

# Expression, Purification, and Characterization of Endo- $\beta$ -*N*-Acetylglucosaminidase H Using Baculovirus-Mediated Silkworm Protein Expression System

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**Abstract** Endo- $\beta$ -*N*-acetylglucosaminidase (Endo H) from *Streptomyces plicatus* hydrolyzes the core di-GlcNAc units of asparagine-linked oligosaccharides and is regarded as an important tool for glycobiology research. In the present study, we established a large-scale system to produce secreted Endo H using a silkworm-baculovirus expression system (silkworm-BES). The recombinant Endo H purified from silkworm hemolymph had activity comparable to that from recombinant *Escherichia coli*. As well as its well-characterized substrate RNase B, the Endo H from silkworm-BES was able to deglycosylate the high-mannose glycoproteins from silkworm hemolymph. Interestingly, the secretion amount of recombinant Endo H was significantly varied among the different silkworm strains, which could provide valuable information for larger-scale protein productions from silkworm-BES.

**Keywords** Endo- $\beta$ -*N*-acetylglucosaminidase · Deglycosylation · BES · Silkworm · Secretion

## Introduction

Post-translational modifications (PTMs) contribute to the control of the biochemical properties of eukaryotic proteins. Glycosylation is one of the most prominent and abundant PTMs, with

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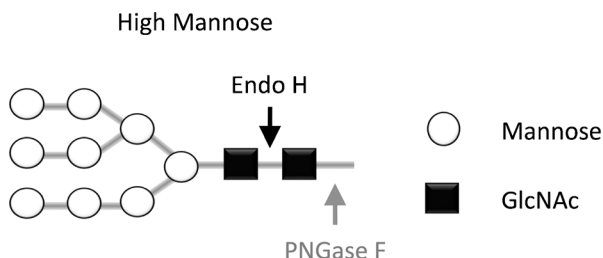
important roles in protein folding, conformation, stability, and activity. For example, in human, the majority of secreted proteins are glycosylated on asparagine (Asn) residue located within a consensus sequence Asn-X-Ser or Asn-X-Thr tripeptide, where X stands for any amino acid except Proline (Pro). The first step in the *N*-glycosylation pathway is the transfer of 14 assembled carbohydrate chains ( $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ ) from dolichol pyrophosphate oligosaccharide to Asn in endoplasmic reticulum (ER) [1]. Following glycosidase digestion of *N*-glycans in ER, two major types of oligosaccharide, the high-mannose type or complex type are generated by several glycosyltransferases in the Golgi apparatus [2]. Despite the apparent contributions of *N*-glycans to the structure and function of glycoprotein, the biological impact of each *N*-glycan structure has not been fully elucidated because of the heterogeneity of *N*-glycans.

To gain a better understanding of the significance of protein glycosylation, it is necessary to analyze the effect of oligosaccharides digestion by endoglycosidases. Among these, peptide-N4-(*N*-acetyl- $\beta$ -glucosaminyl)asparagine amidase (PNGase F) and Endo- $\beta$ -*N*-acetylglucosaminidases H (Endo H) are broadly used for the *N*-linked glycoprotein analysis. PNGase F hydrolyzes the glycosidic bond between core GlcNAc of *N*-glycan and Asn residue, and thus is available for the complete removal of all *N*-glycans except for the core  $\alpha$ -1,3-fucosylated *N*-glycans [3]. In contrast, Endo H hydrolyzes GlcNAc $\beta$ 1-4GlcNAc linkage of high mannose and hybrid type glycans and could not cleave the linkage of complex type glycans (Fig. 1) [4]. Therefore, Endo H can be utilized to deglycosylate glycoproteins for not only for structure analysis but also for monitoring the assembly of mature complex-type *N*-glycans of protein precursors [5–8].

*Streptomyces plicatus* Endo H is a secreted enzyme consisting of a mature peptide of 269 amino acid residues and a leader sequence with 44 amino acid residues [9, 10]. Thus far, recombinant Endo H has been reported to be produced only in the *Escherichia coli* expression system. Most recombinant Endo H expressed in *E. coli* cells remained to be associated with the surface of membrane or located in cytoplasm due to incomplete removal of the leader sequence [11, 12]. Since the original *S. plicatus* Endo H is a secretory protein, other secretory expression systems might be more suitable for the large-scale production of recombinant Endo H.

