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A glycosynthase derived from an inverting GH19 chitinase from the moss *Bryum coronatum*

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BcChi-A, a GH19 chitinase from the moss *Bryum coronatum*, is an endo-acting enzyme that hydrolyses the glycosidic bonds of chitin, $(GlcNAc)_n$ [a β-1,4-linked polysaccharide of GlcNAc (*N*-acetylglucosamine) with a polymerization degree of *n*], through an inverting mechanism. When the wild-type enzyme was incubated with α-$(GlcNAc)_2$-F [α-$(GlcNAc)_2$ fluoride] in the absence or presence of $(GlcNAc)_2$, $(GlcNAc)_2$ and hydrogen fluoride were found to be produced through the Hehre resynthesis–hydrolysis mechanism. To convert BcChi-A into a glycosynthase, we employed the strategy reported by Honda et al. [(2006) J. Biol. Chem. **281**, 1426–1431; (2008) Glycobiology **18**, 325–330] of mutating Ser^{102}, which holds a nucleophilic water molecule, and Glu^{70}, which acts as a catalytic base, producing S102A, S102C, S102D, S102G, S102H, S102T, E70G and E70Q. In all of the mutated enzymes, except S102T, hydrolytic activity towards $(GlcNAc)_6$ was not detected under the conditions we used. Among the inactive BcChi-A mutants, S102A, S102C, S102G and E70G were found to successfully synthesize $(GlcNAc)_4$ as a major product from α-$(GlcNAc)_2$-F in the presence of $(GlcNAc)_2$. The S102A mutant showed the greatest glycosynthase activity owing to its enhanced F^- releasing activity and its suppressed hydrolytic activity. This is the first report on a glycosynthase that employs amino sugar fluoride as a donor substrate.

Key words: chitin oligosaccharide, chitobiose phosphorylase, glycosynthase, α-*N*,*N*′-diacetylchitobiosyl fluoride [α-$(GlcNAc)_2$-F], site-directed mutagenesis.

INTRODUCTION

Chitin oligosaccharides $(GlcNAc)_n$ [a β-1,4-linked polysaccharide of GlcNAc (*N*-acetylglucosamine) with a polymerization degree of *n*] have been drawing attention because of their presumed biological functions, such as inhibition of tumour angiogenesis [1], control of cell growth, differentiation and development in vertebrates [2,3], their anti-oxidative effect [4], and the eliciting of defensive actions in plants [5,6]. However, in spite of these attractive properties, the molecular mechanisms underlying their biological activities remain to be clarified. In this context, it is highly desirable to obtain well-defined chitin oligosaccharides in large quantities. Chitin oligosaccharides have been prepared by enzymatic degradation [7] or acid hydrolysis of chitin followed by purification with an appropriate separation system [8,9]. In both cases, the glycosidic bonds in the chitin chain are cleaved randomly, producing a mixture of chitin oligosaccharides with a wide variety of chain lengths. This leads to a laborious purification procedure. Procedures for synthesizing chitin oligosaccharides are also laborious, because multiple protection, coupling and deprotection reactions are required to control the regio- and stereoselectivity in glycosidic bond formation [10].

Chitinases (EC 3.2.1.14) that catalyse the hydrolysis of chitin are widely distributed in living organisms. The enzymes have been classified into two families, GH (glycoside hydrolase) family 18 (GH18) and 19 (GH19), on the basis of the amino acid sequence of their catalytic modules (http://www.cazy.org/) [11,12]. Anomeric forms of the enzymatic products are retained in the reaction catalysed by GH18 chitinases, but inverted in the reaction by GH19 chitinases. Several GH18 chitinases exhibit significant transglycosylation activity [13,14], that can be utilized for synthesizing a new glycosidic bond [15]. However, yields are usually poor, because of the subsequent break down of the transglycosylation products. Thus mutations of the enzyme proteins have been performed with the aim of increasing the yields. Mutation of aspartic acids of the catalytic triad (DXDXE) in GH18 chitinases was found to successfully enhance the transglycosylation reaction [16,17]. In recent years, glycosynthase-catalysed synthesis has become one of the most attractive strategies for synthesizing the glycosides. The first glycosynthase was reported by Mackenzie et al. [18], who converted a retaining β-glucosidase from *Agrobacterium* sp. into a glycosynthase by introducing a mutation in its catalytic nucleophile (Glu^{358}). Thereafter, a number of retaining GHs were converted into glycosynthases by introducing a mutation in their catalytic nucleophiles [19]. The glycosynthase technique was developed by mimicking the Hehre resynthesis–hydrolysis mechanism [20], in which an inverting GH acts towards the glycosyl fluoride possessing a 'wrong' anomer resulting in a net release of fluorine atom through the two steps: (i) synthesis of a new glycoside with an acceptor molecule in a 'correct' anomer and (ii) hydrolysis of the glycoside formed in the first step. Nevertheless, most of the glycosynthases reported until recently were derived from retaining GHs. In 2006, Honda and Kitaoka [21] reported the conversion of an inverting glycoside hydrolase, Rex (reducing-end xylose-releasing exo-oligoxylanase) from *Bacillus halodurans* C-125, into a glyosynthase. Two mutants, in which the general base Asp^{263} was substituted with cysteine

Abbreviations used: ChBP, chitobiose phosphorylase; 2,5-DHB, 2,5-dihydroxy benzoic acid; GH, glycoside hydrolase; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_n$, a β-1,4-linked polysaccharide of GlcNAc with a polymerization degree of *n*; α-GlcNAc-F, α-GlcNAc fluoride; α-$(GlcNAc)_2$-F, α-$(GlcNAc)_2$ fluoride; La^{3+}-ALC, lanthanum/alizarin complexon; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; Rex, reducing-end xylose-releasing exo-oligoxylanase.

