Identification and characterization of a core fucosidase from the bacterium Elizabethkingia meningoseptica

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All reported α-1-fucosidases catalyze the removal of nonreducing terminal 1-fucoses from oligosaccharides or their conjugates, while having no capacity to hydrolyze core fucosylated glycoproteins directly. Here, we identified an α-fucosidase from the bacterium Elizabethkingia meningoseptica with catalytic activity against core α-1,3-fucosylated substrates, and we named it core fucosidase I (cFase I). Using site-specific mutational analysis, we found that three acidic residues (Asp-242, Glu-302, and Glu-315) in the predicted active pocket are critical for cFase I activity, with Asp-242 and Glu-315 acting as a pair of classic nucleophile and acid/base residues and Glu-302 acting in an as yet undefined role. These findings suggest a catalytic mechanism for cFase I that is different from known α-fucosidase catalytic models. In summary, cFase I exhibits glycosidase activity that removes core α-1,3-fucoses from substrates, suggesting cFase I as a new tool for glycobiology, especially for studies of proteins with core fucosylation.

Fucosylated oligosaccharides are found in many natural substrates, such as cell polysaccharides, milk oligosaccharides, glycoproteins, and glycolipids (1, 2). Although fucose is relatively low in abundance in the biosphere, it is widely distributed in all kingdoms, and fucose-containing glycoconjugates have been proven to be involved in many biological and pathological processes, including tissue development (3, 4), angiogenesis (5), fertilization (6), tumor metastasis (7), and microbe/host interactions (8). Disease-associated fucosylation patterns of oligosaccharides on glycoproteins have been reported (9). Moreover, fucosylated α-fetoprotein (AFP-L3) has been proposed to be a new biomarker for hepatocellular carcinoma (10).

α-1-Fucosidases are exoglycosidases that catalyze the removal of nonreducing terminal 1-fucose residues; these residues are usually attached by α-1,2 linkage to β-galactose (Gal), α-1,3 linkage to β-glucose (Glc), or 1,3/4/6 linkages to N-acetylgalactosamine (GlcNAc) on oligosaccharides, oligolipids, and other glycoconjugates. Early studies indicated that abnormal α-fucosidase activity is related to a number of clinical conditions, such as cancer, inflammation, and cystic fibrosis (3, 11, 12). FUCA1 and FUCA2 are two human GH29 α-1-fucosidases that are both active on 1,2/3/4/6-linked fucosyl oligosaccharides, and as well synthetic pNP-Fuc (13, 14). Inactivation or severe deficiency of FUCA1 leads to a clinical condition named fucosidosis, an autosomal recessive lysosomal storage disease that results in a lethal accumulation of fucosylated glycoconjugates in the lysosomes of most tissues, including the peripheral and central nervous systems (15). FUCA2 has been found to be essential for Helicobacter pylori adhesion during its infection of gastric cells (16). α-Fucosidases are also of considerable clinical interest because the presence of α-fucosidase activity in serum has been used in the diagnosis for hepatocellular cancers for decades (17).

Core fucosylation is a common modification in which α-1,6 and/or α-1,3 linkages connect fucose and the first GlcNAc of the core pentasaccharide in the N-glycan of the glycoproteins (18), such as immunoglobulin G (containing a core α-1,6-fucose) (19), horseradish peroxidase (HRP, containing a core α-1,3-fucose), and phospholipase A2 (PLA2, containing both kinds of core fucosylation) (20, 21). Core α-1,6 fucosylation affects biological processes, including receptor activation, signal transduction, endocytosis, and cell adhesion, and it is involved in the regulation of many physiological and pathological events (22, 23). Core α-1,3 fucosylation is a conserved feature in plant and invertebrate N-linked oligosaccharides (24). Developmental and morphological abnormalities associated with altered shoot gravitropism during vegetative growth were observed for rice plants lacking core α-1,3-fucose (25). Moreover, this epitope was considered to be a neural marker for Drosophila and many other invertebrate species (26, 27). As core α-1,3 fucosylation is not present in mammals, IgE and/or IgG antibodies directly bind to core α-1,3-fucose structures, allowing the detection of these structures directly in the sera of some allergic patients (28). Approximately 30–50% of patients

3 The abbreviations used are: pNP-Fuc, 4-nitrophenyl-α-L-fucopyranoside; cFase I, core fucosidase I; PNGase F, peptide-N-glycosidase; PCMB, p-chloromercuribenzoate; PDB, Protein Data Bank; CBB, Coomassie Brilliant Blue; PLA2, phospholipase A2; AAL, Aleuria aurantia lectin; ESI, electrospray ionization; WB, Western blotting.
**Identification of a core fucosidase**

with insect venom allergies have IgE antibodies that react with both honeybee and yellow jacket venom (24). What is more, the presence of core α-1,3-fucose glyco-epitopes on therapeutic N-glycoproteins that were produced in plant cells raised the immunogenicity in human therapy (29). This result demonstrated that core α-1,3-fucose residues are a common allergic epitope in humans (24, 28, 29). The previous studies showed that the physiological process of core defucosylation is probably initiated with digestion of glycoprotein by proteases or removal of the glycan from glycoproteins by peptide N-glycosidase in the lysosome/vacuole (30, 31). Despite these critical features of core defucosylation, a one-step core defucosylation process has yet to be identified. Although extensive research effort has been applied to α-1-fucosidase for decades, understanding of specific critical biochemical and biologically interesting fucosidase activities are still missing. For example, although characterized α-fucosidases were able to hydrolyze the terminal fucose residue in oligosaccharides, glycolipids, and/or glycoproteins (32–37), none of these α-fucosidases has been proven capable of removing core fucose residues from glycoproteins. To the best of our knowledge, no core α-1,3 fucosidase has been reported yet. Therefore, finding a new α-fucosidase that has core fucosidase activity is required to improve our understanding of core fucosidase in biological systems and processes.

Endo-β-N-acetylgalactosaminidases (F1, F2, and F3) and peptide:N-glycosidase F (PNGase F) have been identified in *Elizabethkingia* spp. and broadly used in glycobiological research (38, 39). In addition, we have identified the novel protein peptide:N-glycosidase F-II (PNGase F-II) from *Elizabethkingia meningoseptica* and revealed that the enzyme has broad substrate specificity, including deglycosylation of core α-1,3-fucosylated N-glycans, that is resistant to PNGase F hydrolysis (40). In this study, we identified a novel fucosidase from the *E. meningoseptica* genome and demonstrated that it has core fucosidase activity.

**Results**

**Bioinformatics analysis of a novel candidate fucosidase gene in the whole-genome sequence of *E. meningoseptica***

*E. meningoseptica* FMS-007 is a clinical strain that was isolated from a T-cell non-Hodgkin’s lymphoma patient in our laboratory (41). Bioinformatics analysis of the whole genome revealed a fucosidase candidate (core fucosidase I, cFase I) that consisted of 480 amino acids with a predicted signal peptide of 25 amino acids. Two of the homologs in the PDB database that were closest in sequence to cFase I were bacterial α-fucosidases from *Thermotoga maritima* (TM0306) and *Bacteroides thetaiotaomicron* (BT2970). Their sequence identities with cFase I were 25 (PDB code 1HL8) and 26% (PDB code 2WVT), respectively. Conserved domain analysis revealed that cFase I was similar to enzymes that belonged to the GH29 class of carbohydrate-active enzymes as defined in the CAZY database. The typical structure of GH29 α-fucosidases is composed of two domains. The N-terminal domain adopts an (α/β)_{10} barrel-like fold, with eight parallel strands that are packed around a central axis and surrounded by six helices and a C-terminal sandwich domain.