The baculovirus expression system (BES) is widely used to express eukaryotic recombinant proteins, because the PTMs and protein-folding systems are similar to those of mammals [13]. In our current study, *S. plicatus* Endo H with silkworm 30 K secreted signal peptide was expressed and purified as a secreted protein form by BES. We then assessed the function of purified recombinant Endo H as an Endo- $\beta$ -*N*-acetylglucosaminidase. Our results demonstrated that the silkworm-BES is efficient for large-scale production of secreted functional Endo H.

**Fig. 1** Diagram of substrate specificities of Endo H. Black and gray arrows represent the cleavage sites of Endo H and PNGase F, respectively. Opened circles mannose, filled squares GlcNAc [23]



## Materials and Methods

### Cells and Silkworms

The NIAS-Bm-oyanagi2 and Bme21 cells were cultured in IPL-41 medium (Sigma, St. Louis, MO) with 10 % fetal bovine serum (Gibco, Grand Island, NY) at 27 °C. The silkworm strains used in this study were supplied by the silkworm stock center of Kyushu University supported by the National BioResource Project (NBRP). The larvae were routinely reared on flesh mulberry leaves at 25–27 °C.

### Construction of Recombinant Baculoviruses

Insect codon adapted coding region of the *S. plicatus* Endo H (DDBJ/GenBank accession number AB829335) were chemically synthesized (Genscript, Tokyo, Japan) and cloned into pUC57 vector. To construct gateway-based entry clones, open-reading frame (ORF) of Endo H was amplified by polymerase chain reaction (PCR) using the primers Endo H NOSP-5 (5'-gcccgccggcggtgaagcaggggccgacc-3') and Endo H *Xho*I-3 (5'-caatctcgaggagttctgacagcttcgc-3'). The amplified Endo H PCR product without native signal peptide was digested with *Xho*I, and inserted into the *Eco*RV and *Xho*I site of the modified pENTR11 (pENTR11L2130KTEVH8STREP) vector. The pENTR11L21TEVH8STREP vector, containing a lobster L21 sequence (for enhancing translation efficiency) and signal peptide from silkworm 30 kDa protein at the N-terminal, and the tobacco etch virus (TEV) protease cleavage site, an 8-histidine (H8)-tag and an eight amino acids Strep (STREP)-tag at the C-terminal, was used from our laboratory stock (see Supplementary Fig. 1). The resulting construct was named pENTRL2130K-EndoH-TEVH8STREP and the nucleotide sequences have been deposited in DDBJ database (Accession No. AB838596). The transfer plasmid pDEST*Polh*30K-EndoH-TEVH8STREP for generating recombinant baculovirus was subsequently constructed between the pENTRL2130K-EndoH-TEVH8STREP and pDEST8 vector (Invitrogen, Carlsbad, California) by the Gateway LR reaction according to the manufacture's protocol.

Recombinant baculovirus was generated using BmNPV/T3 bacmid system as described elsewhere [14]. Briefly, The recombinant transfer plasmid pDEST*Polh*30K-EndoH-TEVH8STREP was transformed into *E. coli* BmT3DH10Bac to generate recombinant bacmid DNAs. After purification using the FlexiPrep kit (Amersham Pharmacia Biotech, Piscataway, NJ), the bacmid DNAs were transfected into NIAS-Bm-oyanagi2 cells using FuGENE HD transfection reagent (Promega, Madison, WI) to yield recombinant virus particles (BmNPV/*Polh*30K-EndoH-TEVH8STREP). Before transfection, the medium was replaced by COSMEDIUM 009 serum-free medium (Cosmo-Bio, Tokyo, Japan). Four days after incubation at 27 °C, cell culture medium containing recombinant P1 viruses were collected and stored at 4 °C in the dark. High-titer virus (P3) stock was prepared by serial infection following the protocols recommended in the manufacturer's manual (Invitrogen).

### Expression of Recombinant Endo H

The expression level of Endo H with 8 × His-tag by viral infection into BmNPV-sensitive Bme21 cells [15] was measured as follows:  $1 \times 10^5$  cells per well in six-well tissue culture plates were infected with the recombinant BmNPV at a multiplicity of infection (MOI) of 1. The culture medium and infected cells were harvested or recovered at the indicated days post-infection (DPI). After centrifugation at 1,000 rpm for 10 min at 4 °C, the supernatant

containing secreted proteins was subjected to Western blotting analysis. The cell pellet was suspended in ice-cold extraction buffer (20 mM Tris–HCl pH 7.4, 0.5 M NaCl, 1 % TritonX-100, 10 % glycerol, 10 mM 2-mercaptoethanol, 1 mM PMSF) and homogenized. The homogenates were centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant containing soluble proteins and the precipitate containing insoluble proteins were also subjected to Western blotting analysis.