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and asparagine, were found to accumulate significant amounts of xylotriose from α-xylobiosyl fluoride and xylose. This was the first report of a glycosynthase derived from an inverting GH, suggesting the availability of inverting GHs for synthesis. Tyr[198] holding the nucleophilic water molecule at the catalytic site was also found to be a mutation target for creating a glycosynthase from Rex [22].

Taira et al. [23] isolated and characterized BcChi-A, a GH19 chitinase from the moss *Bryum coronatum*. Because of its high thermal stability (T_m, up to 70 °C), small molecular size (22 kDa) and relatively high yield of recombinant protein in *Escherichia coli* (100 mg/l of culture), BcChi-A appears to be an appropriate enzyme for basic and applied research, including the creation of a first glycosynthase from the GH19 scaffold. Although various retaining and inverting GHs have been converted into glycosynthases, there have been no reports on glycosynthases derived from enzymes employing the amino sugar fluoride as a donor substrate. We report in the present study the successful conversion of a GH19 chitinase into a glycosynthase by introducing mutations into a serine residue holding a nucleophilic water molecule.

MATERIALS AND METHODS

Chemicals

$(GlcNAc)_n$ ($n = 1$–6) were purchased from Seikagaku Biobusiness. All other reagents were of analytical grade available commercially.

SDS/PAGE and protein measurement

SDS/PAGE was carried out using the method of Laemmli [24] using a 15 % acrylamide gel. Proteins on the gel were stained with Coomassie Brilliant Blue R250. Protein concentrations were determined by reading absorbance at 280 nm, using a molar absorption coefficient of BcChi-A obtained from the equation proposed by Pace et al. [25].

Homology modelling

SWISS-MODEL, a knowledge-based protein modelling tool [26], was employed to construct the tertiary structure of BcChi-A using the known X-ray structure of chitinase G from *Streptomyces coelicolor* A3(2) (PDB code 2CJL) [27] as a template. The modelled structure of BcChi-A was used to select mutation targets for converting the enzyme into a glycosynthase.

Protein expression and purification

Site-directed mutagenesis was performed according to Wang and Malcolm [28] using the QuikChange® site-directed mutagenesis kit (Stratagene). The BcChi-A mutants and the corresponding oligonucleotide primers used in the present study are listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/444/bj4440437add.htm). The wild-type and mutated BcChi-A were successfully produced and purified by methods described previously [23].

Chemoenzymatic synthesis of α-GlcNAc-F (α-GlcNAc fluoride) and α-$(GlcNAc)_2$-F [α-$(GlcNAc)_2$ fluoride]

α-GlcNAc-F was synthesized by the reaction of tetra-*O*-acetyl GlcNAc with pyrydinium poly(hydrogen fluoride), followed by

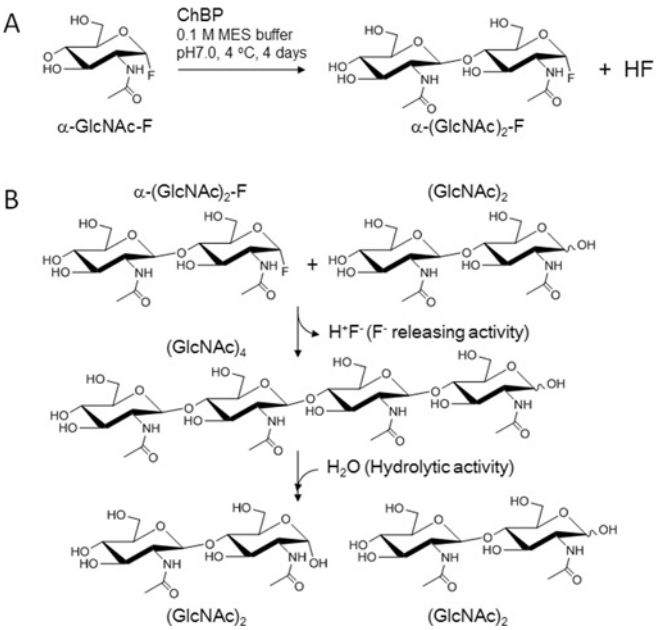

Figure 1 Reaction scheme of the enzymatic reactions used

(**A**) Direct synthesis of α-$(GlcNAc)_2$-F from α-GlcNAc-F by means of ChBP. (**B**) Hehre resynthesis–hydrolysis reaction catalysed by BcChi-A.

