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Enhanced Glycosylation with Mutants of Endohexosaminidase A (Endo A)

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Glycosylation of proteins is the most diverse form of post-translational modification, and can play a key role in protein folding,[1] and can also crucially affect important protein properties.[2–6] However, since the biosynthesis of glycans is not under direct genetic control, glycoproteins are produced intracellularly as heterogeneous mixtures of glycoforms, in which different oligosaccharide structures are linked to the same peptide chain. Access to pure single glycoforms of glycoproteins has now become a major scientific objective[7] since it is not only a prerequisite for more precise biological investigations into the different effects glycans have on protein properties, but also an important commercial goal in the field of glycoprotein therapeutics, which are currently marketed as heterogeneous mixtures of glycoforms.

Access to single glycoforms of glycoproteins can be achieved by total synthesis of both glycan and polypeptide components, and some outstanding achievements in this area have recently been published.[8,9] However, such synthesis approaches are particularly arduous and do not realistically represent a practical approach that could be applied to widespread and large-scale glycoprotein production. Alternative approaches based on bioengineering of cell lines in order to optimise production of glycoproteins that bear particular oligosaccharide structures have also been reported[10,11] and are being exploited commercially, though such approaches have no guarantee of complete glycan homogeneity.

An alternative method for achieving homogeneous protein glycosylation involves the use of enzymatic catalysis,[12] and one particular class of enzyme that displays considerable synthesis potential in this respect comprises the endohexosaminidases.[13] Endohexosaminidases 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, and since they cleave this linkage they can also be used to selectively synthesise it. Two members of this class that have been demonstrated to display useful *synthesis* glycosylation activity are Endo M from *Mucor hiemalis*[14–17] and Endo A from *Arthrobacter protophormiae*.[18,19] However, since these enzyme-catalysed reactions are reversible, competitive product hydrolysis can greatly reduce synthesis efficiency, particularly when transglycosylations are undertaken with unactivated donors.

Seminal work in the field by Shoda and co-workers demonstrated that carbohydrate oxazolines are useful activated glycosyl donors for these enzymes, presumably because they mimic the putative oxazolinium ions, which are proposed intermediates in the enzyme-catalysed hydrolysis reaction.[20] Subsequently, extensive work from the group of Wang has detailed the efficient synthesis of a series of glycopeptides by transglycosylation with Endo A;[21–24] they have also recently reported the synthesis of single glycoforms of ribonuclease B by using this approach.[25]

In order to circumvent the problem of competitive product hydrolysis previous work has focussed on the attempted development of irreversible glycosylation reactions with structurally modified oxazolines as glycosyl donors; the synthesis products of these reactions are generally not hydrolysed by the endohexosaminidase used to promote their synthesis;[26–28] the enzymes therefore act as glycoligases. However, another potential way to circumvent this problem is to either use specifically mutated enzymes or glycosynthases—as developed by Withers[29] and Planas[30]—which are not capable of product hydrolysis.

The term "glycosynthase" was first applied by Withers to retaining glycosidases in which the nucleophilic of the two catalytic acid residues in the enzyme active site had been replaced by site-directed mutagenesis with a nonparticipating residue, for example, by alanine. The use of an activated glycosyl donor, such as a glycosyl fluoride, allows this mutant enzyme to promote a synthesis reaction, but the mutant enzyme is not capable of hydrolysing the product glycosidic linkage as the key nucleophilic residue was absent.

Endo A is a member of family 85 of the glycohydrolases (GH85). These enzymes, though they are retaining glycosidases, are thought to catalyse hydrolysis by a neighbouring-group-participation mechanism in which the carbonyl oxygen of the 2-acetamide of the second GlcNAc residue is the actual nucleophile, rather than an enzyme-bound aspartate or glutamate. These enzymes, therefore, do not possess a nucleophilic residue at the active site, and as such it is not possible to envisage the production of a glycosynthase along the lines of the accepted Withers and Planas precedents. However, Wang et al. have very recently reported[31] the production of a series of mutants of Endo M—another family 85 endohexosaminidase—which they screened for hydrolytic and transglycosylation activity. In particular they identified an N175A mutant of Endo M in which Asn175—a conserved residue in the GH85 family—was replaced by alanine; this mutant displayed glycosynthase activity by using oxazolines as glycosyl donors. Since this N175A mutant displayed only marginal hydrolysis activity it