All samples were electrophoresed on a 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under the reducing condition and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Milford, MA), and further blocked in 5 % skim milk (Wako, Tokyo, Japan) followed by incubation with HisProbe-HRP (1:2,000; Thermo Scientific, Rockford, IL). The protein bands were visualized using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

#### Purification of Recombinant Endo H

Bme21 cells ( $1 \times 10^5$  cells per 1 ml) in 20 ml IPL-41 insect cell culture medium were infected with recombinant BmNPV/*Polh30K-EndoH-TEVH8STREP* at an MOI of 1. The cultured medium containing the secreted proteins was harvested at 4 DPI. After centrifugation at 1,000 rpm for 10 min, the supernatant was consequently employed for protein purification. As for the silkworm-BES, the recombinant virus ( $1 \times 10^5$  plaque-forming unit per larva) was injected into the 5th instar silkworm larvae (day 3) of d17 strain. Four days after inoculation, 10 ml of the hemolymph from 25 silkworm larvae was collected by cutting larval legs into a tube containing 20 mM 1-phenyl-2-thiourea and then centrifuged at 8,000 rpm for 30 min at 4 °C to remove insoluble matter.

The cell culture medium or hemolymph was diluted by binding buffer A (20 mM Tris–HCl pH 7.4, 0.5 M NaCl) containing complete EDTA-free protease inhibitor tablet (1 tablet/100 ml; Roche, Paris, France) and 1 mM PMSF, and then centrifuged at 22,000 rpm for 30 min at 4 °C. After the filtration with a 0.45- $\mu$ m filter (Millipore), the recombinant Endo H was purified by nickel affinity chromatography using HisTrap excel column (GE Healthcare Bioscience, Piscataway, NJ) using 20 and 500 mM imidazole in the binding and elution buffers, respectively. After concentration and the buffer exchange to binding buffer B (100 mM Tris–HCl pH 8.4, 0.15 M NaCl, 1 mM EDTA), the recombinant Endo H was applied to StrepTrap HP column (GE Healthcare), and then eluted by buffer B containing 2.5 mM desthiobiotin.

The purified fractions were visualized with Coomassie Brilliant Blue R-250 or Western blotting analysis after separation through 15 % SDS-PAGE. The purified recombinant Endo H was concentrated again and the buffer was exchanged to storage buffer (20 mM Tris–HCl pH 7.4, 50 mM NaCl, 5 mM Na<sub>2</sub>EDTA) by ultrafiltration using Amicon 10 K filters (Millipore). The purified recombinant Endo H was quantified by YabGelImage software (<https://sites.google.com/site/yabgel/home>) using bovine serum albumin (BSA) as standard.

#### Deglycosylation Assay

The recombinant Endo H from *E. coli* was purchased from New England Biolabs (Beverly, MA) and was represented as rEndo H (*E. coli*). One unit (1 U) is defined as the amount of rEndo H (*E. coli*) required to remove above 95 % of the carbohydrate from 10  $\mu$ g of denatured RNase B for 1 h at 37 °C in a total reaction volume of 10  $\mu$ l. The deglycosidase assay was performed in reaction buffer (50 mM sodium citrate buffer, pH 4.5–7.5) using RNase B as substrate. 2.5  $\mu$ g of RNase B (NEB) and 6.25–31.25 units of rEndo H (*E. coli*), or 10–50 ng of rEndo H (silkworm) were mixed with the above reaction buffer up to a total volume of 20  $\mu$ l.

The amount of rEndo H (Bme21) could not be determined because of the low quantity obtained. The mix solutions were incubated at 27–47 °C for 10–60 min. The deglycosylation assay of glycoproteins in silkworm hemolymph was performed after denaturing at 100 °C for 10 min with denaturing buffer (NEB). The denatured glycoproteins of silkworm hemolymph were deglycosylated by 31.25 units of rEndo H (*E. coli*) (NEB) or 50 ng of rEndo H (silkworm) at 37 °C for 1 h. All samples were visualized by 15 % or 12 % SDS-PAGE, respectively.