O-deacetylation using sodium methoxide in methanol according to a standard procedure [29,30]. To synthesize α-$(GlcNAc)_2$-F, we employed the synthetic reaction catalysed by ChBP (chitobiose phosphorylase) from *Vibrio proteolyticus* [31]. Since α-GlcNAc-F can act as both donor and acceptor substrates for ChBP [32], the direct synthesis of α-$(GlcNAc)_2$-F from α-GlcNAc-F was conducted using ChBP as a catalyst, as shown in Figure 1(A). The enzyme was added to a solution of α-GlcNAc-F in 1 ml of 0.1 M Mes buffer (pH 7.0). After 4 days at 4 °C, the products were concentrated and separated using silica gel chromatography with 4:1 acetonitrile/methanol as the eluent to obtain pure α-$(GlcNAc)_2$-F. NMR spectra were determined using a JEOL ECX-400P spectrometer operating at 298 K at a ^{13}C frequency of 100.53 MHz and a ^{19}F frequency of 376.17 MHz. The NMR data for synthesized α-$(GlcNAc)_2$-F were: ^{13}C-NMR ([$^2H_4$]methanol); δ 174.07 (C = O), 107.22 (d, $J_{C1,F}$ 222.3 Hz), 103.08 (C1′), 80.25, 78.02, 75.59, 74.35, 71.81, 70.32, 65.88, 62.42, 60.96, 57.21, 54.66, 53.75, 23.06 (acetamide), 22.48 (acetamide) and ^{19}F-NMR ([$^2H_4$]methanol); δ −147.89 (dd, $J_{F,H2}$ 27.6 Hz, $J_{F,H1}$ 55.1 Hz).

Reaction of the BcChi-A enzymes towards α-GlcNAc-F and α-$(GlcNAc)_2$-F

The reaction mixture (10 μl), consisting of 4 μM wild-type BcChi-A and 25 mM donor substrate [α-GlcNAc-F or α-$(GlcNAc)_2$-F], was incubated in the absence or presence of 25 mM acceptor substrate [GlcNAc or $(GlcNAc)_2$] in 0.1 M Mops buffer (pH 7.0) at 37 °C for 3 h. The reaction products were analysed by TLC using a silica gel 60 aluminium sheet (Merck). The plate was developed in a solvent system of butan-1-ol/methanol/28.8 % ammonium hydroxide (5:4:2, by vol.), and the carbohydrates were visualized by heating the plate after dipping it in vanillin/H_2SO_4 reagent. The glycosynthase activity of the BcChi-A mutants was examined by incubating the reaction mixture (10 μl) containing 4 μM enzyme and 25 mM α-$(GlcNAc)_2$-F (donor) in the absence or presence of $(GlcNAc)_2$

(acceptor) in 0.1 M Mops buffer (pH 7.0) for 3 h at 37 °C. The reaction products were analysed by TLC as described above. To analyse the reaction products with HPLC, the donor and acceptor concentrations were increased to 0.1 M respectively. After 30, 60, 90, 120 and 180 min, the enzymatic reaction was terminated by adding an equal volume of 0.1 M NaOH solution, and the mixture was applied to a gel-filtration column of TSK-GEL G2000PW (Tosoh). Elution was carried out with distilled water at a flow rate of 0.3 ml/min. Oligosaccharides were detected by ultraviolet absorption at 220 nm.

F^--releasing activity

The F^--releasing activity was assayed colorimetrically using a La^{3+}-ALC (lanthanum/alizarin complexon) solution using the method of Shoda et al. [33]. The enzymatic F^- release reaction was performed in a reaction mixture consisting of 4 μM enzyme (wild-type, S102A, S102C or S102G), 25 mM α-$(GlcNAc)_2$-F and 25 mM $(GlcNAc)_2$ in 0.1 M Mops buffer (pH 7.0) at 37 °C, as shown in Figure 1(B). After incubation for 180 min, 2 ml of 0.5 % La^{3+}-ALC solution containing 50 % (v/v) acetone was added to the mixture (10 μl) and incubated for 90 min at 25 °C. The amount of F^- liberated from α-$(GlcNAc)_2$-F (the first reaction step in Figure 1B) was quantified by measuring the absorbance at 620 nm using the standard curve obtained with authentic sodium fluoride solutions.

Hydrolytic activity of BcChi-A and its mutants towards $(GlcNAc)_6$

The specific activity of wild-type BcChi-A and its mutants was determined using the $(GlcNAc)_6$ substrate. The enzymatic reaction was performed in 50 mM sodium acetate buffer (pH 5.0) at 40 °C. The enzyme and substrate concentrations were 0.2 μM and 4.75 mM respectively. At a given reaction time, the enzymatic reaction was terminated by adding an equal volume of 0.1 M NaOH solution, and the mixture was applied to a gel-filtration column of TSK-GEL G2000PW (Tosoh) as described above. $(GlcNAc)_n$ was detected by ultraviolet absorption at 220 nm. The peak area obtained for $(GlcNAc)_6$ was converted into a molar concentration, which was then plotted against the reaction time. Specific activity was determined from the initial velocity of the $(GlcNAc)_6$ degradation.

MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) of the glycosynthase reaction products

Products obtained from the glycosynthase reaction were identified by MALDI–TOF-MS. The reaction mixture, consisting of 4 μM BcChi-A S102A, 25 mM α-$(GlcNAc)_2$-F and $(GlcNAc)_2$ in 0.1 M Mops buffer (pH 7.0) was incubated for 3 h at 37 °C. A portion (1 μl) of the reaction mixture was mixed with an equal volume of 2,5-DHB (2,5-dihydroxy benzoic acid) [20 mg/ml in acetonitrile/water, 80:20 (v:v)]. After the addition of 0.1 μl of 0.1 % trifluoroacetic acid, the mixture was placed on to a plate in a MALDI micro MX (Waters), and then dried. Mass spectra were obtained in positive-ion reflection mode. $(GlcNAc)_n$ ($n = 1$–6) were used as standard *m*/*z*.

