Streptococcus pneumoniae endohexosaminidase D; feasibility of using *N*-glycan oxazoline donors for synthetic glycosylation of a GlcNAc-asparagine acceptor†‡

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Endohexosaminidase D, a family 85 glycoside hydrolase from *S. pneumoniae* and the first endohexosaminidase to be discovered, is found to be capable of catalysing the glycosylation of a glycosyl amino acid bearing a GlcNAc residue using a variety of *N*-glycan oxazoline donors. Although enzyme-catalysed oxazoline hydrolysis is a significant competing reaction that is not countered by the addition of organic co-solvents or variation of reaction pH, a high yielding synthetic process can be achieved by the sequential addition of multiple equivalents of oxazoline donor, demonstrating the synthetic potential of this enzyme as a biocatalyst for the synthesis of defined glycoconjugates. Notably Endo-D does not appear to hydrolyse the resulting products under the conditions used. The synthetic activity displayed by Endo D implies that other, as yet untested, family GH85 enzymes may display similar synthetic potential. Furthermore since Endo D is capable of cleaving *N*-glycans attached to monoclonal antibodies (mAbs), and also of cleaving glycans that are core-fucosylated, the development of Endo D as a useful biocatalyst for the synthesis of important defined homogeneous complex glycoconjugates may have significant future potential, provided that the limitation of direct oxazoline hydrolysis can be surmounted.

Introduction

Endo-β-*N*-acetylglucosaminidases[1] (endohexosaminidases, or ENGases) are a class of enzyme that specifically cleave the chitobiose core [GlcNAcβ(1–4)GlcNAc] of *N*-linked glycans between the two *N*-acetyl glucosamine residues (Fig. 1). The hydrolytic activity of endohexosaminidase D (Endo D), observed in the culture fluid of *Streptococcus pneumoniae* and reported by Muramatsu in 1971, was in fact the first endohexosaminidase activity to be disclosed.[2] Subsequently, more than 15 other endohexosaminidases have been identified, and several of these enzymes have now been cloned and expressed. According to the CAZy[3] enzyme classification system all of the endohexosaminidases identified to date belong to either family 18 (GH18) or family 85 (GH85) of the superfamily of glycoside hydrolases.

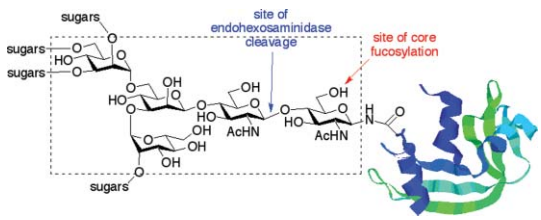

Fig. 1 Structure of the core region of *N*-glycans showing the sites of endohexosaminidase cleavage, core fucosylation and decoration with additional sugars that leads to the typical high mannose, hybrid and complex structures.

Endohexosaminidases, such as endohexosaminidase H (Endo H,[4] a member of family GH18) from *Streptomyces plicatus* (*griseus*)[5] have been long-standing tools used to remove the mixture of heterogeneous *N*-glycans from glycoproteins, either to facilitate protein handling and analysis, or to allow separation of the released oligosaccharides for glycan profiling. However, recently there has been resurgence in interest in endohexosaminidases as potent biocatalytic tools for the chemoenzymatic synthesis of defined homogeneous glycoconjugates, including glycoproteins.

The natural heterogeneity of the glycan portions of glycoproteins produced intracellularly, compounded with the inseparability of these mixtures of protein 'glycoforms', has meant that access to pure homogeneous single glycoforms of glycoproteins has become a major scientific objective.[6] Such access would not only facilitate more precise biological investigation into the effects that different glycan structures have on protein properties, but is also an important commercial goal in the field of glycoprotein therapeutics, since these glycoproteins are currently approved and marketed as heterogeneous mixtures that can be difficult to reproducibly generate. Furthermore the emergence during the past decade of monoclonal antibodies (mAbs) as probably the most important class of new biotherapeutic agent has added considerable

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financial impetus to the development of technologies that permit the production of defined glycoproteins in homogenous form. For example, human IgG antibodies contain *N*-linked glycans at the two Asn-297 residues of the dimeric Fc (constant fragment) region, and extensive work has revealed that the precise identity of the glycans at these sites is key for modulating antibody-mediated responses such as antibody dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[7] Moreover, the efficacy of mAb-based therapies has also been demonstrated to be dependent on polymorphism of the FcR receptor of natural killer (NK) cells. Optimal tuning of mAb glycosylation patterns to take account of such polymorphisms represents a significant opportunity to increase the efficacy of mAb therapies for individuals for whom current therapies fail. Therefore it is not surprising that considerable effort has recently been expended in the attempted optimisation of the glycosylation patterns of mAbs. For example, the Biotech companies GlycoFi[8] and GlyCart[9] have focussed on cell engineering approaches to produce expression systems that can be used to produce mAbs with more 'humanised' glycosylation patterns, although these approaches give no guarantee as to complete glycoprotein homogeneity since they rely on cell biosynthesis.

