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Endohexosaminidase-catalysed glycosylation with oxazoline donors: effects of organic co-solvent and pH on reactions catalysed by Endo A and Endo M

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ABSTRACT

The synthetic efficiency of endohexosaminidase-catalysed glycosylation reactions using N-glycan oxazolines as donors was investigated as two reaction parameters were varied. Both the addition of quantities of an organic co-solvent and modulation of reaction pH between 6.5 and 8.0 were found to have different effects on reactions catalysed by either Endo A (and two available mutants) or Endo M, indicating subtle differences between these two family GH85 enzymes. Fine tuning of reaction pH, or the addition of quantities of an organic co-solvent, resulted in beneficial increases in achievable synthetic efficiency by effecting a reduction in the rate of competitive hydrolytic processes.

1. Introduction

Access to pure homogeneous defined single glycoforms of glycoproteins has now become a major scientific objective.[1] Not only is this a prerequisite to facilitate more precise biological investigation into the effects that different glycans have on protein properties, but it is also an important commercial goal in the field of glycoprotein therapeutics, which are currently marketed as heterogeneous mixtures. Glycoprotein remodelling using endohexosaminidases, a class of enzymes that specifically cleave the chitobiose core of N-linked glycans between the two GlcNAc residues, is an attractive methodology that can allow access to a variety of defined homogenous glycoconjugates including glycoproteins.

The two endohexosaminidase enzymes that have so far shown the most useful *synthetic* glycosylation activity are Endo M from *Mucor Hiemalis*[2] and Endo A from *Arthrobacter protophormiae*,[3] both of which are members of the glycohydrolase (GH) family 85.[4] Initial studies revealed the remarkable ability[5,6] of these enzymes to effect transglycosylation of a variety of acceptor substrates bearing GlcNAc residues using asparagine-linked isolated oligosaccharides as donors. However, because these enzymes naturally display hydrolytic activity the efficiency of such approaches was very limited; typically 100–1000 equiv of glycosyl acceptor was required in order to effect even very moderate yields of product due to competitive hydrolytic processes. Following the disclosure by Shoda[7] and co-workers that carbohydrate oxazolines could be used as activated glycosyl donor substrates for these enzymes, the synthetic efficiency of endohexosaminidase-catalysed glycosylation has been significantly increased. Studies both by ourselves,[8] and by Wang and co-workers[9] have detailed the application and further development of this basic methodology and demonstrations of the utility of both Endo A and Endo M for the transfer of intact oligosaccharides to amino acids,[8a–c] peptides[9a–d] and proteins[8d,9f,g,k] (including monoclonal antibodies)[9j] have now all been reported. Nevertheless, although the use of oxazolines as donor substrates has already proven to be extremely beneficial, the issue of hydrolytic activity remains. In particular with more extended glycan structures such activity can seriously compromise synthetic efficiency.

In an attempt to decrease the natural hydrolytic activity of the enzymes both Wang[9m] and ourselves[8d] have used site-directed mutagenesis to produce 'glycosynthase'[10]-like mutants of Endo A, whilst Wang and Yamamoto have also reported a similar engineering of Endo M.[9g] Indeed recently reported crystallographic data are now available for both wild-type (WT)[11] and Endo A mutants[12] which may facilitate such future optimisation of enzyme production. Although such approaches do result in a further increase in achievable synthetic yield, in the majority of cases, particularly with extended donors, reactions are still far from efficient. Herein the use of multiple additions of an excess of oxazoline donor may be required to achieve a good yield of product. Continued limitation of synthetic efficiency arises because, although mutant

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enzymes may not be able to hydrolyse the products of these glycosylation reactions, they unfortunately can still catalyse direct hydrolysis of the oxazoline donors themselves.

Previous reports on the use of Endo A[13] and Endo M[14] for transglycosylation using asparagine-linked donors have detailed that increased synthetic efficiency could be achieved by the addition of an organic co-solvent to these aqueous reaction mixtures. Since the increased efficiency thereby observed was presumably due to a decrease in the rate of competitive hydrolytic processes, then the addition of quantities of an organic co-solvent to other synthetic reactions catalysed by endohexosaminidases would appear to be a promising avenue for investigation. Moreover throughout the numerous reports on the uses of oxazolines as donors for endohexosaminidase-catalysed glycosylations several reaction parameters have been varied without a specific comment. One of the most notable of these is the reaction pH. The pH/rate dependency for the hydrolytic activity of hexosaminidases is typically a bell-shaped curve centred around pH 5.0–8.0, the precise pH optimum depending on the enzyme.[15] The upper and lower limits of this bell-shaped curve generally correspond with the kinetic p*K*a values of the two catalytic acid residues in the enzyme active site. For example, the pH optimum for Endo H is pH 5.5, and its hydrolytic activity drops rapidly as the pH is either increased from pH 6.0 to pH 8.0, or is decreased from pH 5.0 to pH 4.0.[16] Although family GH85 endohexosaminidasses, such as Endo M and Endo A, possess only one key catalytic acid residue in the active site (E173 for Endo A and E177 for Endo M) similar pH/rate profiles have generally been observed for their hydrolytic activity.[2b,15d]

The non-enzyme-catalysed hydrolysis of carbohydrate oxazolines under acidic conditions in aqueous solution is well established, obviating the use of an oxazoline in any reaction performed at a pH below that of the lower limit of the enzyme's bell-shaped pH/rate curve.[17] However since the hydrolytic activity of these enzymes is dependent on protonation of the glycosidic oxygen by the key catalytic carboxylic acid residue, then moving to a higher reaction pH should curtail the rate of product hydrolysis, in correspondence with the upper limit on the pH/rate curve. In addition an increase in reaction pH may not overly affect the rate of a synthetic reaction employing an oxazoline donor, and thus a further kinetic advantage may be obtained. Moreover working at higher reaction pH will also increase the lifetime of an oxazoline donor in the reaction medium by a reduction in the rate of non-enzyme-catalysed hydrolysis. In light of these possibilities it was considered that an increase in reaction pH employing oxazolines as activated donors may perhaps give a further kinetic advantage to synthetic processes over competing hydrolytic ones. This report therefore details investigations that were undertaken into the effects that both the addition of an organic solvent and variation of reaction pH had on the synthetic efficiency of endohexosaminidase-catalysed glycosylation with oxazolines as donors.