Phylogenetic analysis showed that cFase I was in neither the cluster of the established GH29-A subfamily nor that of GH29-B (Fig. 1). The evolutionary distance of cFase I gene to the GH29-A and GH29-B subfamilies indicated that the ancestral gene of cFase I was in a separate lineage than the current taxonomic lineage of the GH29-A and GH29-B subfamilies, suggesting that it may have different biochemical and biological functions.

**Molecular cloning and expression of cFase I**

A DNA fragment of α-fucosidase that lacked the sequence that encodes its N-terminal signal peptide was amplified by PCR using genomic DNA from *E. meningoseptica* FMS-007 as a template and was then ligated into a pET-28a(+) expression vector to produce a fusion protein with an N-terminal His	extsubscript{6} tag. The purified protein migrated as a single band with an apparent molecular mass of 52 kDa when subjected to SDS-PAGE (data not shown), in agreement with its calculated mass.

**Enzymatic characterization of cFase I with pNPs**

We verified the enzymatic activity of recombinant cFase I by subjecting the purified protein to a colorimetric enzymatic assay that used pNP-Fuc as its leading substrate. Fucosidase activity was detected. The optimum pH of cFase I activity is 4.5 (Fig. 2a), whereas the pH stability assay showed that cFase I was unstable at pH values less than 3.0 (Fig. 2b). We also demonstrated that the optimum temperature of the activity was 55 °C (Fig. 2c); however, a thermostability assay showed that cFase I quickly lost its hydrolytic activity at this temperature (Fig. 2d), indicating that the substrate may help to prevent the denaturation of the enzyme at 55 °C. As a result, 37 °C was chosen as the standard testing temperature in later experiments to prevent the denaturation of the enzyme and potential non-enzymatic hydrolysis of the substrate at higher temperatures.

The effects of various metal ions and reagents on cFase I activity were also investigated in 0.1 M acetate buffer, pH 4.6. At a 1 mM concentration, divalent cations Mg	extsuperscript{2+}, Mn	extsuperscript{2+}, Ca	extsuperscript{2+}, Ni	extsuperscript{2+}, Fe	extsuperscript{2+}, and Cd	extsuperscript{2+} had no significant effect on the enzymatic activity, and Zn	extsuperscript{2+} ions only slightly inhibited the activity; by contrast, the enzyme activity was obviously inhibited by Cu	extsuperscript{2+} ions (Table 1). The inhibition of cFase I by Cu	extsuperscript{2+} was concentration-dependent, with a nonlinear decrease in activity as the concentration of Cu	extsuperscript{2+} ions increased (Fig. 2e). Moreover, the organomercury PCMB severely inactivated the enzyme (Table 1), whereas this inhibitory effect on cFase I was reversible with additional DTT (data not shown). Our observations were similar to those in previous reports on fucosidases (42–44). Furthermore, the fucosidase-specific inhibitor deoxyfuconojirimycin inhibited the activity of cFase I, indicating the fucosidase activity of the enzyme (Fig. 2f). The chelator EDTA had no significant influence on the enzyme activity. The presence of 1 M urea and 10 mM DTT had no effect on the activity of cFase I, whereas 0.1% SDS almost inactivated the enzyme. The inhibitory effect of SDS to cFase I was overcome by Nonidet P-40 (data not shown).

To further define the substrate specificity of cFase I, we tested the enzyme against a panel of other pNP monosaccharides that have been used to assay glycosidase activity and spec-
Identification of a core fucosidase

Table 2

| Enzyme                                      | Accession Number |
|---------------------------------------------|-----------------|
| DmFuca Drosophila melanogaster              | AAM50292.1      |
| FucA2 Homo sapiens                          | NP_14409.2      |
| ClFucA1 Canis lupus familiaris              | AAAS2481.1      |
| FucA1 Homo sapiens                          |                  |
| DdFuca Dictyostelium discoideum             | AAAS1149.1      |
| TM0306 Thermotoga maritima                 | AAO78076.1      |
| SsFuca1 Sulfobolus solfataricus            | AAO51194.1      |
| FgFuca Fusarium gramineae                  | AAO50114.1      |
| BT2970 Bacteroides thetaiotaomicron        | AAO50114.1      |
| ATFB Lactobacillus casei                   | AAAS78076.1     |
| Afc1 Lactobacillus casei                   | AAAS1149.1      |
| cFase I Elizabethkingia meningoseptica     |                   |
| BbAfcB Bilobacterium bifidum               | CAQ67877.1      |
| SsFuc1 Streptomyces sp.                    | CAQ67877.1      |
| Afc2 Clostridium perfringens               | AAO87877.1      |
| BT2192 Bacteroides thetaiotaomicron        | AAO87877.1      |
| AIFUC1 Arabidopsis thaliana                | AAAS1149.1      |
| Axy8 Arabidopsis thaliana                  | AAAS1149.1      |
| LIFuc Lilium longiflorum                   | AAAS1149.1      |
| ANB149.2 Aspergillus nidulans              | AAAS1149.1      |
| BbAfcA Bilobacterium bifidum               | AAAS1149.1      |
| Afc3 Clostridium perfringens               | AAAS1149.1      |
| Blon 2335 Bifobacterium bifidum             | AAAS1149.1      |
| B. longum Blon 2335                        | AAAS1149.1      |

Figure 1. Phylogenetic tree of cFase I and characterized α-L-fucosidases. The amino acid sequences of all enzymes were obtained from GenBank™. Sequence alignment was performed using ClustalW with the MEGA 5.0 program. The tree was constructed using a neighbor-joining method. GenBank™ accession numbers of these proteins are as follows: Drosophila melanogaster (DmFuca; AAAS50292.1); Homo sapiens (FucA2; NP_14409.2); Canis lupus familiaris (ClFucA1; AAAS2481.1); Dictyostelium discoideum (DdFuca; AAAS1149.1); Thermotoga maritima (TM0306; AAO78076.1); Sulfobolus solfataricus (SsFuca1; AAO51194.1); Fusarium gramineae (FgFuca; AAO50114.1); Bacteroides thetaiotaomicron (BT2970; AAO78076.1); Lactobacillus casei (ATFB; CAQ67877.1); Arabidopsis thaliana (Afc1; CAQ67877.1); Elizabethkingia meningoseptica (cFase I; AAAS78076.1); Streptomyces sp. (SsFuc1; AAAS1149.1); Clostridium perfringens (Afc2; AAAS78076.1); B. thetaiotaomicron (BT2192; AAO78076.1); Arabidopsis thaliana (Axy8; CAQ67877.1); Lilium longiflorum (LIFuc; AAO87877.1); Aspergillus nidulans (ANB149.2; AAAS1149.1); B. bifidum (BbAfcA; AAAS1149.1); C. perfringens (Afc3; AAAS78052.1); B. longum (Blon 2335; AAAS78052.1).