An alternative and attractive approach that may allow access to a wide range of glycoproteins bearing homogenous defined glycan structures, including mAbs, is that of *in vitro* glycoprotein remodelling. The remodelling approach involves the initial trimming back of the oligosaccharide chains of naturally produced heterogeneous mixtures of glycoforms to single GlcNAc residues using endohexosaminidases; a process that is routinely performed with enzymes such as Endo H. In a second, and considerably more challenging step, defined oligosaccharides can then be added to these 'GlcNAc handles', using enzymatic catalysis. Two endohexosaminidases have so far shown useful *synthetic* glycosylation activity in this latter respect; Endo M[10] from *Mucor Hiemalis* and Endo A[11] from *Arthrobacter protophormiae*, both of which are members of the family GH85. Early demonstrations of the synthetic potential of these two enzymes relied upon the use of Asn-linked oligosaccharides to act as the glycosyl donors. Since the enzymes naturally operate hydrolytically, competitive product hydrolysis was, not surprisingly, found to compromise achievable product yield; transglycosylations using these 'unactivated donors' were therefore quite inefficient. However the use of carbohydrate oxazolines as donors for family GH85 endohexosaminidase-catalysed glycosylation of acceptors bearing GlcNAc residues, first demonstrated by Shoda and co-workers,[12] has since led to substantial increases in synthetic efficiency. Indeed, subsequently both ourselves[13] and Wang[14] and co-workers have published extensively on the application and further development of both Endo A and Endo M as biocatalysts for the synthesis of a variety of defined glycoconjugates bearing *N*-glycans. Recently acquired crystallographic data for Endo A[15] and its E173Q mutant[16] will greatly facilitate future enzyme engineering in the pursuit of refining these enzymes as efficient biocatalysts.

When considering the future potential applications of endohexosaminidase-mediated glycosylation reactions the therapeutic importance of mAbs highlights these proteins as key substrates for methodological development and application. Indeed such remodelling of a human IgG1-Fc fragment has already been demonstrated by Wang[14i] who used Endo A to catalyze the addition of a $Man_3GlcNAc$ tetrasaccharide using the corresponding oxazoline donor, though almost 100 equivalents of this material were required in order to drive this reaction to completion. In a similar vein, the potential use of Endo D as a selective synthetic biocatalyst, using oxazolines as donors, would appear to be a potentially fruitful line of study for several reasons. Firstly, Endo D, in consort with a panel of other glycoside hydrolases produced by *S. pneumoniae*, naturally hydrolyses complex glycans attached to human IgGs; it may therefore prove to be a particularly suitable biocatalyst for the remodelling of mAbs. Secondly, Endo D is a family GH85 enzyme that is capable of hydrolysing glycans that contain a core fucose, *i.e.* which are fucosylated at the 6-position of the innermost GlcNAc residue. Notably Endo A[11] and Endo M[10b] are not capable of hydrolysing glycans that are fucosylated in this fashion. Therefore the development of Endo D as a synthetic biocatalyst may also allow access to defined glycoconjugates that contain a core fucose. This report therefore details preliminary investigations into the potential use of Endo D to catalyse the glycosylation of acceptors bearing GlcNAc residues using *N*-glycan oxazolines as activated glycosyl donors.

Results and discussion

Following the initial reports of hydrolytic activity, Endo D was subsequently isolated from *S. pneumoniae* by Muramatsu, purified,[17] and its specific hydrolytic activity was also investigated with respect to substrate structure. Much later it was cloned and expressed,[18] and truncation and mutational studies revealed residues in the enzyme active site critical for catalytic activity.[19] Sequence analysis placed Endo D in family GH85, alongside Endo A and Endo M, the two enzymes that have already demonstrated useful synthetic activity with oxazoline donors. Furthermore very recently the crystal structure of the catalytic domain of Endo D (SpGH85)[20] was solved providing key structural insight into the catalytic site and some insight into the oligosaccharide binding motifs of the enzyme. It is envisaged that this structural information will prove extremely valuable for fine-tuning and optimisation of any synthetic activity displayed by this enzyme by future protein engineering.

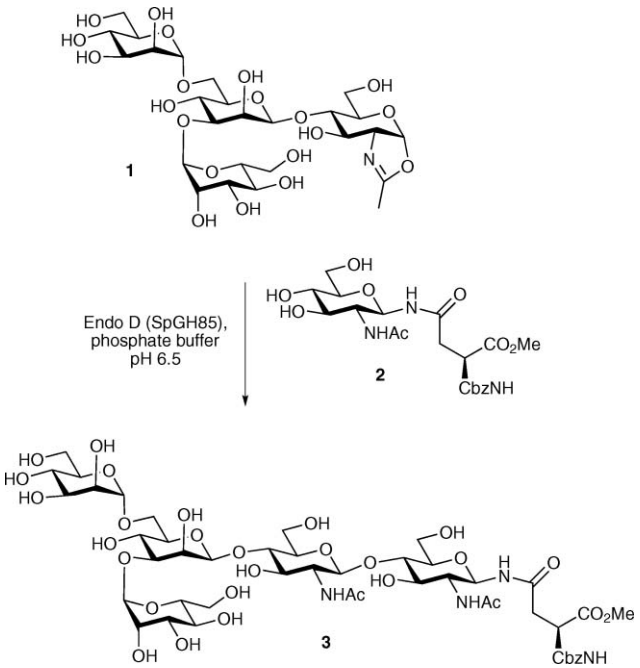
Endo D naturally cleaves complex *N*-glycans, acting in collaboration with neuraminidase, β-galactosidase and *exo*-β-*N*-acetylglucosaminidase enzymes produced by *S. pneumoniae*. Following the sequential action of these other enzymes, Endo D itself cleaves either the core *N*-glycan pentasaccharide ($Man_3GlcNAc_2Asn$, Fig. 1 structure shown within dotted line) or the more extended $Man_5GlcNAc_2Asn$ heptasaccharide structure, either of which may be core fucosylated at the terminal GlcNAc. In particular Endo D requires[17b,17e,21] an exposed 2-hydroxyl group in the α-mannose residue which is linked by an α(1–3) linkage to the inner mannose of the core structure and so, for example, Endo D will not cleave high mannose glycans. It may be expected therefore that Endo D would have a strict tolerance for which donor oxazolines may be processed. Studies to clarify the scope of which donor oxazolines are processed by Endo D were therefore undertaken as described below.

