP were prepared by sequential hydrolysis using *Arthrobacter ureafaciens* neuraminidase (Nacalai Tesque), and jack bean β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase (Seikagaku Corporation, Japan). S-alkylated bovine pancreatic ribonuclease B (S-alkylated RNase B) was prepared as described (*13*). Ten micrograms of S-alkylated RNase B or heat-denatured RNase B were incubated with 3-μM MBP-PNG-1 in 30 μl of reaction buffer at 25°C for 12 h. The reaction mixtures were subjected to SDS–PAGE, and proteins were visualized by Coomassie brilliant blue staining.

Assay for Transglutaminase and Protein Disulphide Reductase—The transglutaminase assay was performed using *N*-carbobenzoxy-Gln-Gly as a substrate (*14*). The protein disulphide reductase assay was carried out using insulin as a substrate (*15*, *16*). Briefly, a 5.0 μM solution of wild-type MBP-PNG-1, MBP-PNG-$1^{C34S/C37S}$ or recombinant yeast thioredoxin 2 (Oriental Yeast, Japan) in reaction mixture (20 mM Tris–HCl, pH 7.5, containing 1 mM EDTA and 0.3 mM DTT; 90 μl volume) was pre-incubated at room temperature for 15 min. The reaction was started by adding 10 μl of 10 mg/ml bovine insulin (Sigma), and the change in absorbance at 595 nm was recorded up to 60 min.

MALDI-TOF MS Analysis—To analyse reaction products of PNGase, matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager Biospectrometry Work-station (PerSeptive Biosystems, USA). Oligosaccharides were analysed in the negative-ion mode using 2,4,6-trihydroxyacetophenone as the matrix, and peptide was analysed in the positive-ion mode.

RESULTS

Identification of PNGase From C. elegans—We first examined whether PNGase activity could be detected in the lysates of *C. elegans* at mixed developmental stages. When DNS-SGP, a dansylated hexapeptide with a single biantennary complex-type *N*-glycan, was incubated with lysates, we found a new peak on HPLC analyses at a retention time corresponding to that of the product generated with bacterial PNGase (PNGase F) treatment; this product could not be detected in experiments using boiled lysate (Fig. 1). This result suggests the presence of PNGase in *C. elegans*. Although there is some possibility of the effect of lysosomal glycosylasparaginase, we assumed that the latter enzyme might not act on SGP containing DNS-hexapeptide under these conditions. We then searched for the gene encoding PNGase from *C. elegans* by BLAST, and assumed F56G4.5 to be an orthologue of the *S. cerevisiae* PNGase, Png1p. The gene F56G4.5, designated as *png-1*, encodes a 606 amino acid polypeptide (Fig. 2A and B). The transglutaminase domain, which contains the catalytic triad (Cys-251, His-278 and Asp-295 residues) required for PNGase activity, was found in the central part (amino acid residues 246–297). Besides this domain, two zinc-binding motifs ($C^{191}PKC^{194}$ and $C^{225}DGC^{228}$) were also conserved in PNG-1. Near the N-terminus, there was a thioredoxin-like domain (amino acid residues 3–106) that is unique to *C. elegans* PNGase among various orthologues. On the contrary, worm PNGase does not contain the PUB domain that is conserved in mammalian and insect PNGases.

Expression of Recombinant PNG-1 in E. coli and Mammalian Cells—We cloned *png-1* cDNA and expressed in *E. coli* as MBP-fused protein. MBP-PNG-1 was purified by two-step chromatographies to give a