The substrate linkage specificity of cFase I was examined by treating a panel of oligosaccharides that contain representative α-linkages to fucose residues. The reaction mixtures were analyzed using a K-FUCOSE kit and ESI-MS. The assay was conducted in 20 mM sodium phosphate buffer, pH 4.6, at 37 °C. The results of the K-FUCOSE assay showed that cFase I was capable of hydrolyzing 3-FL and Lewis X that had a fucose attached by an α-1,3 linkage, but it was inactive against oligosaccharides with other types of linkages, such as 2`-FL, Fucα1–6GlcNAc, 6-fucosyl-N,N′-diacetyltizoibiose, and Lewis A (Table 2). cFase I was more active against Lewis X than 3-FL and pNP-Fuc (Table 2).

ESI-MS was employed to verify the reactivity of cFase I against the oligosaccharides (Fig. 3). In the control experiment that used 3-FL and no cFase I, a molecular ion peak at m/z 511.13, representing a sodium adduct of 3-FL (calculated mass, m/z 511.16), was detected. The peak was significantly attenuated by cFase I treatment; after this treatment, two intense mass ion peaks appeared at m/z 365.08 and 187.05, representing sodium adducts of Gal(β1–4) Glic disaccharide (calculated mass, m/z 315.11) and fucose monosaccharide (calculated mass, m/z 187.06), respectively. A similar result was obtained in the treatment of Lewis X. The peak at m/z 552.17, representing sodium adducts of Lewis X, was replaced by peaks representing a sodium adduct of Gal(β1–4) Glic disaccharide (at m/z 406.12) and a fucose (at m/z 187.03) after cFase I treatment. cFase I did not react with substrates that possessed other types of linkages (data not shown). These results demonstrated that cFase I was an α-1,3-fucosidase.

Sequence analysis of cFase I

A structure-based sequence alignment was conducted to predict the active sites of cFase I (Fig. 4). The overall structures of these characterized α-fucosidases were quite conserved, although the primary sequence identity between cFase I and the other proteins was between 20 and 32%. The main substrate-binding site residues that were identified in TM0306, such as His-34, His-128, His-129, and Tyr-171, were conserved in cFase I and corresponded to the residues His-57, His-132, His-133, and Tyr-181, respectively. The C-6 methyl group in fucose was...
enclosed within a hydrophobic pocket formed by the conserved Phe-32, Tyr-171, Trp-222, and Phe-290 residues, which corresponded to Phe-55, Tyr-181, Trp-240, and Phe-324 in cFase I, respectively (Fig. 4). With the exception of Glu-66 and Trp-67 in TM0306, the other residues that play an important role in the interaction between TM0306 and fucose are conserved in cFase I.

The double displacement model in which a pair of acidic amino acids function separately as a nucleophile and an acid/base is well accepted by the research community to be the catalytic model of GH29 fucosidase activity. The catalytic nucleophile in α-fucosidases was determined to be an aspartic acid residue that is fully conserved in both GH29-A and GH29-B subfamilies (33, 37, 46–49), whereas the catalytic acid/base residue is not fully conserved in the family. The conservation of this catalytic nucleophile strongly indicated that the Asp-242 in cFase I would be a catalytic nucleophile. The catalytic acid/base residue in cFase I was determined to be Glu-302 based on an alignment of TM0306, BT2970, BT2912, and BiAfcB. Two unique features were identified in the analysis. We noticed that another fully conserved Glu (Glu-315) is not required for catalytic activity in most of the identified α-fucosidases but is required for general acid/base catalysis of human α-fucosidase (hsFucA1, FUCA1) (34, 48), indicating that Glu-315 may have a role in catalytic activity. In addition, after substrate binding, a large conformational change at the loop (173–182) that follows the nucleophile (Asp-172) in the sequence of BiAfcB (47) indicated that the loop is probably a structurally flexible region that may be involved in determining substrate specificity. Comparing the established crystal structure of fucosidase indicated that there was an extended loop (249KFTDPTVEWQKTIKVE265) that followed the catalytic nucleophile Asp-242 in the primary sequence (Fig. 4).

**Defining the enzymatic active sites of cFase I**

A mutational study was performed to ascertain the identity of the catalytic residues and the function of the abovementioned loop. Moreover, it has been proved that mercuric ions and organomercury have a strong bonding affinity for the sulfhydryl group (50, 51), and the inactivation of cFase I by PCMB may indicate a potential role of cysteine (Cys-73, Cys-317, and

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**Table 1**

| Action of reagents on cFase I activity |
|--------------------------------------|
| **Reagents** | **Concentration** | **Relative activity**% |
| Mg<sup>2+</sup> | 1 mM | 94.4 |
| Mn<sup>2+</sup> | 1 mM | 95.3 |
| Ca<sup>2+</sup> | 1 mM | 100.7 |
| Zn<sup>2+</sup> | 1 mM | 87.0 |
| Ni<sup>2+</sup> | 1 mM | 93.4 |
| Fe<sup>2+</sup> | 1 mM | 92.2 |
| Cu<sup>2+</sup> | 1 mM | 14.6 |
| Cd<sup>2+</sup> | 1 mM | 95.2 |
| PCMB | 0.5 mM | 0.5 |
| EDTA | 5 mM | 115.2 |

* The activity was measured at 37 °C for 60 min in 0.1 M acetate buffer, pH 4.6, with 1 mM pNP-Fuc as substrate. The relative activity without any reagent was taken to be 100%.
Figure 3. Hydrolysis by recombinant cFase I of fucosylated oligosaccharides on 3-FL and Lewis X. 3-FL (a) and Lewis X (b) were incubated in the absence (a1 and b1) and presence (a2 and b2) of purified cFase I, and the reaction mixtures were subjected to ESI-MS analysis. The molecular ion peaks at m/z 511.13 (a1) and 552.17 (b1) correspond to sodium adducts of 3-FL and Lewis X, respectively. The molecular ion peaks at m/z 365.08 and 406.12 are sodium adducts of lactose (calculated, 365.1) and lacto-N-biose (calculated, 406.1), respectively. The peak at m/z 187.0 is a sodium adduct of fucose monosaccharide.