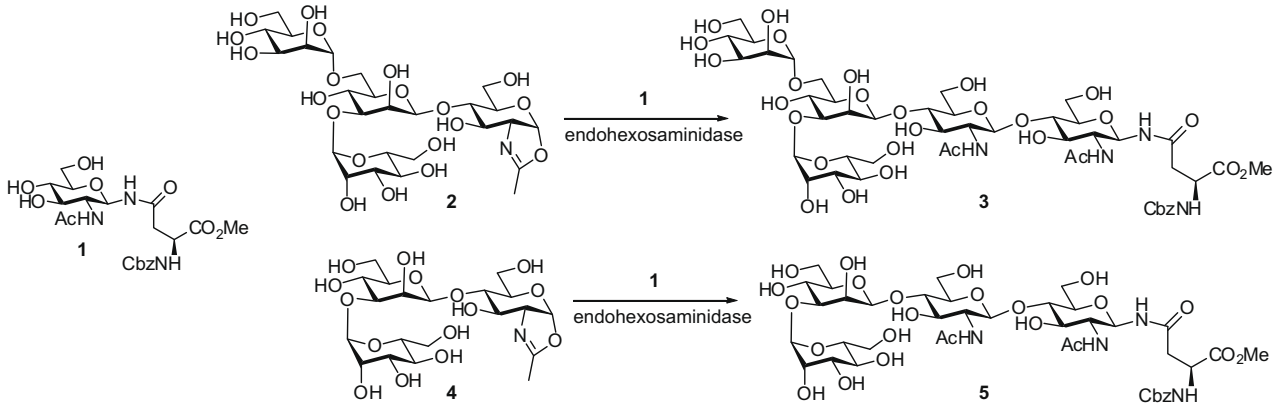
2. Results and discussion

Glycosylation of the glycosyl amino acid acceptor **1** using the tetrasaccharide oxazoline donor **2** to produce the pentasaccharide glycosyl amino acid **3** was used as the standard reaction for investigation of the dependency of synthetic efficiency on reaction parameters (Scheme 1). In addition, for certain reactions catalysed by Endo M the trisaccharide oxazoline donor **4** was also used for glycosylation of **1**, resulting in the formation of tetrasaccharide glycosyl amino acid **5**.

2.1. Effect of an organic co-solvent

The inclusion of an organic co-solvent in the reaction medium has frequently been used to increase the synthetic efficiency of glycosidase-catalysed processes;[18] the two most commonly employed organic co-solvents in this respect being dimethylsulfoxide (DMSO) and acetone. Indeed as discussed previously, the addition of quantities of an organic co-solvent has been shown to improve the synthetic efficiency of both Endo A-[8] and Endo M-[14]catalysed transglycosylation reactions using asparagine-linked N-glycan oligosaccharides as donors. However it is notable that in some of these reactions large excesses of glycosyl acceptor were required. For example, using Endo A, Lee and co-workers typically[8] employed more than 1000 equiv of a GlcNAc acceptor relative to the quantity of the $Man_9GlcNAc_2Asn$ donor used. In contrast, the more modest yields reported by Yamanoi[14] and co-workers for Endo M corresponded to the use of only 2 equiv of a sialylglycopeptide donor. In both cases it was proposed that the improvements in yield were due to both an enhancement of the rate of the transglycosylation process itself and a reduction of the rate of product and starting material hydrolysis by decreasing the effective water concentration. It should be noted, however, that the precise identity of the organic co-solvent was also found to have a differential effect on the extent of transglycosylation, indicating a more subtle role over and above that of simply decreasing effective water concentration. For example, the use of DMF as an additive enhanced transglycosylation activity for Endo A,[8] but completely destroyed such activity for Endo M.[14]

The effect of the addition of an organic co-solvent on the synthetic efficiency of processes carried out using oxazoline donor **1** was investigated using WT Endo A, Endo M, and also two available Endo A mutants, E173H and E173Q. DMSO was selected as a repre-



Scheme 1.

sentative organic co-solvent for these comparative studies, since its use had previously been shown to have a beneficial effect on the synthetic efficiency of transglycosylation for both Endo A and Endo M. A series of experiments was undertaken in which the yield of product **3**, arising from glycosylation of **1** by **2**, was monitored as a function of time. Time course studies of synthetic reactions catalysed by WT Endo A, the E173H and E173Q mutants and Endo M performed in the presence of varying quantities of DMSO as an organic co-solvent in phosphate buffer at pH 6.5 are shown in Figure 1.

In the case of WT Endo A (Fig. 1A) the addition of 5% DMSO by volume to the reaction medium led to a significant improvement in the efficiency of the reaction. Herein quantitative conversion to product was observed after 30 min and no significant amount of product hydrolysis was observed until after 3 h reaction time. After this time slow product hydrolysis could be observed. This result contrasted with the reaction performed in the absence of DMSO; a maximum yield of 88% was observed after 30 min, after which time rapid product hydrolysis ensued. The addition of as little as 5% by volume DMSO as an organic co-solvent therefore resulted in an improved efficiency of the glycosylation process primarily because of a marked decrease in the rate of product hydrolysis. Further experiments in which the DMSO content was increased to 10% and 15% led to further small decreases in the rate of product hydrolysis, but also to corresponding decreases in the rate of the synthetic reaction. For example, with 15% v/v DMSO quantitative product formation was not observed until after 60 min.