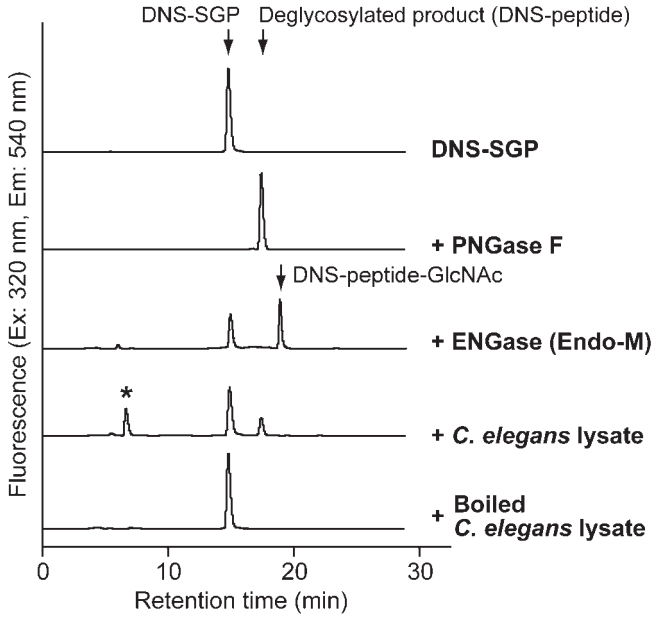

Fig. 1. **PNGase activity in the lysate of *C. elegans***. Each reaction mixture, including DNS-SGP as a substrate, was incubated with PNGase F, endo-β-*N*-acetylglucosaminidase (ENGase) from *Mucor hiemalis* (Endo-M) (*12*) and lysates (native or boiled) of *C. elegans*, and subjected to HPLC. Asterisk indicates unidentified product.

single protein band on SDS–PAGE at a position of around 110 kDa, which is consistent with the calculated mass (Fig. 2C). We used this enzyme preparation for characterization of the enzyme. To elucidate the intracellular localization of PNG-1, we transfected CHO cells with pME-*png-1*-FLAG-GST and analysed them by immunofluorescent microscopy. PNG-1 was detected in the cytosol, and was partially co-localized with an ER membrane protein PIG-X (Fig. 2D). From this result, PNG-1 may localize in the cytosol and ER in the cells of *C. elegans*.

Various Properties of Recombinant PNG-1—To determine the PNGase activity of recombinant PNG-1, we incubated the enzyme with SGP and analysed the reaction mixture by reversed phase HPLC. Two new peaks were detected at a retention time of around 10 min (a1, a2) and one new peak at 19.5 min (b) (Fig. 3A). MALDI-TOF MS analyses of the first two peaks gave the same mass ion peak at m/z 2221.19, which corresponds to a de-protonated form $[M-H]^-$ of the released oligosaccharide (Fig. 3B, panel a, MS chart of the latter peak a2). Such different peaks on HPLC might occur due to different anomeric structures at the reducing ends of the same oligosaccharide. Analysis of peak b on HPLC gave a mass ion peak at m/z 662.66, which corresponds to a protonated form $[M+H]^+$ of the hexapeptide, in which the Asn residue was converted to Asp because of the release of oligosaccharide by PNGase (Fig. 3B, panel b). We then incubated the enzyme with various glycan-trimmed SGP. Asialo- ($Gal_2GlcNAc_2Man_3GlcNAc_2$-), agalactosyl- ($GlcNAc_2Man_3GlcNAc_2$-), trimannosyl- ($Man_3GlcNAc_2$-) and monomanosyl- ($Man_1GlcNAc_2$-) SGP were completely hydrolyzed by PNG-1 under the same conditions above (data not shown). These results indicate that PNG-1 could remove various forms of oligosaccharides including sialo-complex-type from glycopeptides.

Next, we determined some of the properties of PNG-1 using SGP as a substrate. The enzyme showed highest activity at pH 7.5 and at 25°C. The EDTA, Mg^{2+} and Ca^{2+} at a final concentration of 2 mM had hardly affect on its activity (relative activities: 100, 86 and 81%, respectively), whereas Mn^{2+} and Fe^{2+} moderately (44 and 10%) and Zn^{2+}, Cu^{2+} and Co^{2+} almost completely inhibited it (<0.1%). It is known that z-VAD-fmk, an inhibitor of caspases, is a potent irreversible inhibitor of both yeast and mammalian PNGases (*17*). As in other eukaryotic PNGases, PNG-1 was inhibited by z-VAD-fmk at an IC_{50} value of 4.6 μM, whereas bacterial PNGase F was insensitive, even at a concentration of 1 mM (Fig. 4A). Since it was reported that a PNGase homologue in *Arabidopsis thaliana* (AtPng1p) exhibited transglutaminase activity (*18*), we checked whether PNG-1 had transglutaminase activity or not. However, PNG-1 didn't show any detectable transglutaminase activity when *N*-carbobenzoxy-Gln-Gly was used as a substrate (data not shown).