RESULTS

Synthesis of α-$(GlcNAc)_2$-F

Various glycosyl fluorides have been generally synthesized from O-acetate sugars with pyrydinium poly(hydrogen fluoride), followed by O-deacetylation using sodium methoxide or ammonium gas in methanol [20,21,32,34,35]. We first tried to synthesize α-$(GlcNAc)_2$-F by this standard procedure. However, the yield of α-$(GlcNAc)_2$-F was very low. On the other hand, ChBP catalyses reversible phosphorolysis of $(GlcNAc)_2$, producing GlcNAc 1-phosphate and GlcNAc. For the synthetic (reverse) reaction, ChBP employs not only GlcNAc 1-phosphate, but also α-GlcNAc-F as a donor substrate. In addition, α-GlcNAc-F was found to act as both a donor and an acceptor substrate in ChBP-catalysed synthesis [32]. Thus, in the present study, a chemoenzymatic method without protection of the hydroxy group was employed to obtain α-$(GlcNAc)_2$-F directly from α-GlcNAc-F using the synthetic reaction catalysed by ChBP [31] (Figure 1A). After incubation of α-GlcNAc-F (33 mg, 0.15 mmol) with ChBP (23 nmol), the products were separated by silica gel chromatography to obtain pure α-$(GlcNAc)_2$-F (13 mg, 0.03 mmol). Chemical shifts and coupling constants obtained from the NMR spectra of the product were consistent with the structure of α-$(GlcNAc)_2$-F. The yield of α-$(GlcNAc)_2$-F was 20 %.

Reactivity of the wild-type BcChi-A towards α-GlcNAc-F and α-$(GlcNAc)_2$-F

Prior to converting BcChi-A into a glycosynthase, we examined whether the enzyme catalyses the Hehre resynthesis–hydrolysis reaction. The results are shown in Figure 2(A). The wild-type BcChi-A was incubated with α-GlcNAc-F (lane 3), α-GlcNAc-F and GlcNAc (lane 4), or α-GlcNAc-F and $(GlcNAc)_2$ (lane 5). The enzyme was also incubated with α-$(GlcNAc)_2$-F (lane 6), α-$(GlcNAc)_2$-F and GlcNAc (lane 7), or α-$(GlcNAc)_2$-F and $(GlcNAc)_2$ (lane 8). No matter whether the acceptor substrate [GlcNAc or $(GlcNAc)_2$] was present or not in the reaction mixture, α-GlcNAc-F was not consumed even after 3 h of incubation, as was observed from the intensity of the corresponding TLC spots (lanes 3, 4 and 5). In contrast, α-$(GlcNAc)_2$-F was consumed by the enzyme in all cases (lanes 6, 7 and 8). The spots corresponding to α-$(GlcNAc)_2$-F disappeared and $(GlcNAc)_2$ became visible (lanes 6 and 7). In lane 8, the spots of α-$(GlcNAc)_2$-F disappeared, and the TLC spot corresponding to $(GlcNAc)_2$ became more intensive than the spots in lanes 6 and 7. This could be explained by the reaction scheme shown in Figure 1(B); that is, the acceptor $(GlcNAc)_2$ was linked to the donor α-$(GlcNAc)_2$-F, producing hydrogen fluoride and $(GlcNAc)_4$, which was hydrolysed again into $(GlcNAc)_2$. It was assumed that the amount of $(GlcNAc)_2$ in the reaction mixture was doubled by the formation of $(GlcNAc)_2$ from α-$(GlcNAc)_2$-F. It appears that the wild-type BcChi-A catalyses the Hehre resynthesis–hydrolysis reaction. However, at this point, the simple hydrolysis of α-$(GlcNAc)_2$-F by the wild-type BcChi-A could not be ruled out.

Production and hydrolytic activity of the BcChi-A mutants

To convert BcChi-A into a glycosynthase, we tried to find the mutation targets by closely examining the modelled structure of BcChi-A obtained from the crystal structure of a GH19 chitinase G from *S. coelicolor* A3(2) (PDB code 2CJL) as a template [27]. In Figure 3, the modelled structure of BcChi-A (light green) was superimposed on to the crystal structure of chitinase G (cyan) by the aid of the Pair Fitting wizard of the molecular visualization program PyMOL (http://www.pymol.org). From the close-up view of the catalytic cleft, we found that the side chains of Glu^{68} (catalytic acid) and Glu^{77} (catalytic base) in chitinase G fully correspond to those of Glu^{61} and Glu^{70} in BcChi-A. A

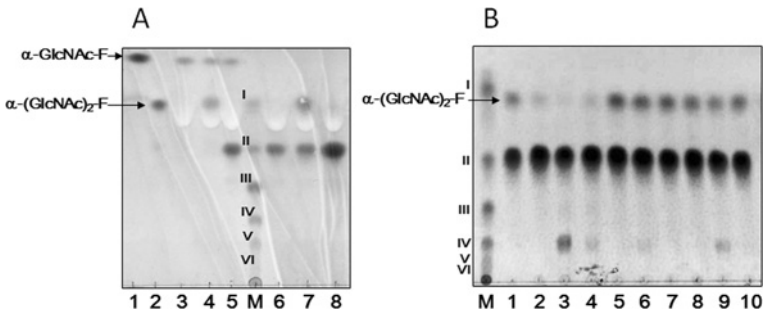

Figure 2 Action of BcChi-A towards α-GlcNAc-F and α-$(GlcNAc)_2$-F in the absence or presence of $(GlcNAc)_n$ ($n = 1$ or 2)