Variation of reaction parameters

The tetrasaccharide oxazoline donor **1** was chosen as a model substrate for initial studies since it corresponds to the core

mannose structure pentasaccharide that is a known substrate for the hydrolytic action of Endo D. The glycosyl amino acid **2**[13a] was used as the model glycosyl acceptor. A truncated version of Endo D, termed SpGH85§ comprised of amino acids 159-807 of the complete protein and which contains the catalytic domain, was used as the catalyst. SpGH85, which has previously been demonstrated to posses all of the catalytic activity of the full-length Endo D, was cloned, expressed and purified as previously described.[20] A series of glycosylation experiments were undertaken in which reaction parameters were systematically varied. The synthetic efficiency of each reaction catalysed by Endo D was assessed by HPLC monitoring of the conversion of acceptor **2** into the corresponding glycosylated product **3** (Scheme 1). Time course studies of these reactions are shown in Fig. 2.

Firstly, although a definition of unit activity for full-length Endo D already existed,[22] this assay was not readily amenable since it required access to ^{14}C labelled acetyl $Man_3GlcNAc_2Asn$. Therefore, an initial study focussed on determining the approximate quantity of enzyme required to efficiently promote any glycosylation reaction. Reactions were conducted in phosphate buffer (0.1 M) at pH 6.5, and the total reaction volume was kept constant at 50 μL. The amount of Endo D used to catalyse the reaction was varied between 0.0116 mg mL^{-1} and 1.16 mg mL^{-1} (corresponding crudely to 0.155–15.5 mU μL^{-1})¶, as shown in Fig. 2A. The use of 1.16 mg mL^{-1} of Endo D produced pentasaccharide **3** in 30% yield after approximately 30 min. This yield remained constant even after extended reaction times, implying either that, whilst the enzyme was capable of processing the tetrasaccharide oxazoline **1** it was incapable of hydrolysing the product **3** at an appreciable



Scheme 1 Endo D catalysed glycosylation of GlcNAc-Asn acceptor **2** with tetrasaccharide oxazoline donor **1**.

§ The terms Endo D and SpGH85 are used interchangeably after this point.
¶ This crude estimation is made on the basis of the assumptions of a similar level of enzyme purity and Muramatsu's 13.4 units mg^{-1} quantification. See ref. 17*d* and 22.

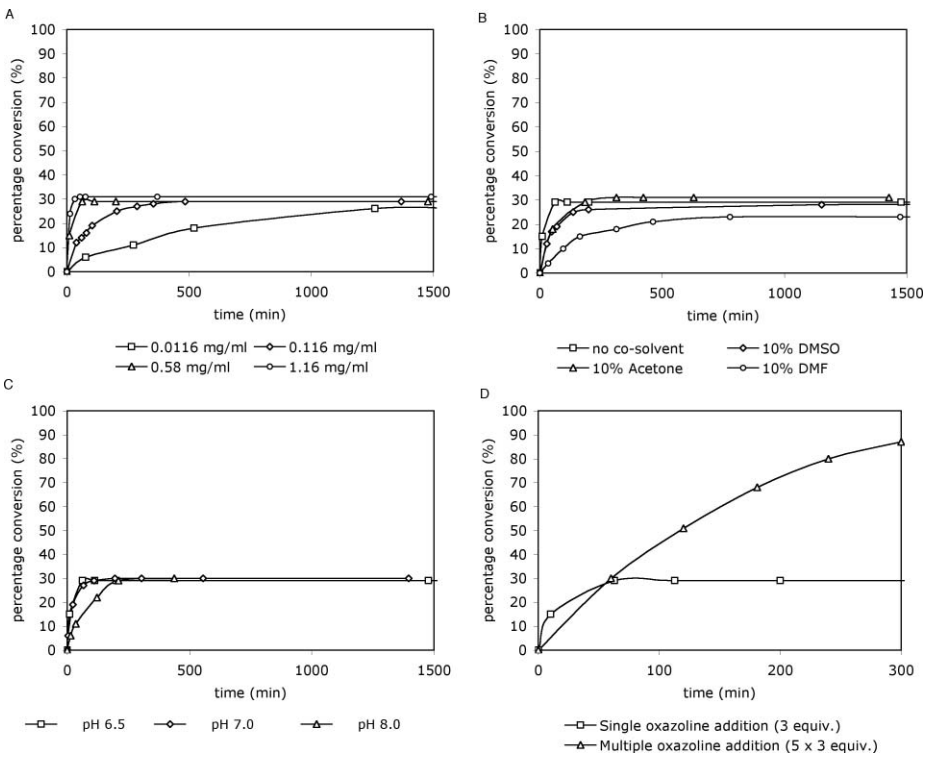


Fig. 2 Time-course studies of glycosylation of acceptor **2** with oxazoline **1** catalysed by truncated Endo D (SpGH85) to produce pentasaccharide glycosyl amino acid **3**. (A) Variation of Endo D concentration; (B) effect of the addition of various organic co-solvents; (C) effect of reaction pH; (D) increased conversion to product by multiple additions of oxazoline donor.

rate, or alternatively, that the enzyme was losing activity over time. Decreasing the concentration of enzyme by a factor of two (to 0.58 mg mL^{-1}) only led to a slight decrease in both the product yield (29%) and also the rate by which it was formed; approximately 1 h was required to achieve this maximum yield. A subsequent decrease in the enzyme concentration by a further factor of five to 0.116 mg mL^{-1} led to a significant decrease in the rate of glycosylation; this reaction took approximately 8 h to produce **3** in 29% yield. Decreasing the amount of enzyme by a further factor of ten to 0.0116 mg mL^{-1} led to a slight reduction in the maximum observable yield of **3** (26%), and to a further increase in the time required to achieve this (approximately 20 h). Since the initial two-fold decrease in the quantity of enzyme from 1.16 to 0.58 mg mL^{-1} had led to minimal differences in both the rate and yield of reaction, it was deemed most appropriate and convenient to use a concentration of 0.58 mg mL^{-1} of Endo D as the standard quantity of enzyme employed in subsequent investigations.