Worm PNGase Cleaves N-glycan From Denatured RNase B—To examine the action of PNG-1 on glycoproteins, we used RNase B, which has a single high mannose-type *N*-glycan, as a substrate (*13*). First, S-alkylated (fully denatured) RNase B was treated with bacterial PNGase F or PNG-1. As shown by SDS–PAGE, PNGase F completely deglycosylated RNase B (Fig. 4B, lane 2). PNG-1 also deglycosylated RNase B almost completely, although the amount of enzyme used was 120 times higher than that of PNGase F (lane 3). Next, we examined whether PNG-1 could remove *N*-glycan from native RNase B, because yeast PNGase was reported to remove *N*-glycan only from denatured glycoproteins (*19*). RNase B was deglycosylated by PNG-1 when native RNase B was pre-incubated at temperatures >70°C for 20 min, which should be sufficient to denature the protein (Fig. 4C). These results indicate that worm PNGase removes high mannose-type *N*-glycan from denatured RNase B, but not from native RNase B.

Protein Disulphide Reductase Activity of Thioredoxin-like Domain—PNG-1 has a putative active site sequence (WCGPC) of thioredoxin near the N-terminus, as described above (Fig. 2A). To examine whether PNG-1 actually has thioredoxin activity or not, an insulin disulphide reducing assay was carried out. In this assay, disulphide reductase activity is monitored by an increase in turbidity caused by the formation of a precipitate of the dissociated insulin B chain, as a result of reduction of the disulphide bonds between insulin A and B chains (*16*). The addition of PNG-1 to the reaction mixture resulted in a rapid increase in turbidity similar to that seen in the addition of recombinant yeast thioredoxin 2 (Fig. 4D). To determine if the reductase activity is catalysed by the putative active site ($WC^{34}GPC^{37}$) in the thioredoxin-like domain, a mutant ($PNG\text{-}1^{C34S/C37S}$) was prepared, in which two Cys residues (Cys-34 and Cys-37) were substituted for