(**A**) Wild-type BcChi-A. The reaction was conducted by incubating a reaction mixture (10 μl) consisting of 4 μM wild-type BcChi-A and 25 mM donor substrate [α-GlcNAc-F or α-$(GlcNAc)_2$-F] in the absence or presence of 25 mM acceptor substrate [GlcNAc or $(GlcNAc)_2$] in 0.1 M Mops buffer (pH 7.0) at 37 °C for 3 h. The reaction products were analysed by TLC. Lanes 1 and 2, α-GlcNAc-F and α-$(GlcNAc)_2$-F respectively, incubated without the enzyme; and lanes 3, 4 and 5, α-GlcNAc-F, α-GlcNAc-F and GlcNAc, and α-GlcNAc-F and $(GlcNAc)_2$ respectively, incubated with the enzyme; lanes 6, 7 and 8, α-$(GlcNAc)_2$-F, α-$(GlcNAc)_2$-F and GlcNAc, and α-$(GlcNAc)_2$-F and $(GlcNAc)_2$ respectively incubated with the enzyme. M is the standard for $(GlcNAc)_n$ ($n = 1$–6). Roman numerals in the profile indicate the degrees of polymerization of $(GlcNAc)_n$. (**B**) Mutant BcChi-A. The reaction was conducted by incubating the reaction mixture (10 μl) containing 4 μM enzyme and 25 mM α-$(GlcNAc)_2$-F (donor) in the absence or presence of $(GlcNAc)_2$ (acceptor) in 0.1 M Mops buffer (pH 7.0) for 3 h at 37 °C. Lane 1, without enzyme; lane 2, wild-type BcChi-A; lane 3, S102A; lane 4, S102C; lane 5, S102D; lane 6, S102G; lane 7, S102H; lane 8, S102T; lane 9, E70G; and lane 10, E70Q. Roman numerals in the profile indicate the degrees of polymerization of $(GlcNAc)_n$.

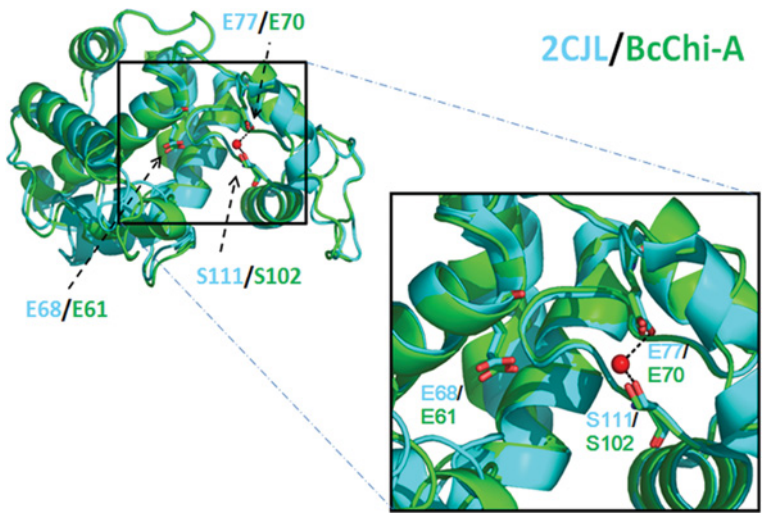

Figure 3 Superimposition of the modelled structure of BcChi-A and the template structure chitinase G from *S. coelicolor* A3(2) (PDB code 2CJL)

The side chains of Glu^{68}, Glu^{77} and Ser^{111} of chitinase G and the corresponding residues of Glu^{61}, Glu^{70} and Ser^{102} of BcChi-A are shown in stick representation. The water molecule that is co-ordinated with Glu^{77} and Ser^{111} by hydrogen bonding (broken lines) is represented as a red sphere. Glu^{70} and Ser^{102} were the mutation targets for creating a glycosynthase.

water molecule represented by a red sphere is co-ordinated with Glu^{77} and Ser^{111} in chitinase G by hydrogen bonding (broken line), and Ser^{111} of chitinase G corresponds to Ser^{102} of BcChi-A. Thus in BcChi-A Glu^{70} and Ser^{102} probably act as the catalytic base and hold a nucleophilic water molecule in the catalytic cleft respectively, and appear to correspond to Asp^{263} and Tyr^{198} respectively of Rex. Therefore we mutated Glu^{70} and Ser^{102} to obtain eight mutated enzymes (Supplementary Table S1). All mutants, E70G, E70Q, S102A, S102C, S102D, S102G, S102H and S102T, were expressed in amounts equivalent to that of the wild-type and purified to homogeneity (Supplementary Figure S1 at http://www.BiochemJ.org/bj/444/bj4440437add.htm). In all of the mutated enzymes, except S102T, hydrolytic activity towards $(GlcNAc)_6$ was not detected under the conditions we employed. A very low residual activity might have been undetected in this assay condition. Only S102T exhibited a significant enzymatic activity, 43 % of that of the wild-type BcChi-A, indicating an importance of the hydroxy group at the 102nd position probably in forming a hydrogen bond with a nucleophilic water molecule. Glu^{70} and Ser^{102} were found to be critically important for the hydrolytic activity, and the mutations of these residues might be an appropriate strategy to suppress the hydrolytic activity, leading to accumulation of the synthetic product shown in the middle of Figure 1(B).

