Transglycosylation toward naringenin-7-*O*-glucoside using an N180H mutant of *Coprinopsis cinerea* endo-β-*N*-acetylglucosaminidase

Takao Ohashi[a, 1], Yu Fujisawa[a], Marc R. Hayes[b], Ryo Misaki[a], Jörg Pietruszka[b, c], Kazuhito Fujiyama[a, *]

[a]International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan

[b]Institut für Bioorganische Chemie, Heinrich-Heine-Universität Düsseldorf im Forschungszentrum Jülich, 52426 Jülich, Germany

[c]Institut für Bio- und Geowissenschaften: Biotechnologie (IBG-1), Forschungszentrum Jülich, 52426 Jülich, Germany

[*]To whom correspondence should be addressed. Email: fujiyama@icb.osaka-u.ac.jp. Tel: +81-6-6879-7453. Fax: +81-6-6879-7454.

[1]Present address: Department of Life Science, Faculty of Science and Engineering, Setsunan University, 17-8 Ikedanaka-machi, Neyagawa, Osaka 572-8508, Japan

Keywords: Endo-CC N180H, flavonoid, SGP, transglycosylation

Abstract

Flavonoids are generally glycosylated, and the glycan moieties of flavonoid glycosides are known to greatly affect their physicochemical and biological properties. Thus, the development of a variety of tools for glycan remodeling of flavonoid glycosides is highly desired. An endo-β-*N*-acetylglucosaminidase mutant Endo-CC N180H, which is developed as an excellent chemoenzymatic tool for creating sialylglycoproteins, was employed for the glycosylation of flavonoids. Endo-CC N180H transferred the sialyl biantennary glycans from the sialylglyco peptide to *p*NP-GlcNAc and narigenin-7-*O*-glucoside. The kinetic parameters of Endo-CC N180H towards SGP and *p*NP-GlcNAc were determined. Flavonoid glucosides harboring a 1,3-diol structure in the glucose moieties acted as substrates of Endo-CC N180H. We proposed that the sialyl biantennary glycan transfer to the flavonoid by Endo-CC N180H could pave the way for the improvement of the inherent biological functions of the flavonoids and creation of novel flavonoid glycoside derivatives for future human health benefits including foods and drugs.

1. Introduction

Flavonoids are one of the major constituents of polyphenols and comprise one of the largest family of plant specialized metabolites, and over 15,000 flavonoid-related compounds have been identified [1]. A variety of flavonoids have been used as pharmaceuticals, nutraceuticals and food additives for human health benefit with promising properties such as antioxidant, antibacterial, antiviral, anti-tumor, anti-inflammatory activities [2,3]. Indeed, numerous *in vitro* studies demonstrated that various types of flavonoids exert anti-tumor activities by a variety of mechanisms such as preventing cell cycles, promoting apoptosis, and inhibiting angiogenesis and metastasis [4,5,6]. However, flavonoids are generally poorly soluble in aqueous solutions due to their hydrophobic benzene ring. Although the low-aqueous solubility hinders their efficient bioavailability from the diet, glycosylation of flavonoids is known to be the one of the solutions to increase their solubility [7,8]. In addition, the different glycan structures provide different biological functions of flavonoid glycosides, such as sweetness, bitterness, and toxicity in human diets [1]. Indeed, deglycosylation by glycosidases is commonly used in the food industry to alter the sensory natures of beverages such as debittering of citrus juices by transforming the bitter naringin to tasteless naringenin-7-*O*-glucoside (N7G, prunin) or naringenin [1]. Numerous laboratory scale methods for glycan modifications by using flavonoid glycosyltransferases and engineered glycosidases as glycosynthases were also reported [9,10]. However, the glycan remodeling method by transferring oligosaccharides to flavonoid glycosides has never been attempted. Therefore, the development of remodeling tools for glycan moieties of flavonoid glycosides by oligosaccharide transfers is highly desired.

An endo-β-*N*-acetylglucosaminidase (ENGase) is an endo-glycosyl hydrolase that cleaves the β1,4-glycosidic linkage of the *N*,*N*'-diacetylchitobiose moiety of *N*-linked oligosaccharide. Some ENGase mutants have been developed and transfer GN1-type oligosaccharides *en bloc* to a variety of glycosyl acceptors carrying a single *N*-acetylglucosamine (GlcNAc) residue [11]. The N180H mutant

of the ENGase originating from the edible mushroom *Coprinopsis cinerea* (Endo-CC N180H) was developed and exhibits a transglycosylation activity towards sialyglycopeptide (SGP) or sialyglyco-oxazoline as glycan donors and mono-*N*-acetylglucosaminyl-RNase B as a glycan acceptor [12,13]. Endo-CC N180H is believed to display mostly the same enzymatic properties as the Endo-M N175Q and N175H mutants except with a superior thermostability and easier production in *Escherichia coli* [12]. A 1,3-diol structure and an equatorial hydroxyl group at the C-4 position of the acceptor sugars are essential whereas the aglycone structures attached to the acceptor sugars have less impact on the transglycosylation reaction by Endo-CC N180H, as was the case for Endo-M N175Q [14,15]. Therefore, we deduced that the flavonoid glycosides with the mentioned-above 1,3-diol structures could be potential acceptor substrate for Endo-CC N180H.