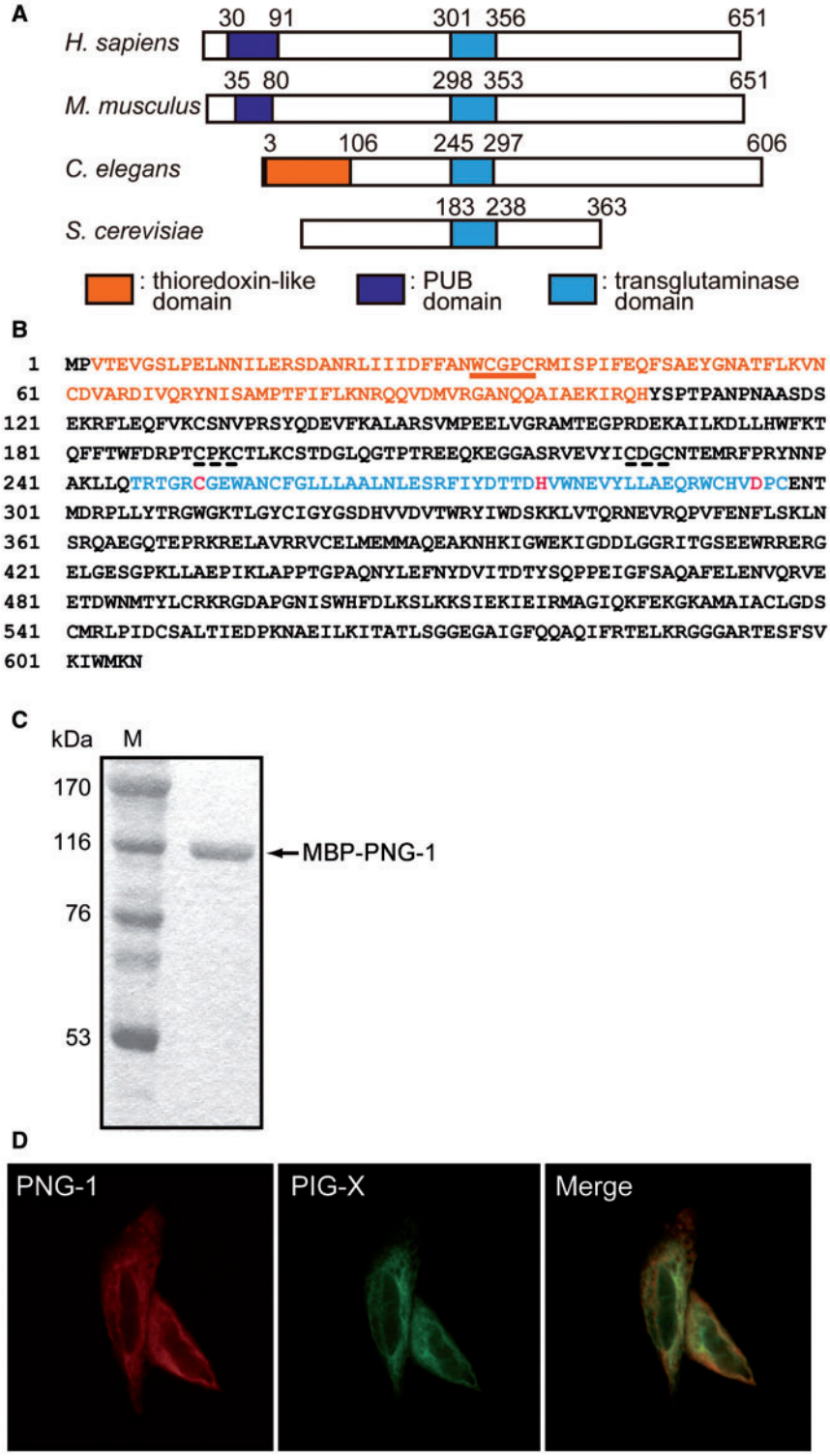

Fig. 2. **Expression of the recombinant PNG-1**. (**A**) Schematic representation of PNG-1 and its orthologues. (**B**) Amino acid sequence of PNG-1 from *C. elegans*. Coloured letters represent the thioredoxin-like domain (orange) and the transglutaminase domain (cyan). The solid orange line indicates the active site of the thioredoxin-like domain; dashed lines indicate the conserved zinc-binding motif; and red letters indicate the catalytic triad of the transglutaminase domain (Cys-251, His-278, and Asp-295 residues). (**C**) SDS–PAGE of MBP-PNG-1 expressed in *E. coli*. M, molecular mass marker. (**D**) Localization of PNG-1 expressed in CHO cells. PNG-1 tagged with FLAG-GST and an ER protein PIG-X tagged with GFP were co-expressed and stained.