#### N-Linked Glycan Analysis Using MALDI-TOF Mass Spectrometry

75 µg of RNase B (NEB) alkylated by 17.5 mM IAA was digested by approximately 400 unit of trypsin (Roche) for 1 h at 37 °C. After heating at 90 °C for 5 min, RNase B was deglycosylated by 500 units of rEndo H (silkworm) for 5 h. The released *N*-linked glycans were purified and labeled with *N*-[(aminooxy)acetyl]tryptophanylarginine methyl ester (aoWR) using BlotGlyco (Sumitomo Bakelite, Tokyo, Japan) according to the manufacturer's instructions. The aoWR-labeled *N*-linked glycans were then analyzed using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) performed on AXIMA-CFR Plus (Shimadzu, Japan) using 2,5-dihydroxybenzoic acid dissolved in acetonitrile/water (3:7 v/v). Exact mass of glycans was calculated by the following formulation: [observed *m/z* (numbers at the peak)] + [H<sub>2</sub>O (18.01)] – [aoWR (447.22)] – [H (1.00)].

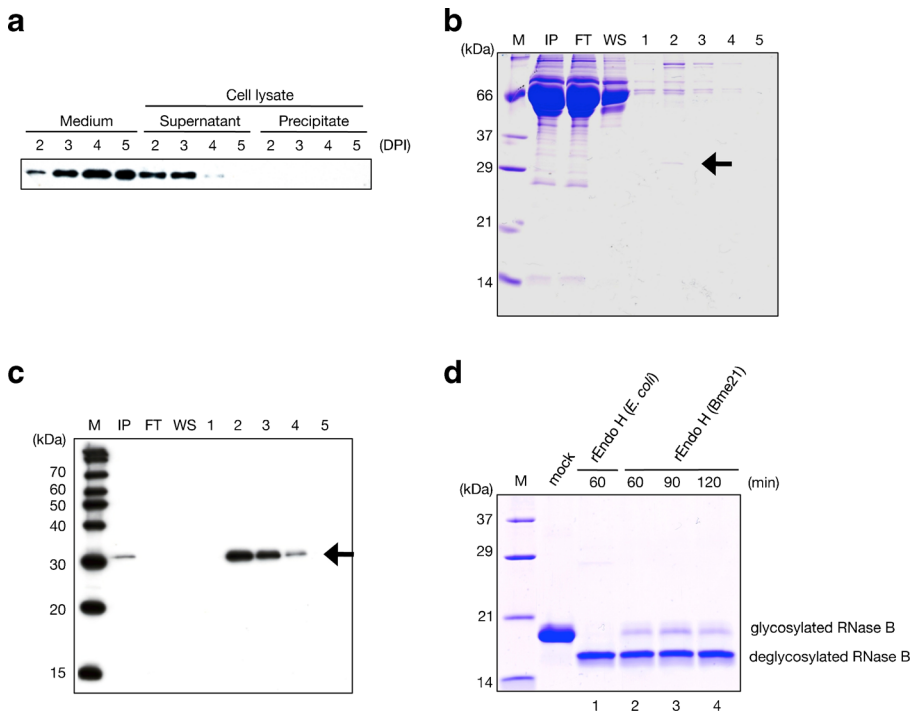
#### Screening of Silkworm Strains for Efficient Secretion of rEndo H (Silkworm)

The 26 silkworm strains were used for the expression screening of Endo H (Fig. 7). The BmNPV/*Polh*30K-EndoH-TEVH8STREP-infected silkworm larvae of each strain at 4 DPI were dissected and hemolymph were collected on ice separately. After the centrifugation at 5,000 rpm for 10 min, 0.5 µl of the supernatants were resolved on 15 % SDS-PAGE. The expression levels of randomly selected three samples of each infected strain were analyzed by Western blotting.

## Results and Discussion

#### Secretion of Recombinant *S. plicatus* Endo H from BmNPV-Infected Cultured *B. Mori* Cells

The signal peptide of *S. plicatus* Endo H was reported to be 44 amino acids residues [10]. To secrete the protein into cell culture medium or silkworm hemolymph, 30 K signal peptide from *B. mori* [16] was inserted into the N-terminus of Endo H cDNA instead of the native signal sequence. For the efficient two-step purification, 8 × His-tag and Strep-tag were added to the C-terminus of Endo H. The secreted mature Endo H was predicted to contain 306 amino acid residues with a molecular weight of 32.9 kDa. The recombinant baculovirus carrying the Endo H expression cassette (BmNPV/*Polh*30K-EndoH-TEVH8STREP) was generated using the BmNPV/T3 bacmid system as described in the “[Materials and Methods](#)” section. To optimize the conditions for Endo H expression, Bme21 cells were infected with recombinant BmNPV encoding Endo H. As shown in Fig. 2a, almost all the Endo H protein was secreted by 30 K signal peptide at 4 DPI, although a significant amount of Endo H protein was accumulated inside the cell in soluble form at 3 DPI. The insoluble Endo H protein could be detected slightly at higher exposure levels (data not shown).