The addition of DMSO as an organic co-solvent resulted in a reduction of the rate of synthetic processes catalysed by both the Endo A mutants. In the absence of DMSO the E173H mutant catalysed production of **3** in 99% yield after 150 min; subsequent product hydrolysis was, as expected based on the designed incorporated mutation, extremely slow and only occurred in a significant amount after 6 h. The addition of 5% DMSO to the reaction mixture resulted in a slower synthetic reaction; 98% conversion to product was only achieved after 520 min. This yield of product then remained essentially constant, dropping slightly to 96% after three days (Fig. 1B). Increasing the DMSO content further to 10% v/v and then to 15% v/v led to progressive further reductions in the rate of the synthetic reaction. However no reduction was observed in the maximum achievable yield of the synthetic reaction, and significantly product hydrolysis was also completely curtailed in these cases. In the absence of DMSO as an organic co-solvent the E173Q mutant was able to catalyse the glycosylation reaction in

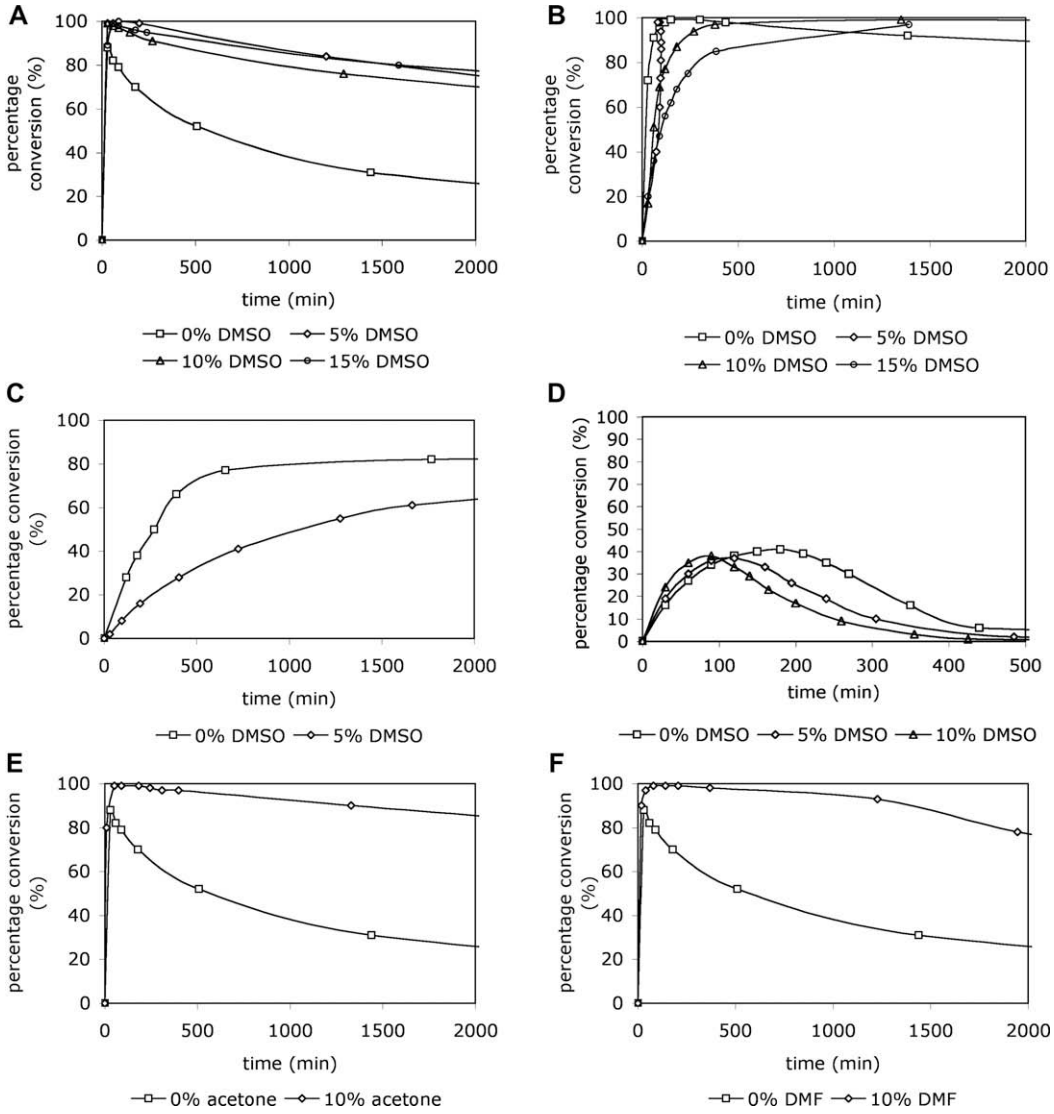


Figure 1. Time correlations of product yield for glycosylations of acceptor **1** with donor **2** in the presence of an organic co-solvent with (A) WT Endo A with DMSO; (B) Endo A E173H with DMSO; (C) Endo A E173Q with DMSO; (D) WT Endo M with DMSO; (E) WT Endo A with acetone and (F) WT Endo A with DMF.