As mentioned above, the maximum yield of the glycosylation process using a single addition of 3 equivalents of oxazoline donor was a rather modest 30%. This compared somewhat unfavourably with results obtained using either Endo A or Endo M under similar conditions; Endo A afforded **3** in a maximum yield of 88% after 30 min, after which point product hydrolysis led to a progressive decrease in the efficiency of the reaction, whilst Endo M afforded **3** in a maximum yield of 41% after 3 h, though again after this time, product hydrolysis led to a progressive decrease in the efficiency of the reaction. The observation that the enzyme was still active at 20 h, coupled with the absence of any time-dependent decrease in product formation, strongly suggests that incomplete conversion to product was due to competitive enzyme-catalysed hydrolysis of the oxazoline donor **1**, rather than to product hydrolysis. These data reveal a significant difference between the activities of these different family GH85 enzymes.

Recent investigations into the synthetic utility of Endo M and Endo A have shown that both a variation in reaction pH and the addition of an organic co-solvent to the reaction medium may have a beneficial effect on the efficiency of the synthetic glycosylation reaction.[23] A brief survey of these two reaction parameters was therefore carried out to determine whether the modest yield of the Endo D catalysed reaction could be improved by effecting a decrease in the rate of direct oxazoline hydrolysis relative to that of the desired synthetic reaction.

Firstly the use of three different organic co-solvents was investigated: DMSO, DMF and acetone (Fig. 2B). The addition of 10% DMSO led to a decrease in the rate of the reaction, but did not alter the overall yield of **3** (28% yield after around 5 h). In contrast, the addition of 10% DMF had a more detrimental effect on the reaction; the reaction rate was reduced considerably such that the maximum product yield was not achieved until after 12 h, and the maximum observable yield was also reduced to 23%. Finally, the addition of 10% acetone to the reaction mixture again led to a decrease in the rate of the reaction, but actually led to a very slight increase in the observed yield of **3** (31%, reached after 5 h), although this minor increase is within the bounds of experimental error. Once again, in all three of these instances no product hydrolysis was observed during extended reaction times. However, since no significant increase in product yield was achieved, it appears that the addition of organic co-solvent has no particularly beneficial effect on the yield of glycosylations catalysed by Endo D.

Variation of reaction pH was also investigated (Fig. 2C). Endo D has been shown to have a pH optimum of 6.5 for hydrolysis, with half maximal values of 5.3 and 8.2.[17a] A series of reactions were conducted at pH 6.5, pH 7.0 and pH 8.0 using a phosphate buffer (0.1 M). However as can be seen from Figure 3C, increasing the reaction pH had no beneficial effect on the yield of the glycosylation reaction; rather an increase in pH merely caused a slight decrease in the rate of the reaction.

It was concluded that unfortunately oxazoline hydrolysis catalyzed by Endo D (SpGH85) competes significantly with glycosylation and is unavoidable under the conditions tested here. To investigate if this limit could be overcome and push the conversion of acceptor **2** to product **3** towards completion the addition of further equivalents of the oxazoline donor **1** after particular time intervals was attempted. This multiple addition procedure worked satisfactorily as shown in Fig. 2D. Herein, the addition of 3 further equivalents of oxazoline donor **1** every hour for 4 h (*i.e.* a total of 15 equivalents of donor) allowed production of **3** in 87% yield. Although such a procedure was rather inefficient with respect to the quantity of oxazoline donor required, it did demonstrate that reactions catalysed by Endo D may be driven to near completion, ruling out "product" inhibition and also supporting the view that competitive oxazoline hydrolysis was the major factor which limited synthetic efficiency. Studies with donor **1** were concluded by investigating if de-glycosylated ribonuclease B (dRNase B), which has previously been used as a model protein acceptor for enzymatic glycosylations using both Endo A and Endo M, could be glycosylated with Endo D. However, no appreciable amount of glycosylation of dRNase B was observed using the previously applied conditions, even when a large excess of donor **1** was used. The precise reasons for the lack of glycosylation activity in this instance are as yet unclear, particularly as both Endo A and Endo M are capable of glycosylating dRNase B with oxazoline donor **1**. We have established that Endo D itself does not catalyse hydrolysis of commercially available ribonuclease B (RNase B), but presumably this lack of hydrolytic activity is primarily due to the fact that RNase B is itself decorated with high mannose glycans that Endo D cannot cleave, rather than to any innate inability of Endo D to hydrolyse glycans that are attached to RNase B. It is therefore most probable that the lack of synthetic activity so far observed with dRNase B is because direct competitive oxazoline hydrolysis is a much more rapid process than glycosylation of the GlcNAc residue attached to the protein under the conditions investigated to date. Further kinetic investigations will be required in order to confirm this fact, and more importantly to develop a reaction process in which useful synthetic activity can be observed.