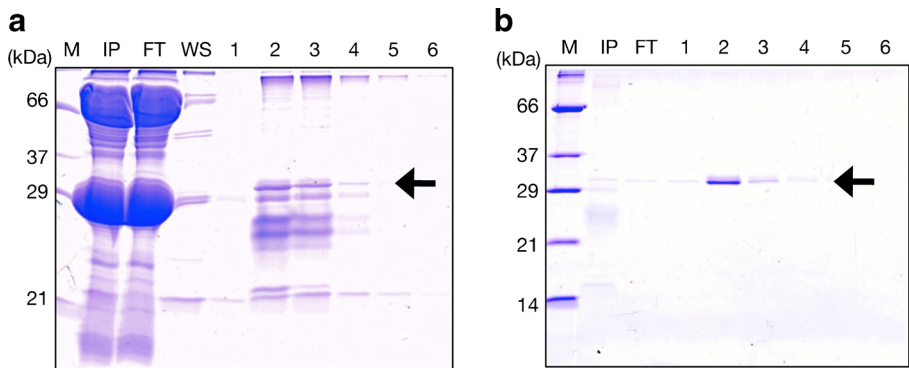
**Fig. 2** Purification and characterization of Endo H from BmNPV-infected Bme21 cells. **a** Time course of Endo H expression in Bme21 cells infected with the recombinant BmNPV. The cells and culture medium were harvested at 2, 3, 4 and 5 days post-infection (DPI). Recombinant Endo H was detected by Western blotting using HisProbe-HRP. **b** Purification of Endo H by nickel affinity chromatography. *M* molecular mass markers, *IP* input, *FT* flow-through, *WS* wash fraction, *lanes 1–5* elution fractions by 500 mM imidazole. Recombinant Endo H was visualized by Coomassie Brilliant Blue R-250 staining. **c** The second and third fractions of panel **b** were concentrated and subjected to Strep-tag affinity chromatography purification. *Lanes 1–5* elution fractions by 2.5 mM desthiobiotin. Recombinant Endo H from Bme21 [rEndo H (Bme21)] was detected by Western blotting using HisProbe-HRP. The arrows indicate the bands of recombinant Endo H. **d** Endo- $\beta$ -*N*-acetylglucosaminidase activity assay. RNase B incubated with rEndo H (*E. coli*) (lane 1) and rEndo H (Bme21) for indicated times (*lanes 2–4*) were resolved by 15 % SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 staining. Lane *mock* represents RNase B without enzyme treatment. The molecular weight of glycosylated RNase B was 17.0 kDa

#### Purification and Characterization of Endo H from Bme21 Cells Infected with the Recombinant BmNPV

For the purification of secreted Endo H from Bme21 cells [rEndo H (Bme21)], culture medium was harvested at the 4 DPI. As the first purification step, 20 ml of the cell culture medium was purified through nickel affinity chromatography (Fig. 2b). Subsequently, the fractions 2 and 3 eluted in the 500 mM imidazole were combined and then subjected to the second purification process by Strep-tag affinity chromatography (Fig. 2c). The Western blotting analysis showed the band which is close to the expected size of the rEndo H (Bme21).