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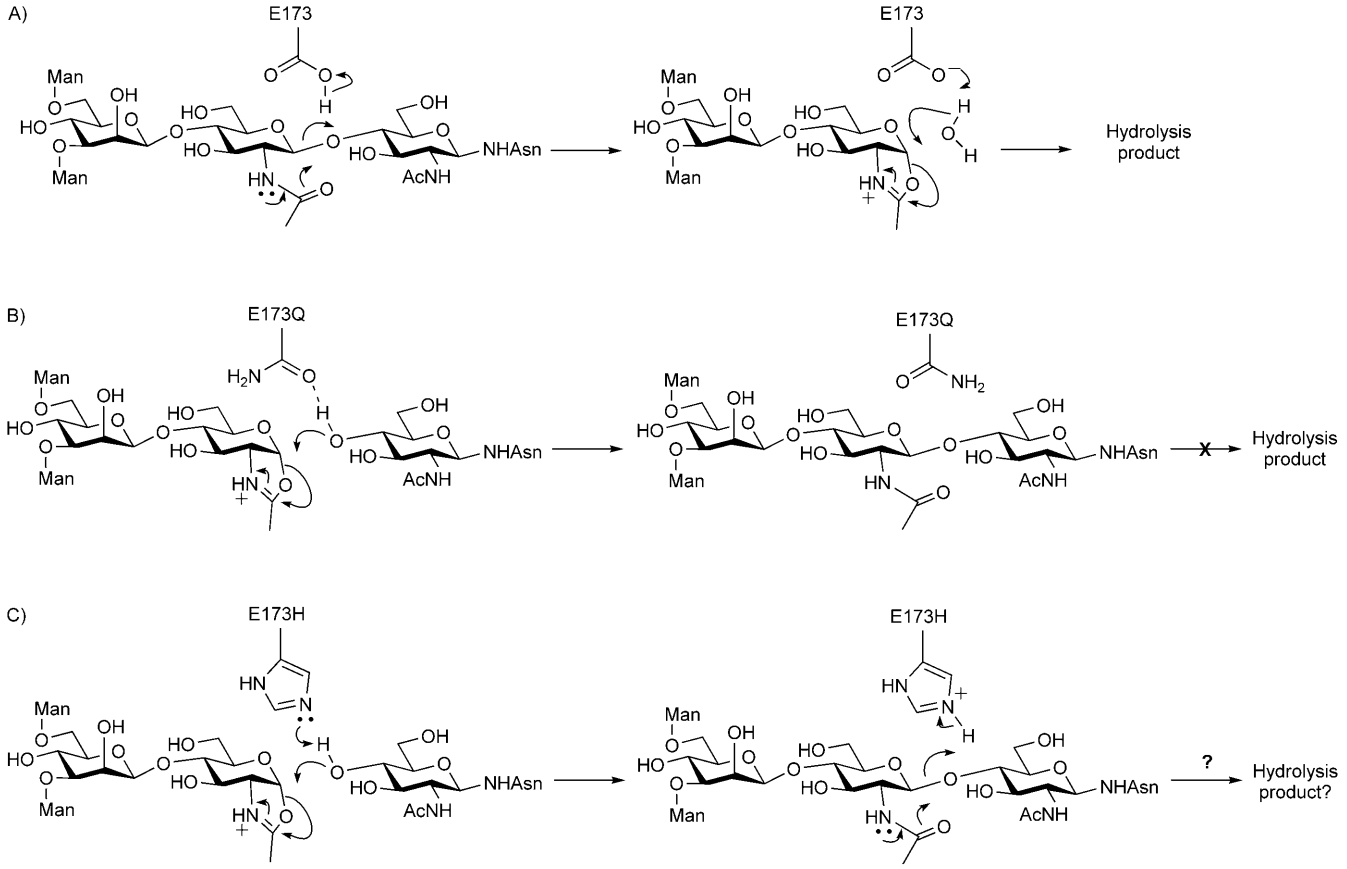
was concluded that Asn175 plays a key role in promoting oxazolinium ion intermediate formation during the hydrolytic mechanism, though not by acting as the proton donor (identified for Endo M as Glu177).

