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α-1,4-Glucan Lyase Performs a Trans-Elimination via a Nucleophilic Displacement Followed by a Syn-Elimination

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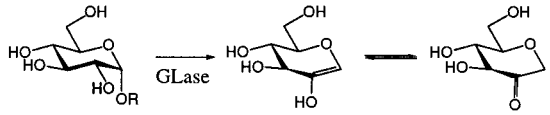
α-1,4-Glucan lyase (EC 4.2.2.13, GLase), which occurs widely in eukaryotes, degrades α-1,4-glucans and maltooligosaccharides via a nonhydrolytic pathway to release 1,5-D-anhydrofructose (1) from the nonreducing end (Scheme 1).[1] The enzyme is mechanistically interesting since, in contrast to other polysaccharide lyases which cleave uronic acid-containing polymers via an elimination reaction of the C4−O1′ bond facilitated by the acidity of the proton adjacent to the acid, no such activation is available in α-glucans. Nonetheless, GLases cleave the bond between C1′ and O1′ of the nonreducing sugar residue of α-glucans to generate a monosaccharide product with a double bond between C1′ and C2′.[1] In this communication we present a mechanistic study of the GLase including the trapping of a covalent glycosyl-enzyme intermediate and measurement of kinetic isotope effects.

Several lines of evidence indicate that GLases are related to retaining α-glucosidases.[1,2,3] For example, oxocarbenium ion-like transition states are suggested on the basis of inhibition by the known α-glucosidase inhibitors, acarbose and 1-deoxynojirimycin, while inactivation by a carbodiimide suggests a role for a carboxylic acid.[2,3] Amino acid sequence analysis reveals that GLases share sequence similarity with retaining α-glucosidases of glycoside hydrolase family 31.[1] These enzymes employ a two-step catalytic mechanism involving the formation and hydrolysis of a covalent glycosyl-enzyme intermediate via oxocarbenium ion-like transition states.[4] Two carboxylic acids are involved in this mechanism, one acting as a nucleophile, the other as an acid/base catalyst. If the GLase closely mimics these glycosidases, its mechanism may involve the formation of a covalent glycosyl-enzyme intermediate via an oxocarbenium ion-like transition state followed by an E1-type syn-elimination. Alternatively, the GLase may directly cleave the substrate via a trans-elimination mechanism that likely involves substantial E1 character. Distinction between these two options could be achieved via a search for a covalent glycosyl enzyme intermediate and through measurement of kinetic isotope effects with substrates deuterated at C1 and C2.

The mechanism-based glycosidase inactivators, 5-fluoro-α-D-glucopyranosyl fluoride (5FGlcF) and 5-fluoro-β-L-idopyranosyl fluoride (5FIdoF), have been used to trap covalent glycosyl-enzyme intermediates on α-glucosidases.[5−7] In many cases, epimers of fluorosugars inverted at C5 have turned out to be more effective and indeed 5FIdoF is the more potent α-glucosidase inactivator.[6,8] Thus, α-1,4-glucan lyase from *Gracilariopsis* was incubated with 5FIdoF. Time-dependent inactivation according to pseudo-first-order kinetics was observed, yielding an inactivation rate constant, k_i, of 0.61 ± 0.02 min^{-1} and an inactivator dissociation constant, K_i, of

Scheme 1



1

28.3 ± 2.1 mM. Active site binding of the inactivator is shown by protection experiments in which the competitive inhibitor acarbose (0.2 μM, $K_i = 0.02$ μM) reduced the pseudo-first-order rate constant for inactivation by 30 mM 5FIdoF from 0.31 min^{-1} to 0.055 min^{-1}, consistent with the value predicted on the basis of competitive binding ($k = 0.053$ min^{-1}). Further, the catalytic competence of the intermediate is indicated by the first-order reactivation observed ($k_{react.} = 0.036$ min^{-1}) after removal of excess inactivator.

Fully inactivated GLase along with a control sample was subjected to peptic digestion followed by LC/ESI MS[9] comparative mapping, which revealed that a peptide fragment corresponding to *m*/*z* 1206 was detected only in the inactivated sample while a fragment of *m*/*z* 1025, lower in mass by the amount expected for the 5-fluoroidosyl moiety (*m*/*z* 181), was detected in the control sample. This strongly suggests that the fragment of *m*/*z* 1206 is the active site peptide labeled by 5FIdoF and therefore contains the catalytic nucleophile. This fragment was isolated by LC and sequenced by ESI MS/MS (Figure 1). The daughter ion spectrum (Figure 1b) reveals a fragment corresponding to mass 1025 (mass difference of *m*/*z* 181 from the parent ion) arising from the peptide without label. However, interpretation of the rest of the fragmentation pattern was not fruitful. Fortunately, an excellent fragmentation pattern was observed in the daughter ion spectrum of the species of *m*/*z* 1025 generated by orifice fragmentation (Figure 1a). This readily yielded a sequence of FVWQDMTV and revealed that the uninterpretable peaks in Figure 1b arose from the loss of water or a hydroxyl from the peptide. Inspection of the daughter ion spectrum further reveals fragments of *m*/*z* 645, 989, and 1089 which are consistent with peptides DMTV, FVWQDM, and FVWQDMT, each bearing the 5FIdo moiety. Consensus amino acid residues within the smallest peptide correspond to Asp 553 and Met 554 in the sequence.[1] Asp 553 is absolutely conserved in all family 31 enzymes including GLases and this equivalent residue has been proposed previously as the catalytic nucleophile in family 31 α-glucosidases.[1,7,10]

These results indicate that Asp 553 is the catalytic nucleophile of GLases, which strongly suggests that GLases adopt a mechanism very similar to that of retaining α-glucosidases involving formation of a covalent intermediate followed by a syn-elimination rather than a direct trans-elimination. Further evidence for this mechanism is derived from kinetic isotope effect (KIE) analysis using two