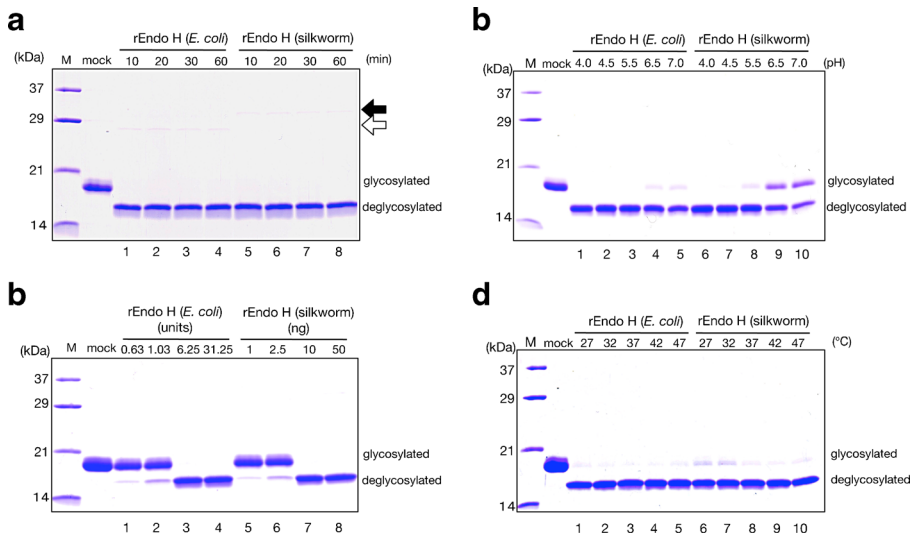
To measure the deglycosylation activity of secreted Endo H, RNase B, a protein that does not require denaturing treatments for deglycosylation [11], was used as a substrate glycoprotein. Although the mobility change in RNase B was observed in the rEndo H (Bme21) treatment, a 1-h reaction time was not sufficient for complete deglycosylation (Fig. 2d). These



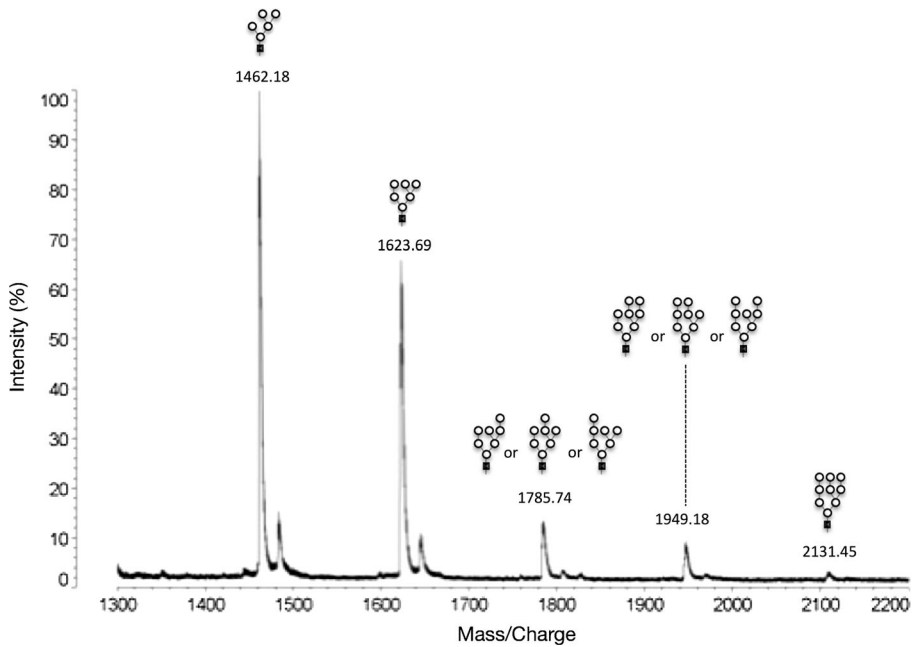


**Fig. 3** Purification of Endo H from hemolymph of silkworm larvae. **a** The recombinant Endo H proteins purified by nickel affinity chromatography. *M* molecular mass markers, *IP* input, *FT* flow-through, *WS* wash fraction, lanes 1–6 elution fractions by 500 mM imidazole. **b** All elution fractions by 500 mM imidazole were concentrated and further purified by Strep-tag affinity chromatography. *M* molecular mass markers, *IP* input, *FT* flow-through, *WS* wash fraction, lanes 1–6 elution fractions by 2.5 mM desthiobiotin. All samples were detected with Coomassie Brilliant Blue R-250, and the arrows indicate the bands of recombinant Endo H

data suggested that Endo H produced by the BES has the biological activity, while a greater amount of the enzyme was required for rapid deglycosylation.