Sialic acids are acidic sugar molecule, which generally occupy the non-reducing ends of *N*-linked complex type glycans on glycoproteins. Sialic acids are involved in various biological events such as fertilization, development, proliferation, differentiation, malignancy and host-pathogen recognition *via* the interaction with sialic acid-specific ligand proteins [16]. In addition, sialic acid also biophysically contributes to the aqueous solubility of sialic acid-attaching molecules [17]. Here, we report the transglycosylation of a sialyl-biantennary glycan to flavonoids by Endo-CC N180H and the determination of kinetic parameters and the substrate preference of Endo-CC N180H.

2. Materials and methods

2.1. Materials for Endo-CC N180 transglycosylation reaction

Endo-CC N180H, sialyl glycopeptide (SGP, H-Lys-Val-Ala-[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$-]Asn-Lys-Thr-OH) and asialo-SGP (H-Lys-Val-Ala-[(Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$-]Asn-Lys-Thr-OH) were purchased from Fushimi Pharmaceutical Co., Ltd. (Kagawa, Japan). N7G, neohesperidin, quercetin-7-*O*-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside were purchased from Extrasynthese (Genay, France). Naringin, narirutin and rutin were

purchased from Wako (Osaka, Japan). *p*-Nitrophenyl (*p*NP-) GlcNAc was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Transglycosylation assay for Endo-CC N180H

A reaction mixture containing 750 μunits of Endo-CC N180H, 25 mM potassium phosphate buffer, pH 7.5, 2.5 mM SGP or asialo SGP and 0.5 mM of the respective flavonoid, in a total volume of 10 μL, was incubated for 30 min at 30 °C. The reaction was terminated by boiling at 100 °C for 3 min and the reaction products were collected in the supernatant by centrifugation at 10,000 *g* for 2 min.

The reaction products were analyzed by reversed-phase (RP-) HPLC. A COSMOSIL 5C_{18}-AR-II RP-HPLC column (6.0 × 250 mm; Nacalai, Kyoto, Japan) was equilibrated with 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.9 mL min^{-1}. After injecting the sample, the absorbed materials were eluted by two types of linear gradients, 0 – 20 % acetonitrile in 0.1 % TFA for the reaction with *p*NP-GlcNAc or 0 – 30 % acetonitrile in 0.1 % TFA for the reaction with flavonoids. UV detection was carried out at 290 nm.

2.3. The determination of kinetic values

The kinetic values of Endo-CC N180H towards SGP were determined under the standard assay conditions with various concentrations of SGP (0.1, 0.25, 0.5, 0.75, 1.0, 2.5 and 5.0 mM) in the presence of 10 mM *p*NP-GlcNAc. The kinetic values towards *p*NP-GlcNAc were also determined under the standard assay conditions with various concentrations of pNP-GlcNAc (0.01, 0.015, 0.02, 0.1, 1.0 and 10 mM) in the presence of 5 mM SGP. Aliquots were analyzed by RP-HPLC. The kinetic values are estimated using a Lineweaver-Burk plot.

2.4. Electrospray ionization-ion trap-mass spectrometry

An electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS) analysis was performed essentially as described previously [18].