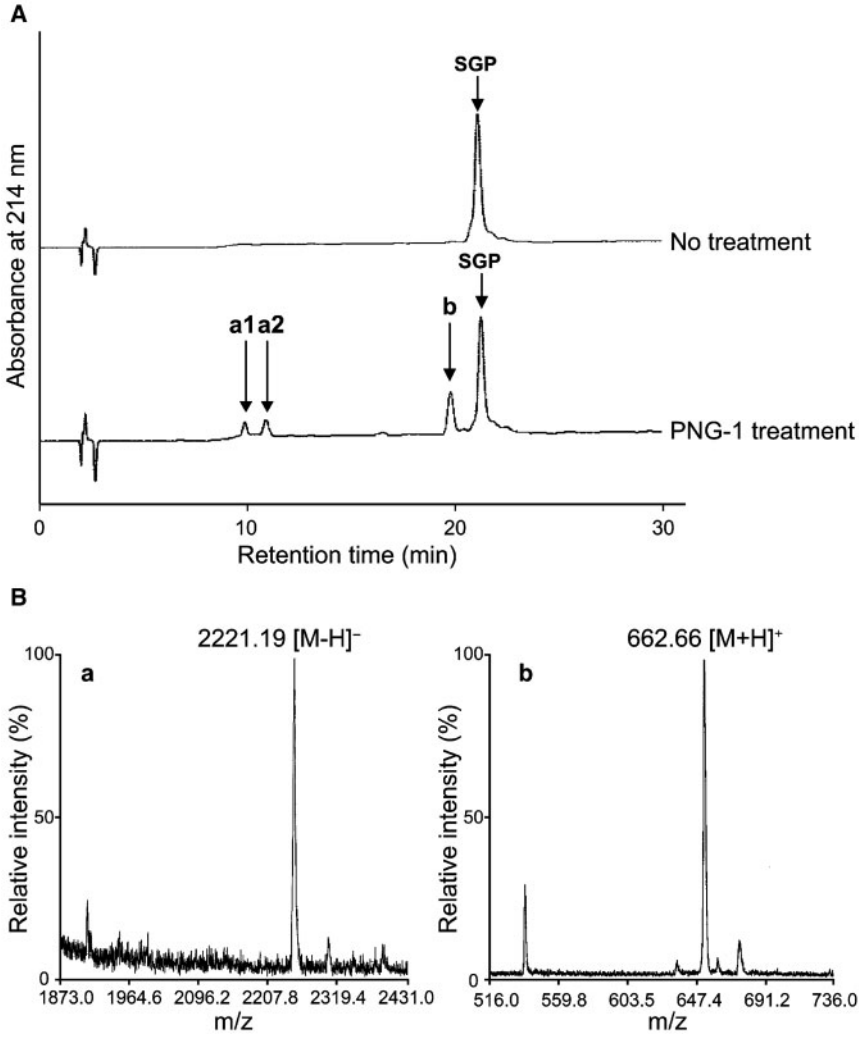

Fig. 3. **Deglycosylation of SGP by PNG-1**. (**A**) HPLC profiles of SGP only (upper chart) and SGP treated with PNG-1 (lower chart). (**B**) MALDI-TOF MS analyses of peaks a2 (panel **a**) and b (panel **b**). Mass ion peaks at m/z 2221.19 in panel a and 662.66 in panel b correspond to oligosaccharide and hexapeptide from SGP, respectively.

Ser residues. It was confirmed that these mutations caused no change in either conformation or PNGase activity, by analysis of CD spectroscopy and enzyme assay using SGP as a substrate, respectively (data not shown). As expected, the mutant failed to reduce the disulphide bond of insulin (Fig. 4D), indicating that the Cys residues in $WC^{34}GPC^{37}$ are responsible for the insulin disulphide reductase activity of PNG-1. These results show that PNG-1 of *C. elegans* is a unique bifunctional enzyme having both thioredoxin activity and deglycosylation activity.

DISCUSSION

In this study, we identified that F56G4.5 in *C. elegans* encodes PNGase (PNG-1). Although several other genes in the genome of *C. elegans*, such as W04G5.5 and Y50D4B.7, show some similarity to PNG-1, a catalytic triad is not found in their transglutaminase domains. Thus, we excluded their biochemical characterizations. Recombinant PNG-1 was functionally expressed in *E. coli*, and released both high mannose-type and complex-type *N*-glycans. However, since PNGase is involved in ERAD, its natural substrates may be nascent glycoproteins bearing high mannose-type *N*-glycan. In fact, Endo-CE, an *N*-glycan-processing enzyme that acts following the release of *N*-glycan by PNG-1 in the cytosol, prefers high mannose-type *N*-glycan (*7*). In contrast to broad glycan specificity, PNG-1 could only act on denatured glycoprotein or short glycopeptide. This fact suggests that the enzyme may not recognize distal part of glycans and instead recognizes proteins in the unfolded state, or peptides (Figs. 3, 4B and C).

Although the general properties of PNG-1 are largely similar to those of yeast PNGase, the effects of Zn^{2+} and EDTA on deglycosylation activity were quite different. Yeast PNGase requires Zn^{2+} for its activity and stability, and the chelation of Zn^{2+} by EDTA results in a loss of its activity (*20*). On the other hand, PNG-1 was inactivated by Zn^{2+} and insensitive to EDTA, even