Work detailed in this paper, which was performed concurrently with the complimentary approach of Wang et al., was concerned with the development of glycosynthase mutants of Endo A. Previous studies on Endo A revealed that Trp216 was key for the transglycosylation activity,[32] and very recently it has been proposed[33] that Glu173 is the catalytic residue that acts as a general acid to protonate the glycosidic oxygen during the hydrolytic step, and as a general base to deprotonate the incoming hydrolytic water. Replacement of Glu173 with glycine, aspartate and glutamine resulted in either extremely significant or complete loss of hydrolytic activity,[32] whilst replacement with alanine produced a hydrolytically inactive mutant, the activity of which could be rescued by the addition of azide or formate.[33] However, we reasoned that mutants of Endo A that lack the general acid Glu173 might still be able to process activated glycosyl donors, and in particular an oxazoline could still serve as a transition-state mimic and allow a synthesis reaction to occur. In the first step of the hydrolytic mechanism Glu173 acts as the general acid responsible for protonation of the outgoing β-glycosidic oxygen; this results in the formation of an intermediate oxazolinium ion (Scheme 1 A). In the second step, Glu173 acts as a general base and deprotonates the incoming water molecule. Without a proton donor residue at position 173 all hydrolytic activity should be curtailed since glycosidic bonds cannot be broken without prior protonation of the anomeric oxygen atom. However, an oxazoline might still be able to enter into the mechanistic pathway and could still be glycosylated by an incoming alcohol (or hydrolysed by incoming water) in the second step. Such a mutant enzyme would therefore be a glycosynthase—all hydrolytic activity is curtailed, but an activated oxazoline donor would still be processed.

It was decided to produce two mutants of Endo A in which Glu173 had been replaced by alternative amino acids; these were chosen to be glutamine, a substitution which in fact had already previously been made and which had resulted in total loss of hydrolytic activity,[32] and histidine. These choices were made on the basis of the following rationale. Glutamine is a nonacidic residue and, therefore, should be incapable of promoting the hydrolytic reaction; the caveat being that it would also not be able to act as a general base and facilitate the synthesis reaction by aiding deprotonation of the incoming nucleophile. However, it could still act as a hydrogen-bond acceptor and facilitate nucleophilic attack on the oxazoline (Scheme 1 B). Alternatively, histidine could either act as a general acid or a general base, depending on its protonation state. The pK_a of histidine is ~6.0 as compared to ~4.1 for the side chain acid of glutamic acid; the hope, therefore, was that a histidine residue



Scheme 1. Catalytic mechanisms of family 85 endohexosaminidases. A) Hydrolytic mechanism of WT Endo A; B) putative synthesis mechanism of mutant E173Q with an oxazoline donor; C) putative synthesis/hydrolytic mechanism of mutant E173H with an oxazoline donor.

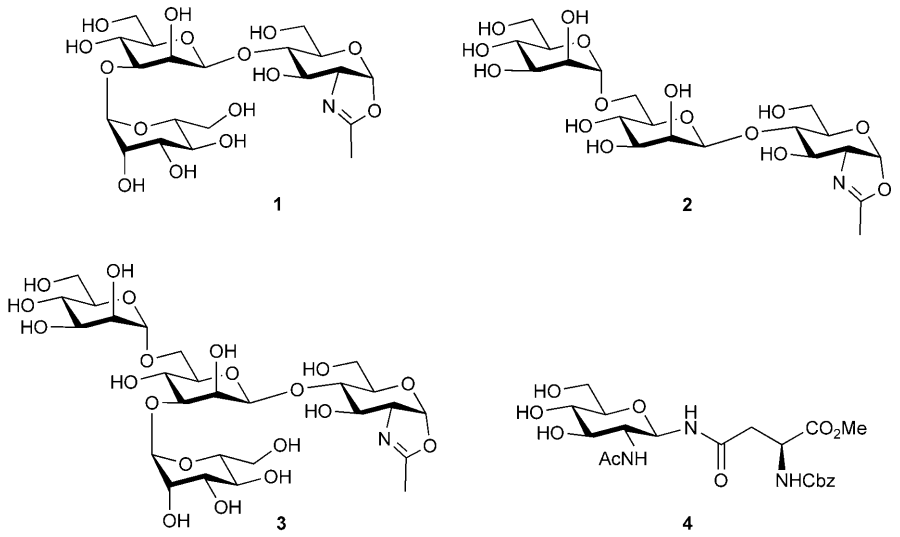
at position 173 would perhaps still act as a general base and facilitate the synthesis reaction, but that its lower pK_a could mean a reduction in its ability to act as a general acid, with an accompanying reduction in the hydrolytic capability of the enzyme (Scheme 1 C).

Two mutants of Endo A, E173Q and E173H, in which Glu173 was exchanged for glutamine and histidine, respectively, were produced by site-directed mutagenesis, and the proteins were over-expressed in *E. coli* and then purified. The three oxazolines **1**–**3** were accessed as glycosyl donors as previously described,[26,28] and these were then used together with glycosyl amino acid **4**[26] (Scheme 2) in a series of enzyme-catalysed reactions with wild-type (WT) Endo A, and the two mutants E173H and E173Q.