3. Results and discussion

3.1. Transglycosylation of narigenin-7-*O*-glucoside by Endo-CC N180H

To examine whether Endo-CC N180H catalyzes the transglycosylation towards N7G as a flavonoid glycoside, we conducted an *in vitro* transglycosylation assay using N7G as the glycan acceptor substrate and SGP as the glycan donor substrate, as well as using *p*NP-GlcNAc as a positive control as reported in literature [14] (Fig. 1A). The new peaks appearing after the transglycosylation were designated as peaks a and b, then collected and analyzed by ESI-IT-MS. MS analysis of the collected peaks showed the *m/z* values of 2343.8 and 2458.7 for peaks a and b, respectively (Fig. 1B). These corresponded to the masses of the sialyl-biantennary-glycosylated *p*NP and naringenin, therefore indicating that Endo-CC N180H transfers the sialyl biantennary complex-type glycan to the N7G flavonoid. Furthermore, to quantitatively characterize the transglycosylation by Endo-CC N180H, the kinetic parameters towards SGP and *p*NP-GlcNAc were determined (Table 1). The K_m and the k_{cat} values for SGP and *p*NP-GlcNAc were lower than the reported values for Endo-M N175Q and N175H [19]. In addition, the specificity constant (k_{cat}/K_m) towards SGP of Endo-CC N180H were higher than those of Endo-M N175Q and N175H, while the specificity constant towards *p*NP-GlcNAc was approximately comparable to those of Endo-M N175Q and N175H. Sugar oxazolines, activated intermediates of the hydrolytic pathway, are known to be efficient donor compounds for transglycosylation by ENGases [11]. The specificity constants towards sialylglyco (SG-) oxazolines were reported to be higher than those towards SGP for Endo-M N175Q and N175H [19]. Endo-CC N180H was also reported to utilize SG-oxazolines for transglycosylation [13]. Therefore, it is worth

investigating the kinetic parameters for SG-oxazolines to quantitatively characterize the transglycosylation by Endo-CC N180H using these donors.

3.2. Substrate specificities of Endo-CC N180H

To examine the influence of the primary-attaching glycosylation pattern of the flavonoids and the effects of Rha attachments to the Glc moieties on the transglycosylation by Endo-CC N180H, we carried out the transglycosylation assay using a range of flavonoid glycosides (N7G, naringin, narirutin, neohesperidin, quercetin-3-*O*-glucoside, rutin, quercetin-7-*O*-glucoside and quercetin-3-*O*-galactoside) along with SGP as the glycan donor. The initial transglycosylation activities are summarized in Table 2. Transglycosylation activities were observed towards both 7-*O*- and 3-*O*-glucosylated flavonoids (for example, quercetin-3-*O*-glucoside and quercetin-7-*O*-glucoside) with a similar transglycosylation ratio, suggesting that Endo-CC is tolerant towards the binding sites of Glc residues on the flavonoid aglycones. The flavonoid glycosides with Glc moieties modified by Rha residue at the C6 position (narirutin and rutin) did not act as substrates. In contrast to the quercetin-3-*O*-glucoside, the quercetin-3-*O*-galactoside was not converted by the enzyme. These results are consistent with the previous finding that the minimum required structure for the transglycosylation by Endo-M and Endo-CC N180H corresponds to the 1,3-diol structure with C4-secondary and C6-primary free hydroxyl groups of sugar moiety [11,14]. All the flavonoid glycoside acceptors converted by Endo-CC N180H acted as better acceptors than *p*NP-GlcNAc. The effects of applying asialo-SGP as the donor in the transglycosylation was also examined for the above-mentioned flavonoid glycosides. The asialo-SGP was a better donor than SGP in combination with *p*NP-GlcNAc and all examined flavonoid glycoside acceptors, increasing the enzyme activity 1.3 – 2.2-fold. Examination of the transglycosylation activities of Endo-CC N180H towards the smaller glycan-attaching glycoproteins may help reveal the mechanism of the glycan donor preference of Endo-CC N180H.

ENGases including Endo-CC N180H are widely applied for glycan remodeling of glycoproteins with heterogenous glycoforms, creating a homogenous glycoform especially for pharmaceutical proteins [11]. Moreover, Endo-CC N180H has also been proven useful for introducing the radioisotopic ^{13}C- or ^{3}H-labbled glycans or chemically active functional groups, such as an azide, modifying glycans of not only glycoproteins but also a wide range of small molecules such as *p*NP glycosides, Asn-linked GlcNAc, 4-methylumbelliferyl glycosides and glycopeptides [14]. In this study, we found that Endo-CC N180H was able to transfer the sialyl biantennary glycans to flavonoid glycosides. Modification of the acidic sialyl glycans should provide a further improved solubility of flavonoids in aqueous solution, although the flavonoid aglycones are known to show high water insolubility [7]. In addition, flavonoid glycosides harboring human-type sialyl biantennary glycans would be non-allergic and might be helpful to broaden the availability of the edible flavonoid glycosides for human health benefits. For instance, apigenin and luteolin inhibit TNF-induced proinflammatory NF-κB, IRF and Akt signaling pathways in murine intestinal epithelial cells and TNF-induced interleukin-8 production in human colon epithelial cells, respectively [20,21]. These flavonoids are typically conjugated as 7-*O*-glucosides in plants, which is the favorable form for Endo-CC N180H. An isoflavone glycoside, genistin, is known to broadly show health benefits such as decreasing the risk of cancer, osteoporosis and apoptosis, and additionally exhibits antioxidative properties [22]. Various glycosides with antidiabetic potential have been isolated from plants and caused attention as lead compounds [23]. Furthermore, a number of flavonoids have been reported to have pharmaceutical effects. It is possible to enhance their medicinal effects by adding sugars such as 7-*O*-glucoside and then *N*-glycans. A sodium taurocholate cotransporting polypeptide (NTCP) has been identified as a functional receptor for the hepatitis B virus (HBV) infection to hepatocytes. Some of the digitalis-like compounds known as a traditional cardiac triterpene glycoside has shown inhibitory effects on the *in vitro* HBV infection assumingly by inhibiting the binding between the HBV and NTCP [24,25]. Among these digitalis-like compounds, proscillaridin A and convallatoxin both