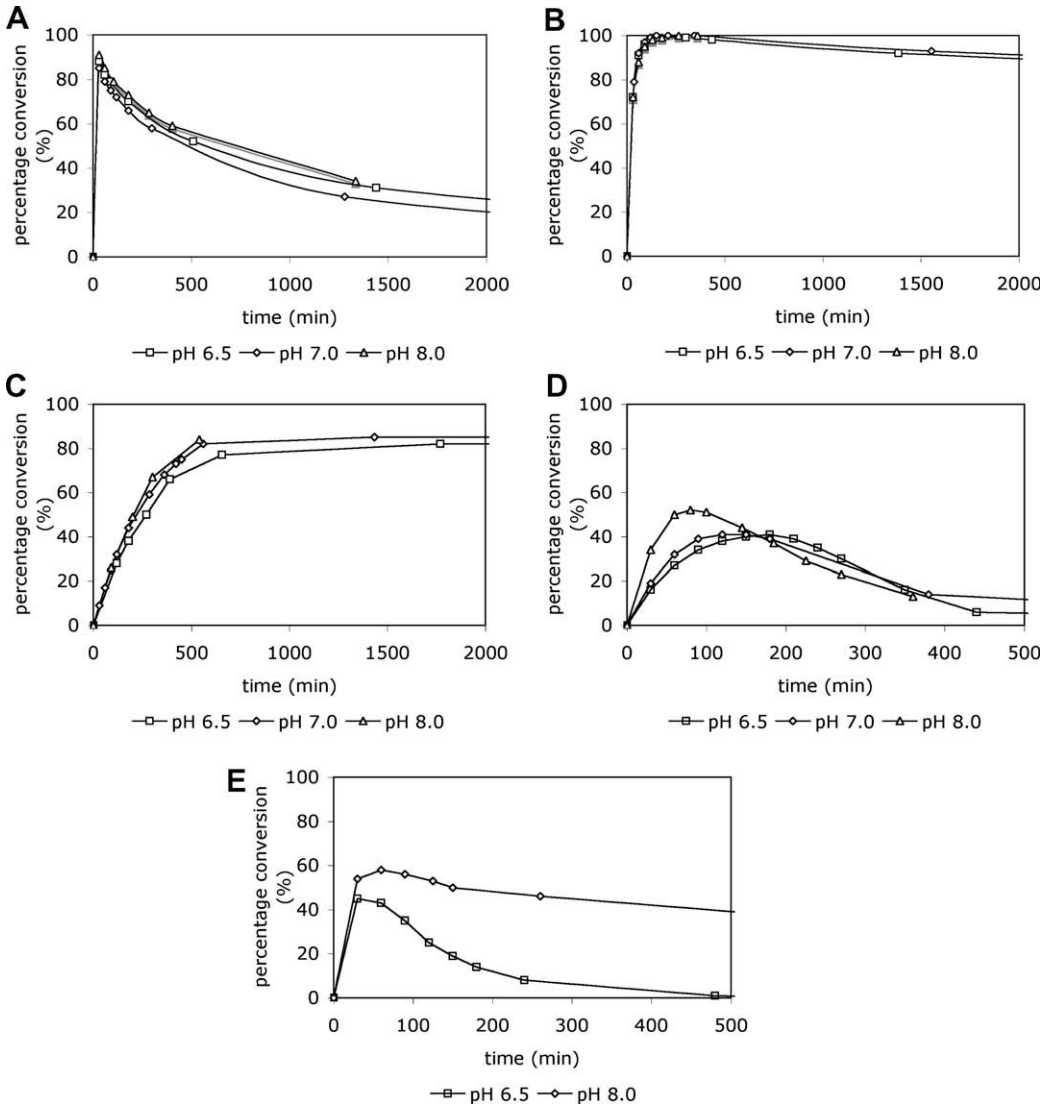


Figure 2. Time correlations of product yield for glycosylation of acceptor **1** at varying pH with: (A) WT Endo A and donor **2**; (B) Endo A E173H and donor **2**; (C) Endo A E173Q and donor **2**; (D) WT Endo M and donor **2**; and (E) WT Endo M and donor **4**.

82% in an irreversible fashion; the mutant enzyme being completely incapable of hydrolysing the product. However the synthetic reaction was itself slow, and took around 30 h to reach this maximum value (Fig. 1C). The addition of 5% DMSO by volume as a co-solvent again led to a further decrease in the rate of the synthetic reaction and correspondingly to a decrease in the maximum amount of **3** produced (73% yield after 90 h). The lower synthetic efficiency observed in the latter case can be attributed to direct competitive hydrolysis of the oxazoline donor itself during the extremely protracted reaction.

The results obtained with Endo A contrasted markedly with those obtained with Endo M (Fig. 1D).[†] In the case of Endo M an increase in the DMSO content actually led to a slight *increase* in the rate of the synthetic reaction. However correspondingly the rate of product hydrolysis was also *increased* by the addition of DMSO. These observations using oxazoline **2** as the donor are in fact somewhat contradictory to the results previously reported by Yamanoi and co-workers.[14] In that study the rate of transglycosylation reactions catalysed by Endo M using a complex biantennary sialoglycopeptide (SGP) as the donor was not significantly affected by the addition of an organic co-solvent in quantities of 30% or lower by volume. Moreover Yamanoi and co-workers[14] reported that the rate of product hydrolysis was reduced by the addition of an organic co-solvent, resulting in a higher achievable yield. In the present study with oxazoline **2** as the donor the maximum yields obtained using Endo M as a catalyst were 41% after 3 h (no added DMSO), 37% after 2 h (5% DMSO) and 38% after 90 min (10% DMSO). The maximum yield obtainable was therefore not altered by the addition of an organic co-solvent, but the rate by which it was achieved was increased with increased DMSO content. Likewise the rate of product hydrolysis also showed a small increase with increased DMSO content.