Table 2
Linkage specificity of cFase I

| Substrate                     | Abbreviation | Structure                                      | Relative activity |
|-------------------------------|--------------|------------------------------------------------|------------------|
| p-Nitrophenyl-α-L-fucoside    | pNP-Fuc      | Fucα-pNP                                       | 100%             |
| 6-Fucosyl-N-acetylglicosamine | 6-Fuc        | Galβ1–4(Fucα1–3)Glc                            | ND               |
| 2'-Fucosyllactose             | 2'-FL        | Fucα1–2Galβ1–4Glc                             | ND               |
| 3-Fucosyllactose              | 3-FL         | Galβ1–4(Fucα1–3)Glc                            | 1328.6           |
| Lewis A trisaccharide         | Le*          | Galβ1–3(Fucα1–4)Glc                            | ND               |
| Lewis X trisaccharide         | Le*          | Galβ1–4(Fucα1–3)Glc                            | 15448.5          |
| 6-Fucosyl-N,N'-diacetylttobiose | N2F         | GlcNAcβ1–4(Fucα1–6)Glc                         | ND               |

a The substrate was used at a concentration of 1.0 mM.
b The activity on pNP-Fuc was taken to be 100%.
c ND means not detectable.
Figure 4. Sequence analysis of cFase I. Structure-based sequence alignment of some GH29 family members. The 3D structures of *T. maritima* α-fucosidase (TM0306, Protein Data Bank code 1HL8), *B. thetaiotaomicron* α-fucosidas (BT2970 and BT2192, Protein Data Bank codes 2WVV and 3EYP, respectively), *B. longum* α-fucosidase (BiAfcB, Protein Data Bank code 3UES), and *E. meningoseptica* α-fucosidase (cFase I) were aligned using PROMALS3D, and the figure was produced using ESPript. The secondary structures of TM0306 and BiAfcB are indicated above and below the sequence, respectively. Identical residues are shown in white letters on a red background, and similar residues are shown in red letters on a white background. The residues (in TM0306) that are important for Fuc binding are indicated by magenta circles. The purple star denotes the conserved nucleophile. Green stars indicate experimentally confirmed acid/base residues and/or acid/base candidates in cFase I. A ligand-induced mobile loop (173–182 in BiAfcB) is boxed in yellow, and the corresponding extended loop of cFase I is highlighted with yellow squares.
Cys-467) in the catalytic activity. Alanine mutants of Asp-242, Glu-302, and Glu-315, the serine or alanine mutants of Cys-73, Cys-317, and Cys-467, and the deletion mutant 249Δ265 (deletion of residues 249–265) were prepared as His-tagged fusion proteins. The CD spectra of the mutants and of wildtype cFase I were analyzed to check the consistency of their secondary structures. The results showed that there was no significant perturbation in the global structure (Fig. S2). In addition, their ability to catalyze the hydrolysis of different types of substrates was investigated.

The 249Δ265 mutant enzyme retained the ability to catalyze the hydrolysis of substrates with slightly different $k_{cat}$ and $K_m$ values (Table 3). In contrast to the activity of the deletion mutant, all three alanine replacement mutants exhibited greatly reduced activity against pNP-Fuc, 3-FL, and Lewis X (Table 3). The D242A mutant showed no activity against 3-FL and dramatically decreased activity against pNP-Fuc and Lewis X. The value of $k_{cat}/K_m$ decreased 1000- and 1928-fold for reactions with pNP-Fuc and Lewis X, whereas the $K_m$ increased just 28- and 7-fold, respectively (Table 3). The E302A mutant hydrolyzed pNP-Fuc and Lewis X with a 736- and 900-fold decrease in $k_{cat}/K_m$, whereas the value of $K_m$ increased 2- and 10-fold, respectively. To our surprise, E315A also lost its enzymatic activity, evidencing no activity against the chromogenic substrate. The $k_{cat}/K_m$ values of E315A mutant activity against 3-FL and Lewis X were 473- and 585-fold lower than that of the wildtype, although there was no significant change in $K_m$ (Table 3).

To address the potential function of thiol group(s) for cFase I activity, a mutagenesis assay was conducted, and the result demonstrated that C73S and C467S substitutions had no impact on cFase I activity, whereas the activity of C317S mutant on pNP-Fuc, 3-FL, and Lewis X decreased 2-, 3-, and 2-fold, respectively (Table 3). In addition, we also found that the $k_{cat}/K_m$ values of C317A mutant against pNP-Fuc, 3-FL, and Lewis X decreased 9-, 4-, and 6-fold, respectively (Table 3).

ESI-MS analysis was conducted to verify the catalytic activity of all mutants against oligosaccharides (data not shown), and the results were consistent with the aforementioned results.

**Chemical rescue the activity of the cFase I mutants**

It is well documented that replacing the pair of catalytic residues with alanines in active sites of glycosidases creates cavities in which small anions can be accommodated to rescue the catalytic ability of the enzyme by acting as a nucleophile or a general base (34, 37, 46, 48, 52–55). To distinguish the potential roles of Glu-302 and Glu-315 in the catalytic pocket, a chemical rescue assay was conducted for the mutants. Sodium azide (3 M, buffered at pH 4.6) and sodium formate (3 M, buffered at pH 4.6) were used in the rescue assay. When pNP-Fuc (1 mM) was used as the substrate, in the presence of sodium azide (750 mM) and sodium formate (200 mM), the activity of E315A was enhanced 8- and 7-fold, respectively (Fig. 5); this range of recovery was consistent with the 2–10-fold level of activity rescue in previous reports (54). In contrast with the results on E315A, neither sodium azide nor sodium formate had a significant rescue effect on E302A, even at concentrations of 1.5 M. The results indicated that three acidic amino acids were involved in the catalytic...
Identification of a core fucosidase

Figure 5. Chemical rescue of the mutants (D242A, E302A, and E315A) of cFase I. The effect of varying concentrations of sodium azide (a) and sodium formate (b) on the rate of enzyme-catalyzed cleavage is shown. Reactions were performed at 37 °C, pH 4.6, with pNP-fucose as the substrate.

activity of cFase I, with Asp-242 and Glu-315 functioning as the nucleophile and acid/base residues that were reported in studies of other fucosidases; an additional acidic residue (Glu-302) is present, with an as yet undefined role.

Core fucosidase activity of cFase I on PLA2 and HRP

To test whether the enzyme was active against core-fucosylated glycoproteins, we subjected PLA2 (containing core α-1,3- and α-1,6-fucosylated N-glycans) to cFase I assays. The core α-1,3-fucosylated glycans on PLA2 were resistant to PNGase F digestion (Fig. 6, lane 3, CBB, Fig. 6a), whereas PNGase F-II can release N-glycans, including core α-1,3 and α-1,6-fucoses (lane 5, CBB, Fig. 6a). After cFase I treatment, the cFase I-treated PLA2 was sensitive to PNGase F digestion, indicating the removal of the core α-1,3-fucosylated glycans from PLA2 (lane 4, CBB, Fig. 6a). The signal in Western blotting (WB) experiments with a polyclonal antibody against core α-1,3-fucose/β-1,2-xylene on HRP disappeared after cFase I treatment both with and without PNGase F (lanes 2 and 4, WB, Fig. 6a), demonstrating core α-1,3-fucosidase activity of cFase I. The intensity of the AAL blot upon cFase I (lane 2, LB, Fig. 6a) and PNGase F (lane 3, LB, Fig. 6a) treatment minimally changed, whereas the signal almost disappeared with a sequential treatment of cFase I and PNGase F. The result indicated that cFase I functioned on core α-1,3-fucosylated glycans.

