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Improvement of Endo-β-*N*-acetylglucosaminidase H production using silkworm–baculovirus protein expression system

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ABSTRACT

Endo-β-*N*-acetylglucosaminidase H (Endo H) catalyzes cleavage between the GlcNAc residues of the chitobiose core of *N*-linked glycans, leaving one GlcNAc residues attached to asparagine. Endo H cleaves high mannose and hybrid, but not complex, *N*-linked oligosaccharides on glycoproteins. Because of its unique specificity, Endo H is widely used for the structural and functional analyses of glycoproteins. In our previous study, the recombinant Endo H was produced as a secreted protein using silkworm–baculovirus expression system, but the yield was low (30 μg Endo H/10 ml larval hemolymph) compared to that of *Escherichia coli*. In this study, we purified active recombinant Endo H as an intracellular protein from fat body of silkworm infected with the recombinant baculovirus expressing Endo H without the exogenous signal peptide. Remarkably, the yield (9.3 mg from 20 silkworm larvae) was about 310-fold higher than that secreted into larval hemolymph as reported previously. In addition, we screened the silkworm strains maintained in Kyushu University and identified n17 as a high-level expression strain for Endo H.

Introduction

Glycosylation is one of the most ubiquitous post-translational modifications, with significant effects on protein folding, conformation, stability, and function (Imperiali and O'Connor, 1999; Bertozzi and Kiessling, 2001). Proteins in secretory pathway are usually modified by specific glycotransferases with *N*-linked and/or *O*-linked oligosaccharides in endoplasmic reticulum (ER) and/or Golgi apparatus. In particular, *N*-linked glycans, which are modified at asparagine (Asn) residues of a consensus sequence Asn–X–Ser/Thr (where X is any amino acid except proline), are widely spread among the eukaryotic organisms. It has been predicted that more than half of the eukaryotic proteome is glycosylated with about 90% of these are likely to be *N*-glycosylated (Apweiler et al., 1990). In spite of the significance of carbohydrate chains, their detail structures and functions have not yet been well characterized due to their remarkable heterogeneity.

Oligosaccharide-cleaving enzymes are used for investigating oligosaccharide structures, structure–function relationships, and glycoprotein biosynthesis by virtue of releasing intact glycans from their host proteins without destruction of the peptide itself. Most common *N*-linked carbohydrates can be removed by peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase F (PNGase F, EC3.5.1.52) or Endo-β-*N*-acetylglucosaminidase H (Endo H, EC 3.2.1.96).

The PNGase F is a 34.8 kDa (314 amino acids) enzyme secreted by *Flavobacterium meningosepticum* (Plummer et al., 1984). PNGase F catalyzes the hydrolysis of *N*-glycosidic bond between the innermost GlcNAc and Asn residues of high mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins (Elder and Alexander, 1982). During the removal of intact glycans, the modified Asn residue is converted to aspartic acid (Asp). PNGase F cleaves nearly all types of *N*-linked glycans, but does not release glycans when the innermost GlcNAc residue is linked to an α-1,3-fucose residue (Chu, 1986; Tretter et al., 1991). On the other hand, Endo H is a 29.0 kDa (269 amino acids) glycosidase secreted by *Streptomyces plicatus* (Tarentino and Maley, 1974; Robbins et al., 1984). The Endo H cleaves between the GlcNAc residues in the di-*N*-acetylchitobiose core of *N*-linked oligosaccharides, generating a truncated sugar molecule with one GlcNAc residue remaining on the Asn. Endo H cleaves high mannose and certain types of hybrid glycans, whereas the complexes with more than 4 different sugar types per glycan chain including the GlcNAc and paucimannose sugars are resistant to hydrolysis (Tarentino et al., 1989; Trimble and Tarentino, 1991; also see Fig. 1A). Owing to the limited substrate range of Endo H, it has been utilized to monitor glycosylation processes in Golgi apparatus (Guo et al., 2011; Taner et al., 2011).