Finally two other co-solvents were investigated for the WT Endo A-catalysed glycosylation of acceptor **1** by donor **2**. In line with the previous report on the effect of added co-solvents for Endo A-catalysed transglycosylation using Asn-linked donors[8] the use of either 10% acetone by volume (Fig. 1E) or 10% DMF by volume (Fig. 1F) was found to have a beneficial effect; in both cases the rate of the synthetic reaction was not greatly affected, but the rate of product hydrolysis was significantly diminished. Indeed product hydrolysis was most retarded with 10% acetone as the

[†] It should also be noted that the unit activity definitions of Endo A and Endo M used in this study are different. See Section 4 for precise definitions.

co-solvent; quantitative product formation was observed after 50 min (Fig. 1E), and less than 3% hydrolysis was observed until after 400 min. It can therefore be concluded that the use of 10% acetone by volume probably represents the optimal reaction solvent for WT Endo A-catalysed glycosylation using oxazoline donors.

2.2. Effect of reaction pH

Published data for Endo M[2b] reveal a pH rate maximum for hydrolytic activity in the range pH 6.0–6.5; at higher pH the activity dropped markedly. In contrast, the pH rate profile of Endo A was reported to have a very broad optimum, most active in the range pH 5.0–9.0, though the rate of hydrolysis was little reduced even at pH 11.[3c] Furthermore the optimum pH for Endo A-catalysed transglycosylation using asparagine-linked donors has been reported to be pH 6.0, though no precise data were shown.[3b] An investigation was undertaken to elucidate how synthetic efficiency varied with reaction pH for both of these enzymes using oxazolines as donors, the rationale being that moving to higher reaction pH may curtail the hydrolytic activity of the enzyme. Figure 2 shows the pH dependency of glycosylation reactions catalysed by WT Endo A (Fig. 2A) and the two mutants E173H (Fig. 2B) and E173Q (Fig. 2C). As can be seen there was no significant variation in product yield, or even in the time dependency of yield, as the pH was increased from 6.5 to 8.0.[‡] This result reflects the 'broad pH optimum' that was described for the hydrolytic activity of Endo A.[3c] The only minor variation observed in these comparative experiments was in the case of the E173Q Endo A mutant, where an increase in reaction pH led to a small increase in product yield. Unfortunately it was not possible to extend the current study to include reactions performed above pH 8.0 since decomposition of the acceptor **1** occurred at these higher pH values.

In contrast to the results obtained for Endo A, the synthetic efficiency of glycosylation reactions catalysed by Endo M, also shown in Figure 2, showed a more marked dependence on reaction pH. At pH 6.5 glycosylation of acceptor **1** with donor **2** produced **3** in a maximum yield of 41% after 3 h; after this time product hydrolysis rapidly decreased the synthetic efficiency of the reaction (Fig. 2D). At pH 7.0 pentasaccharide **3** was produced more rapidly; the maximum yield of 41% was reached after 2 h, though again after this time product hydrolysis was rapid. Increasing the pH further to 8.0 led to a further increase in the maximum obtainable yield of **3** (52% after 80 min) though again after this time product hydrolysis led to a rapid decrease in yield. These results indicated that, at least for Endo M, increasing the reaction pH increased the rate of the synthetic reaction relative to that of competitive hydrolytic processes. The fact that the synthetic reaction appeared to be accelerated at higher pH is somewhat surprising, particularly since a previous report indicated that the hydrolytic activity of Endo M should be approximately 50% lower at pH 8.0 than at pH 6.5.[2b] However Figure 2D clearly shows that an increase in pH resulted in increases in both the rate of product formation and the maximum achievable yield.

Puzzled by this somewhat unexpected result, a second series of reactions was carried out using the (1→3)-linked trisaccharide oxazoline **4** as the glycosyl donor, again with glycosyl amino acid **1** as the acceptor (Fig. 2E), to produce tetrasaccharide glycosyl amino acid **5**. At pH 6.5 the maximum yield of product **5** observed was 45%; this maximum was reached after only 30 min and after this time very rapid product hydrolysis ensued. When a similar reaction was conducted at pH 8.0 the rate of product formation was similar, though significantly the rate of product hydrolysis was dramatically decreased, in line with the expected reduction in hydrolytic activity of Endo M at this higher pH. Thus an increased maximum yield of 58% of **5** was achieved after 60 min, and after 500 min 40% of the product **5** was actually still present in the reaction mixture. By way of comparison at pH 6.5 effectively all of **5** had been hydrolysed after this time. These results suggest that in the case of the (1→3)-linked trisaccharide donor **4** the low yield of the transglycosylation product is primarily due to competitive product hydrolysis, and that the rate of this hydrolysis reaction is suppressed at higher pH in a similar manner. It can be concluded therefore that whilst close control of reaction pH in the range 6.5–8.0 is not critical for obtaining the maximum synthetic yield for reactions catalysed by Endo A, working at higher pH does increase the synthetic efficiency of reactions catalysed by Endo M. These results are broadly in line with the different pH/rate dependences and maxima previously described for these two enzymes.