Variation of oxazoline donors

As mentioned previously, Endo D has been reported to possess quite strict substrate requirements for hydrolytic activity towards Asn-linked *N*-glycans. It may therefore have been expected that the synthetic activity of Endo D could also be quite limited with respect to the oxazoline structures it was able to process. The caveat to this presumption is that the ability of an endohexosaminidase to catalyse hydrolysis of, or glycosylation of an acceptor with, an *N*-glycan oxazoline donor does not necessarily correlate with its corresponding hydrolytic activity towards the product of that synthetic reaction. Such variation may stem from the

consideration that the normal glycoside bond cleavage reaction catalyzed by Endo D is a two-step process involving the transient formation of a relatively high-energy oxazoline intermediate and its subsequent hydrolysis. The hydrolysis of a high-energy oxazoline intermediate, however, is only the second half of the overall process for glycoside bond cleavage. Therefore, it is quite possible that Endo D would be capable of hydrolysing an oxazoline intermediate, since this could be a fast step in the normal glycoside bond cleavage reaction, and yet be unable to form the same oxazoline from the glycoside substrate, since formation of the oxazoline could be the slow rate determining step. Given this uncertainty, the flexibility of the Endo D with respect to oxazoline substrates it is able to process was investigated by assaying a variety of alternative oxazoline donors as synthetic substrates using the standard reaction conditions detailed previously (pH 6.5, no co-solvent, single addition of 3 equivalents of oxazoline donor, Scheme 2).

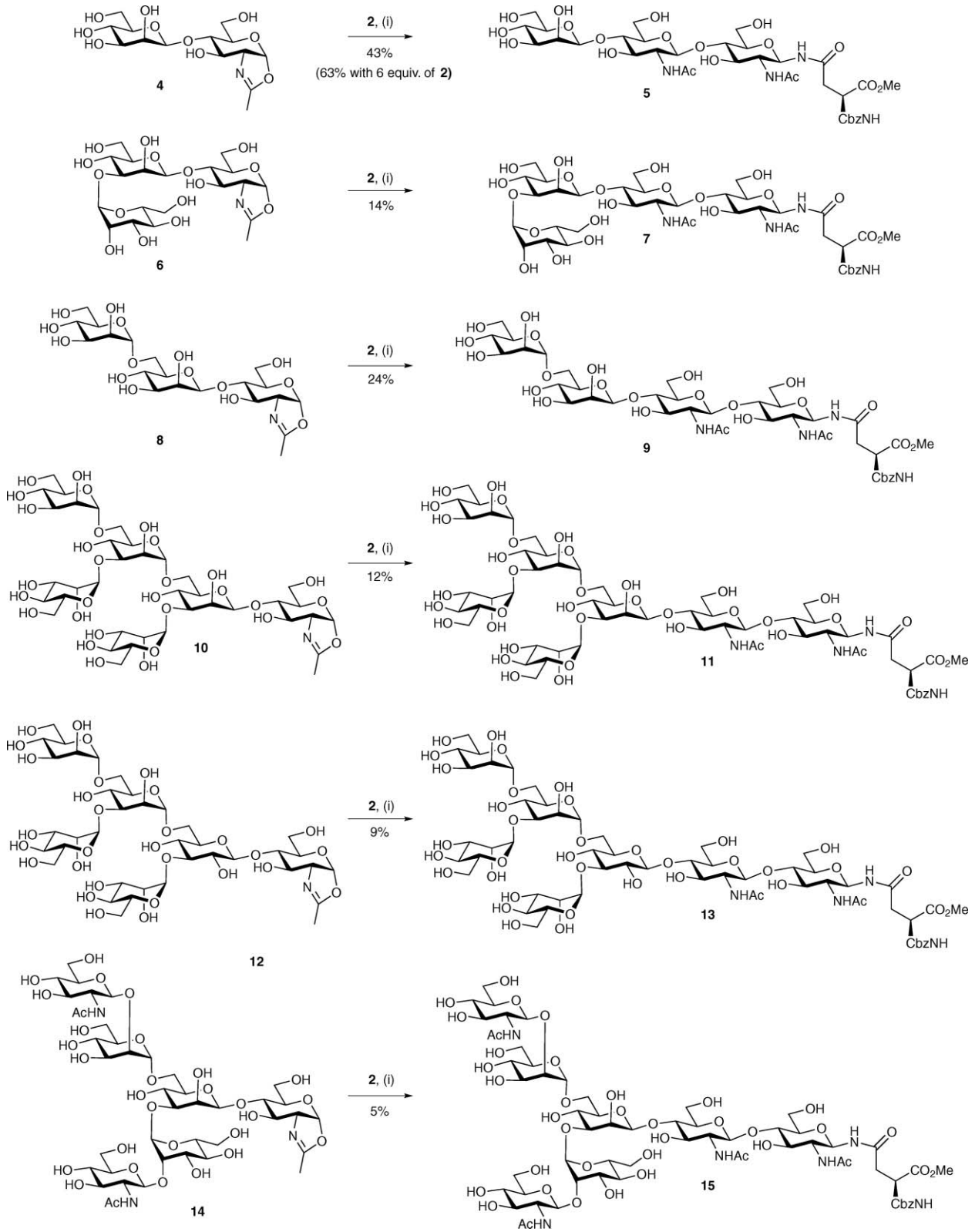
Firstly, use of the disaccharide oxazoline donor **4** produced the trisaccharide **5** in a maximum 43% yield after around 2.5 h. Interestingly, if this reaction was left for an extended period of time the yield of product **5** was found to decrease slightly to 38%. However, rather than being due to product hydrolysis, it was found that this reduction in yield was in fact due to a second glycosylation of **5**. Close inspection of the HPLC trace revealed the formation of a new minor peak, and when this product was collected and subjected to mass spectrometric analysis it was identified as a pentasaccharide, presumably arising from glycosylation of the 4-hydroxyl of the terminal mannose residue of **5** by a second equivalent of the disaccharide oxazoline **4**. Previous studies on both Endo A[11b] and Endo M[24] have revealed the ability of sugars other than GlcNAc to act as acceptors, including mannose. Moreover Wang has recently demonstrated[14k] the potential for Endo A to effect multiple glycosylations and so yield oligomers, albeit for an acceptor/donor having a terminal *glucose* unit. It appears that a similar situation operates here, and that Endo D can also glycosylate an acceptor other than GlcNAc using an oxazoline donor. The addition of a further 3 equivalents of oxazoline donor **4**, in an attempt to push the reaction towards completion did result in an increase in the yield of product **5** obtained (63%), but there was also a corresponding increase in the yield of the pentasaccharide product arising from double glycosylation.