Reaction progress in all cases was carefully monitored by HPLC. Figure 1 details time-course studies of the yield of glycosylated product formed for each of the three donors catalysed by each of the three enzymes. In terms of kinetics, all reactions catalysed by WT Endo A were faster than those catalysed by the two mutant enzymes. For WT Endo A the maximum product formation was reached after less than 30 min with all donors investigated, and there was little difference between the rates of reactions with the different donors (Figure 1 A, D and G). Reactions catalysed by the E173H mutant were much more substrate dependent. With the (1–3)-linked trisaccharide donor **1**, maximum product formation was achieved only after 18 h (Figure 1 B), whilst with the (1–6)-linked trisaccharide donor **2** it was achieved after 4 h (Figure 1 E). The reaction with the tetrasaccharide donor **3** was the fastest, and maximum yield was achieved after about 2.5 h (Figure 1 H). Glycosylations catalysed by the E173Q mutant were even slower, and reaction times of about 20 h were required in order to reach the maximum yield with all of the donors investigated (Figure 1 C, F and I).

The dependence of the rate of reaction on the donor structure, which was particularly observed with the E173H mutant, probably reflects the importance of the different parts of the oxazoline donor for binding to the enzyme active site. For example, the presence of an α-mannose residue at position six of the central mannose (donor **2**) led to a much faster enzymatic reaction than with an α-mannose residue solely at position three (donor **1**), whilst the tetrasaccharide donor **3**, which possesses α-mannose residues at both positions, reacted much faster than the trisaccharides. This relative ranking of the importance of the presence of mannose residues at branch points also correlated with the rates of product hydrolysis. Wild-type Endo A hydrolysed all of the products **5**, **6** and **7** at a significant rate, and reproduced acceptor **4** and released the donor as the free reducing sugar. However, the rate of hydrolysis increased in the order **5** < **6** < **7** (Figures 1 A, D and G)—an order that corresponded with the relative rates of glycosylation of the donors **1** < **2** < **3** observed with mutant E173H. This rank order was also reflected in the maximum yields obtained for product formation (**5** < **6** < **7**) for glycosylations catalysed by both of the mutant enzymes (Table 1).

Figure 1 also clearly shows that the two mutations did indeed suppress the ability of the enzyme to hydrolyse the products. Replacement of Glu173 by histidine resulted in a mutant that still retained some hydrolytic activity (Figures 1 B, E and H), but for these substrates this was considerably reduced compared to that of WT Endo A (Figures 1 A, D and G). Indeed an assay with ribonuclease B (RNase B) as the substrate revealed the hydrolytic activity of the E173H mutant to be approximately 20% that of WT Endo A. Replacement of Glu173 by glutamine completely abolished product hydrolysis (Figures 1 C, F and I), and no hydrolytic activity was observed towards RNase B; this confirms the previous report of Fujita and Takegawa.[32] However, this E173Q mutant had also been found not to have any transglycosylation activity when chitobiose–Asn linked oligosaccharides were used as donors.[32] The findings here, therefore, once again underline the usefulness and higher activity of glycosyl oxazolines as donors in enzymatic glycosylations catalysed by endohexosaminidases. The E173Q mutant, previously considered to be inactive for transglycosylation, was in fact capable of processing these oxazolines efficiently although the overall efficiency of the process was substrate dependent (Table 1). With the (1–3)-linked trisaccharide donor **1**, the maximum obtainable yield of product **5** was a very modest 17%, but with the (1–6)-linked trisaccharide donor **2** the synthesis efficiency improved, and product **6** could be obtained in 66% yield. Moreover with the tetrasaccharide donor **3**, synthesis efficiency improved once more and 82% yield of product **7** could be ob-



Scheme 2. Oxazoline donors **1**–**3** used for glycosylation of GlcNAcAsn amino acid acceptor **4** with WT Endo A and mutants E173H and E173Q.