3. Conclusions

The two sets of comparative experiments with WT Endo M and WT Endo A (taken together with the E173H and E173Q mutants) indicate subtle yet significant differences between the two enzymes. In the first instance the addition of DMSO as an organic co-solvent had a far greater and opposite effect on reactions catalysed by Endo A as opposed to those catalysed by Endo M. In this case Endo A-catalysed reactions (both synthetic and hydrolytic) were slowed by increased amounts of an organic co-solvent, whilst for Endo M the rates of both processes were actually slightly enhanced. The use of either 10% acetone or 10% DMF with WT Endo A was also found to be particularly useful; in both cases the rate of the synthetic reaction was not greatly affected, but the rate of product hydrolysis was greatly diminished. Secondly variation of the reaction pH from 6.5 to 8.0 had little effect on reactions catalysed by Endo A, or its mutants, in line with the broad pH/rate profile described for Endo A.[3c] However, increased reaction pH had a significant effect on reactions catalysed by Endo M, and led to a substantial improvement in product yield, particularly with one of the two oxazoline donors used. This result is in line with the strong pH rate dependence described for Endo M, with maximal hydrolytic activity being observed in the range pH 6.0–6.5.[2b] Extension of investigations to include higher reaction pH, which may prove beneficial for both enzymes, was unfortunately not possible in the current study due to instability of the glycosyl acceptor **1**. Subtle differences between Endo A and Endo M therefore mean that it is not possible to arrive at a definitive set of reaction conditions that will be optimal for any GH85 family endohexosaminidase-catalysed process. However it is clear that fine tuning of reaction pH or the addition of quantities of an organic co-solvent may result in beneficial increases in synthetic efficiency by effecting a reduction in the rate of competitive hydrolytic processes.

4. Experimental

4.1. General

HPLC grade dimethylsulfoxide (DMSO), acetone and dimethyl formamide (DMF) were purchased from Sigma–Aldrich. Glycosyl amino acid acceptor **1**[8a] and oxazoline donors **2**[8c] and **3**[8c] were synthesised as described previously. Recombinant Endo M was supplied by Professor Kenji Yamamoto (Kyoto). Recombinant Endo A and the Endo A E173H and E173Q mutants were produced using the pET23d-Endo-A plasmid supplied by Professor Kaoru Takegawa (Kagawa), as previously described.[8d] Unit definitions of enzyme activity used in this report are as follows. Endo A activity was

[‡] Quantitative analysis of experiments performed at pH 8.0 for reaction times of greater than 7 h was not always possible due to acceptor decomposition; broad peaks developed in the HPLC trace obviating accurate peak integration.

assayed with RNase B as the substrate, applying the unit definition of Endo H activity provided by New England Biolabs. One unit of Endo A is defined as the amount of enzyme required to remove >95% of the carbohydrate from 10 μg of denatured RNase B in a total reaction volume of 10 μL in 1 h at 37 °C. Endo M activity was assigned as supplied by Professor Yamamoto; 1 unit of Endo M is defined as the amount of enzyme yielding 1 μmol of GlcNAc-Asn-DNS from $Man_6GlcNAc_2$-Asn-DNS in 1 min at 37 °C.

4.2. Analysis of enzymatic glycosylation

Endohexosaminidase-catalysed glycosylations were monitored by HPLC 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 5μ C-18 column, 250 × 4.6 mm) was used to monitor the reactions, with 2 μL aliquots taken at appropriate time intervals. The column was eluted with 22% MeCN/H_2O. The product yield was determined by integration of the product and acceptor peaks.

4.3. Typical procedures for enzymatic glycosylation

4.3.1. Method 1: variation of co-solvent

Glycosyl amino acid acceptor **1** (100 μg, 621 nmol) and oxazoline donor **2** (3 equiv) 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: 50 μL; overall buffer concentration 0.1 M). Endo M (10 mU) or Endo A (50 mU) or mutant enzyme (5 μg) was added and the temperature was maintained at 23 °C. Reaction progress was monitored by HPLC analysis and the product yield was determined by integration of the product and acceptor peaks.

4.3.2. Method 2: variation of reaction pH

Glycosyl amino acid acceptor **1** (100 μg, 621 nmol) and oxazoline donor (**2** or **4**, 3 equiv) were dissolved in sodium phosphate buffer (0.1 M solution of appropriate pH, total reaction volume: 50 μL). Endo M (10 mU) or Endo A (50 mU) or mutant enzyme (5 μg) was added and the temperature was maintained at 23 °C. Reaction progress was monitored by HPLC analysis and the product yield was determined by integration of the product and acceptor peaks.