Endo D was also found to be capable of effecting glycosylation of the acceptor **2** with both the (1–3) linked trisaccharide donor **6**, which gave the tetrasaccharide product **7** in a maximum yield of 14%, and the (1–6) linked trisaccharide donor **8**, which gave tetrasaccharide **9** in a maximum yield of 24%. Although the efficiency of both of these processes appeared to be significantly lower than when the tetrasaccharide donor **1** had been employed, both reactions were complete within one hour. In both cases the product yield did not decrease after prolonged reaction times, indicating that Endo D was unable to hydrolyse the products **7** or **9** at an appreciable rate, and again implying that the modest yields were due to competitive oxazoline hydrolysis.

Glycosylations were also undertaken using three different hexasaccharide oxazolines **10**, **12** and **14** (Scheme 2). Under the standard reaction conditions the high-mannose type hexasaccharide **10** gave heptasaccharide **11** in 12% yield; the reaction was complete after approximately 3.5 h, and no product hydrolysis was observed after this time. The donor **12**, a non-natural *gluco*-modified analogue of **10**, was also processed by the enzyme and afforded heptasaccharide **13** in 9% yield after approximately 9 h. It has been shown previously that both Endo M[13b] and Endo A[13c] are able to tolerate a *manno*- or *gluco*pyranose at this internal position, using oxazoline donors to synthesise products that these enzymes are then unable to hydrolyse. The fact that **13** was produced in this study indicated that Endo D can also process oxazoline donors containing a central *gluco*pyranose unit, though in this instance the rate of reaction was significantly decreased. The fact that product **13** was not hydrolysed by Endo D was in this instance perhaps less surprising than the analogous observations made using either Endo A or Endo M, since in these experiments Endo D did not display hydrolytic activity against any of the reaction products. Finally, hexasaccharide **14**, an oxazoline structure that corresponds to a truncated complex-type hexasaccharide, was also processed by Endo D and gave heptasaccharide **15**, albeit in a very modest 5% yield. In this case the reaction was complete within 30 min, and again no product hydrolysis was observed. This final result is of particular interest since Endo D has been reported to be unable to hydrolyse complex *N*-glycans before removal of all the external residues, including all of the GlcNAc units. In this instance the successful, yet inefficient, synthesis of **15** indicates that the previously identified requirement[17b,18e,21] for Endo D activity for an exposed 2-hydroxyl group on the α(1–3)-linked mannose is not strictly adhered to when using an oxazoline donor. However, the very low product yield means that this process is not currently synthetically useful.

Investigation of enzyme kinetics

In order to confirm the hypothesis that direct oxazoline hydrolysis was the main competing side reaction, a brief investigation of the kinetics of Endo D catalysed oxazoline and product hydrolysis was undertaken using one representative oxazoline. Thus, the rates of enzyme-catalysed hydrolysis of the (1–6)-linked trisaccharide oxazoline **8** and the corresponding synthetic product of the enzymatic glycosylation reaction, tetrasaccharide amino acid **9** (which could be a substrate for the enzyme) were measured. Kinetic studies were performed by the use of HPLC analysis of aqueous reaction mixtures in which either material (**8** or **9**) was treated with Endo D. The rate of hydrolysis was determined by the use of an evaporative light scattering detector (ELSD) in the case of **8**, or a UV detector in the case of **9**, to analyze reaction mixtures that were quenched after 5 min. For the hydrolysis of oxazoline **8**, Michaelis–Menten analysis (see the ESI‡) revealed K_m to be 9 mM (±3). The hydrolytic rate constant k_{cat} was measured as 220 min^{-1} (±20), and the apparent second order rate constant of the hydrolytic process represented by k_{cat}/K_m was therefore estimated to be 24 min^{-1} mM^{-1}. Kinetic studies on the hydrolysis of the corresponding synthetic product **9** (the product obtained from glycosylation of acceptor **2** with oxazoline **8**) were also attempted. However the rate of hydrolysis of **9** was found to be so slow that measurement of kinetic parameters proved to be impracticable. For example, no hydrolysis of **9** was observed after 60 min under equivalent reaction conditions and concentrations that had led to significant hydrolysis of oxazoline **8** after 5 min. Subsequent incubation of tetrasaccharide **9** with Endo D for extremely long periods of time revealed that only ~10% hydrolysis was achieved after 7000 min of reaction time; a result that contrasted with the



Scheme 2 Reagents and conditions: (i) Endo D (SpGH85), Na_2HPO_4 (100 mM), pH 6.5, 23 °C.

observation of ~50% hydrolysis of **8** under identical conditions after merely 5 min. Thus k_{cat}/K_m for the hydrolysis of synthetic product **9** by Endo D was crudely estimated to be at least three orders of magnitude lower than that for hydrolysis of oxazoline **8**.