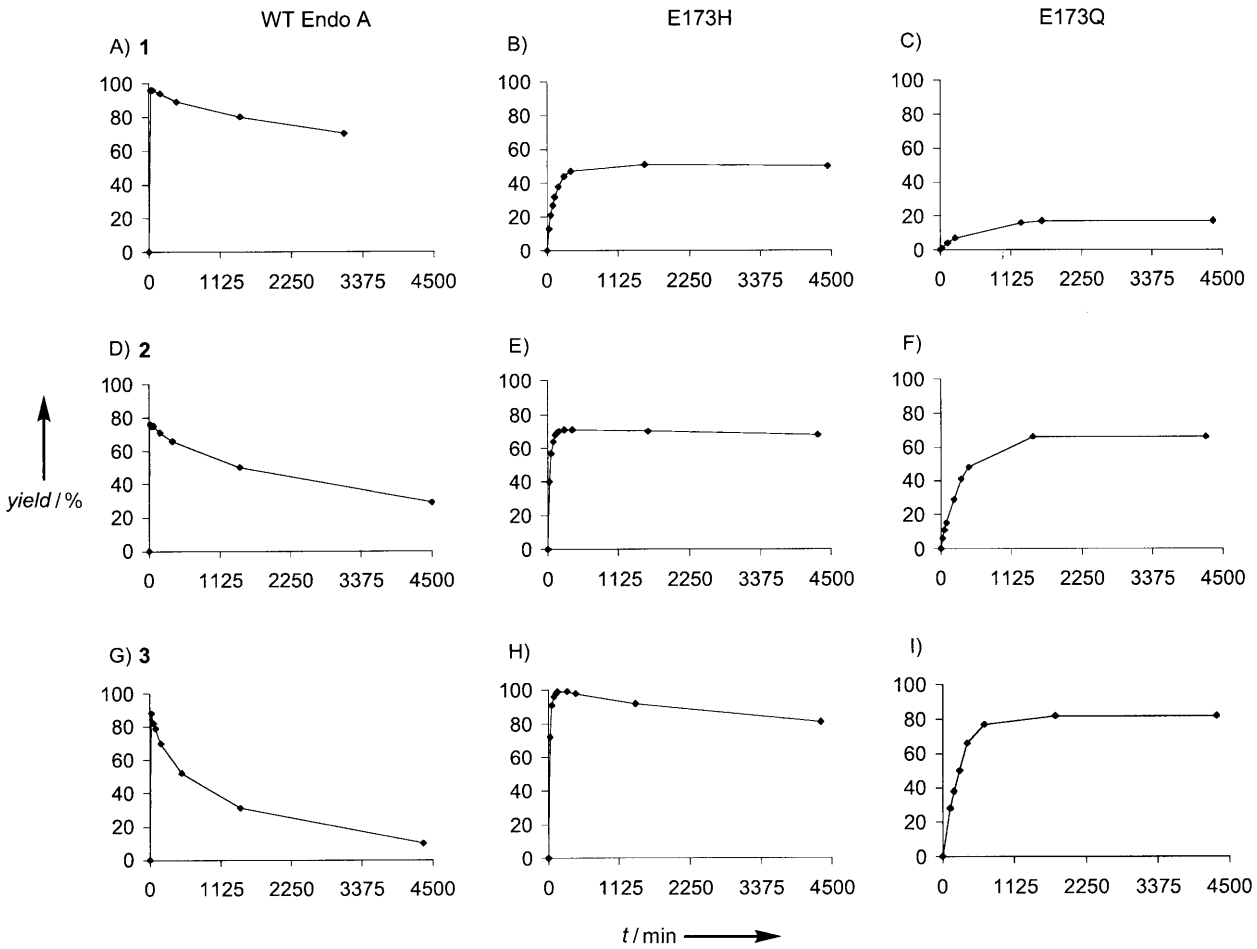
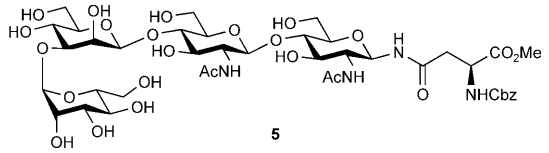
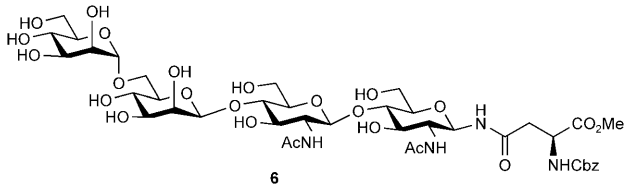
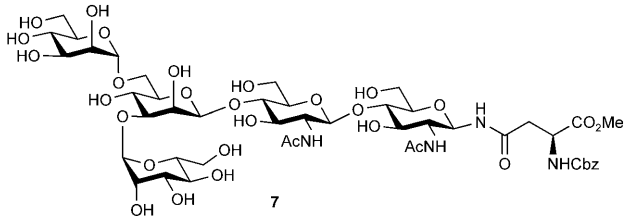


Figure 1. Time correlations of product yield for glycosylations of acceptor **4** with donors: A–C) **1**, D–F) **2** and G–I) **3** by using WT Endo A, mutant E173H and mutant E173Q.