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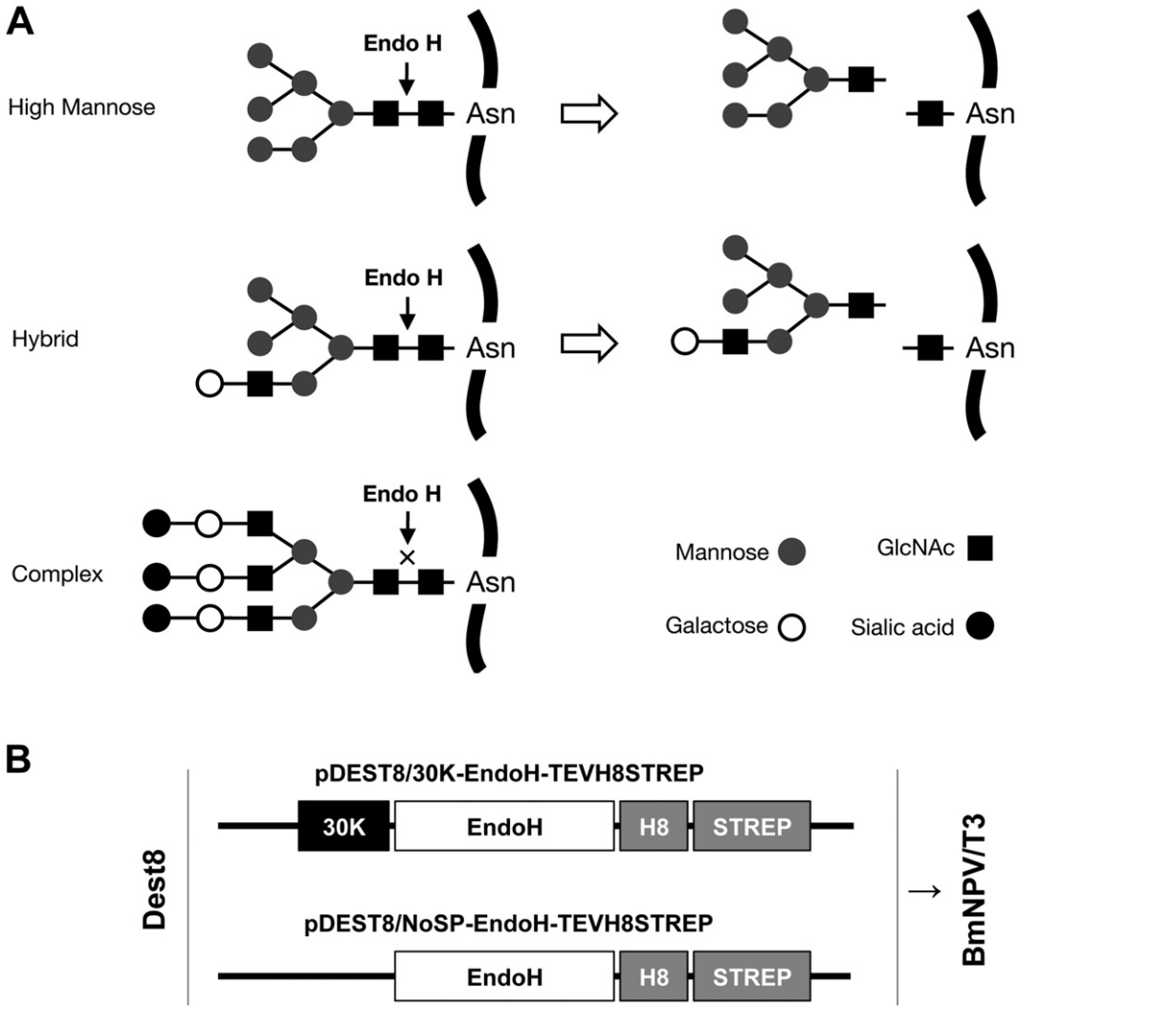

Fig. 1. Schematic diagram of the substrate specificities of Endo H and the expression constructs in this study (A) The enzyme reaction results in the release of the *N*-linked oligosaccharides. *Black arrow* represents the cleavage site of Endo H. *Gray circles*, mannose; *Black squares*, GlcNAc; *White circles*, Galactose; *Black circle*, Sialic acid (B) Schematic representation of recombinant BmNPV-T3. The plasmid pDEST8/30K-EndoH-TEVH8STREP and pDEST8/NoSP-EndoH-TEVH8STREP were generated from its corresponding pENTR and pDEST8 vector by Gateway reaction. *30K*, signal peptide of silkworm 30 kDa protein; *Endo H*, Endo-ß-*N*-acetylglucosaminidase H; *H8*, 8 × histidine-tag; *STREP*, Strep-tag.

In spite of the usefulness, the recombinant Endo H (rEndo H) has been expressed only in *Escherichia coli* for a long time (Trimble and Maley, 1984; Trumbly et al., 1985). In our previous work, Endo H was produced using silkworm–baculovirus expression system (silkworm–BES) (Mitsudome et al., 2014), which is widely utilized for the secretion of the recombinant proteins at extremely high levels. In addition, the protein expression using the silkworm larvae or pupae is approximately 10- to 100-fold higher than that using silkworm cells (O'Reilly et al., 1992; Luckow et al., 1993; Motohashi et al., 2005; Kato et al., 2010; Mon et al., 2013). The Endo H with silkworm 30 K signal peptide was expressed and 30 μg of pure and active Endo H was recovered from 10 ml of silkworm hemolymph (Mitsudome et al., 2014), but the yield was relatively poor compared to that from *E. coli* system (23 mg/4 l of culture) (Trumbly et al., 1985).

In this study, the non-secreted form of Endo H without a signal peptide was expressed and purified from silkworm fat body. The resulting yield was 310-fold higher than that of secreted form (9.3 mg/20 silkworm larvae). The enzymatic activity was comparable to the commercial Endo H. We also screened the silkworm strains for efficient production of intracellular Endo H, indicating that n17 strain is a promising bioreactor for intracellular expression of foreign proteins.

Materials and methods

Cells and silkworms

The NIAS-Bm-oyanagi2 (kindly provided from Dr. Imanishi) cells were cultured in IPL-41 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). The silkworm strains used in this study were obtained from the Institute of Genetic Resources, Kyushu University Graduate School. The larvae were reared routinely with mulberry leaves at 25–27 °C.

Construction of expression vectors

The pENTRL21-30K-EndoH-TEVH8STREP vector (DDBJ accession No. AB838596) (Mitsudome et al., 2014), which was inserted the codon-optimized Endo H to the downstream of 30 K signal peptide, was used in this study. The 30 K signal peptide sequence was excised directly by inverse PCR using the primers L21-atg-3 (5′-catggtggcggtttttttaggagttgcctgc-3′) and EndoH-NoSP-5 (5′-gccccggcc ccggtgaagcaggggccgacc-3′), and the resulting construct was named pENTRL21-NoSP-EndoH-TEVH8STREP.