Mass spectrometry analysis was conducted to verify cFase I activity on PLA2 (Fig. 6c). PLA2 was first treated with or without cFase I; then, all the N-glycans were released by PNGase F-II separately. The N-glycan mass spectra pattern of cFase I-treated PLA2 showed several specific downshifts (compared with the PLA2/PNGase F-II control) that matched those expected for defucosylation (146 or 292 Da). Peaks at m/z 1631.50, 1485.46, 1225.36, 1079.30, 1063.33, and 917.26, which represented glycans that had been reported in PLA2 with core fucosylated glycans, were replaced by new peaks at m/z 1339.31. What is more, peaks at m/z 1225.36 and 1063.33, which corresponded to core α-1,3- and α-1,6-fucosylated glycans, disappeared, demonstrating that the existence of core α-1,6-fucoses did not affect the hydrolysis of core α-1,3-fucoses. The treatment had limited impact on signals at m/z 1079.30 and 917.26. This result confirmed that PLA2 N-glycans contained both α-1,3- and/or α-1,6-linked core fucoses and that cFase I was only active against core α-1,3-fucosylated N-glycans. Moreover, we observed that cFase I was inactive against core α-1,6-fucosylated IgG (Fig. S3). Our further study indicated that cFase I was fully active on free core α-1,3-fucosylated glycans released from HRP, whereas poor activity against 2-anthranilamide (2-AB)-labeled glycans was detected (Fig. S4).

The core α-1,3 defucosylation activity of cFase I was confirmed with HRP (Fig. 6, b and d). The Western blotting by anti-HRP and lectin blotting by AAL lectin before and after cFase I treatment have been conducted (Fig. 6b). The result showed that the treatment by cFase I had a minimal but clear impact on the signal intensity, indicating a core α-1,3-fucosidase activity of cFase I. We also conducted a mass spectrometry analysis to verify the removal of core fucose from HRP by cFase I treatment (Fig. 6d). Digesting HRP with cFase I caused a complete loss of all of its fucose-containing glycans (e.g. m/z 1414.40, 1211.32, 1079.38, and 1049.29). In addition, the formation of new bands or augmentation of existing bands that represented defucosylated glycans at m/z 1268.31 (Man$_n$GlcNAc$_m$Xyl$_l$), 1065.27 (Man$_n$GlcNAc$_m$Xyl$_l$), 933.28 (Man$_n$GlcNAc$_m$), and 903.27 (Man$_n$GlcNAc$_m$Xyl$_l$) occurred. All of these results demonstrated that cFase I had the capacity to hydrolyze the core α-1,3-fucose in HRP. Moreover, the relative activity of cFase I against HRP was about 3.0 and 0.24% compared with substrate 3-FL and Lewis X, respectively, as determined by ESI-MS analysis (data not shown).

Moreover, the assay that measured the core α-fucosidase activity of the mutants (D242A, E302A, E315A, and 249Δ265) revealed significantly reduced catalytic activity in the mutants (D242A, E302A, and E315A) against core-fucosylated glycoproteins; by contrast, the mutant 249Δ265 retained the wild-type level of core α-fucosidase activity against both HRP and PLA2 (Fig. 7). However, the other mutants found still retained the core α-fucosidase activity (data not shown).

As core α-1,3-fucose is a key part of the epitope recognized by IgE from insect and/or plant allergic individuals (24, 56). We tested the impact of cFase I treatment on IgE-mediated allergic-
Identification of a core fucosidase
Identification of a core fucosidase

Although α-fucosidases have been identified, an example with core α-1,3-fucosidase activity on glycoprotein is still missing. Eukaryotic fucosidases from humans and Fusarium graminearum as well as bacterially produced enzymes from B. thetaiotaomicron, Bifidobacterium bifidum, and other organisms have been proven to be active only against oligosaccharides or terminal fucose residues on glycoprotein (13, 14, 32, 35, 42, 60). Almond α-fucosidase is inactive on free oligosaccharide that is chemically released from HRP with a core-associated fucose (Fuca3-GlcNAc) (61, 62). Although certain types of Lewis fucoses have been removed with an α-fucosidase from Arabidopsis, the core α-1,3-fucoses were resistant to the treatment (62). Moreover, a rice α-fucosidase has been proved to hydrolyze both the α-1,4-fucosyl linkage in the Lewis-type of plant complex N-glycans and the α-1,3-fucosyl linkage in lacto-N-fucopentaose III but not α-1,3-fucosyl linkage in the core of plant N-glycans (63). Although it has been demonstrated that an α-fucosidase from Charonia lampas was active against a free oligosaccharide (Fuca3-GlcNAc) that had been chemically released from HRP, only high concentrations of the enzyme acted on the oligosaccharide, and it demonstrated no hydrolytic activity on oligosaccharides that were still conjugated to glycoproteins (61). It has been proven that NixE protein from plant pathogen Xanthomonas campestris pv. campestris has poor fucosidase activity on core-fucosylated glycoprotein pre-treated with NixK (α-1,3-mannosidase) and NixL (β-1,2-xylosidase) (64). In this study, we used a combination of assays (a core fucose-specific Western blotting and a fucose-specific lectin blotting) and a comparative MS analysis of free oligosaccharides that were released by PNGase F-I and sequential digestion by cFase I and PNGase F-II to demonstrate that cFase I can remove core α-1,3-fucose residues from HRP and PLA2, two well-known substrates with core fucosylation that are derived from plants and insects, respectively. Enzymatic characterization revealed that cFase I functioned well in acidic buffer conditions (pH 4–6) and was sensitive to Cu²⁺ ions and PCMB; these properties are consistent with reports of other α-fucosidases (42, 44). To the best of our knowledge, cFase I is the first enzyme shown to be capable of removing core α-1,3-fucose.

Our results also demonstrated that cFase I is more active on Lewis X than on 3-FL trisaccharide, suggesting a preference for GlcNAC/fucose linkages over Glc/fucose linkages. The N-acetyl (acetamido) group at the C2 position of the substrate may act as an intramolecular nucleophile to assist the attack on the anomeric carbon. This substrate-assisted mechanism has been found in several GH enzyme families (GH18, GH20, GH56, GH84, and GH85) (65). Recently, a report indicated that the α-fucosidase AfcB from B. bifidum also prefers the linkage on GlcNAc over that of Glc (42).

Discussion

In this study, we reported the molecular cloning, expression, and functional characterization of a novel core fucosidase (cFase I) from the E. meningoseptica genome. The bioinformatics analysis indicated that cFase I belonged to the GH29 family and possessed the domains and residues that were conserved for fucose binding and catalytic activity. Different types of enzymatic assays with substrates, including synthetic compounds, native oligosaccharides, and glycoproteins, clearly demonstrated that cFase I is a newly identified α-1,3-fucosidase that exhibits a unique and previously unreported activity against core α-1,3-fucoses on glycoproteins.

In summary, cFase I hydrolyzed core α-1,3-fucoses in glycoproteins but had no activity or limited activity against core α-1,6-fucosylated glycans. Therefore, the candidate fucosidase was named core fucosidase I or cFase I.