Based on previous structure–activity relationships reported for the hydrolytic activity of Endo D it is unsurprising that Endo D is unable to effect hydrolysis of **9**; tetrasaccharide **9** does not contain a mannose unit at the 3-position of the central branching mannose residue. However the rapid hydrolysis of the corresponding oxazoline structure **8**, which also does not possess a mannose unit at the 3-position of the central branching mannose, confirms the considerable discrepancy that may exist between the rates of oxazoline and product hydrolysis. In this instance the high energy oxazoline intermediate **8** is hydrolysed by Endo D rapidly, whereas the corresponding synthetic reaction product **9** is effectively inert to hydrolysis.

This discrepancy in the ability of Endo D to process the oxazoline intermediate by transfer to either water or a glycosyl acceptor, as compared to its inability to hydrolyse the corresponding glycosylation product, suggests that the Endo D-catalyzed glycosylation reaction has potential scope for improvement. Currently, the problem presented here is that Endo D does not discriminate effectively between water or acceptor **2** during the processing of the oxazolines studied here. This lack of selectivity in the group transfer reaction may stem from the fact that acceptor **2** is itself not recognized efficiently by Endo D, which is consistent with the fact that glycoside **9** is a very poor substrate for this enzyme. Two interesting lines of study that we are now pursuing, following this initial feasibility study, is that inclusion of fucose on the acceptor may favour partitioning of the oxazoline toward transfer to the glycan acceptor rather than to water. The second idea is that mutation of Endo D to favour partitioning to a glycan acceptor over water should also be feasible.

Conclusions

The above studies demonstrate for the first time that Endo D, like Endo A and Endo M, two other family 85 glycoside hydrolase enzymes, is capable of catalysing the glycosylation of an acceptor bearing a GlcNAc residue using *N*-glycan oxazolines as activated donors. Methodological studies revealed that synthetic efficiency is somewhat lower than for Endo A or Endo M, and that the major competing process is direct enzyme-catalysed oxazoline hydrolysis. The significant rate of direct oxazoline hydrolysis was confirmed by kinetic studies in one instance. These studies revealed that direct hydrolysis of an oxazoline occurred rapidly even though Endo D was not capable of hydrolysing the corresponding product that was produced by the synthetic reaction employing this oxazoline together with a GlcNAc acceptor. Unfortunately attempted Endo D-catalyzed glycosylation of de-glycosylated RNase B (dRNase B) was unsuccessful, presumably due to the high rate of direct competing oxazoline hydrolysis. However, it is pertinent to note that Endo D is capable of employing a variety of structurally different oxazolines as donors and, as alluded to above, tolerated structures are not limited to known hydrolytic substrates for the enzyme. Indeed in the present study none of the glycosyl amino acid products were found to be hydrolysed by Endo D at an appreciable rate. The synthetic capabilities demonstrated by Endo D indicate therefore that other family GH85 enzymes related to Endo D should also display the ability to glycosylate acceptors using oxazolines as donors, and thus themselves may be useful catalysts for the synthesis of defined glycoconjugates. Furthermore, the recently reported crystal structure of Endo D[20] will undoubtedly provide key structural insight that will allow the future fine-tuning and optimisation of the synthetic activity of Endo D by protein engineering; for example by effecting a reduction in the rate of competing oxazoline hydrolysis. Such mutational studies on Endo D, and investigations into the effect of core fucosylation on the transfer reaction are currently in progress, and the results will be reported in due course.

Experimental

General

Enzymatic experiments were performed at 23 °C and monitored by reverse phase HPLC by one of two methods. Method A: using a Hewlett-Packard 1050 HPLC instrument using Clarity software (version 2.4.1.43) connected to an Agilent 1100 variable wavelength detector at 257 nm wavelength. An analytical HPLC (Phenomenex Gemini 5 μ C-18 column, 250 × 4.6 mm) was used to monitor the synthetic reactions, with 2 μL aliquots taken at appropriate time intervals. The column was eluted with 22% MeCN–H_2O for 20 min at a flow rate of 1 mL min^{-1}. The yield was determined by integration of the product and acceptor peaks. Method B: using a Dionex Ultimate 3000 instrument using Chromeleon software (version 6.80, build 2212) connected to a Dionex Ultimate 3000 variable wavelength detector at 207, 220, 254 and 280 nm wavelengths. An analytical HPLC (Grace Platinum 3 μ C-18 column, 100 × 4.6 mm) was used to monitor the reactions, with 1 μL aliquots taken at appropriate time intervals and diluted into 10 μL of water. The column was eluted by a linear gradient of 25-35% MeCN containing 0.3% TFA for 15 min, followed by a 95% MeCN wash for 2 min and reequilibration at 25% MeCN for 3 min, all at a flow rate of 1 mL min^{-1}.

Materials

The catalytic domain (SpGH85) of Endo D was produced in *E. coli* (BL21) and purified as previously described.[20] *N*-glycan oxazoline donors **1**, **4**, **6**, **8**, **10**, **12** and **14** and the Asn-GlcNAc glycosyl amino acid acceptor **2** were all synthesised as previously described.[13a,13c,13e] Glycosyl amino acid products **3**, **5**, **7**, **9**, **11**, **13** and **15** exhibited spectral data identical to those reported previously,[13c,13e] and which are provided in the ESI.§

Typical glycosylation procedures

Method 1: variation of the quantity of Endo D employed, and the use of various oxazoline donors. Glycosyl amino acid acceptor **2**[13a] (100 μg, 621 nmol) and oxazoline donor (3 eq.) were dissolved in sodium phosphate buffer (0.1 M solution, pH 6.5, total reaction volume: 50 μL). Endo D (5 μL of a 5.8 mg mL^{-1} solution; 390 mU or overall concentration of 7.8 mU μL^{-1})[17d,22] was added and the temperature maintained at 23 °C. Reaction progress was monitored by HPLC analysis (method A) and the yield determined by UV integration of the product and acceptor peaks. To drive the reaction between **1** and **2** towards completion the reaction was conducted as described above except that 3 further equivalents

of donor **1** were added after every hour for 4 h (*i.e.* to a total of 15 equivalents of donor). The progress of the reaction was monitored by HPLC analysis of aliquots taken immediately before each subsequent addition.