tained in an irreversible reaction. Even better yields were achieved by using the E173H mutant, though again synthesis efficiency was substrate dependent. Whilst the yield of **5** obtained with the (1–3)-linked trisaccharide donor **1** was only 51%, use of the (1–6)-linked trisaccharide donor **2** produced **6** in 71% yield, in an essentially irreversible process (Figure 1E). Most impressively use of the E173H mutant with tetrasaccharide donor **3** gave the pentasaccharide product **7** in quantitative yield, and with a dramatically reduced rate of subsequent hydrolysis as compared to WT Endo A. The time-course plot of the reaction (Figure 2) shows that the yield of product remained practically constant over a period of 6 h; this illustrates that as long as there was oxazoline donor left in solution, product formation proceeded and was much faster than hydrolysis—only when all of the donor was exhausted did the yield decrease. This finding also indicates that the ratio of donor to acceptor used, which in these investigations was 3:1 (donor/acceptor), could probably be reduced significantly without product yield being reduced; thus the overall efficiency of the process could be improved.

It should be borne in mind that the maximum yields that can be achieved with any of these enzymatic reactions are dependent on the relative rates of three processes: one synthesis and two hydrolytic. These are the rate of glycosylation, which forms the desired product, the rate of enzyme catalysed product hydrolysis and the rate of direct hydrolysis of the oxazoline donor, either by water alone[34] or catalysed by the enzyme. Suppression of product hydrolysis alone is therefore not sufficient to guarantee a good product yield, as can be seen from some of the present examples. For example, during glycosylation of donors **1** and **2** catalysed by the E173H mutant it is apparent that after a certain period of time product formation ceased (Figures 1B and E); this presumably indicates that no oxazoline remains in solution, and was rather hydrolysed instead of being transferred onto the acceptor. The improved synthesis efficiency observed by using larger oxazoline donors might indicate that the relative rate of glycosylation versus direct oxazoline hydrolysis becomes more favourable as more extended oxazoline donors are used. Under normal circumstances product hydrolysis is also faster with more extended oligosaccharides, and so the overall efficiency of the synthesis processes becomes limited by product hydrolysis. However, because product hydrolysis is totally suppressed with mutant enzymes, such as E173Q, and to a lesser extent with E173H,

Table 1. Glycosylation of **4** with donors **1**–**3** catalysed by WT Endo A and mutants E173H and E173Q.

Oxazoline donor	Product	Maximum yield [%][a]		
		WT	E173H	E173Q
1	 **5**	96 ↓	51 ↓	17 →
2	 **6**	76 ↓	71 ↓	66 →
3	 **7**	88 ↓	99 ↓	82 →

[a] Yields determined by integration of acceptor and product peaks; →: yield stayed constant; ↓: yield decreased after reaching the stated maximum due to product hydrolysis.

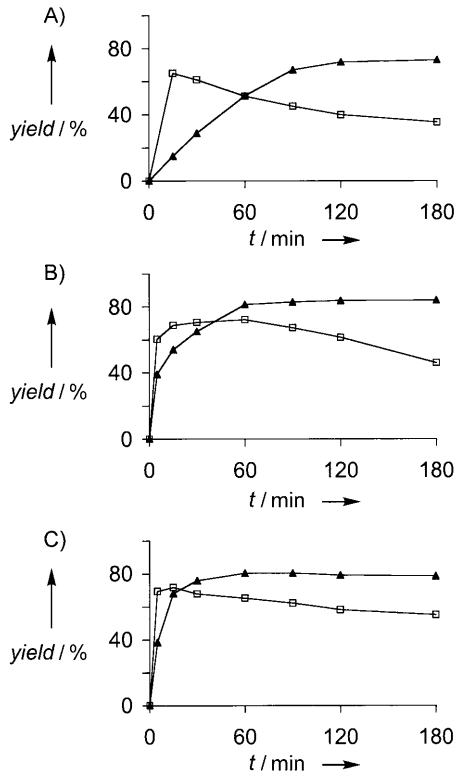


Figure 2. Time-course studies of the extent of glycosylation of dRNase B with oxazoline **3** catalysed by WT Endo A (□) and the E173H mutant (▲) at different substrate concentrations. Reactions were carried out in potassium phosphate buffer (50 mM, pH 6.5) at 37 °C with oxazoline **3** in a 20-fold excess over dRNase B; Endo A and dRNase B were at concentrations of: A) 18 μM and 0.46 mM, B) 50 μM and 1.3 mM and C) 35 μM and 1.8 mM, respectively.