Figure 7. Core defucosylation of PLA2 and HRP by cFase I mutants. Enzyme-digested glycoprotein was subjected to CBB staining, and Western blot (WB) analysis with rabbit polyclonal anti-HRP antibodies. Control, only substrate; D242A, substrate treated with D242A mutant of cFase I; E302A, E315A, and 249A265, substrate treated with E302A, E315A, and 249A265 mutants, respectively. WT, substrate treated with wildtype cFase I.

Figure 6. Defining cFase I activity by MALDI-TOF and other analyses. Core defucosylation of PLA2 (a and c) and HRP (b and d) by cFase I is shown. Enzyme-digested glycoprotein was subjected to CBB staining (top), Western blot (WB) analysis with rabbit polyclonal anti-HRP antibodies (middle), and lectin blot (LB) analysis with biotinylated AAL (bottom). Control (lane 1), only substrate; cFase I (lane 2), substrate treated with purified cFase I; PNGase F (lane 3), substrate treated with PNGase F-II, used as a positive control. N-Glycan profile of PLA2 (c) and HRP (d) treated without (top) and with (bottom) cFase I, N-glycans of untreated or treated substrate were released by PNGase F-II. All the peaks were annotated with GlycoMod server and referenced to the previous study; all molecular ions are present in sodiated form ([M + Na]+). The peak of a potassium adduct ([M + K]+) was evident beside that of a sodiated form, with ~16 Da addition that was not labeled in the graph. The glycan structure of peak m/z 1485.46 was verified to contain an outside chain fucose by MS/MS.

Figure 8. Core fucosidase activity by cFase I mutants. Enzyme-digested glycoprotein was subjected to CBB staining (top), Western blot (WB) analysis with rabbit polyclonal anti-HRP antibodies (middle), and lectin blot (LB) analysis with biotinylated AAL (bottom). Control (lane 1), only substrate; cFase I (lane 2), substrate treated with purified cFase I; PNGase F (lane 3), substrate treated with PNGase F-II, used as a positive control. N-Glycan profile of PLA2 (c) and HRP (d) treated without (top) and with (bottom) cFase I, N-glycans of untreated or treated substrate were released by PNGase F-II. All the peaks were annotated with GlycoMod server and referenced to the previous study; all molecular ions are present in sodiated form ([M + Na]+). The peak of a potassium adduct ([M + K]+) was evident beside that of a sodiated form, with ~16 Da addition that was not labeled in the graph. The glycan structure of peak m/z 1485.46 was verified to contain an outside chain fucose by MS/MS.
Our results demonstrated that three acidic residues (Asp-242, Glu-302, and Glu-315) in the predicted catalytic pocket are essential for cFase I activity, which is similar to the case of the fucosidase from Sulfolobus solfataricus (SsR-fuc) (66). A structure-based sequence alignment indicated that Asp-242 is the nucleophile residue that is found in most fucosidases. A 100-fold decrease in $k_{cat}/K_m$ was observed toward substrate pNP-Fuc in the D242A mutant of cFase I, which is consistent with the behavior of the nucleophile mutant of TM0306 α-fucosidase (37). Activity of the D242A mutant against Lewis X was partially restored by sodium azide (data not shown), supporting the possible role of Asp-242 as a nucleophile. Interestingly, activity rescue was not observed with substrates pNP-Fuc and 3-FL. This result may due to a relatively low specificity of cFase I for pNP-Fuc (14 min$^{-1}$ mM$^{-1}$) and 3-FL (107 min$^{-1}$ mM$^{-1}$) compared with Lewis X (967 min$^{-1}$ mM$^{-1}$). The behavior of D242A was consistent with that in early reports of a glycosidase from S. solfataricus and arabinofuranosidase/xylosidase from Geobacillus stearothermophilus (67, 68).

Studies were conducted to define the roles of Glu-302 and Glu-315 in the catalytic reaction. The catalytic activity of E302A and E315A on all three tested substrates was significantly lower than that of the wildtype, indicating that both Glu residues were critical for enzymatic activity. It has been proven that the function lost in mutants of fucosidases and other glycosidases could be partially recovered by the addition of an external nucleophile (46, 48, 69). Our results demonstrated that the exogenous small ions azide and formate could partially recover the activity of the E315A mutant against all three substrates (data for 3-FL and Lewis X were not shown), indicating that Glu-315 may act as the classical acid/base residue that was established in the conventional model. By contrast, activity of the E302A mutant was not enhanced by the presence of either azide or formate, which indicated that Glu-302 was unlikely to be the classical acid/base catalytic residue in cFase I.

The bioinformatics analysis indicated that cFase I is a new member of the GH29 fucosidase family; the catalysis of most members in this family follows a double-displacement mechanism that is mediated by two carboxylic acid residues at the active site (34). Our results indicated that three acidic acids in the active pocket are essential for cFase I activity, with Asp-42/Glu-315 acting as a pair of nucleophile and acid/base residues, respectively, and Glu-302 as an essential and novel component of the catalytic process. In the first step (fucosylation), one carboxylic acid residue (Asp-242) serves as a nucleophile that attacks the aglycone center of the glycone, and the other carboxylate residue (Glu-315) simultaneously acts as an acidic catalyst that protonates the fucosidic oxygen to promote the departure of the aglycone oxygen, generating a covalent fucosyl-enzyme intermediate. In the second step (defucosylation), the general acid in the first step becomes a general base that assists the decomposition of the fucosyl-enzyme intermediate, proceeding through a general base-catalyzed attack of water at the anomic center to release the fucose. Early studies demonstrated that the rate-limiting steps for different substrates differ; for activated substrate pNP-Fuc, the rate-limiting step of hydrolysis is certainly the defucosylation of the fucosyl intermediate (the second step) because the 4-nitrophenol group is a good leaving group (pK$a$ = 7.18) and the fucosylation of the enzyme requires less assistance from the acid catalyst (34, 48). However, fucosylation (the first step) is the rate-limiting step in reactions with the substrates 3-FL and Lewis X, which form worse leaving groups (52). The fact that E302A was more active against pNP-Fuc than 3-FL and that the kinetic behavior of E302A was similar to that of D242A (Table 3) led us to speculate that Glu-302 is more likely to be involved in the first step. Unfortunately, the mechanism of the catalytic triad remains unclear because the 3D crystal structure of Ssα-fuc and cFase I is unsolved.

PCMB strongly inactivated the activity of cFase I. Moreover, the turnover number and $K_m$ value of cFase I for pNP-Fuc decreased 1144- and 3.6-fold, respectively (data not shown), indicating that the inhibitory effect of PCMB on cFase I is mainly on its hydrolysis process. However, C73S and C467S substitutions had no impact on cFase I activity. Both C317S and C317A mutants of cFase I displayed hydrolytic activity for three substrates, although the activity was reduced. These results indicated that Cys-73 and Cys-467 are not involved in the enzy-
Identification of a core fucosidase

matic activity, whereas Cys-317 has limited impact on cFase I. Together, we concluded that thiol groups on cFase I may not play a major role in the activity of cFase I, and the interaction of Cys-317 in the active pocket with PCMB may create a barrier to impede the acid/base function of Glu-315 in the hydrolytic process.