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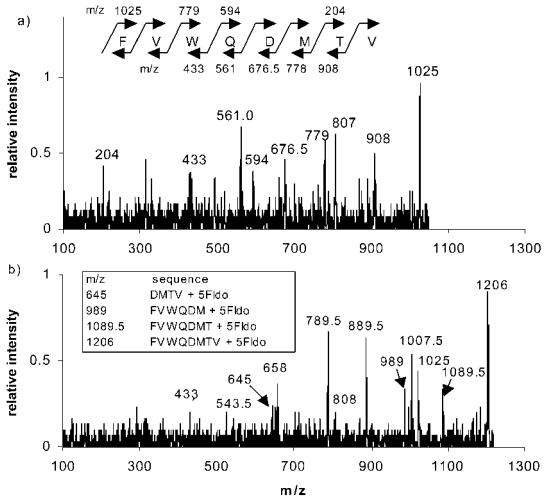
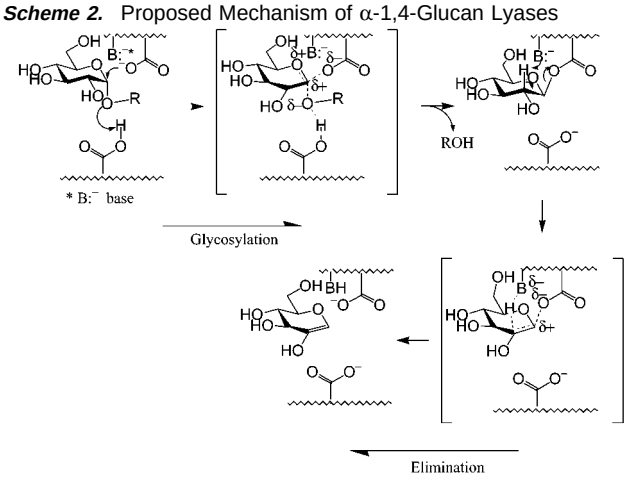


Figure 1. ESI MS/MS daughter ion spectrum along with interpretation: (a) daughter ion spectrum of *m*/*z* 1025 peak from spectrum b; (b) daughter ion spectrum of *m*/*z* 1206 peak from labeled peptide.

Scheme 2. Proposed Mechanism of α-1,4-Glucan Lyases



substrates, *p*-nitrophenyl α-D-glucopyranoside (PNPαGlc, k_{cat} = 0.38 s^{-1}, K_m = 2.0 mM) and α-D-glucopyranosyl fluoride (αGlcF, k_{cat} = 505 s^{-1}, K_m = 28 mM), substituted with deuterium, separately at the 1- and 2-positions. KIEs upon k_{cat} of 1.19 ± 0.02 and 1.06 ± 0.01 were measured with PNP[1-^{2}H]αGlc and its [2-^{2}H] substrate, respectively, while KIEs upon k_{cat}/K_m of 1.16 ± 0.01 and 1.07 ± 0.01 were measured with [1-^{2}H]αGlcF and its [2-^{2}H] substrate. Since PNPαGlc is cleaved over 1000-fold slower than αGlcF, it is highly likely that its k_{cat} value reflects the formation of the glycosyl-enzyme intermediate: a common deglycosylation step cannot be rate-limiting in the two cases. Values of KIEs at each center are essentially identical for the two substrates. This in fact makes sense since k_{cat}/K_m monitors the first irreversible step, presumably the formation of the glycosyl-enzyme intermediate, thus the KIEs measured on PNPαGlc (k_{cat}) and αGlcF (k_{cat}/K_m) both probe the structure of the first transition state. KIEs from 1-deuterio substrates correspond to α-secondary KIEs, thus the large values measured indicate that there is significant oxocarbenium ion character at the glycosylation transition state. Similarly large α-secondary KIEs (1.12−1.21) have been reported previously for family 31 glycosidases.[11] More diagnostically useful are the KIEs measured for the 2-deuterio substrates. If GLases adopted a direct elimination reaction mechanism, a significant primary kinetic isotope effect would be expected. However, the observed values (1.06−1.07) are not big enough for primary KIEs and presumably represent *β*-secondary KIEs. The absence of a primary KIE on the 2-deuterio substrates therefore confirms that a direct trans-elimination mechanism is not in operation but rather a two-step mechanism in which the rate-determining step involves formation of the covalent glycosyl-enzyme intermediate. The α-secondary deuterium KIEs measured confirm that the glycosylation step has substantial oxocarbenium ion character.

In conclusion, we suggest that GLases catalyze the breakdown of α-1,4-glucans via a mechanism involving the formation of a covalent glycosyl-enzyme intermediate which then undergoes a syn-elimination (Scheme 2). The base involved is likely an enzymatic group, and could well be the carboxyl group of Asp 553 as it departs from the anomeric center. Such a mechanism would be consistent with proposals for the mechanism of hydration of glycals by glycosidases in which a syn addition of the protonated nucleophile occurs, followed by hydrolysis of the glycosyl-enzyme intermediate.[12] Other examples of enzymatic syn-eliminations are found in crotonase[13] and UDP−*N*-acetylglucosamine 2-epimerase.[14] Interestingly, earlier work by Wolfenden on nucleoside 2-deoxyribosyltransferase demonstrated that the elimination product D-ribal was occasionally released in the absence of acceptor.[15] Indeed, it is quite easy to imagine this modified mechanism having arisen in a glycosidase through mutations that suppress hydrolysis and favor deprotonation at C2.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Abbreviations: LC, liquid chromatography; ESI MS, electrospray mass spectrometry; ESI MS/MS, electrospray tandem mass spectrometry.
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