Glycosynthase activity of the BcChi-A mutants

Since α-GlcNAc-F was not consumed by the wild-type BcChi-A (Figure 2A), the synthetic ability of the BcChi-A mutants was tested with only α-$(GlcNAc)_2$-F as the donor substrate in the presence of $(GlcNAc)_2$ as the acceptor substrate. As seen in the TLC profile in Figure 2(B), the spot corresponding to α-$(GlcNAc)_2$-F disappeared, and the spot corresponding to $(GlcNAc)_4$ appeared in lanes 3, 4, 6 and 9. This indicates that the S102A, S102C, S102G and E70G mutants exhibited glycosynthase activity and that the $(GlcNAc)_2$ production from α-$(GlcNAc)_2$-F observed in Figure 2(A) is resulting from the Hehre resynthesis–hydrolysis reaction catalysed by the wild-type BcChi-A. In lane 2, only the spot of α-$(GlcNAc)_2$-F became faint, indicating consumption of the substrate by the wild-type.

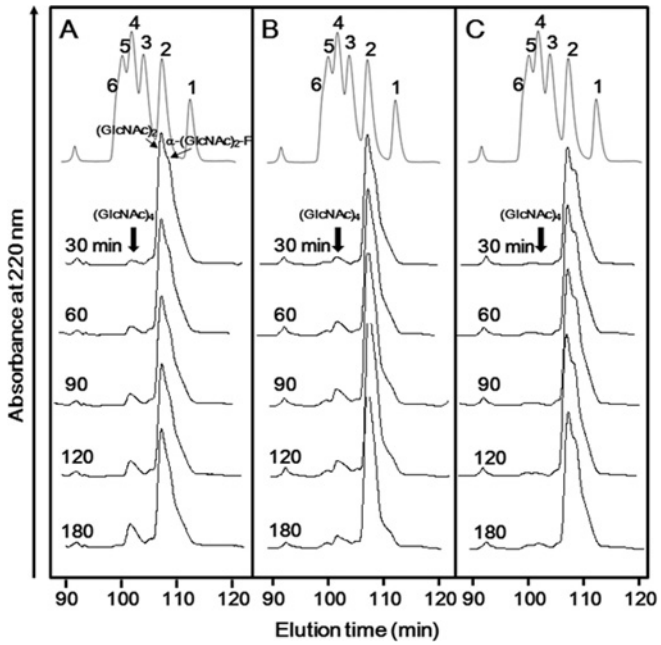

Figure 4 Time-dependent HPLC profiles showing the glycosynthase reaction catalysed by the BcChi-A Ser^{102} mutants

The reaction was conducted by incubating the reaction mixture, containing 4 μM enzyme and 0.1 M α-$(GlcNAc)_2$-F (donor) in the presence of 0.1 M $(GlcNAc)_2$ (acceptor) in 0.1 M Mops buffer (pH 7.0) at 37 °C. Aliquots (12 μl) of the reaction mixture were withdrawn at given sampling times and analysed by HPLC using a column of G2000PW (Tosoh). Elution was conducted with distilled water at a flow rate of 0.3 ml/min. S102A (**A**); S102C (**B**); and S102G (**C**). Individual peaks were identified using the authentic solutions of $(GlcNAc)_n$ ($n = 1$–6) and α-$(GlcNAc)_2$-F as the standards.

In other lanes (5, 7, 8 and 10), the spots of α-$(GlcNAc)_2$-F remained intact and no products were detected, indicating no glycosynthase activity for S102D, S102H, S102T and E70Q. The spot of $(GlcNAc)_4$ in lane 3 was the most intensive, indicating that S102A possesses the highest glycosynthase activity among the mutants tested. Further analysis was not conducted for E70G, because Honda et al. [22] proposed that the mutation of a residue holding a nucleophilic water molecule is more practical than that of a catalytic base for creating a glycosynthase from inverting GHs. Only the Ser^{102} mutants were examined in further detail.

Time course of the glycosynthase reaction monitored by HPLC

The time course of the glycosynthase reaction catalysed by the S102A, S102C and S102G mutants was monitored by HPLC (Figure 4). In an early stage of the reaction, a single major fraction of $(GlcNAc)_2$ combined with a minor fraction of α-$(GlcNAc)_2$-F appeared at the elution time of 108–109 min. The profile of the S102A mutant shows that with the progress of the enzymatic reaction α-$(GlcNAc)_2$-F gradually decreased, but $(GlcNAc)_4$ was accumulated (Figure 4A). The S102C mutant synthesized $(GlcNAc)_4$, like the S102A mutant, up to 90 min, but thereafter the peak area for $(GlcNAc)_4$ gradually decreased (Figure 4B), suggesting that the S102C mutant still retained a low residual hydrolytic activity and the synthetic product $(GlcNAc)_4$ was cleaved into $(GlcNAc)_2$ eventually. The S102G mutant yielded a much smaller amount of $(GlcNAc)_4$ than the S102A mutant, indicating the lowest glycosynthase activity of this mutant (Figure 4C).

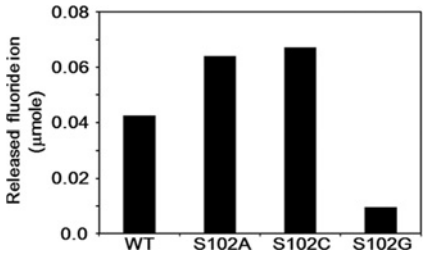

Figure 5 F$^-$-releasing activity of the wild-type BcChi-A and the Ser^{102} mutants

The reaction was conducted in a mixture consisting of 4 μM enzyme (wild-type, S102A, S102C or S102G), 25 mM α-$(GlcNAc)_2$-F and 25 mM $(GlcNAc)_2$ in 0.1 M Mops buffer (pH 7.0) at 37 °C. After 180 min of incubation, the amount of F^- liberated from α-$(GlcNAc)_2$-F was quantified by the method described in the Materials and methods section.