In conclusion, we identified a novel α-L-fucosidase that is active on core α-1,3-fucose on glycoproteins by using a simple and practical method based upon a comparative MS analysis of oligosaccharides that had been released by PNGase F-II digestion alone and a sequential digestion by cFase I and then PNGase F-II. The established method (cFase I/PNGase F-II versus PNGase F-II assays) could be used in the discovery and characterization of new glycosidases, and the reported enzyme cFase I is a potential tool that can be used to develop a deeper understanding of core fucosylation. In addition, our results indicated that three acidic acids in the predicted active pocket are essential for cFase I activity and may act through a dynamic nucleophile and acid/base process that deserves further characterization.

Experimental procedures

Bioinformatics analysis

The DNA sequence of the putative α-fucosidase gene was obtained from the clinical isolate of E. meningoseptica FMS-007 (renamed as Elizabethkingia anopheles FMS-007). Homologous sequences and the conserved domain of the putative α-fucosidase were identified using the Basic Local Alignment Search Tool (BLASTp) in the PDB and NCBI nonredundant sequence databases from the National Center for Biotechnology Information (NCBI) website. The signal peptide was predicted with SignalP version 4.1. Protein sequences for the other α-fucosidases used in the sequence alignment and phylogenetic tree analysis were obtained from UniprotKB. Phylogenetic tree was constructed using the neighbor-joining method in the Molecular Evolutionary Genetics Analysis (MEGA version 5.2) software. The .pdb files for proteins with known structures were downloaded from the Protein Data Bank. Structure-based multiple sequence alignments were performed with PROMALS3D (70), and the figure was produced using ESPript (71). The mass spectrometry was annotated by GlycanMod tool online from ExPASy.

Bacterial strains, media, chemicals, and human sera

E. meningoseptica FMS-007 was isolated from a patient with T-cell non-Hodgkin’s lymphoma. Luria-Bertani broth medium (5 ml, with 10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl, 37 °C) was routinely used for the cultivation of E. meningoseptica and Escherichia coli strains. All the p-nitrophenyl monosaccharides and fucosylated oligosaccharides used in this study, except 6-fucosyl-α-N,N-diacetylgalactosamine (GlcNAc1–4(Fuc1–6)GlcNAc, N2F) were purchased from Carbosynth (Compton, UK). N2F was purchased from Dextra Laboratories Ltd. (Reading, UK). Deoxyxucononjirimycin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Free fucose was assayed with the K-FUCOSE kit from Megazyme International (Dublin, Ireland). PLA2 from honey bee venom and peroxidase (HRP) from horseradish were obtained from Sigma. Biotin-conjugated Aleuria aurantia lectin (AAL) was purchased from Vector Laboratories; anti-HRP polyclonal antibody was purchased from LifeSpan BioSciences (LS-C153975). PNGase F was obtained from New England Biolabs; PNGase F-II was cloned and purified in our laboratory as described previously (40). All other reagents were purchased from Thermo Fisher Scientific or Sigma and were used without further purification. Sera were obtained from patients who visited the allergy clinic for inhalant allergies at the hospital of traditional Chinese and Western medicine of NanChang. After venous blood was drawn, serum was separated and stored at −80 °C until analysis.

Cloning and mutagenesis

The predicted cFase I gene on E. meningoseptica without signal peptide was amplified by PCR from genomic DNA using primers containing NheI and XhoI restriction sites, and the PCR product was subcloned into the pET28a expression vector (kanr; Novagen, Darmstadt, Germany) with N-terminal His6 tag fused as cFase I wildtype. All the cFase I mutants (D242A, E302A, E315A, C73S, C317A, C317S, C467S, and 249Δ265) were generated with the QuikChange mutagenesis kit (Stratagene) from the pET28a-cFase I (WT) plasmid by following the instructions of the manufacturer and were then verified by DNA sequencing. All the primers are listed in Table S1.

Protein expression and purification

The constructed cFase I expression plasmid was transformed into E. coli BL21(DE3), and the transformants were cultured at 37 °C for 16–18 h on kanamycin Luria-Bertani agar plate, and then single clones were cultured in Luria-Bertani medium containing 50 μg/ml kanamycin. This culture (5 ml) was inoculated into the same medium (500 ml) supplemented with kanamycin (50 μg/ml). Cells were grown in a shaker at 200 rpm (Innova 4000; New Brunswick Scientific, Edison, NJ) until they reached an A600 nm of 0.8 and were induced with 1 mM isopropyl-β-D-galactopyranoside for 12 h at 18 °C. The cells were harvested by centrifugation at 4000 × g (Beckman Coulter Allegra™ 25R centrifuge, TA-14-250 rotor) for 10 min, and the pellet was resuspended in a lysis buffer (20 mM sodium phosphate, 0.5 mM NaCl, 25 mM imidazole, pH 7.4). The suspended cells were disrupted using a high-pressure homogenizer (Shanghai LiTu Mechanical Equipment Engineering Co., Ltd., Shanghai, China). The debris was removed by centrifugation (nickel-Sepharose™ 6 Fast Flow column (GE Healthcare)). The target protein-loaded column was washed twice with a wash buffer (20 mM sodium phosphate, 0.5 mM NaCl, 25 mM imidazole, pH 7.4), and the candidate protein was eluted in a gradient elution buffer (20 mM sodium phosphate, 0.5 mM NaCl, 50–500 mM imidazole, pH 7.4). The purity of the protein was judged by SDS-PAGE. Fractions containing the enzyme of interest were dialyzed against cold PBS three times at 4 °C and concentrated using 30-kDa MWCO centrifugal filters (Amicon Ultra, Millipore). Fresh columns were used for each mutant to avoid cross-contamination. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce) with bovine serum albumin as a standard.

1254 J. Biol. Chem. (2018) 293(4) 1243–1258

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Enzymatic assay

All kinetic studies were performed in 20 mM sodium dihydrogen phosphate buffer if not otherwise stated. The pH for optimum α-fucosidase activity was determined by measuring the activity at pH values that were set from 3.0 to 11.0 with phosphate buffer (3.0–8.0) and glycine-NaOH buffer (9.0–11.0). Relative activity at different pH values was determined by incubating 1 μl (2 mg/ml) of α-fucosidase in a 60-μl reaction volume with 1 mM pNP-Fuc in 96-well microwell plates at 37 °C for 60 min. Reactions were stopped by adding a 1.5-fold reaction volumes of 1 mM Na2CO3, and absorbance at 405 nm was determined using a Tecan Infinite® M200 microplate reader. The optimum temperature for enzymatic activity was determined by incubating reactions with pNP-Fuc at 4, 20, 30, 37, 45, 55, 65, and 75 °C, and the relative activity was determined from A405 reads. The assay to test the effects of metal ions was performed under the assay conditions in 0.1 M acetate buffer, pH 4.6, at 37 °C. The enzyme was mixed with 1 mM final concentrations of different metal ions (Mg2+, Mn2+, Ca2+, Zn2+, Ni2+, Fe2+, Cu2+, and Cd2+) and 0.5 mM concentration of PCMB, with or without the presence of EDTA. To determine the values of kinetic constants, from 0.05 to 4 mM pNP-Fuc was coincubated with a constant amount of each enzyme at 37 °C. The time course for each reaction was previously determined to fulfill steady-state assumptions, and reaction rates were determined under initial conditions. The amount of pNP that was generated in each reaction was estimated using a standard curve. Nonlinear regression was used to determine K_m and V_max values by using the program GraphPad Prism software version 5.0 (GraphPad Software, San Diego). The enzyme activity in the hydrolysis of other β-nitrophenyl α/β-glycosides (pNP-β-1-Fuc, pNP-β-d-Fuc, pNP-β-d-Glc, pNP-α-d-Glc, pNP-α-d-Gal, pNP-β-d-Gal, pNP-β-d-Lac, pNP-β-d-Xyl, pNP-α-d-Xyl, pNP-α-d-Man, pNP-β-d-Man, pNP-β-d-GlcNac, pNP-α-d-GlcNac, pNP-α-d-GalNac, pNP-β-d-GalNac, and pNP-α-d-NeuNac) was measured in the same manner as mentioned above. All reactions were triplicated for statistical evaluations.