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References

1. Rich, J. R.; Withers, S. G. *Nat. Chem. Biol.* **2009**, *5*, 206–215.
2. (a) Kadowaki, S.; Yamamoto, K.; Fujisaki, M.; Kumagai, H.; Tochikura, T. *Agric. Biol. Chem.* **1988**, *52*, 2387–2389; (b) Kadowaki, S.; Yamamoto, K.; Fujisaki, M.; Izumi, K.; Tochikura, T.; Yokoyama, T. *Agric. Biol. Chem.* **1990**, *54*, 97–106; (c) Yamamoto, K.; Kadowaki, S.; Watanabe, J.; Kumagai, H. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 244–252; (d) Haneda, K.; Inazu, T.; Yamamoto, K.; Kumagai, H.; Nakahara, Y.; Kobata, A. *Carbohydr. Res.* **1996**, *292*, 61–70; (e) Yamamoto, K.; Fujimori, K.; Haneda, K.; Mizuno, M.; Inazu, T.; Kumagai, H. *Carbohydr. Res.* **1998**, *305*, 415–422; (f) Mizuno, M.; Haneda, K.; Iguchi, R.; Muramoto, I.; Kawakami, T.; Aimoto, S.; Yamamoto, K.; Inazu, T. *J. Am. Chem. Soc.* **1999**, *121*, 284–290; (g) Fujita, K.; Kobayashi, K.; Iwamatsu, A.; Takeuchi, M.; Kumagai, H.; Yamamoto, K. *Arch. Biochem. Biophys.* **2004**, *432*, 41–49.
3. (a) Takegawa, K.; Nakoshi, M.; Iwahara, S.; Yamamoto, K.; Tochikura, T. *Appl. Environ. Microbiol.* **1989**, *55*, 3107–3112; (b) Takegawa, K.; Yamaguchi, S.; Kondo, A.; Kato, I.; Iwahara, S. *Biochem. Int.* **1991**, *25*, 829–835; (c) Takegawa, K.; Tabuchi, M.; Yamaguchi, S.; Kondo, A.; Kato, I.; Iwahara, S. *J. Biol. Chem.* **1995**, *270*, 3094–3099; (d) Takegawa, K.; Yamabe, K.; Fujita, K.; Tabuchi, M.; Mita, M.; Izu, H.; Watanabe, A.; Asada, Y.; Sano, M.; Kondo, A.; Kato, I.; Iwahara, S. *Arch. Biochem. Biophys.* **1997**, *338*, 22–28; (e) Fujita, K.; Takegawa, K. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 680–686.
4. http://www.cazy.org/fam/GH85.html.
5. For some leading references on synthetic applications of Endo A see: (a) Fan, J.-Q.; Quesenberry, M. S.; Takegawa, K.; Iwahara, S.; Kondo, A.; Kato, I.; Lee, Y. C. *J. Biol. Chem.* **1995**, *270*, 17730–17735; (b) Fan, J.-Q.; Huynh, L. H.; Reinhold, B. B.; Reinhold, V. N.; Takegawa, K.; Iwahara, S.; Kondo, A.; Kato, I.; Lee, Y. C. *Glycoconjugate J.* **1996**, *13*, 643–652; (c) Takegawa, K.; Fujita, K.; Fan, J.-Q.; Tabuchi, M.; Tanaka, N.; Kondo, A.; Iwamoto, H.; Kato, I.; Lee, Y. C.; Iwahara, S. *Anal. Biochem.* **1998**, *257*, 218–223; (d) Fujita, K.; Tanaka, N.; Sano, M.; Kato, I.; Asada, Y.; Takegawa, K. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 134–138.
6. (a) Yamamoto, K.; Fujimori, K.; Haneda, K.; Mizuno, M.; Inazu, T.; Kumagai, H. *Carbohydr. Res.* **1997**, *305*, 415–422; (b) Haneda, K.; Inazu, T.; Mizuno, M.; Iguchi, R.; Yamamoto, K.; Kumagai, H.; Aimoto, S.; Suzuki, H.; Noda, T. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1303–1306; (c) Yamanoi, T.; Tsutsumida, M.; Oda, Y.; Akaike, E.; Osumi, K.; Yamamoto, K.; Fujita, K. *Carbohydr. Res.* **2004**, *339*, 1403–1406; (d) Osumi, K.; Makino, Y.; Akaike, E.; Yamanoi, T.; Mizuno, M.; Noguchi, M.; Inazu, T.; Yamamoto, K.; Fujita, K. *Carbohydr. Res.* **2004**, *339*, 2633–2635; (e) Yamanoi, T.; Yoshida, N.; Oda, Y.; Akaike, E.; Tsutsumida, M.; Kobayashi, N.; Osumi, K.; Yamamoto, K.; Fujita, K.; Takahashi, K.; Hattori, K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1009–1013; (f) Haneda, K.; Takeuchi, M.; Tagashira, M.; Inazu, T.; Toma, K.; Isogai, Y.; Hori, M.; Kobayashi, K.; Takeuchi, M.; Takegawa, K.; Yamamoto, K. *Carbohydr. Res.* **2006**, *341*, 181–190; (g) Makimura, Y.; Watanabe, S.; Suzuki, T.; Suzuki, Y.; Ishida, H.; Kiso, M.; Katayama, T.; Kumagai, H.; Yamamoto, K. *Carbohydr. Res.* **2006**, *341*, 1803–1808; (h) Murakami, D.; Shimada, Y.; Kamiya, S.; Yamazaki, K.; Makimura, Y.; Ito, K.; Minamiura, N.; Yamamoto, K. *Arch. Biochem. Biophys.* **2008**, *477*, 299–304.
7. Fujita, M.; Shoda, S.-I.; Haneda, K.; Inazu, T.; Takegawa, K.; Yamamoto, K. *Biochim. Biophys. Acta* **2001**, *1528*, 9–14.