F$^-$-releasing activity of BcChi-A and the mutants possessing glycosynthase activity

The amounts of F^- released from α-$(GlcNAc)_2$-F by the wild-type BcChi-A, S102A, S102C and S102G, were determined using La^{3+}-ALC. As shown in Figure 5, the amount of F^- released by the S102A and S102C mutants was approximately 1.5-fold larger than that released by the wild-type, indicating that the first reaction step, shown in Figure 1(B), was enhanced by these mutations. In contrast, the F^--releasing activity was considerably suppressed in the S102G mutant.

MALDI–TOF-MS of the glycosynthase reaction products

MALDI–TOF-MS was used to analyse the products obtained by the glycosynthase reaction of the S102A mutant, using α-$(GlcNAc)_2$-F as a donor substrate and $(GlcNAc)_2$ as an acceptor substrate. After a 3-h reaction with the S102A mutant, the mass spectra of the products showed three distinct mass signals at m/z values of 446.8, 853.4 and 1259.7, corresponding to the values of sodium adduct ions of $(GlcNAc)_2$, $(GlcNAc)_4$ and $(GlcNAc)_6$ respectively (Figure 6B).

DISCUSSION

For synthesizing β-glycosides by the glycosynthase reaction, α-glycosyl fluorides possessing a fluoride in the opposite anomeric configuration to that of the normal substrates have been used as the donor substrate [18,20,21]. Therefore as the first step of the present study we synthesized α-GlcNAc-F using a standard chemical procedure and then synthesized α-$(GlcNAc)_2$-F enzymatically from the chemically synthesized α-GlcNAc-F using ChBP (Figure 1A). This chemoenzymatic method was successful and appears to be a versatile tool in the synthesis of various derivatives of glycosides of $(GlcNAc)_2$.

Prior to the glycosynthase reaction of the mutated BcChi-A enzymes, we examined the reactivity of the wild-type enzyme towards α-GlcNAc-F and α-$(GlcNAc)_2$-F in the absence or presence of the acceptor substrate, GlcNAc or $(GlcNAc)_2$, by TLC (Figure 2A). α-GlcNAc-F was not consumed by the enzyme at all, regardless of the presence of the acceptor substrate (Figure 2A, lanes 3, 4 and 5). Previously, BcChi-A was shown to hydrolyse chitin oligosaccharides having a degree of polymerization larger than 3 [23,36]. Therefore α-GlcNAc-F was assumed to be too short to be consumed by the enzyme. The Hehre resynthesis–hydrolysis reaction shown in Figure 1(A) only took place when α-$(GlcNAc)_2$-F was employed as a donor substrate. α-$(GlcNAc)_2$-F was consumed by the enzyme in all cases

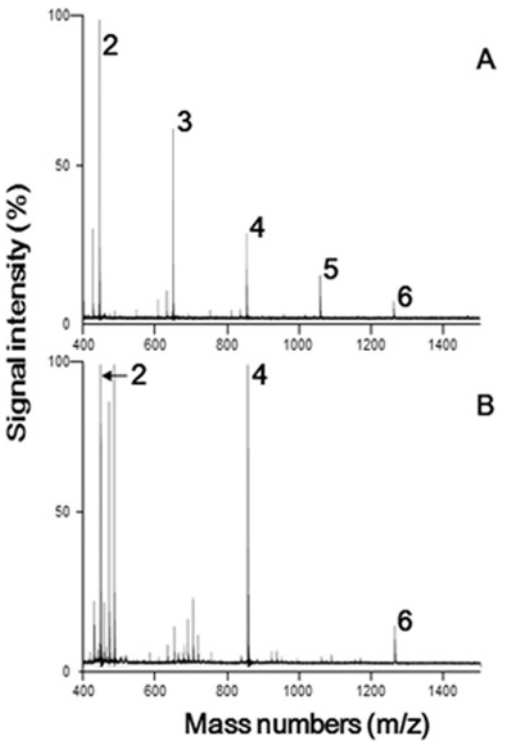

Figure 6 MALDI–TOF-MS of the glycosynthase reaction products

(**A**) Standard $(GlcNAc)_{2–6}$ in 0.1 M Mops buffer (pH 7.0). (**B**) Reaction products. The reaction mixture, consisting of 4 μM BcChi-A S102A, 25 mM α-$(GlcNAc)_2$-F and $(GlcNAc)_2$ in 0.1 M Mops buffer (pH 7.0), was incubated for 3 h at 37°C. The standard and the reaction mixture were mixed with 2,5-DHB on target, and then dried. The numerals 2–6 indicate $(GlcNAc)_{2–6}$.

(Figure 2A, lanes 6, 7 and 8), indicating that α-$(GlcNAc)_2$-F can be both a donor and an acceptor substrate in the enzymatic reaction.