**Fig. 4** Characterization of Endo H from silkworm larvae [rEndo H (silkworm)]. RNase B—incubated with **a** 31.25 units of rEndo H (*E. coli*) (lanes 1–4) or 50 ng of rEndo H (silkworm) (lanes 5–8) for indicated times at 37 °C and pH 5.5, **b** indicated units of rEndo H (*E. coli*) (lanes 1–4) or amounts of rEndo H (silkworm) (lanes 5–8) for 1 h at 37 °C and pH 5.5, **c** 6.25 units of rEndo H (*E. coli*) (lanes 1–5) or 10 ng rEndo H (silkworm) (lanes 6–10) for 1 h at 37 °C and indicated pH, and **d** 6.25 units of rEndo H (*E. coli*) (lanes 1–5) or 10 ng rEndo H (silkworm) (lanes 6–10) for 1 h at indicated temperatures and pH 5.5—was resolved by 15 % SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 staining. Lane mock represents RNase B without enzyme treatment. The filled and open arrows indicate the bands of rEndo H (silkworm) with 8×His-tag and Strep-tag and rEndo H (*E. coli*), respectively. The bands of glycosylated or deglycosylated RNase B are indicated. *M* molecular mass markers

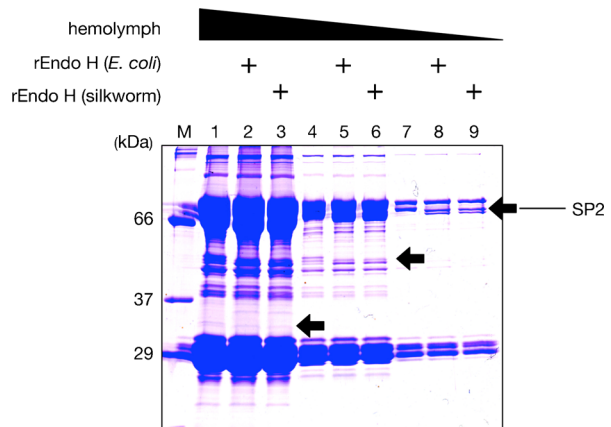


**Fig. 5** MALDI-TOF MS analysis of *N*-linked glycans released from RNase B by rEndo H (silkworm). The filled squares and opened circles represent GlcNAc and mannose, respectively. The numbers at the peak represent molecular masses of the labeled glycans

### Purification and Enzymatic Characterization of Recombinant Endo H from Silkworm Larvae

To obtain a large amount of recombinant Endo H, silkworm-BES was employed as described in the “Materials and Methods” section. The hemolymph of silkworm larvae infected with the

**Fig. 6** Deglycosylation of hemolymph glycoproteins in silkworm larvae. Silkworm hemolymph (1  $\mu$ l) was incubated without (lanes 1, 4, and 7) or with 6.25 units of rEndo H (*E. coli*) (lanes 2, 5, and 8) or 50 ng of rEndo H (silkworm) (lanes 3, 6, and 9). 0.5  $\mu$ l (lanes 1–3), 0.1  $\mu$ l (lanes 4–6), and 0.02  $\mu$ l (lanes 7–9) of hemolymph were resolved on 12 % SDS-PAGE. The arrows indicate the bands of deglycosylated silkworm hemolymph proteins. *M* molecular mass markers, SP2 silkworm storage protein 2



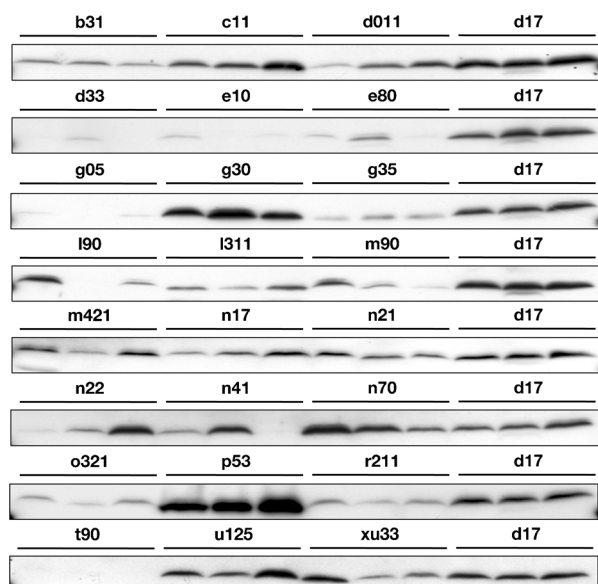


recombinant BmNPV-expressing Endo H was harvested at 4 DPI. SDS-PAGE analysis showed the purified proteins after nickel affinity chromatography (Fig. 3a) and Strep-tag system purifications (Fig. 3b). Approximately 30 µg of Endo H was recovered from 10 ml of silkworm hemolymph at high purity. Our results suggested that the secretion of Endo H into silkworm hemolymph could be effective for mass production of the protein.