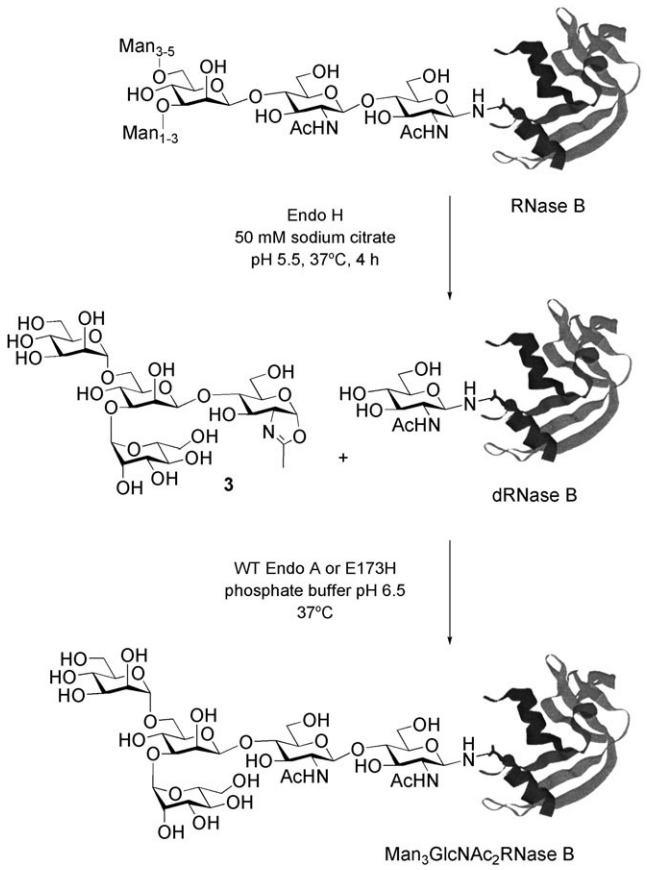
the reaction profile and overall synthesis efficiency improves, and is especially good for the largest oxazoline donor that has so far been investigated.

Attention then turned to the potential application of these enzymes for protein glycosylation. Ribonuclease B (RNase B) was recently remodelled by Wang and co-workers by using WT Endo A,[25] and in this respect would serve as a good system for comparison of the ability of mutant and wild-type enzymes to effect glycoprotein remodelling. The studies detailed above indicated that of the oxazoline donors available tetrasaccharide **3** was the most useful. Furthermore, they also indicated that kinetically the E173H mutant was the more efficient of the two mutants so far produced.

Commercially available bovine RNase B was first enzymatically trimmed back by treatment with Endo H to produce dRNase B—a single protein glycoform that bears a GlcNAc residue at the sole N-linked glycosylation site. Glycoform dRNase B was then used as a substrate for glycosylation with tetrasaccharide oxazoline **3** by using both WT Endo A and the E173H mutant (Scheme 3).

In both cases the enzyme was able to effect production of a single glycoform product, $Man_3GlcNAc_2$RNase B, which was characterised by mass spectrometry (Figure 3; calculated mass of the $Man_3GlcNAc_2$ glycoprotein 14575; found 14575). The progress of the reaction was monitored in both cases, and at three different concentrations of the dRNase B substrate (Figure 2).

Both enzymes efficiently catalysed the formation of the $Man_3GlcNAc_2$ glycoform of RNase B; the maximum yields obtained were 72 and 84% with WT Endo A and the E173H



Scheme 3. Enzymatic remodelling of a mixture of RNase B glycoforms to a single $Man_3GlcNAc_2$ glycoform by using WT Endo A and the E173H mutant.