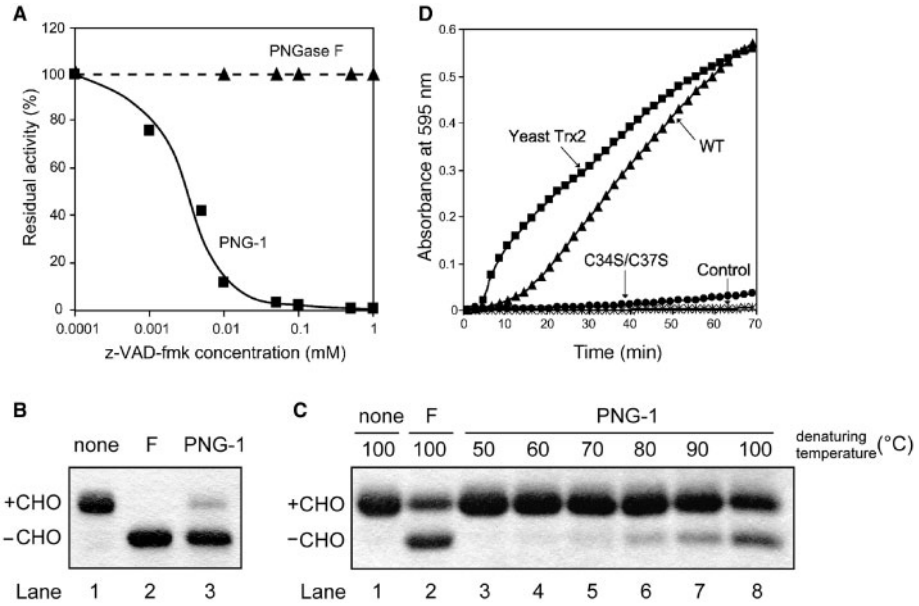

Fig. 4. **Properties of PNG-1**. (**A**) Effect of z-VAD-fmk on deglycosylation activities of PNG-1 (square) and PNGase F (triangle). The reaction was carried out using SGP as a substrate at pH 7.5 and 25°C, for 90 min. (**B**) Deglycosylation of S-alkylated RNase B. After enzyme treatment, proteins were analysed by SDS–PAGE. CHO indicates oligosaccharide. Lane 1, no enzyme; lane 2, PNGase F treatment; lane 3, PNG-1 treatment. (**C**) Deglycosylation of heat-denatured RNase B. Native RNase B was pre-incubated at indicated temperature for 20 min before enzyme treatment. Lane 1, no enzyme; lane 2, PNGase F treatment; lanes 3–8, PNG-1 treatment. (**D**) Insulin disulphide reductase activity of PNG-1. Insulin was incubated with wild-type PNG-1 (triangle), PNG-$1^{C34S/C37S}$ (circle) or yeast thioredoxin 2 (Trx2) (square). Control (no enzyme) is shown with a cross. Turbidity was monitored every 2 min by the absorbance at 595 nm.

though the Zn^{2+}-binding motifs (two CxxC motifs; $C^{191}PKC^{194}$ and $C^{225}DGC^{228}$, Fig. 2) are conserved in PNG-1 as well as in orthologues of yeast and higher eukaryotes. The effect of cations on worm PNGase is rather similar to that on mammalian PNGase (*21*). We also found that an inhibitor of yeast PNGase, z-VAD-fmk, also inhibited the deglycosylation activity of PNG-1 with an IC_{50} of 4.6 μM at 1.5 μM PNG-1 (Fig. 4A). This result indicates that worm PNGase is more sensitive to z-VAD-fmk than yeast PNGase [IC_{50} of 50 μM when yeast PNGase was at 52 nM (*17*)].

Concerning the thioredoxin-like domain of PNG-1, we found that wild-type PNG-1 had protein disulphide reductase activity, whereas the PNG-$1^{C34S/C37S}$ mutant did not (Fig. 4D), indicating that the $WC^{34}GPC^{37}$ motif constitutes the active site of reductase. Why does PNG-1 have disulphide reductase activity in addition to deglycosylation activity? Recently, it was reported that some ERAD substrate proteins are dislocated into the cytosol in their fully native form (*22*). Therefore, one possibility is that the reduction of disulphide bond(s) within the substrate glycoprotein by disulphide reductase destroys its native form, facilitating the ability of the core domain of PNG-1 to access the substrate for deglycosylation. To examine this possibility, we compared the deglycosylation activities of wild type and PNG-$1^{C34S/C37S}$ mutant for RNase B and yeast carboxypeptidase Y as substrates, both of which have *N*-glycan(s) and disulphide bonds within their molecules. However, we could not detect significant difference in the deglycosylation rate between these enzymes *in vitro* (data not shown). Moreover, further addition of the expressed thioredoxin domain did not promote the deglycosylation rate of PNG-1 (data not shown). This result suggests that the thioredoxin-like domain may not be involved in the denaturation of substrates for PNG-1. However, the possibility remains that some chaperone-like proteins cooperate in the denaturation of glycoproteins *in vivo*, or that the *in vitro* addition of DTT, which is used as a substitute for a system of thioredoxin reductase and NADPH to reduce oxidized thioredoxin, may not be suitable. Otherwise, protein disulphide reductase might function independently from deglycosylation activity. There is also a possibility that PNG-1 might participate in other physiological phenomena, as thioredoxin proteins are involved in many thiol-dependent cellular processes.

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