In order to evaluate the availability of the enzymatic activity of purified rEndo H (silkworm), the RNase B was deglycosylated by 50 ng of rEndo H (silkworm). 50 ng of Endo H was enough for cleaving *N*-linked oligosaccharides of 2.5 µg of the RNase B for 10 min (Fig. 4a). As shown in Fig. 4b, 10 ng of the rEndo H (silkworm) was sufficient for the complete deglycosylation in the 1-h reaction and comparable to 6.25 units of rEndo H (*E. coli*). Consequently, approximately 18,750 units of rEndo H (silkworm) were purified from 10 ml of silkworm hemolymph. Additionally, the enzymatic activity of the rEndo H (silkworm) was retained at pH 4.0–7.0 (Fig. 4c) and at 27–47 °C (Fig. 4d). These results indicated that the stability and activity of rEndo H (silkworm) were comparable to those of *S. plicatus* Endo H and rEndo H (*E. coli*) [9].

To validate the specificity of rEndo H (silkworm), the *N*-linked glycans released from RNase B by rEndo H (silkworm) were subjected to MALDI-TOF MS. The mass of each *N*-linked glycan was measured as the additional mass (447.22) due to labeling with *N*-[(aminooxy)acetyl]tryptophanylarginine methyl ester (aoWR) for high-sensitivity detection. Bovine pancreas RNase B oligosaccharides have been characterized to be Man<sub>5</sub>GlcNAc<sub>2</sub> through Man<sub>9</sub>GlcNAc<sub>2</sub> (Supplementary Fig. 2) [17]. The MALDI-TOF MS measurement for the aoWR-labeled glycans were detected as five major peaks corresponding to Man<sub>5</sub>GlcNAc-(aoWR), Man<sub>6</sub>GlcNAc-(aoWR), Man<sub>7</sub>GlcNAc-(aoWR), Man<sub>8</sub>GlcNAc-(aoWR), and Man<sub>9</sub>GlcNAc-(aoWR), respectively (Fig. 5). This result clearly proved that rEndo H (silkworm) cleaves within the chitobiose core of high-mannose oligosacchrides from RNase B.

**Fig. 7** Screening of silkworm strains for expression of recombinant Endo H. 0.5 µl of silkworm hemolymph infected with recombinant BmNPV was resolved in 15 % SDS-PAGE, and Endo H was detected by Western blotting using HisProbe-HRP. Three individual samples were prepared from each strain, and the d17 hemolymph was used as the control



## Deglycosylation of Glycoproteins in the Hemolymph of Silkworm Larvae by Recombinant Endo H

The enzymatic activity of rEndo H (silkworm) to RNase B has been shown. However, the deglycosylation of only RNase B was insufficient to demonstrate the availability of rEndo H (silkworm) to glycosylation research. In order to validate the activity of rEndo H (silkworm) to other glycoproteins, we tried to release high-mannose type *N*-glycans from the endogenous glycoproteins using rEndo H (*E. coli*) and rEndo H (silkworm) (Fig. 6). The mobility shifts of at least three proteins were detected with rEndo H (*E. coli*) or rEndo H (silkworm) treatment. One of the three glycoproteins might be the storage protein 2 (SP2), which is a major protein secreted into silkworm hemolymph with a molecular mass of about 70 kDa [18]. The SP2 was reported to have a high-mannose-type *N*-glycan, Glc<sub>1</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> [19]. As demonstrated in Fig. 6, it was deglycosylated by both *E. coli* Endo H and BES Endo H. Our results suggested that purified rEndo H (silkworm) could be used to remove *N*-glycans attached to glycoproteins consisting in secreted proteins.

## Screening of Strains for Large-Scale Expression and Secretion of Recombinant Endo H

The secreted recombinant Endo H produced by silkworm-BES was shown to be useful for *N*-linked glycoprotein analysis. This result encouraged us to screen the silkworm strains showing high expression levels of the secreted foreign protein. The d17 strain is highly permissive to BmNPV [20] and suitable for the large-scale expression of foreign proteins [21, 22]. As shown in Fig. 7, although several strains generated secreted Endo H, the secretion levels were lower than that from the d17 strain. Interestingly, two strains, g30 and p53, showed significantly higher secreted amounts of Endo H (Fig. 7). We presented the first-ever screening analysis for expressing secreted proteins from silkworm-BES. These results indicated that these two strains might be effective for the larger-scale production of Endo H.

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