Referring to the strategy proposed by Honda et al. [21,22], we mutated Glu^{70} and Ser^{102} of BcChi-A corresponding to Asp^{263} and Tyr^{198} of Rex respectively. As shown in Figure 2(B), a TLC spot corresponding to $(GlcNAc)_4$ was observed in lanes 3, 4, 6 and 9, indicating that S102A, S102C, S102G and E70G exhibited glycosynthase activity, producing $(GlcNAc)_4$ from α-$(GlcNAc)_2$-F and $(GlcNAc)_2$. This is the first report on a glycosynthase that employs amino sugar fluoride as a donor substrate. Judging from the intensity of the spots corresponding to the product $(GlcNAc)_4$, the glycosynthase activities of the Ser^{102} mutants decreased in the following order: S102A>S102C>S102G. Honda and Kitaoka [21] proposed two mechanistic requirements for creating a glycosynthase from inverting GHs: one is to enhance F^--releasing activity (the first step in Figure 1B), and the other is to suppress hydrolytic activity (the second step in Figure 1B). The F^--releasing activity was enhanced by the S102A and S102C mutations, whereas the activity was suppressed in S102G (Figure 5). Thus the glycosynthase activity of the S102 mutants might be mainly controlled by the F^--releasing activity. However, the glycosynthase activity of S102A was significantly higher than that of S102C, although the F^--releasing activities are almost the same between these two mutants. The difference in glycosynthase activity between S102A and S102C might be derived from the difference in residual hydrolytic activity, which could not be evaluated under the conditions for activity determination. In fact, the synthesized $(GlcNAc)_4$ was hydrolysed again by S102C, but not by S102A, as shown in Figure 4. In S102G, a small amount of $(GlcNAc)_4$ was accumulated (Figure 2B, lane 6), although the F^--releasing activity was suppressed as compared with the wild-type enzyme. This might be due to a complete inactivity of S102G for chitin oligosaccharide hydrolysis. Considering both the enhanced F^--releasing activity and the residual hydrolytic activity, S102A was regarded as the best glycosynthase among the mutants we tested. X-ray crystallography of BcChi-A and its mutants is now in progress to specify the structural factors controlling the glycosynthase activity.

MALDI–TOF-MS of the products formed by the S102A mutant showed three mass signals corresponding to the values of sodium adduct ions of $(GlcNAc)_2$, $(GlcNAc)_4$ and $(GlcNAc)_6$ (Figure 6). This result also revealed that BcChi-A was successfully converted into a glycosynthase, forming $(GlcNAc)_4$ from α-$(GlcNAc)_2$-F and $(GlcNAc)_2$. It should be noted that a mass signal for $(GlcNAc)_6$ was also detected in the reaction products. This might be interpreted by the glycosynthetic cycles; that is, $(GlcNAc)_4$ formed by 'the first cycle' was consumed as the acceptor together with α-$(GlcNAc)_2$-F as the donor in 'the second cycle', producing a small but significant amount of $(GlcNAc)_6$.

Enzymatic synthesis of novel sugar compounds was conducted by means of transglycosylation of retaining GH18 chitinases [37,38]. Enhancement of the transglycosylation activity was also conducted by site-directed mutagenesis [16,17] and by controlling the effective water concentration [39]. Chemoenzymatic synthesis of chitin oligosaccharides by the combined use of an activated donor substrate, sugar oxazoline and a deactivated GH18 chitinase from *Bacillus circulans* WL-12 was also reported by Kohri et al. [40]. Our first glycosynthase derived from a GH19 chitinase expands the methodology for the enzymatic synthesis of chitin oligosaccharides and could be useful for synthesizing new sugar derivatives containing a GlcNAc moiety.

AUTHOR CONTRIBUTION

Takayuki Ohnuma, Yuji Honda and Tamo Fukamizo designed the study; Takayuki Ohnuma, Tatsuya Fukuda and Satoshi Dozen performed the experiments; Takayuki Ohnuma, Yuji Honda, Motomitsu Kitaoka and Tamo Fukamizo analysed the data; and Takayuki Ohnuma, Yuji Honda and Tamo Fukamizo wrote the paper.

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SUPPLEMENTARY ONLINE DATA

A glycosynthase derived from an inverting GH19 chitinase from the moss *Bryum coronatum*

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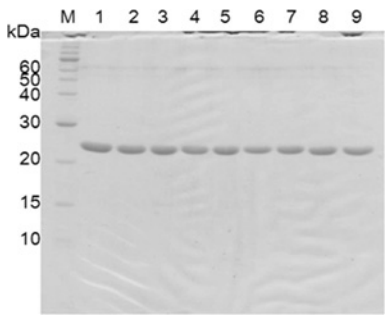

Figure S1 SDS/PAGE of BcChi-A and its mutants

The samples were subjected to SDS/PAGE and protein bands were stained with Coomassie Brilliant Blue R-250. M, Marker; lane 1, BcChi-A; lane 2, S102A; lane 3, S102C; lane 4, S102D; lane 5, S102G; lane 6, S102H; lane 7, S102T; lane 8, E70G; and lane 9, E70Q. Molecular mass is given in kDa on the left-hand side.

Table S1 BcChi-A mutants and the sequences of their oligonucleotide primers

The mutation sites are underlined.

Mutant	Oligonucleotide primer (5′→3′)
S102A	CGTGGTCCAATCCAACTCGCATGGAACTACAACTATGGCG
S102C	CGTGGTCCAATCCAACTCTGCTGGAACTACAACTATGGCG
S102D	CGTGGTCCAATCCAACTCGACTGGAACTACAACTATGGCG
S102G	CGTGGTCCAATCCAACTCGGCTGGAACTACAACTATGGCG
S102H	CGTGGTCCAATCCAACTCCACTGGAACTACAACTATGGCG
S102T	CGTGGTCCAATCCAACTCACATGGAACTACAACTATGGCG
E70G	GGGTTGCAGTTTATCCAAGGGCAAAACCCACAGAGTGATT
E70Q	GGGTTGCAGTTTATCCAACAGCAAAACCCACAGAGTGATT

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