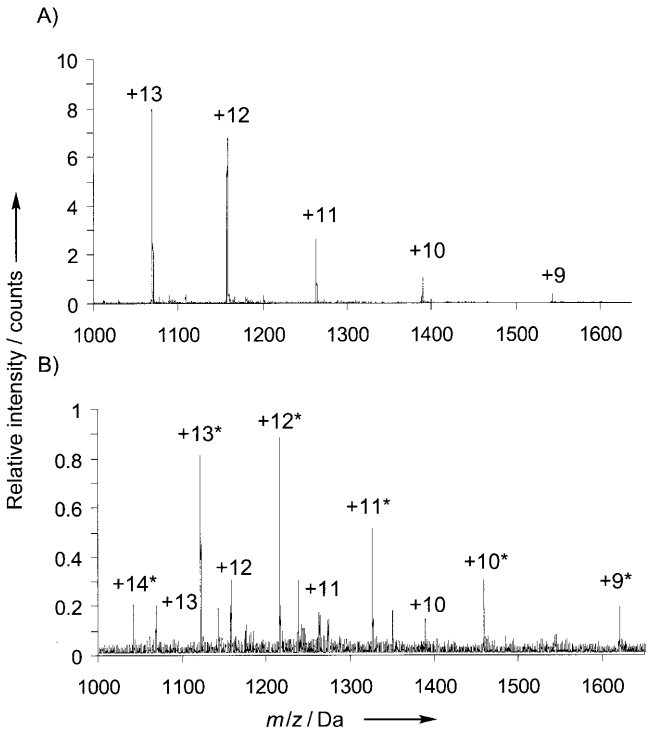


Figure 3. ESI-MS spectra of dRNase B before and after treatment with oxazoline. A) dRNase B; charges for each peak indicate that the protein molecular mass is 13885 Da (expected size 13885 Da). B) Product after treatment of dRNase B with oxazoline **3** in the presence of Endo A E173H. Charged species due to glycosylated RNase B are marked with asterisks and indicate a protein with a molecular mass of 14575 Da (expected size 14575 Da). Charged species without asterisks are due to dRNase B.

mutant, respectively. The time-course study revealed that WT Endo A effected transglycosylation of dRNase B more rapidly than the E173H mutant. However, WT Endo A catalysed hydrolysis of the product glycoprotein, whereas the E173H mutant did not. Therefore, after a certain time period, which was dependent on the concentrations of substrates used, the E173H mutant became the more efficient catalyst, and ultimately allowed formation of the glycoprotein product in a higher yield.

Some comparative comments between these studies and the recently reported approach of Wang et al.[31] are appropriate at this point. Firstly, comparison of the activity of their Endo M N175A mutant and our Endo A mutants indicates that the kinetics of transglycosylation are reduced in all of these mutant enzymes with respect to the WT enzymes. In this study the largest donor we investigated was the $Man_3GlcNAc$–oxazoline, which was the smallest donor investigated by Wang et al. with their N175A mutant, and, therefore, represents the only case in which direct comparison is currently possible. In their report the N175A mutant gave approximately 25% yield of a glycosylated glycopeptide product after 60 min, but the maximum yield obtained in this case with more extended reaction times was not given. With more extended donors their N175A mutant gave yields of glycosylated product in the 50–80% range, and in particular a $Man_9GlcNAc_2$ glycopeptide—a product that previously could only be accessed in ~10% yield with WT Endo A—was synthesised in 72% yield by using 15% DMSO as cosolvent, though the time required for complete reaction was not quoted. In comparison, with $Man_3GlcNAc$–oxazoline **3** the Endo A E173H and E173Q mutants gave product **7** in 99 and 82% yields, respectively (maximum obtainable yields, Table 1), though notably the E173H mutant was capable of slowly hydrolysing product **7**. In terms of comparative kinetics, after 60 min product **7** was formed in 91% yield with the E173H Endo A mutant, and in 15% yield by the E173Q Endo A mutant (c.f., ~25% yield after 60 min in an analogous reaction with the N175A Endo M mutant).

It is particularly noteworthy that in their studies on Endo M, Wang et al. reported[31] that an E177A mutant, in which the key Endo M proton donor residue Glu177 was replaced by alanine, was not capable of promoting transglycosylation even when sugar oxazolines were used as donors. The studies reported here, albeit on Endo A, seem to imply that alanine is not a good choice as the replacement residue; this validates the H-bond acceptor rationale presented in Scheme 1. Indeed it will be interesting to investigate in the future whether the E177H and E177Q mutants of Endo M display glycosynthase activity with oxazolines as donors. The identification of the Endo M N175A glycosynthase mutant by Wang et al.[31] and the useful synthesis activity that they have demonstrated, taken together with this research, clearly indicates that there is much scope for further optimisation of glycosynthase activity in the future. Moreover, the report from Wang et al. of the increased trans-

glycosylation activity of both a Y217F mutant of Endo M and a Y205F mutant of Endo A hints at the potential future advantages of constructing double mutants of both Endo A (e.g., E173H, Y205F) and Endo M (e.g., E177H, Y217F).

In conclusion it has been demonstrated that replacement of the key catalytic residue Glu173 of Endo A allowed production of mutant enzymes for which hydrolytic activity had been either totally suppressed or significantly reduced, but which still possessed the ability to process oxazolines as donors; this allowed the transglycosylation of substrates with GlcNAc residues. The judicious choice of replacement residues as those that are capable of acting as H-bonding acceptors was vindicated as key to the success of the endeavour by the subsequent report of an inactive E177A Endo M mutant. The potential advantage of reduced hydrolytic activity was demonstrated in the production of a single glycoform of RNase B in a more efficient manner than was possible with WT Endo A. Such mutant enzymes might prove to be very useful as glycosynthases for the construction of glycopeptides and biologically important glycoproteins that bear large oligosaccharide structures when competitive product hydrolysis by wild-type enzymes becomes the limiting factor in terms of achievable synthesis efficiency.

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