Method 2: variation of co-solvent. Oxazoline donor **1** (214 μg, 310 nmol 3 eq.) and glycosyl amino acid acceptor **2** (50 μg, 103 nmol) were dissolved in a mixture of the appropriate organic solvent (HPLC grade), water and sodium phosphate buffer (0.4 M solution, pH 6.5, total reaction volume: 25 μL; overall buffer concentration 0.1 M). Endo D (2.5 μL of a 5.8 mg mL^{-1} solution; 195 mU or overall concentration of 7.8 mU μL^{-1}) was added and the temperature maintained at 23 °C. Reaction progress was monitored by HPLC analysis (method A) and the yield determined by UV integration of the product and acceptor peaks.

Method 3: variation of reaction pH. Similarly **2** (50 μg, 103 nmol) and oxazoline donor **1** (214 μg, 310 nmol, 3 eq.) were dissolved in sodium phosphate buffer (0.1 M solution, pH 6.5, total reaction volume: 25 μL). Endo D (2.5 μL of a 5.8 mg mL^{-1} solution; 195 mU or overall concentration of 7.8 mU μL^{-1}) was added and the temperature maintained at 23 °C. Reaction progress was monitored by HPLC analysis (method A) and the yield determined by UV integration of the product and acceptor peaks.

Method 4: attempted glycosylation of dRNase B. Deglycosylated RNase B (prepared previously) (200 μg, 14.4 nmol) and oxazoline donor **1** (50 μg, 72 nmol, 5 eq.) were dissolved in sodium phosphate buffer (0.1 M solution, pH 6.5, total reaction volume: 12 μL). Endo D (1.0 μL of a 5.8 mg mL^{-1} solution; 78 mU or overall concentration of 6.5 mU μL^{-1}) was added and the temperature maintained at 23 °C. Reaction progress was monitored by HPLC analysis (method B).

Kinetic studies

HPLC analysis for enzyme kinetics was carried out by one of two methods. Method A: using a Dionex Ultimate 3000 HPLC system using Chromeleon software (version 6.80 SP2 Build 2284) and a Phenomenex Gemini 5 μ C-18 column, 250 × 4.6 mm. The column was eluted with 5% MeCN–H_2O over a 5 min period for each analysis. This was followed by a gradient to 95% MeCN over 1 min, 95% MeCN for 2 min, gradient to 5% MeCN over 1 min and re-equilibrate for 5 min. Detection was carried out using a Polymer Laboratories PL-ELS 2100 Ice system. The non-linear response of the evaporative light scattering detector (ELSD) was accounted for by separate calibration for each compound involved.[25] Method B: using a Hewlett-Packard 1050 HPLC instrument using Clarity software (version 2.4.1.43) connected to an Agilent 1100 variable wavelength detector at 257 nm wavelength. An analytical HPLC (Phenomenex Gemini 5 μ C-18 column, 250 × 4.6 mm) was used to monitor the synthetic reactions by analysing 1 μL aliquots. The column was eluted with 22% MeCN–H_2O for 20 min at a flow rate of 1 mL min^{-1}. The yield was determined by integration of the product and acceptor peaks.

Hydrolysis of oxazoline 8

Hydrolytic reactions were performed at various concentrations of oxazoline **8** (4, 8, 12, 16, 20, 25, 32, 40 and 50 mg ml^{-1}) at 23 °C in sodium phosphate buffer (0.1 M solution, pH 6.5, total reaction volume: 8 μL) in the presence of Endo D (5.8 ng, 1 μL of a 5.8 mg ml^{-1} solution of enzyme; molecular weight, 73 828.48; final enzyme concentration: 9.82×10^{-6} M). The extent of oxazoline hydrolysis was measured after 5 min by HPLC analysis (method A). Samples were spiked with a known quantity of methionine to act as internal standard, and quantities of oxazoline and hydrolytic product were obtained by using the calibration curve data for each component. The initial rate of reaction (mol min^{-1}) was obtained by division by 5. The kinetic parameters, k_{cat}, K_m, and V_{max}, were then obtained by fitting this experimental data to the Michaelis–Menten model using the non-linear regression program Graphpad Prism™: $k_{cat} = 219.5$ min^{-1} (±18.3); $K_m = 0.0089$ M (±0.003); and $V_{max} = 0.002156$ mol min^{-1}.

Hydrolysis of tetrasaccharide 9

Hydrolytic reaction reactions were investigated using tetrasaccharide **9** (2 μL of an 11 mg ml^{-1} solution; overall substrate concentration 3.67 mg ml^{-1}) at 23 °C in sodium phosphate buffer (3 μL of a 0.1 M solution, pH 6.5; total reaction volume: 6 μL) in the presence of Endo D at two enzyme concentrations: (a) 0.58 ng, 1 μL of a 0.58 mg ml^{-1} solution of enzyme; molecular weight, 73 828.48; final enzyme concentration: 1.3×10^{-6} M; (b) 5.8 ng, 1 μL of a 5.8 mg ml^{-1} solution of enzyme; final enzyme concentration: 13.1×10^{-6} M). In both cases no appreciable amount of hydrolysis of tetrasaccharide **9** was observed after 60 min by HPLC analysis (method B). After 4 d, 17 h and 15 min (6795 min) of reaction time analysis of (b) indicated that 10% of tetrasaccharide **9** had been hydrolysed.

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