8. (a) Rising, T. W. D. F.; Claridge, T. D. W.; Davies, N.; Gamblin, D. P.; Moir, J. W. B.; Fairbanks, A. J. *Carbohydr. Res.* **2006**, *341*, 1574–1596; (b) Rising, T. W. D. F.; Claridge, T. D.; Moir, J. W. B.; Fairbanks, A. J. *ChemBioChem* **2006**, *7*, 1177–1180; (c) Rising, T. W. D. F.; Heidecke, C. D.; Moir, J. W. B.; Ling, Z.; Fairbanks, A. J. *Chem. Eur. J.* **2008**, *14*, 6444–6464; (d) Heidecke, C. D.; Ling, Z.; Bruce, N. C.; Moir, J. W. B.; Parsons, T. B.; Fairbanks, A. J. *ChemBioChem* **2008**, *9*, 2045–2051; (e) Parsons, T.; Moir, J. W. B.; Fairbanks, A. J. *Org. Biomol. Chem.* **2009**, *7*, 3128–3140.
9. (a) Li, B.; Zeng, Y.; Hauser, S.; Song, H. J.; Wang, L.-X. *J. Am. Chem. Soc.* **2005**, *127*, 9692–9693; (b) Li, H.; Li, B.; Song, H.; Breydo, L.; Baskakov, I. V.; Wang, L.-X. *J. Org. Chem.* **2005**, *70*, 9990–9996; (c) Wang, L.-X.; Song, H. J.; Liu, S. W.; Lu, H.; Jiang, S. B.; Ni, J. H.; Li, H. G. *ChemBioChem* **2005**, *6*, 1068–1074; (d) Li, H.; Singh, S.; Zeng, Y.; Song, H.; Wang, L.-X. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 895–898; (e) Zeng, Y.; Wang, J. S.; Li, B.; Hauser, S.; Li, H. G.; Wang, L.-X. *Chem. Eur. J.* **2006**, *12*, 3355–3364; (f) Li, B.; Song, H.; Hauser, S.; Wang, L.-X. *Org. Lett.* **2006**, *8*, 3081–3084; (g) Umekawa, M.; Huang, W.; Li, B.; Fujita, K.; Ashida, H.; Wang, L.-X.; Yamamoto, K. *J. Biol. Chem.* **2008**, *283*, 4469–4479; (h) Wang, L.-X. *Carbohydr. Res.* **2008**, *343*, 1509–1522; (i) Huang, W.; Ochiai, H.; Zhang, X.; Wang, L.-X. *Carbohydr. Res.* **2008**, *343*, 2903–2913; (j) Wei, Y.; Li, C.; Huang, W.; Li, B.; Strome, S.; Wang, L.-X. *Biochemistry* **2008**, *47*, 10294–10304; (k) Ochiai, H.; Huang, W.; Wang, L.-X. *J. Am. Chem. Soc.* **2008**, *130*, 13790–13803; (l) Ochiai, H.; Huang, W.; Wang, L.-X. *Carbohydr. Res.* **2009**, *344*, 592–598; (m) Huang, W.; Li, C.; Li, B.; Umekawa, M.; Yamamoto, K.; Zhang, X.; Wang, L.-X. *J. Am. Chem. Soc.* **2009**, *131*, 2214–2223.
10. (a) Mackenzie, L. F.; Wang, Q. R.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **1998**, *120*, 5583–5584; (b) Malet, C.; Planas, A. *FEBS Lett.* **1998**, *440*, 208–212.
11. Yin, J.; Li, L.; Shaw, N.; Li, Y.; Song, J. K.; Zhang, W.; Xia, C.; Zhang, R.; Joachimiak, A.; Zhang, H.-C.; Wang, L.-X.; Liu, Z.-J.; Wang, P. *PLoS ONE* **2009**, *4*, e4658.
12. PDB code 2vtf. See: Suits, M. D. L.; Ling, Z.; Bingham, R. J.; Bruce, N. C.; Davies, G. J.; Fairbanks, A. J.; Moir, J. W. B.; Taylor, E. J. *J. Mol. Biol.* **2009**, *389*, 1–9.
13. Fan, J.-Q.; Takegawa, K.; Iwahara, S.; Kondo, A.; Kato, I.; Abeyguanawardana, C.; Lee, Y. C. *J. Biol. Chem.* **1995**, *270*, 17723–17729.
14. (a) Akaike, E.; Tsutsumida, M.; Osumi, K.; Fujita, M.; Yamanoi, T.; Yamamoto, K.; Fujita, K. *Carbohydr. Res.* **2004**, *339*, 719–722; (b) Akaike, E.; Yamanoi, T. *Trends Glycosci. Glyc.* **2006**, *18*, 63–71.
15. (a) Synstad, B.; Gaseidnes, S.; Van Aalten, D. M.; Vriend, G.; Nielsen, J. E.; Eijsink, V. G. *Eur. J. Biochem.* **2004**, *271*, 253–262; (b) Williams, S. J.; Mark, B. L.; Vocadlo, D. J.; James, M. N.; Withers, S. G. *J. Biol. Chem.* **2002**, *277*, 40055–40065; (c) Cetinbas, N.; Macauley, M. S.; Stubbs, K. A.; Drapala, R.; Vocadlo, D. J. *Biochemistry* **2006**, *45*, 3835–3844; (d) Abbott, D. W.; Macauley, M. S.; Vocadlo, D. J.; Boraston, A. B. *J. Biol. Chem.* **2009**, *284*, 11676–11689.
16. Schmidt, B. F.; Ashizawa, E.; Jarnagin, A. S.; Lynn, S.; Noto, G.; Woodhouse, L.; Estell, D. A.; Lad, P. *Arch. Biochem. Biophys.* **1994**, *311*, 350–353.
17. Jha, R.; Davis, J. T. *Carbohydr. Res.* **1995**, *277*, 125–134.
18. (a) Yoon, J. H.; Rhee, J. S. *Carbohydr. Res.* **2000**, *327*, 377–383; (b) Crout, D. H.; Vic, G. *Curr. Opin. Chem. Biol.* **1998**, *2*, 98–111; (c) Vic, G.; Thomas, D.; Crout, D. H. G. *Enzyme Microb. Tech.* **1997**, *20*, 597–603; (d) Sauerbrei, B.; Thiem, J. *Tetrahedron Lett.* **1992**, *33*, 201–204; (e) Guisan, J. M.; Rodriguez, V.; Soler, G.; Santana, C.; Fernandez-Lafuente, R.; Bastida, A.; Rosell, C. M.; Lopez, R.; Fernandez-Mayoralas, A.; Martin-Lomas, M. *J. Mol. Catal.* **1993**, *84*, 373–379; (f) Petit, J.-M.; Paquet, F.; Beau, J.-M. *Tetrahedron Lett.* **1991**, *32*, 6125–6128.