Enzyme activity against oligosaccharides

To a 0.2-ml PCR tube, 20 μl of reaction buffer (20 mM sodium phosphate, pH 4.6) was added, followed by 2 μl of a standard oligosaccharide sample (10 mM) and 1 μl of enzyme (2 mg/ml) solution. The reaction mixture was incubated at 37 °C in a water bath for 1 h. The amount of fucose that was released in the reaction mixture was quantified with a fucose kit according to the manual. Briefly, 10 μl of reaction mixture was incubated with buffer and NADP (both used as supplied) in a 96-well plate. When the reaction reached a constant absorbance at 340 nm (A340), 1-fucose dehydrogenase (as supplied) was added to the mixture, and the solution was incubated for ~10 min at 37 °C, followed by continuous measurement of the increase in A340 every 2 min until the absorbance no longer changed. The activity of the enzyme against each substrate was calculated using a standard curve.

ESI-MS, which was used to verify the hydrolysis activity against each substrate, was conducted on a TSQ Quantiva™ triple quadrupole mass spectrometer system (Thermo Fisher Scientific). A 10-μl reaction mixture sample was diluted in 200 μl of 18.2 megohms of ultrapure water and injected into a mass spectrometer at 20 μl/min with a microsyringe pump (pump 22; Harvard Apparatus). The instrument was operated in full scan mode, from m/z 100 to 600 for 1 min in positive mode.

Enzyme activity against glycoproteins

Type VI HRP (from horseradish), which has core α-1,3 fucosylation, and PLA2 (from bee venom), which has both core α-1,3 and core α-1,6 fucosylation, were chosen as substrate glycoproteins for the testing of enzyme activity against core-fucosylated glycoproteins. 100 μg of PLA2 or HRP was added to 400-μl of reaction buffer (20 mM sodium phosphate, pH 4.6) that contained 10 mM DTT, and then the glycoprotein was denatured in a 95 °C water bath for 15 min, followed by the addition of 5 μl (10 μg) of cFase I enzyme. The reaction was carried out at 37 °C for 36 h and then heated to denature the cFase I after digestion was accomplished, and the buffer (acetic acid, pH 4.5) of the sample was then changed by ultrafiltration with a membrane with a molecular weight cutoff (MWCO) of 3 kDa (Millipore, Bedford, MA) at 10,000 × g for 5 min three times. The sample remained in a minimal salt acidic buffer for subsequent PNGase F-II digestion and MS analysis. To accomplish this digestion, we added PNGase F-II to the treated PLA2 and HRP and incubated the samples at 37 °C for 12 h to release the glycans, and the released glycans were collected by ultrafiltration using a 3-kDa MWCO filter and dried with a centrifugal evaporator for MALDI-TOF analysis. MALDI-TOF MS experiments were carried out on an AB Sciex 5800 MALDI-TOF mass spectrometer (AB Sciex). The dried glycan was resuspended in 10 μl of saturated 2,5-dihydroxybenzoic acid, after which 1 μl of resuspended glycan solution was deposited on the MALDI plate using the dried droplet method. Profiling of the glycan was performed in reflector positive mode with the UV laser set at a 400-Hz repetition rate and a wavelength of 355 nm. The enzymatic activity against PLA2 and HRP was also analyzed by SDS-PAGE, Western blotting, and lectin blotting. The glycoprotein substrate was denatured as described above, and the solution was then separated equally into 0.2-ml PCR tubes. Either buffer or cFase I was added, and the samples were incubated at 37 °C for 36 h. The cFase I-treated sample was buffered to pH 8.0 for subsequent PNGase F digestion. Finally, the samples were analyzed by SDS-PAGE, Western blotting, and lectin blotting. The Western blotting experiments used rabbit anti-HRP polyclonal antibodies as the primary antibody, largely to detect the removal of core α-1,3 fucoside from the glycoprotein (56). The lectin blotting assay used biotin-conjugated AAL lectin to evaluate the defucosylation activity after α-fucosidase treatment.

Chemical rescue of enzyme activity

The chemical rescue of the activity of cFase I mutants (D242A, E302A, E315A, and 249D/H) was performed with sodium azide (0–1.5 M) and sodium formate (0–1.5 M). Sodium azide and sodium formate (3 M, both buffered to pH 4.6) were added at varying concentrations, and the rate of product released was monitored. Assays of the cFase I mutant enzymes
Identification of a core fucosidase

with pNP-Fuc was conducted as described above at a temperature of 37 °C. Assays with 3-FL and Lewis X as substrates were performed in a similar manner, with exogenous nucleophile concentrations in the range of 0–1.5 M.

The reaction time of chemical rescue assay was 60 min for WT and 249A265 mutant; 600–720 min for D242A, E302A, and E315A mutants. The reactions were terminated by freezing the reaction mixture with liquid nitrogen for 3-FL and Lewis X, and by mixing with 1.5-fold reaction volumes of 1 M sodium carbonate for pNP-Fuc.

### Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously (58, 59). Briefly, PLA2 or defucosylated PLA2 was dissolved in 50 mM carbonate buffer, pH 9.6, and coated (10 μg/ml) onto 96-well half-area microtiter plates (Costar Co., New York) overnight at 4 °C. Then, the plates were blocked with 1% BSA in PBS containing 0.05% Tween 20 for 2 h at room temperature. Diluted serum (1:3) in blocking buffer was allowed to react overnight at 4 °C. IgE was detected by incubation with biotinylated anti-human IgE antibody (BioLegend, 325504) for 1 h at room temperature, followed by incubation with streptavidin-conjugated AP (Thermo Fisher Scientific, 21324) for 1 h. After one more round of washing, staining of ELISA plates was performed by incubation with 50 μl/well of AP substrate (Sigma, P7998). The absorbance at 405 nm was read after 1 h. The assay was permitted by the Ethics Committee of the Hospital of Traditional Chinese and Western Medicine of NanChang.

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