carry rhamnose in their glycan residues. Thus, once the glycan moiety of proscillaridin A and convallatoxin are remodeled to the human-type biantennary complex type glycans by applying the Endo-CC N180H, we may be able to expect improved pharmacological properties by increasing the aqueous solubility, the half-life in the blood stream, the targeting efficiency to the hepatocyte and the binding ability to NTCP.

In summary, we revealed that Endo-CC N180H transfer the sialyl and asialo biantennary glycans to flavonoid glycosides for the first time. As previously reported, the 1,3-diol structure harboring the C4-secondary and C6-primary free hydroxyl groups in the sugar moiety is important for glycan transfer by Endo-CC N180H. These findings could be applied to the production of the artificial novel flavonoid glycosides harboring human type complex glycans with improved human health benefits for foods and drugs.

Acknowledgements

This work was partially supported by the JSPS Japanese-German Graduate Externship program and the DFG funded Research Training Program SeleCa. We are grateful for a scholarship from the Jürgen Manchot Stiftung (to MRH).

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Scheme legend

Scheme 1. Transglycosylation of flavonoid glycosides by Endo-CC N180H.

Figure legend

Fig. 1. Transglycosylation of N7G by Endo-CC N180H.

A. HPLC profiles of the transglycosylation products after incubation with the pNP-GlcNAc or N7G and SGP for 30 min in the presence of Endo-CC N180H. Peaks a and b were collected and lyophilized before subjected to the ESI-IT-MS analysis. B. The lyophilized peaks a and b were dissolved in 50 % (*v/v*) acetonitrile and directly injected using a syringe pump at a flow rate of 4 μL min^{-1}. Negative ion spectra of peaks a and b were obtained by a Bruker HCT plus ion-trap mass spectrometer. An asterisk indicates the unidentified *m/z* signal.

Table 1. Comparison of kinetic values of Endo-CC N180H with Endo-M mutants.

Substrate	SGP			*p*NP-GlcNAc		
	K_m [a]	k_{cat} [a]	k_{cat}/K_m	K_m [a]	k_{cat} [a]	k_{cat}/K_m
	mM	min^{-1}	$min^{-1}\ mM^{-1}$	*mM*	min^{-1}	$min^{-1}\ mM^{-1}$
Endo-CC N180H	2.3 ± 0.5	17 ± 2.9	7.5	0.50 ± 0.09	12 ± 2.4	24
Endo-M N175Q	100 ± 1.5 [b]	239 ± 7.3 [b]	2.4 [b]	2.1 ± 0.033 [b]	77 ± 0.24 [b]	37 [b]
Endo-M N175H	87 ± 0.65 [b]	143 ± 0.53 [b]	1.6 [b]	1.9 ± 0.054 [b]	40 ± 0.11 [b]	21 [b]

[a] The K_m and k_{cat} values represent mean ± SD (n = 3).

[b] These values are derived from the previous report [19].

Table 2. Initial velocity of Endo-CC N180H towards various substrates.

Acceptor [a]	Initial velocity [b] *mmol h^{-1} $unit^{-1}$*	
	SGP	Asialo SGP
N7G	155 ± 4	229 ± 18
Naringin	141 ± 4	207 ± 4
Narirutin	0	0
Neohesperidin	177 ± 5	259 ± 13
Quercetin-3-*O*-Glc	142 ± 19	262 ± 16
Rutin	0	0
Quercetin-7-*O*-Glc	239 ± 5 [c]	309 ± 8 [c]
Quercetin-3-*O*-Gal	0	0

[a] Structures of the flavonoid compounds can be found in scheme 1.

[b] The initial velocity values represent mean ± SD (n = 3)

[c] 25 mM HEPES-NaOH (pH 7.5) was used instead of 25 mM potassium phosphate buffer (pH 7.5) in the reaction mixture.